

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 Involved¹

Jee Y. Chung, Chuanshu Huang, Xiaofeng Meng, Zigang Dong, and Chung S. Yang²

Laboratory for Cancer Research, College of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854 [J. Y. C., X. M., C. S. Y.], and Hormel Institute, University of Minnesota, Austin, Minnesota 55912 [Z. D., C. H.]

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 H₂O₂ 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-NH₂-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 sinensis*. 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 EGCG³ 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.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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).

² To whom requests for reprints should be addressed, at Laboratory for Cancer Research, College of Pharmacy, Rutgers, The State University of New Jersey, 164 Frelinghuysen Road, Piscataway, NJ 08854-8020. Phone: (732) 445-5361; Fax: (732) 445-0687; E-mail: csyang@rci.rutgers.edu.

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-NH₂-terminal kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography.

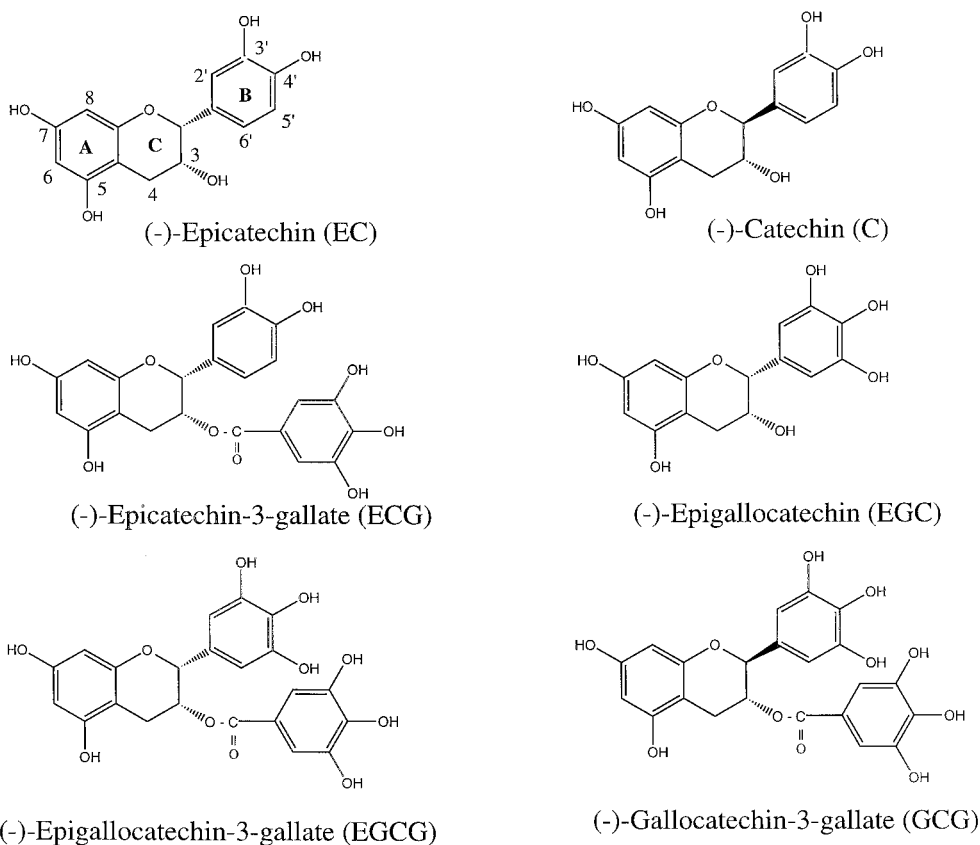
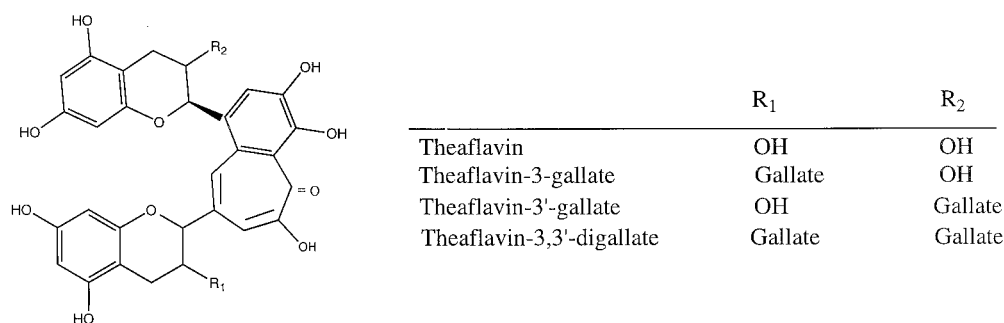
Green Tea Polyphenols**Black Tea Polyphenols**

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 H₂O₂ 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 C₁₈; 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

5% FBS, 2 mM L-glutamine, and 25 $\mu\text{g}/\text{ml}$ gentamicin (Life Technologies, Inc., Rockville, MD) and maintained at 37°C, 95% humidity, and 5% CO₂.

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} , $\sim 30 \mu\text{M}$), 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 – $35 \mu\text{M}$), 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).

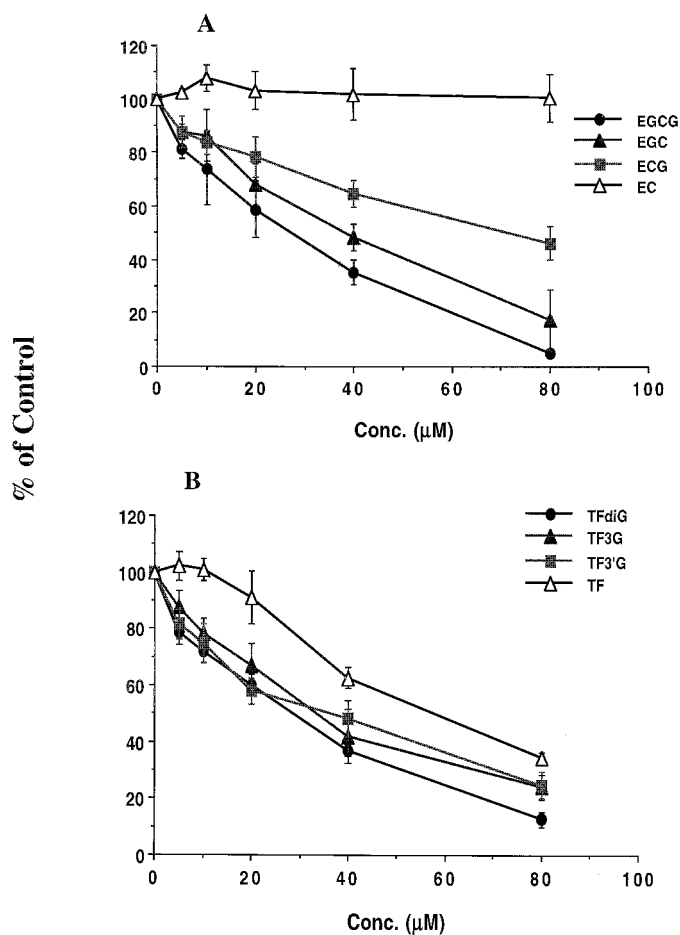


Fig. 2. Effects of tea polyphenols on the growth of *ras*-transformed cells. 30.7b Ras 12 cells (1×10^5) were seeded for 24 h in 5% FBS MEM. The cells were then starved with 0.1% FBS MEM for 24 h before treatment with different concentrations of tea polyphenols for 24 h. After 24 h, cells were trypsinized and counted using the trypan blue exclusion method. A, green tea polyphenols. B, black tea polyphenols. The values are the mean \pm 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. 3B).

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. 4A). However, GCG showed a slightly higher inhibitory activity than EGCG, which was significant at lower concentrations ($P < 0.05$; Fig. 4B). 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

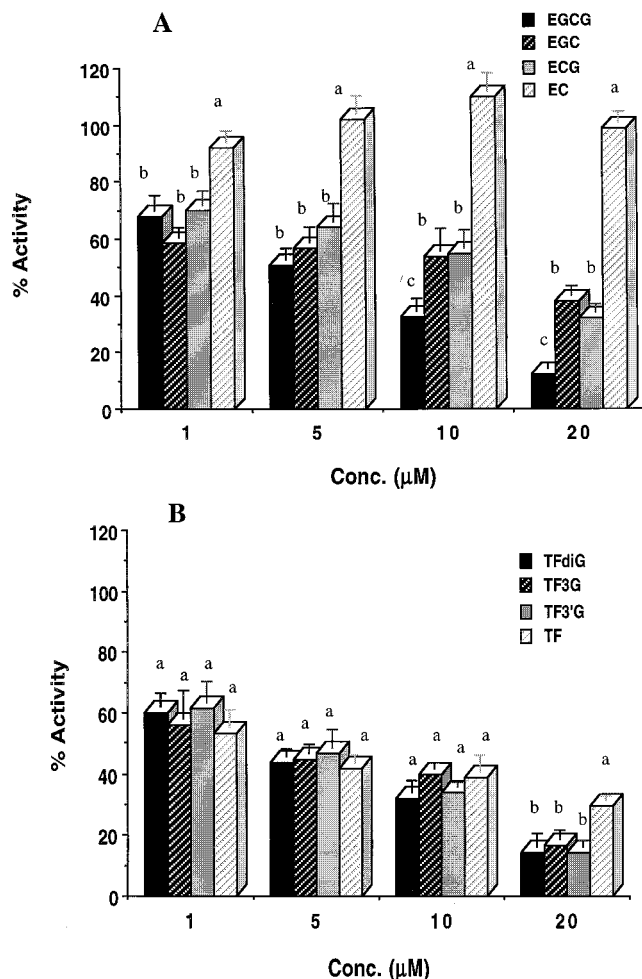


Fig. 3. Effects of green and black tea polyphenols on AP-1 activity. 30.7b Ras 12 cells (2.0×10^5) were seeded for 24 h in 5% FBS MEM. The cells were then placed in 0.1% FBS MEM for 12 h before treatment with various tea polyphenols. After 24 h, the cells were lysed, and luciferase activity was measured in a scintillation counter. A, green tea polyphenols. B, black tea polyphenols. The values are the mean \pm 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. 6A). 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

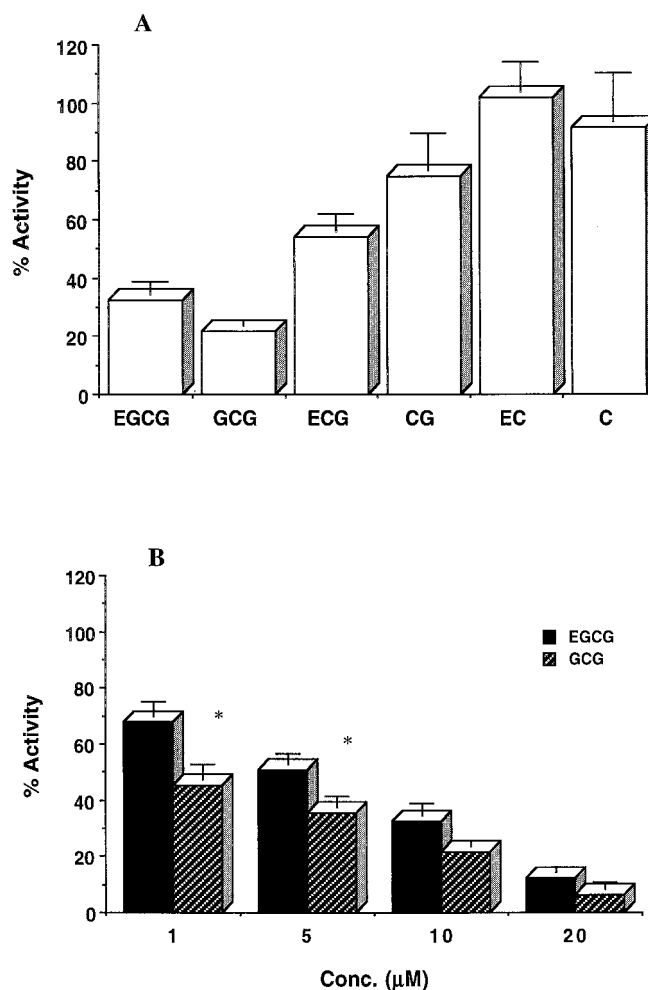


Fig. 4. Comparison of AP-1 inhibition by EGCG, ECG, EC, and their epimers. 30.7b Ras 12 cells (2.0×10^5) were seeded for 24 h in 5% FBS MEM. The cells were then placed in 0.1% FBS MEM for 12 h before treatments with GCG, CG, (-)-catechin (C), EGCG, ECG, and EC. A, comparison of AP-1 activities at 10 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 \pm 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.

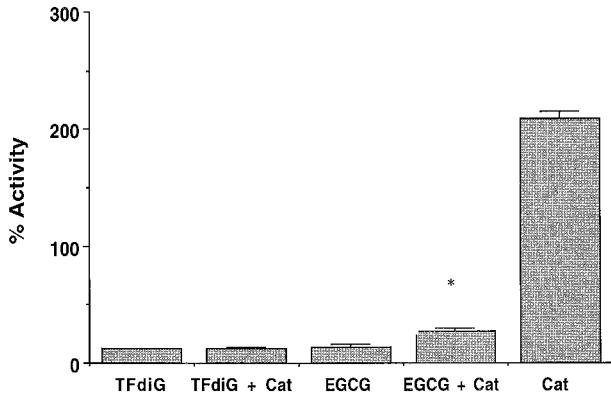


Fig. 5. Effects of catalase on the inhibition of AP-1 activity by EGCG and TFdiG. 30.7b Ras 12 cells (2.0×10^5) were seeded for 24 h in 5% FBS MEM. The cells were then placed in 0.1% FBS MEM for 12 h before the treatments with catalase (50 units/ml) and EGCG or TFdiG ($20 \mu\text{M}$). After 24 h, the cells were lysed, and luciferase activity was measured in a scintillation counter. The values are the mean \pm 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 $20 \mu\text{M}$ 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).

Mehta *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

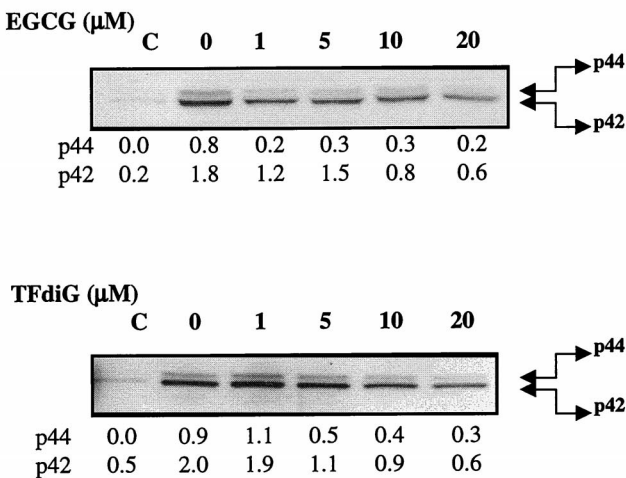
H-ras gene, but the expression of fra-1 was dramatically increased in comparison with the nontransformed 30.7b control cells. This is consistent with published reports that examined the components of AP-1 in transformed cells (19, 33–35). Therefore, fra-1 protein levels were examined in cells that were treated with EGCG or TFdiG for 4 h. TFdiG ($20 \mu\text{M}$) decreased fra-1 protein levels by as much as 73%, whereas fra-1 was not decreased by EGCG (Fig. 8).

DISCUSSION

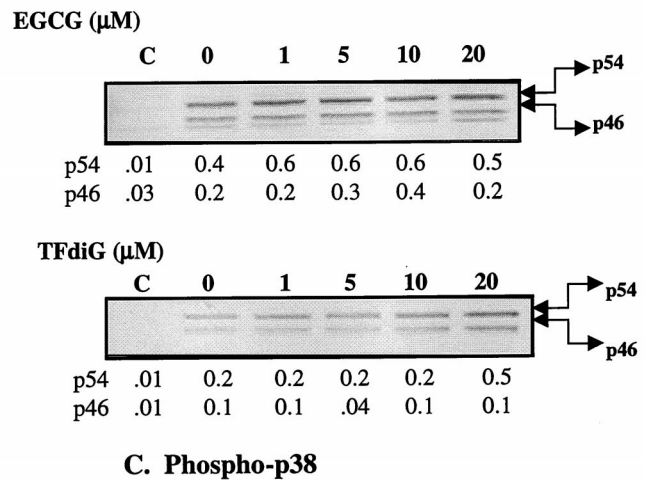
Comparisons of the inhibitory activities of EGCG *versus* ECG, GCG *versus* CG, and EGC *versus* EC indicate that the presence of the galloyl structure on the B ring consistently showed higher inhibition against AP-1 activity and cell growth. The presence of the gallate moiety is also important for the 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-

A. Phospho-ERK



B. Phospho-JNK



C. Phospho-p38

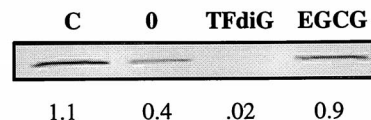


Fig. 6. Effects of EGCG and TFdiG on the active state of MAPK. Total cell lysates from 30.7b Ras 12 cells treated with different concentrations of EGCG and TFdiG for 4 h were analyzed with (A) phospho-specific ERK antibody, (B) phospho-specific JNK, and (C) phospho-specific p38. The 30.7b nontransformed cells were used as a control (C). The concentration of EGCG and TFdiG used in C was $20 \mu\text{M}$. 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.

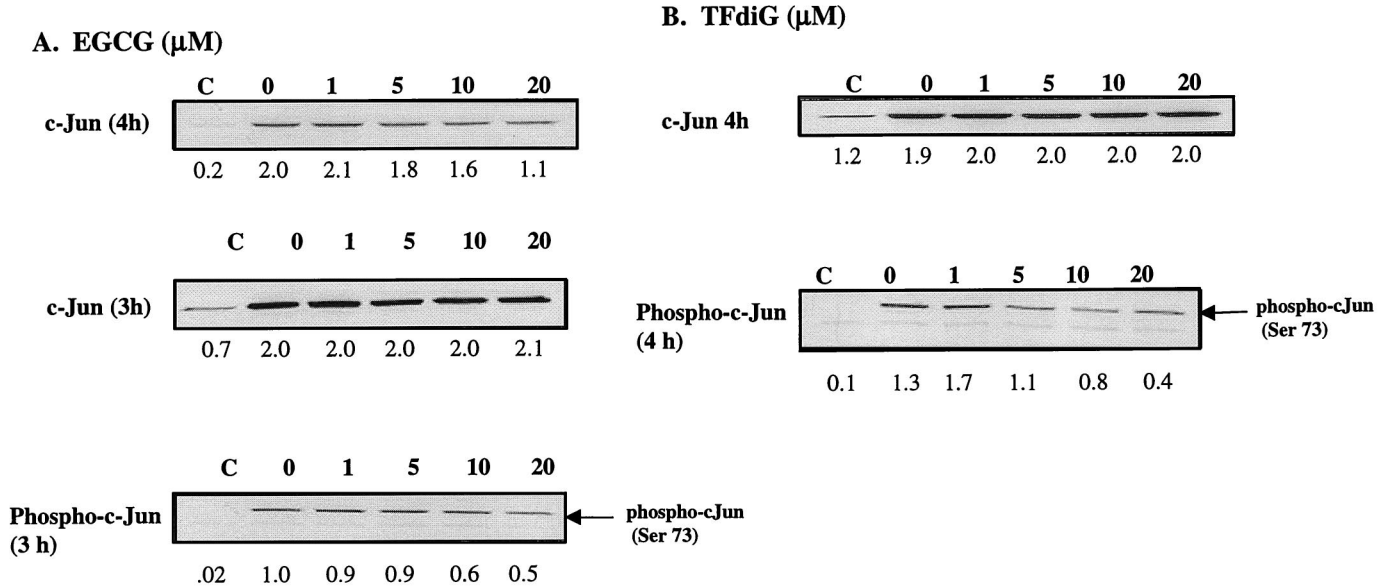


Fig. 7. Effects of EGCG and TFdiG on c-jun and phospho-c-jun. Total cell lysates from 30.7b Ras 12 cells treated with different concentrations of EGCG and TFdiG for 3 or 4 h were analyzed with c-jun and phospho-c-jun (Ser-73) antibodies. A, EGCG; B, TFdiG; C, 30.7b nontransformed control. The values 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. 6A and 7), which indicates that EGCG and TFdiG can both inhibit the phosphorylation of ERK and c-jun, without affecting phospho-JNK levels (Fig. 6C). 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.

Our results show that EGCG and TFdiG both inhibit phosphorylation of the c-jun-NH₂-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. 6C). 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 G₁ 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-

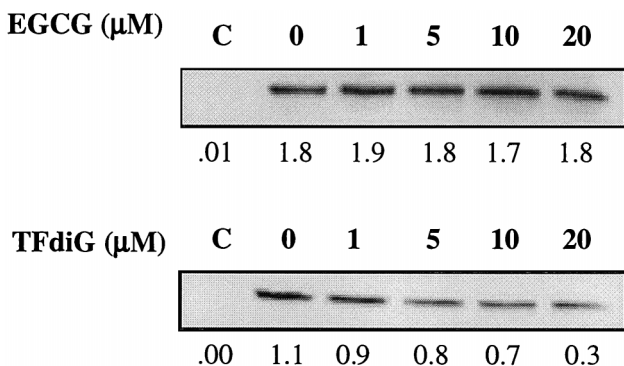


Fig. 8. Effects of EGCG and TFdiG on fra-1. Total cell lysates from 30.7b Ras 12 cells treated with different concentrations of EGCG and TFdiG for 4 h were analyzed with fra-1 antibody. C, 30.7b nontransformed control. The values below the protein bands are given in arbitrary units of intensities as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

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.

REFERENCES

- Yang, C. S., and Wang, Z. Y. Tea and cancer. *J. Natl. Cancer Inst.*, 85: 1038–1049, 1993.
- Katiyar, S. K., and Mukhtar, H. Tea in chemoprevention of cancer: epidemiologic and experimental studies. *Int. J. Oncol.*, 8: 221–238, 1996.
- Yang, G. Y., Wang, Z. Y., Kim, S., Liao, J., Seril, D. N., Chen, X., Smith, T. J., and Yang, C. S. Characterization of early pulmonary hyperproliferation and tumor progression and their inhibition by black tea in a 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis model with A/J mice. *Cancer Res.*, 57: 1889–1894, 1997.
- Huang, M. T., Xie, J. G., Wang, Z. Y., Ho, C. T., Lou, Y. R., Wang, C. X., Hard, G. C., and Conney, A. H. Effects of tea, decaffeinated tea, and caffeine on UVB light-induced complete carcinogenesis in SKH-1 mice: demonstration of caffeine as biologically important constituent of tea. *Cancer Res.*, 57: 2623–2629, 1997.
- Lu, Y. P., Lou, Y. R., Xie, J. G., Yen, P., Huang, M. T., and Conney, A. H. Inhibitory effect of black tea on the growth of established skin tumors in mice: effects on tumor size, apoptosis, mitosis, and bromodeoxyuridine incorporation into DNA. *Carcinogenesis (Lond.)*, 18: 2163–2169, 1997.
- Balentine, D. A., Wiseman, S. A., and Bouwens, L. C. M. The chemistry of tea flavonoids. *Crit. Rev. Food Sci. Nutr.*, 37: 693–704, 1997.
- Yang, C. S., Chen, L., Lee, M. J., and Landau, J. M. Effects of tea on carcinogenesis in animal models and humans (edited under the auspices of the American Institute for Cancer Research). *In: Dietary Phytochemicals in Cancer Prevention and Treatment*, pp. 51–61. New York: Plenum Press, 1996.
- Yang, G. Y., Liao, J., Kim, K., Yurkow, E. J., and Yang, C. S. Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis (Lond.)*, 19: 611–616, 1998.
- Chen, Z. P., Schell, J. B., Ho, C. T., and Chen, K. Y. Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Lett.*, 129: 173–179, 1998.
- Nakayama, M., Suzuki, K., Toda, M., Okubo, S., Hara, Y., and Shimamura, T. Inhibition of the infectivity of influenza virus by tea polyphenols. *Antivir. Res.*, 21: 289–299, 1993.
- Dong, Z. G., Ma, W. Y., Huang, C., and Yang, C. S. Inhibition of tumor promoter-induced activator protein-1 activation and cell transformation by tea polyphenols, (–)-epigallocatechin gallate and theaflavins. *Cancer Res.*, 57: 4414–4419, 1997.
- Karin, M. The regulation of AP-1 activity by mitogen-activated protein kinases. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, 351: 127–134, 1996.
- Karin, M., Liu, Z. G., and Zandi, E. AP-1 function and regulation. *Curr. Opin. Cell Biol.*, 9: 240–246, 1997.
- Dong, Z. G., Birrer, M. J., Watts, R. G., Matrisian, L. M., and Colburn, N. H. Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. *Proc. Natl. Acad. Sci. USA*, 91: 609–613, 1994.
- Dong, Z. G., Watts, R. G., Sun, Y., Zhan, S. N., and Colburn, N. H. Progressive elevation of AP-1 activity during preneoplastic-to-neoplastic progression as modeled in mouse JB6 cell variants. *Int. J. Oncol.*, 7: 359–364, 1995.
- Huang, C. S., Ma, W. Y., Young, M. R., Colburn, N. H., and Dong, Z. G. Shortage of mitogen-activated protein kinase is responsible for resistance to AP-1 transactivation and transformation in mouse JB6 cells. *Proc. Natl. Acad. Sci. USA*, 95: 156–161, 1998.
- Angel, P., and Karin, M. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. *Biochim. Biophys. Acta*, 1072: 129–157, 1991.
- Huang, C. S., Ma, W. Y., Dawson, M. I., Rincon, M., Flavell, R. A., and Dong, Z. G. Blocking activator protein-1 activity, but not activating retinoic acid response element, is required for the antitumor promotion effect of retinoic acid. *Proc. Natl. Acad. Sci. USA*, 94: 5826–5830, 1997.
- Risse-Hackl, G., Adamkiewicz, J., Wimmel, A., and Schuermann, M. Transition from SCLC to NSCLC phenotype is accompanied by an increased TRE-binding activity and recruitment of specific AP-1 proteins. *Oncogene*, 16: 3057–3068, 1998.
- Dumont, J. A., Bitonti, A. J., Wallace, C. D., Baumann, R. J., Cashman, E. A., and Cross-Doersen, D. E. Progression of MCF-7 breast cancer cells to antiestrogen-resistant phenotype is accompanied by elevated levels of AP-1 DNA-binding activity. *Cell Growth Differ.*, 7: 351–359, 1996.
- Saez, E., Ruberg, S. E., Mueller, E., Oppenheim, H., Smoluk, J., Yuspa, S. H., and Spiegelman, B. M. c-Fos is required for malignant progression of skin tumors. *Cell*, 82: 721–732, 1995.
- Dong, Z. G., Crawford, H. C., Lavrovsky, V., Taub, D., Watts, R., Matrisian, L. M., and Colburn, N. H. A dominant negative mutant of jun blocking 12-O-tetradecanoylphorbol-13-acetate-induced invasion in mouse keratinocytes. *Mol. Carcinog.*, 19: 204–212, 1997.
- Domann, F. E., Levy, J. P., Finch, J. S., and Bowden, G. T. Constitutive AP-1 DNA binding and transactivating ability of malignant but not benign mouse epidermal cells. *Mol. Carcinog.*, 9: 61–66, 1994.
- Lamb, R. F., Hennigan, R. F., Turnbull, K., Katsanakis, K. D., MacKenzie, E. D., Birnie, G. D., and Ozanne, B. W. AP-1 mediated invasion requires increased expression of hyaluronan receptor CD44. *Mol. Cell. Biol.*, 17: 963–976, 1997.
- Crawford, H. C., and Matrisian, L. M. Mechanisms controlling the transcription of matrix metalloproteinase genes in normal and neoplastic cells. *Enzyme Protein*, 49: 20–37, 1996.
- Wright, J. H., McDonnell, S., Portella, G., Bowden, G. T., Balmain, A., and Matrisian, L. M. A switch from stromal to tumor cell expression of stromelysin-1 mRNA associated with the conversion of squamous to spindle carcinomas during mouse skin tumor progression. *Mol. Carcinog.*, 10: 207–215, 1994.
- Zachos, G., and Spandidos, D. A. Expression of ras proto-oncogenes: regulation and implications in the development of human tumors. *Crit. Rev. Oncol.-Hematol.*, 26: 65–75, 1997.
- Laemmli, U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*, 227: 680–685, 1970.
- Meyer, M., Pahl, H. L., and Baeuerle, P. A. Regulation of the transcription factors NF- κ B and AP-1 by redox changes. *Chem-Biol. Interact.*, 91: 91–100, 1994.
- Meyer, M., Schreck, R., and Baeuerle, P. A. H₂O₂ and antioxidants have opposite effects on activation of NF- κ B and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. *EMBO J.*, 12: 2005–2015, 1993.
- Denhardt, D. T. Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. *Biochem. J.*, 318: 729–747, 1996.
- Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.*, 7: 2135–2148, 1993.
- Mechta, F., Lallemand, D., Pfarr, C. M., and Yaniv, M. Transformation by ras modifies AP1 composition and activity. *Oncogene*, 14: 837–847, 1997.
- Okimoto, T., Kohno, K., Kuwano, M., Gopas, J., Kung, H.-F., and Ono, M. Regulation of epidermal growth factor receptor by activated H-ras and V-myc oncogenes in mouse Balb/3T3 cells: possible roles of AP-1. *Oncogene*, 12: 1625–1633, 1996.
- Battista, S., Nigris, F., Fedele, M., Chiappetta, G., Scala, S., Vallone, D., Pierantoni, G. M., Megar, T., Santoro, M., Viglietto, G., Verde, P., and Fusco, A. Increase in AP-1 activity is a general event in thyroid cell transformation *in vitro* and *in vivo*. *Oncogene*, 17: 377–385, 1998.
- Minden, A., Lin, A., Claret, F. X., Abo, A., and Karin, M. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell*, 81: 1147–1157, 1995.
- Barthelmann, M., Bair, W. B., Stickland, K. K., Chen, W. X., Timmerman, N., Valcic, S., Dong, Z. G., and Bowden, G. T. (–)-Epigallocatechin-3-gallate inhibition of ultraviolet B induced AP-1 activity. *Carcinogenesis (Lond.)*, 19: 2201–2204, 1998.
- Mehansho, H., Butler, L. G., and Carlson, D. M. Dietary tannins and salivary proline-rich proteins: interaction, induction, and defense mechanisms. *Annu. Rev. Nutr.*, 7: 423–440, 1987.

39. Murray, N. J., Williamson, M. P., Lilley, T. H., and Haslam, E. Study of the interaction between salivary proline-rich proteins and a polyphenol by ¹H-NMR spectroscopy. *Eur. J. Biochem.*, 219: 923–935, 1994.
40. Charlton, A. J., Baxter, N. J., Lilley, T. H., Haslam, E., McDonald, C. J., and Williamson, M. P. Tannin interactions with a full-length human salivary proline-rich protein display a stronger affinity than with single proline-rich repeats. *FEBS Lett.*, 382: 289–292, 1996.
41. Hanks, S. K., and Hunter T. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. *FASEB J.*, 9: 576–596, 1995.
42. Cohen, P. The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Cell Biol.*, 7: 353–361, 1997.
43. Paul, A., Wilson, S., Belham, C. M., Robinson, C. J. M., Scott, P. H., Gould, G. W., and Plevin, R. Stress-activated protein kinases: activation, regulation, and function. *Cell. Signalling*, 9: 403–410, 1997.
44. Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., and Greenberg, M. E. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science (Washington DC)*, 270: 1326–1331, 1995.
45. Knight, R. J., and Buxton, D. B. Stimulation of c-Jun kinase and mitogen-activated protein kinase by ischemia and reperfusion in the perfused rat heart. *Biochem. Biophys. Res. Commun.*, 218: 83–88, 1996.
46. Bogoyevitch, M. A., Gillespie-Brown, J., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., Marshall, C. J., and Sugden, P. H. Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. P38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ. Res.*, 79: 162–173, 1996.
47. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., White, J. R., Adams, J. L., and Young, A. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature (Lond.)*, 372: 739–746, 1994.
48. Beyaert, R., Cuenda, A., Berghe, W. V., Plaisance, S., Lee, J. C., Haegeman, G., Cohen, P., and Fiers, W. The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis in response to tumour necrosis factor. *EMBO J.*, 15: 1914–1923, 1996.
49. Bergers, G., Graninger, P., Braselmann, S., Wrighton, C., and Busslinger, M. Transcriptional activation of the *fra-1* gene by AP-1 is mediated by regulatory sequences in the first intron. *Mol. Cell. Biol.*, 15: 3748–3758, 1995.
50. Kovary, K., and Bravo, R. Existence of different Fos/Jun complexes during the G₀-to-G₁ transition and during exponential growth in mouse fibroblasts: differential role of Fos proteins. *Mol. Cell. Biol.*, 12: 5015–5023, 1992.
51. Domann, F. E., Levy, J. P., Birrer, M. J., and Bowden, G. T. Stable expression of a c-jun deletion mutant in two malignant mouse epidermal cell lines blocks tumor formation in nude mice. *Cell Growth Differ.*, 5: 9–16, 1994.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

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

Jee Y. Chung, Chuanshu Huang, Xiaofeng Meng, et al.

Cancer Res 1999;59:4610-4617.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/59/18/4610>

Cited articles This article cites 47 articles, 14 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/59/18/4610.full#ref-list-1>

Citing articles This article has been cited by 22 HighWire-hosted articles. Access the articles at:
<http://cancerres.aacrjournals.org/content/59/18/4610.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cancerres.aacrjournals.org/content/59/18/4610>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.