[CANCER RESEARCH 59, 4610–4617, September 15, 1999]

Inhibition of Activator Protein 1 Activity and Cell Growth by Purified Green Tea and Black Tea Polyphenols in H-*ras*-transformed Cells: Structure-Activity Relationship and Mechanisms Involved[1]

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ABSTRACT

***ras* gene mutation, which perpetually turns on the growth signal transduction pathway, occurs frequently in many cancer types. The mouse epidermal JB6 cell line has been transfected with a mutant H-*ras* gene to mimic carcinogenesis *in vitro*. These transformed cells (30.7b Ras 12) are able to grow in soft agar, exhibiting anchorage independence and high endogenous activator protein 1 (AP-1) activity, which can be detected by a stable AP-1 luciferase reporter. The present study investigated the ability of different pure green and black tea polyphenols to inhibit this ras signaling pathway. The major green tea polyphenols (catechins), (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin, (−)-epicatechin-3-gallate, (−)-epicatechin, and their epimers, and black tea polyphenols, theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate (TFdiG), were compared with respect to their ability to inhibit the growth of 30.7b Ras 12 cells and AP-1 activity. All of the tea polyphenols except (−)-epicatechin showed strong inhibition of cell growth and AP-1 activity. Among the catechins, both the galloyl structure on the B ring and the gallate moiety contributed to the growth inhibition and AP-1 activity; the galloyl structure appeared to have a stronger effect on the inhibitory action than the gallate moiety. The epimers of the catechins showed similar inhibitory effects on AP-1 activity. The addition of catalase to the incubation of the cells with EGCG or TFdiG did not prevent the inhibitory effect on AP-1 activity, suggesting that H_2O_2 does not play a significant role in the inhibition by tea polyphenols. Both EGCG and TFdiG inhibited the phosphorylation of p44/42 (extracellular signal-regulated kinase 1 and 2) and c-jun without affecting the levels of phosphorylated-c-jun-NH_2-terminal kinase. TFdiG inhibited the phosphorylation of p38, but EGCG did not. EGCG lowered the level of c-jun, whereas TFdiG decreased the level of fra-1. These results suggest that tea polyphenols inhibited AP-1 activity and the mitogen-activated protein kinase pathway, which contributed to the growth inhibition; however, different mechanisms may be involved in the inhibition by catechins and theaflavins.**

INTRODUCTION

Studies with animal carcinogenesis models have demonstrated that green and black tea can inhibit tumor formation as well as tumor progression (1–5). To examine the anticarcinogenic mechanism of green and black tea, the possible active components have been purified and investigated. Green and black tea are two major types of tea manufactured from the leaves of the plant *Camellia sinesis*. Green tea is made by drying fresh tea leaves, which inactivates the enzyme polyphenol oxidase and preserves the polyphenolic constituents in tea (6). It has been found that 30–42% of the dry weight of solids in brewed green tea is composed of polyphenols, of which EGCG[3] is the most abundant (6). Other polyphenols, EGC, ECG, and EC, are also important green tea polyphenols. Fig. 1 shows the chemical structures of these tea polyphenols. These compounds are generally known as tea catechins. Black tea is made by a process known as fermentation, in which tea leaves are crushed to release the polyphenol oxidase for catalyzing the enzymatic oxidation and polymerization of tea catechins. This process results in the production of TFs, thearubigens, and other oligomers (6). TFs are a group of compounds consisting of TF, TF3G, TF3′G, and TFdiG (Fig. 1) that account for 2–6% of the dry weight of solids in brewed black tea (6). The remaining catechins account for 3–10% of the dry weight. Thearubigens, which are of higher molecular weights and are poorly characterized in chemical structure and biological activity, may account for more than 20% of the dry weight of black tea.

EGCG is the most extensively studied tea polyphenol (1). The reported biological activities for EGCG include antioxidative activities, inhibition of tumor promotion-related activities, inhibition of cell growth, and antiviral activities (1, 2, 7–10). Recently, EGCG and TFs were also shown to inhibit the activity of a transcription factor, AP-1, through the inhibition of MAPK, specifically, the JNK in JB6 cells (11). The mechanisms of action of other tea polyphenols are poorly understood.

AP-1 transcription factor is a protein dimer composed of members of the basic region leucine zipper protein superfamily, specifically, the Jun, Fos, and activating transcription factor proteins (12, 13). AP-1 activity has been implicated in various cellular functions including proliferation, transformation, differentiation, and apoptosis (14–17). High AP-1 activity has also been shown to be involved in the tumor promotion (18) and progression of various types of cancers, such as lung (19), breast (20), and skin cancer (21–24). AP-1 regulates many genes that contain the specific DNA sequences in the promoter region collectively called the TPA response element (17). One class of genes that AP-1 regulates is matrix metalloproteinases, which catalyze the proteolytic cleavage of extracellular matrix components; AP-1 activity has been associated with invasive and metastatic characteristics of cancer cells (25, 26). Dumont *et al.* (20) have shown that in the MCF-7 breast cancer cell line, increased metalloproteinase secretion and AP-1 activity are also associated with the development of antiestrogen-resistant phenotype.

Various human cancers have been found to have mutations and overexpression of *ras* genes as an early or late event in the carcinogenesis process (27). In the present study, a mutant H-*ras* gene-transformed JB6 cell line, 30.7b Ras 12, was used to examine the inhibition of cell proliferation and AP-1 activity by pure green tea

Received 4/2/99; accepted 7/21/99.

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[1] Supported by NIH Grants CA56673 and CA81064 and National Institute of Environmental Health Sciences Center Grant ES05022. This study served to partially fulfill a doctorate thesis requirement (for J. Y. C.) in the graduate program for Cellular and Molecular Pharmacology, UMDNJ-Robert Wood Johnson Medical School (Piscataway, New Jersey).

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[3] The abbreviations used are: EGCG, (−)-epigallocatechin-3-gallate; EGC, (−)-epigallocatechin; ECG, (−)-epicatechin-3-gallate; EC, (−)-epicatechin; GCG, (−)-gallocatechin-3-gallate; CG, (−)-catechin-3-gallate; TF, theaflavin; TF3G, theaflavin-3-gallate; TF3′G, theaflavin-3′-gallate; TFdiG, theaflavin-3,3′-digallate; AP-1, activator protein 1; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-jun-NH_2-terminal kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography.

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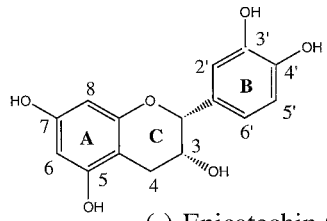
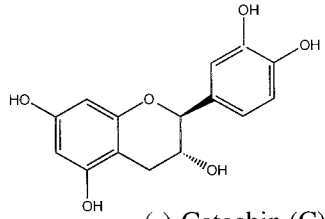
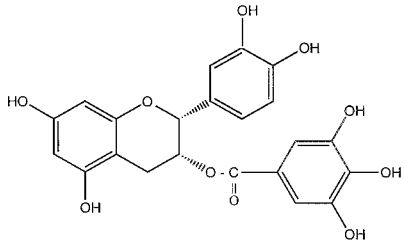
Green Tea Polyphenols



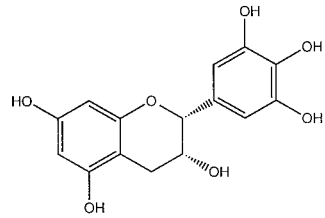
(-)-Epicatechin (EC)



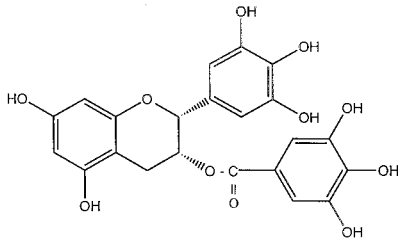
(-)-Catechin (C)



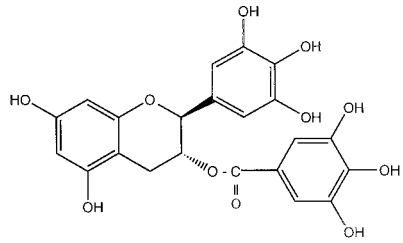
(-)-Epicatechin-3-gallate (ECG)



(-)-Epigallocatechin (EGC)

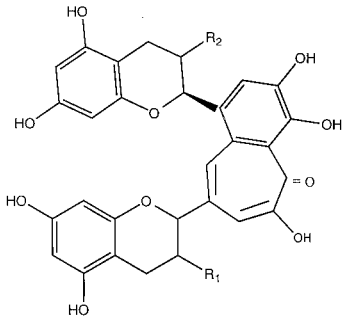


(-)-Epigallocatechin-3-gallate (EGCG)



(-)-Gallocatechin-3-gallate (GCG)

Black Tea Polyphenols



	R_1	R_2
Theaflavin	OH	OH
Theaflavin-3-gallate	Gallate	OH
Theaflavin-3'-gallate	OH	Gallate
Theaflavin-3,3'-digallate	Gallate	Gallate

Fig. 1. Structures of green tea and black tea polyphenols.

polyphenols and their epimers, as well as by the recently purified black tea polyphenols. To further understand the mechanisms that underlie the inhibitory actions, EGCG and TFdiG were chosen as representative green and black tea polyphenols to study their effect on MAPK and the constituents of the AP-1 complex. In addition, catalase was used to examine the possible involvement of H_2O_2 in our experimental system.

MATERIALS AND METHODS

Purified Green and Black Tea Polyphenols. The purified green tea polyphenols EGCG, EGC, ECG, and EC were generous gifts from the Thomas J. Lipton Company (Englewood, NJ). Dr. C. T. Ho (Rutgers University, Piscataway, NJ) and Mitsui Norin Co. Ltd. (Fujied, Japan) generously provided purified TF and TFdiG, respectively. TF3G and TF3′G were purified from a mixture of TFs at Rutgers University by reverse-phase HPLC using an ODS column (MCM-pak C_{18}; 4.6 × 150 mm; ESA Inc.). HPLC was performed under isocratic conditions using solvent A [1.75% acetonitrile, 0.15% tetrahydrofuran, 0.5% acetic acid, and 97.6% water (pH 3.0)] and solvent B [58% acetonitrile, 12.5% tetrahydrofuran, 0.5% acetic acid, and 29% water (pH 3.4)] at a ratio of 65% solvent A:35% solvent B. The flow rate was 1 ml/min. The purity of TF3G, TF3′G, and other tea polyphenols was determined to be >98% by HPLC. CG, (−)-catechin, and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO). All of the green tea polyphenols were dissolved in 0.85% NaCl, and all of the black tea polyphenols were dissolved in DMSO at a 0.1% final concentration.

Cell Culture. JB6 30.7b cells were transfected with RSV-Ha-*ras* 12 expression vector and AP-1-luciferase vector to generate the transformed 30.7b Ras 12 cell line. Similarly, JB6 30.7b cells were transfected with the vector lacking the Ha-*ras* gene insert to generate a nontransformed 30.7b cell line. Both 30.7b Ras 12 and 30.7b cells were cultured in Eagle's MEM containing

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5% FBS, 2 mM L-glutamine, and 25 μg/ml gentamicin (Life Technologies, Inc., Rockville, MD) and maintained at 37°C, 95% humidity, and 5% CO_2.

Growth Inhibition Assay. 30.7b Ras 12 cells (1.0×10^5) were seeded onto a 6-well plate for 24 h in 5% FBS MEM. The cells were serum-starved for 24 h in 0.1% FBS MEM before tea polyphenol treatments. The cells were then treated with different tea polyphenols (1–80 μM) in 5% FBS MEM. Cells that could exclude the trypan blue dye were counted as viable cells.

AP-1 Activity Assay. The AP-1 transactivation assay was performed using the Ha-*ras* transformed 30.7b Ras 12 cells, which contain a stable AP-1-luciferase reporter gene construct. The cells (2.0×10^5) were seeded onto a 6-well plate in 5% FBS MEM. After a 24-h incubation in 5% FBS MEM, the cells were cultured in 0.1% FBS MEM for 12 h before tea polyphenol treatments. The cells were treated with different tea polyphenols (1–20 μM) for 24 h. Similarly, for the catalase experiment, cells were incubated with both catalase (Sigma) and EGCG or TFdiG (20 μM) for 24 h. The cells were then washed twice with cold PBS and lysed using the cell lysis buffer provided in a luciferase assay kit (Promega, Madison, WI). Luciferase activity was measured by adding an equal volume of the luciferase assay buffer, which contains luciferin, and cell lysate. The luciferase activity was then measured using a scintillation counter.

Western Blot Analysis. The active states of MAPK proteins ERK1/ERK2, p38, and JNK were determined using phospho-specific antibodies (New England Biolabs, Beverly, MA). Total cell lysates used to determine the levels of phosphorylated ERK1/ERK2, p38, JNK, c-jun, and fra-1 were prepared following the method of New England Biolabs. In brief, 30.7b Ras 12 cells (1.5×10^6) were seeded onto a 100-mm tissue culture dish in 5% FBS MEM. The cells were incubated in 0.1% FBS MEM for 48 h before treatment with EGCG (1–20 μM) or TFdiG (1–20 μM) for 3 or 4 h. After the incubation with EGCG or TFdiG, the cells were washed twice with cold PBS and lysed with cell lysis buffer (New England Biolabs, Beverly, MA) containing 1 mM phenylmethylsulfonyl fluoride. The cell lysate was sonicated and centrifuged at 14,000 rpm for 10 min at 4°C. The protein concentration of the supernatant was determined using a BCA protein assay kit (Pierce, Rockford, IL). For Western blot analysis, 50 μg of protein were used for SDS-PAGE on a 12% polyacrylamide gel under the conditions described previously (28). The proteins were then transferred onto polyvinylidene difluoride membranes and probed with phospho-specific antibodies for MAPKs or phospho-c-jun (Ser-73; New England Biolabs). Fra-1 protein levels were detected with anti-fra-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The Western blots were visualized using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). The levels of MAPK, c-jun, and fra-1 proteins were quantified using the Bio Image Intelligent Quantifier program (Ann Arbor, MI).

Statistical Analysis. ANOVA and Fisher's exact test were performed on AP-1 activity and growth inhibition data using the StatView program (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Antiproliferation Activity of Tea Polyphenols. The growth inhibition of the H-*ras*-transformed cells after a 24-h incubation with green and black tea polyphenols was examined (Fig. 2). Among the tea catechins, EGCG exhibited the strongest growth-inhibitory activity (IC_{50}, ~30 μM), followed by EGC and ECG, which was significant at the higher concentrations ($P < 0.05$). No significant growth-inhibitory activity was observed with EC between the concentrations of 1 and 80 μM ($P > 0.05$). Among the black tea polyphenols, TFdiG, TF3G, and TF3′G showed strong growth-inhibitory activity (IC_{50}, ~30–35 μM), followed by TF. Longer incubation (48 and 72 h) with the tea polyphenols did not increase the inhibitory effect, and the order of potency remained the same (data not shown). It was also observed that when cells with higher densities were used in the experiment, lower inhibitory activities by tea polyphenols were observed. For example, at cell densities of 5×10^4, 1×10^5, and 2×10^5, 10 μM EGCG inhibited growth by 40%, 30%, and 10%, respectively (data not shown).

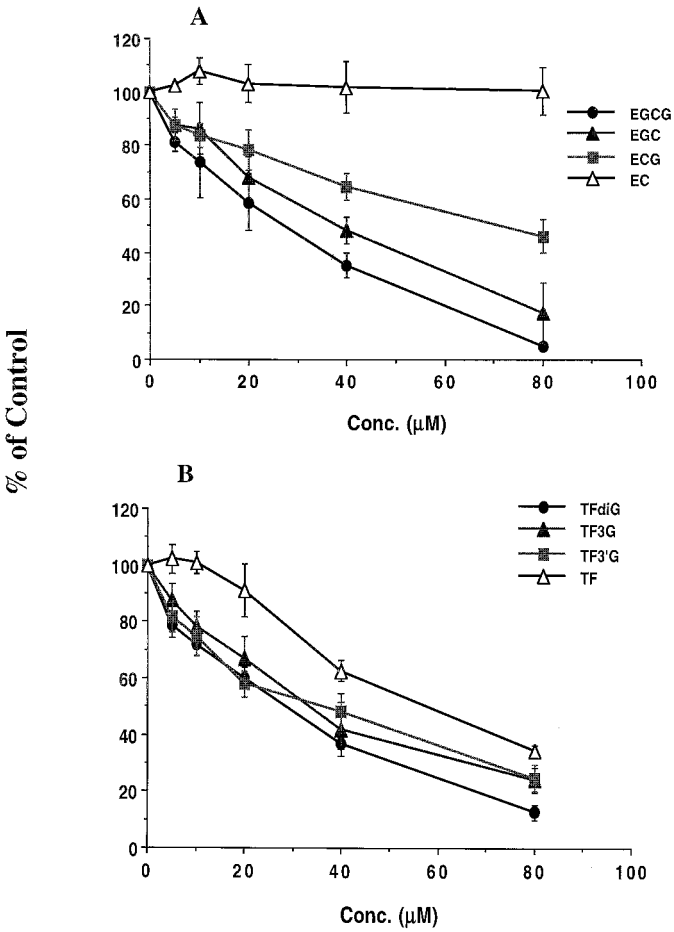

Fig. 2. Effects of tea polyphenols on the growth of *ras*-transformed cells. 30.7b Ras 12 cells (1×10^5) were seeded for 24 h in 5% FBS MEM. The cells were then starved with 0.1% FBS MEM for 24 h before treatment with different concentrations of tea polyphenols for 24 h. After 24 h, cells were trypsinized and counted using the trypan blue exclusion method. *A*, green tea polyphenols. *B*, black tea polyphenols. The values are the mean ± SE of three separate determinations.

Effects of Green Tea and Black Tea Polyphenols on AP-1 Activity. The major pure green and black tea polyphenols, EGCG, EGC, ECG, EC, TF, TF3G, TF3′G, and TFdiG, were examined for their ability to inhibit AP-1 activity (Fig. 3). Among the green tea polyphenols, the most active inhibitor was EGCG, followed by EGC and ECG, with estimated IC_{50} values of 5, 10, and 15 μM, respectively. Inhibition of AP-1 activity was not observed with EC in the concentration range examined (1–20 μM) but was observed at a higher concentration (50% at 100 μM; data not shown). All of the black tea polyphenols showed a strong inhibitory effect on AP-1 activity with an estimated IC_{50} value of 5 μM for TF3G, TF3′G, and TFdiG. TF was less effective than its gallate derivatives at 20 μM (Fig. 3*B*).

The structure-activity relationship in the inhibition of AP-1 activity was further examined with green tea polyphenols together with their epimers, GCG, CG, and (−)-catechin. Comparisons between EGCG, EGC, ECG, and EC showed that the presence of a galloyl structure on the B ring or gallate moiety produced a higher inhibitory activity against AP-1. The inhibitory action of GCG, CG, and (−)-catechin against AP-1 activity was similar to that of their epimers (*i.e.*, EGCG, ECG, and EC, respectively; Fig. 4*A*). However, GCG showed a slightly higher inhibitory activity than EGCG, which was significant at lower concentrations ($P < 0.05$; Fig. 4*B*). To test the activity of the gallate moiety, cells were treated with gallic acid for 24 h. Gallic acid was active against AP-1 activity with a 40% and a 70% inhibition at

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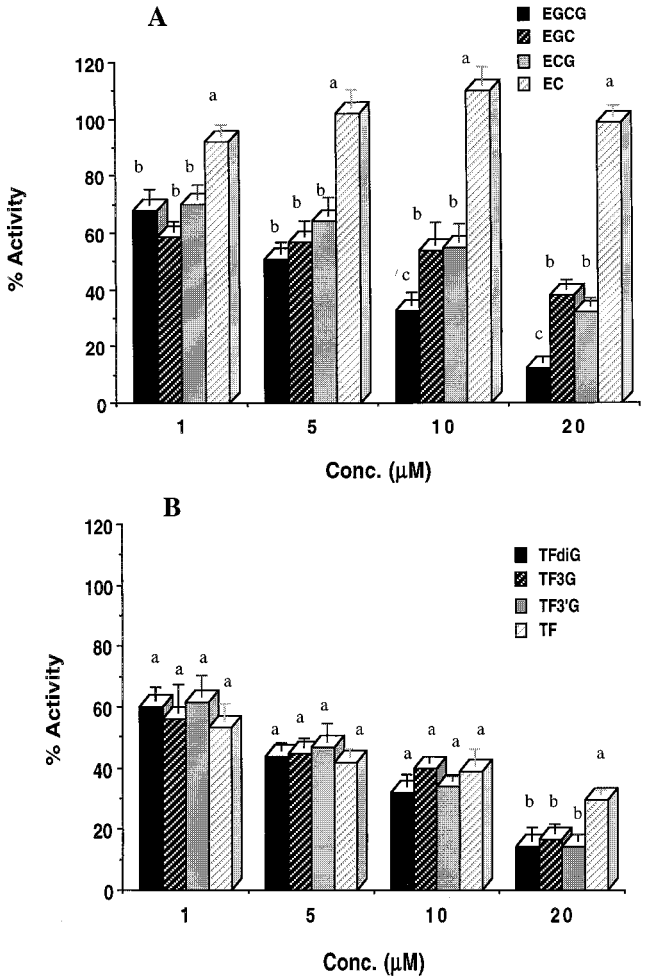

Fig. 3. Effects of green and black tea polyphenols on AP-1 activity. 30.7b Ras 12 cells (2.0×10^5) were seeded for 24 h in 5% FBS MEM. The cells were then placed in 0.1% FBS MEM for 12 h before treatment with various tea polyphenols. After 24 h, the cells were lysed, and luciferase activity was measured in a scintillation counter. *A*, green tea polyphenols. *B*, black tea polyphenols. The values are the mean ± SE of three separate determinations. Values with different subscripts (*a*, *b*, and *c*) are significantly different ($P < 0.05$) from each other as determined by ANOVA followed by Fisher's exact test.

10 and 20 μM, respectively (data not shown). The activity of gallic acid was comparable to that of ECG.

Effects of Catalase on the Inhibition of AP-1 Activity by EGCG and TFdiG. The regulation of transcription factors has also been reported to be affected by redox changes that occur within a cell (29). Specifically, prooxidant conditions have been shown to inhibit AP-1 activity (30). We previously reported (8) that incubation of EGCG with lung cancer cells produced H_2O_2 and induced apoptosis, and that the addition of catalase abolished the induction of apoptosis. To test whether H_2O_2 was the mediator for the inhibition of AP-1 activity by tea polyphenols, catalase (50 units/ml) was added to the cell culture medium along with TFdiG or EGCG (20 μM) for 24 h. The presence of extracellular catalase had no effect on the inhibition of AP-1 activity by TFdiG (Fig. 5). The results suggest that H_2O_2 does not play a significant role in the inhibition of AP-1 by TFdiG. In the incubation with EGCG, however, the addition of catalase slightly prevented the inhibition of AP-1 activity, suggesting that H_2O_2 may play a small role in the inhibition of AP-1 by EGCG; this is uncertain, because the addition of catalase to the cell culture medium (in the absence of tea polyphenols) significantly enhanced AP-1 activity.

Effects of EGCG and TFdiG on MAPK and AP-1 Components. The activation of a signal transduction pathway stimulated by either growth factors, stress, or an oncogene such as *ras* has been shown to involve MAPKs, ERK, JNK, and p38 (31). These MAPKs are activated upon phosphorylation, which then allows them to phosphorylate and activate transcription factors such as AP-1 (32). When EGCG or TFdiG was added to 30.7b Ras 12 cells to examine their effect on MAPKs, we observed a decrease in the level of phosphorylated ERK (Fig. 6*A*). An antibody that recognizes total ERK protein levels showed that this decrease in phosphorylated ERK was not due to a decrease in ERK protein (data not shown). The quantification of the phospho-ERK bands showed that EGCG and TFdiG decreased the protein levels by as much as 67% and 70%, respectively, at a 20 μM concentration. However, the phosphorylated levels of JNK and p38 were not decreased by EGCG. In contrast, TFdiG (20 μM) strongly decreased phosphorylated p38 and appeared to increase the phosphorylated levels of JNK (Fig. 6, *B* and *C*). Basal levels of these activated MAPKs were present in the nontransformed 30.7b cells.

The different effects of EGCG and TFdiG on MAPKs led us to examine the components of AP-1 in 30.7b Ras 12 cells. After the cells were treated with either EGCG or TFdiG for 4 h, c-jun protein was

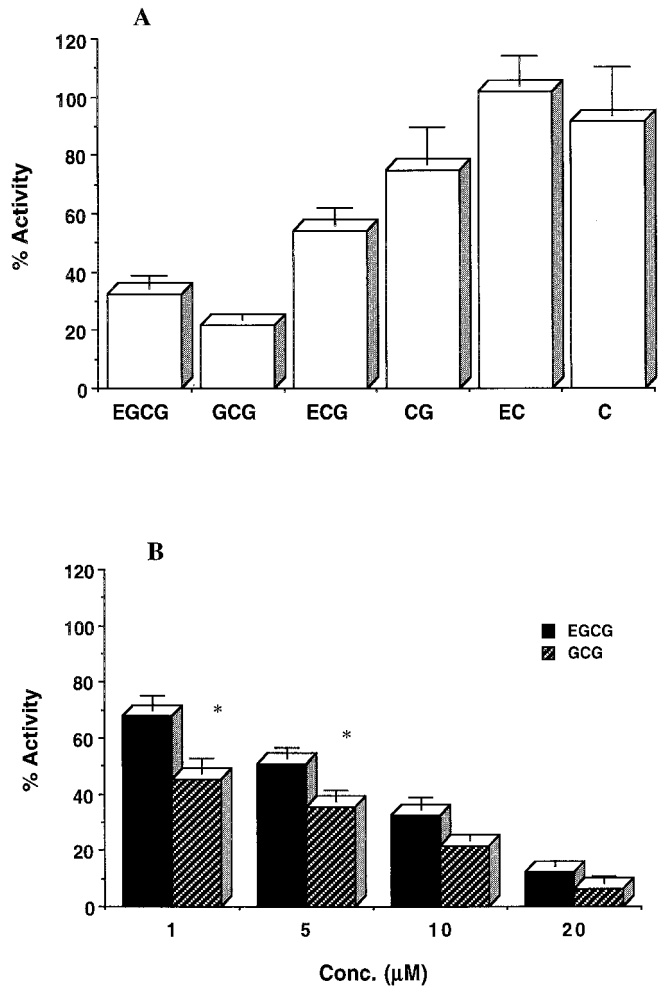

Fig. 4. Comparison of AP-1 inhibition by EGCG, ECG, EC, and their epimers. 30.7b Ras 12 cells (2.0×10^5) were seeded for 24 h in 5% FBS MEM. The cells were then placed in 0.1% FBS MEM for 12 h before treatments with GCG, CG, (−)-catechin (*C*), EGCG, ECG, and EC. *A*, comparison of AP-1 activities at 10 μM. B, dose-response pattern of the inhibitory activity of GCG on AP-1 in comparison to that of EGCG. The values are the mean ± SE of three separate determinations. Values with an *asterisk* are significantly different ($P < 0.05$) from those of the epimers as determined by ANOVA followed by Fisher's exact test.

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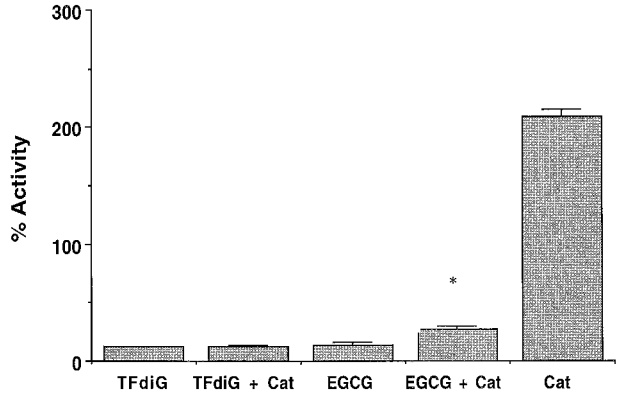

Fig. 5. Effects of catalase on the inhibition of AP-1 activity by EGCG and TFdiG. 30.7b Ras 12 cells (2.0×10^5) were seeded for 24 h in 5% FBS MEM. The cells were then placed in 0.1% FBS MEM for 12 h before the treatments with catalase (50 units/ml) and EGCG or TFdiG (20 μM). After 24 h, the cells were lysed, and luciferase activity was measured in a scintillation counter. The values are the mean ± SE of three separate determinations. Values with an *asterisk* are significantly different ($P < 0.05$) from those without catalase treatment as determined by ANOVA followed by Fisher's exact test.

detected by Western blot analysis. EGCG at 10 and 20 μM decreased the level of c-jun by 20% and 45%, respectively, whereas TFdiG had no appreciable effect on the level of c-jun protein (Fig. 7, *A* and *B*). The phosphorylation state of c-jun was then examined. Because the c-jun level was not significantly decreased by EGCG at 3 h, this time point was selected to examine the phosphorylation state of c-jun. The results showed that both EGCG and TFdiG decreased the levels of phospho-c-jun at 3 and 4 h, respectively (Fig. 7).

Mechta *et al.* (33) reported that in cells transformed by either the H-*ras* or K-*ras* oncogene, the composition of the AP-1 dimer changes from the more stable c-jun and c-fos dimer to a c-jun and fra-1 dimer. Initially, both c-fos and fra-1 were investigated in the 30.7b Ras 12 and the nontransformed 30.7b cells. The results showed that c-fos expression was not significantly increased by the transfection of the H-*ras* gene, but the expression of fra-1 was dramatically increased in comparison with the nontransformed 30.7b control cells. This is consistent with published reports that examined the components of AP-1 in transformed cells (19, 33–35). Therefore, fra-1 protein levels were examined in cells that were treated with EGCG or TFdiG for 4 h. TFdiG (20 μM) decreased fra-1 protein levels by as much as 73%, whereas fra-1 was not decreased by EGCG (Fig. 8).

DISCUSSION

Comparisons of the inhibitory activities of EGCG *versus* ECG, GCG *versus* CG, and EGC *versus* EC indicate that the presence of the galloyl structure on the B ring consistently showed higher inhibition against AP-1 activity and cell growth. The presence of the gallate moiety is also important for the inhibitory activity, as observed in the comparisons of EGCG *versus* EGC and ECG *versus* EC and for gallic acid. EC lacks both the gallate moiety and galloyl structure and is a poor inhibitor. EGCG with both the gallate moiety and galloyl structure is a strong inhibitor of AP-1 activity and growth among the green tea polyphenols. EGCG has a slightly lower inhibitory activity than its epimer, GCG, which shows that the stereochemical position of the B ring galloyl structure may also play a small role. On the other hand, ECG has the same potency as its epimer, CG, in the inhibition of AP-1 activity.

The four TFs studied vary from each other by the number and position of the attached gallate moieties. TF, which does not contain any gallate moiety, is still a potent inhibitor of AP-1 activity. This result is different from the behavior of catechins; EC, which does not have a gallate structure, shows minimal inhibitory activity against AP-1. This may suggest that the increased number of hydroxyl groups and the bulkiness of the structure facilitate interactions with proteins and thus affect the function of AP-1 and cell proliferation. The TF structure may be mainly responsible for the strong AP-1 and growth-inhibitory activity observed for TF3G, TF3′G, and TFdiG. The addi-

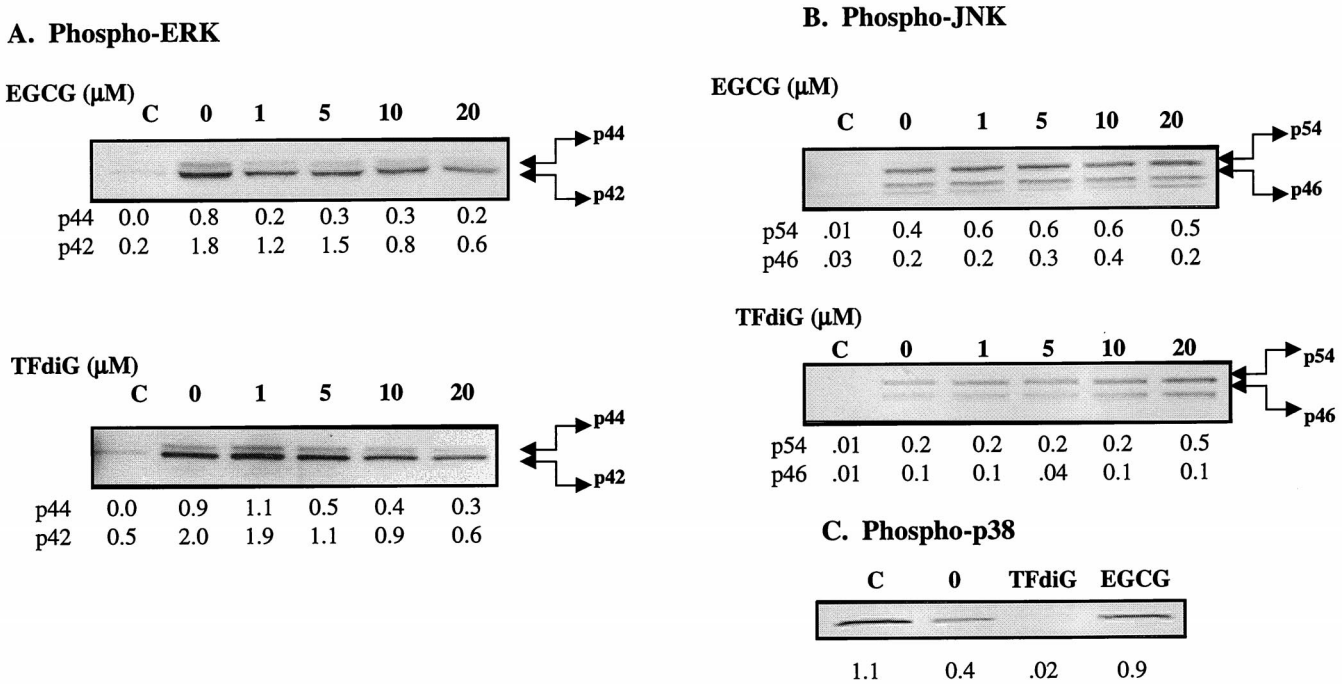

Fig. 6. Effects of EGCG and TFdiG on the active state of MAPK. Total cell lysates from 30.7b Ras 12 cells treated with different concentrations of EGCG and TFdiG for 4 h were analyzed with (*A*) phospho-specific ERK antibody, (*B*) phospho-specific JNK, and (*C*) phospho-specific p38. The 30.7b nontransformed cells were used as a control (*C*). The concentration of EGCG and TFdiG used in *C* was 20 μM. The values below the protein bands are given in arbitrary units of intensities as determined by the Bio Image Intelligent Quantifier program. The experiment was repeated at least two times, and similar results were obtained for each replicate.

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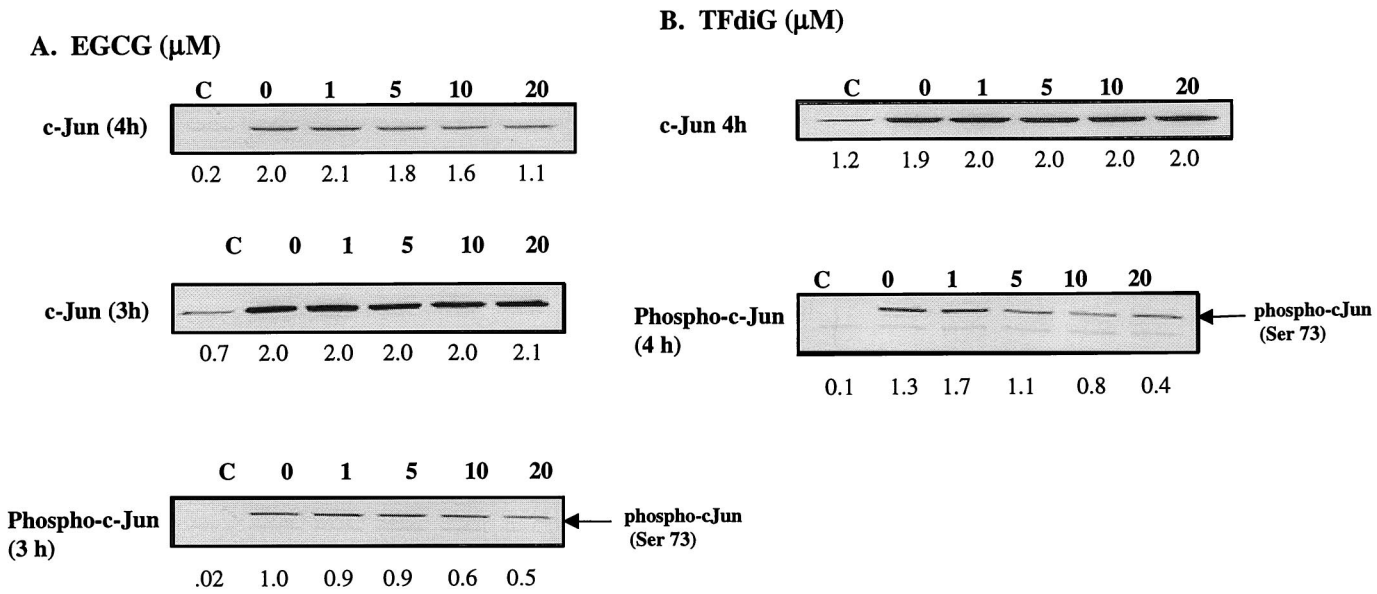

Fig. 7. Effects of EGCG and TFdiG on c-jun and phospho-c-jun. Total cell lysates from 30.7b Ras 12 cells treated with different concentrations of EGCG and TFdiG for 3 or 4 h were analyzed with c-jun and phospho-c-jun (Ser-73) antibodies. *A*, EGCG; *B*, TFdiG; *C*, 30.7b nontransformed control. The values below the protein bands are given in arbitrary units of intensities as determined by the Bio Image Intelligent Quantifier program. The experiment was repeated at least two times, and similar results were obtained for each replicate.

tional gallate structure did not significantly enhance the activity of TFdiG in comparison with TF3G and TF3′G. However, the gallate structure did enhance the growth-inhibitory activities of TF3G, TF3′G, and TFdiG in comparison with TF at higher concentrations (Fig. 2).

Due to its strong activity as an inhibitor and the presence of two gallate moieties,TFdiG was chosen as the representative black tea polyphenol to compare with EGCG in determining the molecular mechanism for the inhibition of AP-1 activity and cell growth. The well established ras signal transduction pathway involves the activation of protein kinases (Raf and MEK), which leads to the phosphorylation of MAPK, ERK1, and ERK2 at threonine and tyrosine residues (31). Ras can also activate JNK and p38 by a similar activation of the protein kinase phosphorylation cascade through rac and cdc42, which are small GTP-binding proteins (36). Our results showed that EGCG and TFdiG decreased phospho-ERK and phospho-c-jun levels (Figs. 6*A* and 7), which indicates that EGCG and TFdiG can both inhibit the phosphorylation of ERK and c-jun, without affecting phospho-JNK levels (Fig. 6*C*). These results are consistent with previous reports, which showed that EGCG inhibited JNK activity in TPA or UVB-induced AP-1 activity (11, 37). It is tempting to propose that the physical binding of EGCG and TFdiG to proteins such as c-jun inhibits its phosphorylation by JNK. It has been reported that tea catechins can bind to proteins that are rich in prolines (38–40), and, interestingly, MAPKs are known as the proline-directed protein kinases because they phosphorylate serine/threonine residues that are within a proline-rich region (41). Thus, it is possible that EGCG or TFdiG can interfere with MAPK activity by interacting with their substrates.

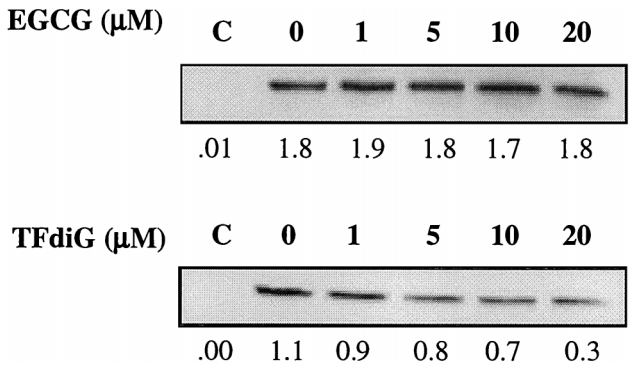

Fig. 8. Effects of EGCG and TFdiG on fra-1. Total cell lysates from 30.7b Ras 12 cells treated with different concentrations of EGCG and TFdiG for 4 h were analyzed with fra-1 antibody. *C*, 30.7b nontransformed control. The values below the protein bands are given in arbitrary units of intensities as determined by the Bio Image Intelligent Quantifier. The experiment was repeated at least two times, and similar results were obtained for each replicate.

Our results show that EGCG and TFdiG both inhibit phosphorylation of the c-jun-NH_2-terminal region and thus quickly turn off AP-1 activity. However, EGCG and TFdiG diverge at the regulation of the third MAPK, p38. It was observed that phospho-p38 formation was inhibited by TFdiG, but not by EGCG (Fig. 6*C*). It is interesting to note that unlike ERK and JNK, phospho-p38 is highly activated in the nontransformed 30.7b cells. The p38 kinase has mostly been implicated in cellular processes associated with cellular stress, such as osmotic shock and UV irradiation (42, 43). However, there is emerging evidence that implicates p38 in a broader role as a mediator of apoptosis (44), regrowth and repair (45, 46), cytokine synthesis (47, 48), and the expression of inducible cyclooxygenase-2 and nitric oxide synthase (43). The inhibitory effect of TFdiG on phospho-p38 in our system needs to be studied further to determine the specific role of p38 and the consequence of its inhibition.

Another difference in the activities of EGCG and TFdiG is at the level of AP-1 components; EGCG decreases the level of c-jun protein, but TFdiG decreases the level of fra-1 protein. It is interesting to observe the differential regulation of c-jun and fra-1 by EGCG and TFdiG because both *c-jun* and *fra-1* genes can be autoregulated by AP-1 (17, 49). It has been reported that in a H-*ras*-driven cellular transformation process, fra-1 plays a role that is equally as important as that of c-jun (33). In addition, Kovary and Bravo (50) have demonstrated that fra-1 expression is involved in cellular proliferation by promoting G_1 to S-phase transition in exponentially growing cells. Therefore, the decrease in fra-1 protein by TFdiG may be one mechanism by which growth is inhibited.

In our current cell culture system with H-*ras*-induced transforma-

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tion, we observed that, in general, tea polyphenols inhibited the growth of these cells in the same order of potency as seen for their inhibition of AP-1 activity. The inhibitory concentrations for AP-1 activity by tea polyphenols (with estimated IC_{50} values of 5–15 μM) were lower in comparison with the concentrations for growth inhibition (estimated IC_{50}, 30–70 μM). The reason for this difference in the effective concentrations of tea polyphenols for growth and AP-1 inhibition is not known. One possibility is that AP-1 activity acts primarily in the pathway leading to cellular transformation and invasive characteristics of cells (14, 22, 24, 25, 51) and is not the rate-limiting factor in regulation of cell growth. This is supported by our previous work (11), which showed that the inhibition of TPA-induced transformation by EGCG and TFs was not due to the inhibition of growth within a 1–20 μM concentration range. Another factor contributing to this difference is the different serum concentrations used for the AP-1 assay (0.1%) and the cell proliferation assay (5%). When 5% serum was used to study the inhibition of AP-1 by EGCG, the estimated IC_{50} increased from 5 to 10 μM. Nevertheless, this factor is not enough to account for the difference observed. Additional studies are needed to elucidate the mechanisms of growth inhibition by EGCG and TFdiG.

In summary, our results show that green and black tea polyphenols can inhibit AP-1 activity as well as cell proliferation. The mechanisms of the inhibitory actions of EGCG and TFdiG on AP-1 activity and growth acted through the inhibition of phospho-ERK and phospho-c-jun formation and, subsequently, through the decreased levels of c-jun and fra-1 by EGCG and TFdiG, respectively. Although EGCG is the most abundant and most active compound among all of the tea polyphenols, other catechins such as EGC and ECG and TFs are also expected to contribute to the growth- and AP-1-inhibitory activity of green and black tea. Additional studies are needed to determine the exact mechanisms by which these compounds affect the MAPK pathways, AP-1 activity, and cell proliferation.

ACKNOWLEDGMENTS

We are grateful to Dr. Theresa J. Smith for inspiring discussions, helpful suggestions, and critical review of this manuscript. We thank Dr. Chi-Tang Ho for generously providing TF, and we gratefully acknowledge Petar Hinic for technical assistance and Dorothy Wong for excellent secretarial assistance.

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Inhibition of Activator Protein 1 Activity and Cell Growth by Purified Green Tea and Black Tea Polyphenols in H-*ras*-transformed Cells: Structure-Activity Relationship and Mechanisms Involved

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