Modulation of AP-1 by Natural Chemopreventive Compounds in Human Colon HT-29 Cancer Cell Line

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Purpose. Activator protein-1 (AP-1) has been implicated as playing important roles in apoptosis and cancer development. In this work, we studied several natural chemopreventive compounds for their potential chemopreventive properties in the modulation of AP-1 signaling pathway in HT-29 colon cancer cells.

Methods. The HT-29 cells were transfected with AP-1–luciferase reporter gene, and one of the stable clones (C-4) was used for subsequent experiments. The HT-29 C-4 cells were treated for 1 h with various natural chemopreventive agents and challenged with AP-1 stimulators such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or hydrogen peroxide (H_2O_2) for 6 h. The c-Jun N-terminal kinase (JNK) was examined to understand the effect of these compounds on the upstream signaling activator of AP-1. The protein expression level of endogenous cyclin D1, a gene that is under the control of AP-1, was also analyzed after treatments with the agents. In addition, cell death induced by these compounds was evaluated by MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-arboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt].

Results. TPA and H_2O_2 treatments strongly induced AP-1–luciferase activity as expected. Phenethyl isothiocyanate, sulforaphane, curcumin, and resveratrol increased AP-1–luciferase activity dose-dependently and then decreased at higher doses in the presence or absence of TPA. Allyl isothiocyanate and (−)-epigallocatechin-3-gallate (EGCG) increased AP-1–luciferase activity dose-dependently up to 50 and 100 μM. Other tea catechins and procyanidin dimers, however, had little or no effect on AP-1–luciferase activity. The JNK activity was induced by the isothiocyanates and EGCG. Most of the chemopreventive compounds induced cell death in a dose-dependent manner, with the exception of epicatechin (EC) and the procyanidins, which had little effect. The expression of endogenous cyclin D1 protein was well correlated with those of AP-1–luciferase assay.

Conclusion. Taken together, these results suggest that natural chemopreventive compounds may have differential biological functions on the signal transduction pathways such as AP-1 in the intervention of colon cancer progression and carcinogenesis.

KEY WORDS: activator protein-1(AP-1); c-jun N-terminal kinase (JNK); chemopreventive compounds; cyclin D1; HT-29 cell.

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ABBREVIATIONS: AP-1, activator protein-1; AITC, allyl isothiocyanate; CUR, curcumin; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate; H_2O_2, hydrogen peroxide; ITC, isothiocyanate; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PCB1, procyanidin B1; PCB2, procyanidin B2; PEITC, phenethyl isothiocyanate; RES, resveratrol; SUL, sulforaphane; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

INTRODUCTION

Activator protein-1 (AP-1) has been implicated as playing important roles in various biological processes including apoptosis and cancer development. AP-1 is a heterodimeric protein complex composed of members of the basic leucine zipper proteins including Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra-1, and Fra 2), Maf (c-Maf, MafB, MafA, MafG/F/K, and Nrl), and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, and JDP2) subfamilies (1). It is known to bind to a palindromic DNA sequence such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-responsive element (TRE) present within the regulatory region of variety of genes, including c-Jun (2). AP-1 activity has been involved in many diverse cellular processes including biological apoptosis, proliferation, transformation, and differentiation, although the exact outcome may be highly dependent on tissue and developmental stage (3–5).

AP-1 activity can be regulated by many mechanisms, one of which is the activation of mitogen-activated protein kinase (MAPK) pathways (6). The members of MAPK belong to the superfamily of serine/threonine kinases. To date, at least seven MAPK members have been identified in mammalian cells. Three of them have been well studied: extracellular signal-regulated protein kinases (ERK), c-jun N-terminal kinases (JNK), and p38. For example, activation of JNK leads to the phosphorylation and activation of c-Jun and ATF2, which, in turn, activate c-Jun transcription through the TPA-responsive element (6). Activation of MAPK pathways appears to be not only to stimulate the transcriptional activities of AP-1 components, but also to increase the abundance of their proteins (7).

Colon cancer is the second leading cause of cancer death and the third most common cancer among both men and women in the United States. It has become one of most preventable types of cancer during the past two decades (8). During the last several decades, a large number of chemopreventive and chemotherapeutic agents used in cancer treatment have been discovered from natural products (9,10). Of the natural compounds, isothiocyanates (ITCs) in cruciferous vegetables, catechins in green tea, resveratrol in grape seeds and red wine, curcuminoids in turmeric, and procyanidins in various fruits and nuts have gained much attention as potential chemopreventive agents (Fig. 1). However, the chemopreventive mechanisms of these compounds, in particular their roles in signal transduction pathways, are not fully understood. In the current study, we established a new model system using the human colon cancer cell line HT-29 stably transfected with a AP-1–luciferase reporter gene to evaluate and compare the effects of several natural chemopreventive agents on this signaling pathway. We further examined the relationship between MAPK JNK activation and AP-1 reporter gene expression as well as cell viability induced by these chemopreventive compounds, which may offer insights into their potential chemopreventive effects. In this study, we found that ITCs and curcumin at low concentrations induced the expressions of AP-1 reporter gene as well as endogenous cyclin D1 protein but decreased their expressions at higher concentration, and that the phenolic compounds such as ECG and EGCG at higher concentration induced the expression of

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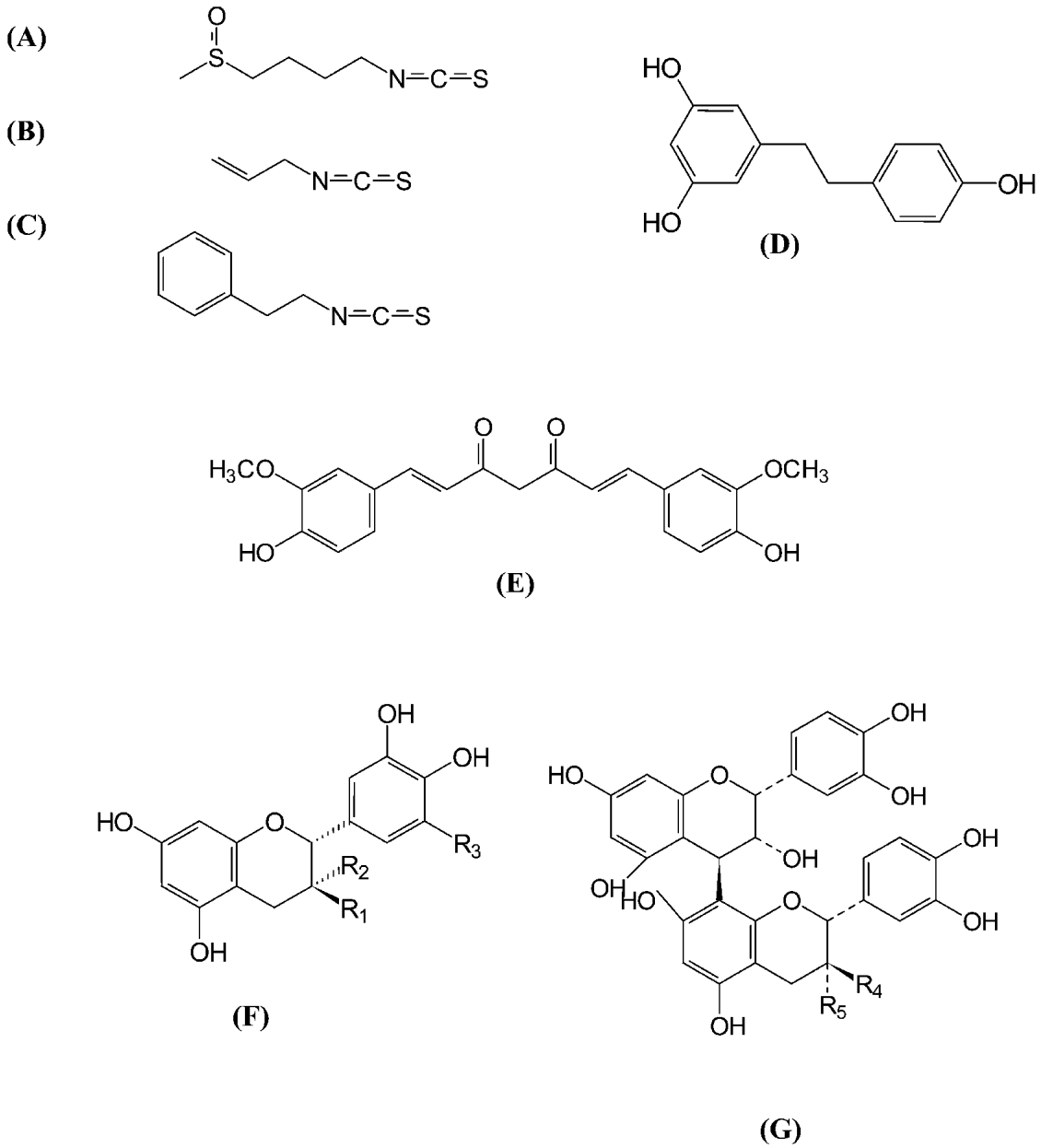

Fig. 1. Structures of various chemopreventive compounds used in this study. (A) sulforaphane, (B) allyl isothiocyanate, (C) phenylethyl isothiocyanate, (D) resveratrol, (E) curcumin, (F) (–)-epicatechin (R_1 = H, R_2 = OH, R_3 = H); (–)-epicatechin-3-*O*-gallate (R_1 = H, R_2 = *O*-galloyl, R_3 = H), (–)-epigallocatechin (R_1 = H, R_2 = OH, R_3 = OH), (–)-epigallocatechin 3-gallate (R_1 = H, R_2 = *O*-galloyl, R_3 = OH), and (G) procyanidin B1 (R_4 = OH, R_5 = H), procyanidin B2 (R_4 = H, R_5 = OH).

the AP-1 reporter gene and endogenous cyclin D1 protein whereas they had little effect at lower concentrations.

MATERIALS AND METHODS

Chemicals

Phenethyl isothiocyanate (PEITC), allyl isothiocyanate (AITC), sulforaphane (SUL), curcumin (CUR), resveratrol (RES), (–)-epicatechin (EC), (–)-epicatechin-3-gallate (ECG), (–) epigallocatechin (EGC), (–)-epigallocatechin-3-gallate (EGCG), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemicals Co. (St Louis, MO, USA).

Procyanidins B1 (PCB1) and B2 (PCB2) were obtained from Extrasynthese (Genay, France). All other chemicals were of analytical grade and were purchased from commercial sources.

Cell Culture and Stable Transfection

HT-29 human colon cancer cell line was obtained from American Type Culture Collections (Manassas, VA, USA) and maintained in Minimal Essential Medium supplemented with 10% fetal bovine serum and antibiotics at 37°C with 5%

CO_2. AP-1– luciferase construct containing five copies of AP-1 consensus binding site (–TGACTCA-) (2,11) and pcDNA3.1 neomycin plasmid were co-transfected into HT-29 cells by Lipofectamine 2000 (LF2000, Invitrogen Life Technology, Carlsbad, CA, USA), following the manufacturer's instructions, and stable clones were selected with 0.5 mg/ml of G418 sulfate (Invitrogen Life Technology). The AP-1–luciferase construct was kindly provided by Drs. Anning Lin (University of Chicago, Chicago, IL, USA) and Michael Karin (University of California, San Diego, CA, USA) (2,11).

AP-1 Reporter Gene Activity Assay

One of the HT-29 AP-1 clones (named HT-29 C4) was subcultured in 24-well plates at a density of 10^5cells/well, incubated for 24 h, and the cells were pretreated for 1 h with various chemopreventive agents at different concentrations. The cells were then challenged with a stimulator, TPA (100 nM), followed by 6 h incubation. Luciferase activity was assayed with a luciferase kit from Promega (Madison, WI, USA) by using a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany) as described by Chen *et al.* (12). The luciferase activity was normalized against protein amount and expressed as fold induction over the control cells. All reporter gene experiments were performed at least three times in triplicate.

MTS Assay for Cell Viability

HT-29 C4 cells were seeded in 24-well plates at a density of 10^5cells/well. After 24 h, the cells were treated with different doses of chemopreventive compound for 48 h. The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-arboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay was performed with CellTiter 96 Aqueous nonradioactive cell proliferation assay kit (Promega) by the manufacturer's instructions. After 2 h, the absorbance was measured at 490 nm with μQuant ELISA reader (BIO-TEK Instruments, Inc., Madison, WI, USA). The cell viability was determined by the percentage of treated over the control that was treated with DMSO (0.1%). The results from at least three separate experiments were analyzed with Student's *t* test for the statistically significant difference.

Western Blot Analysis of JNK and Cyclin D1

After treatments for 1 h with various chemopreventive compounds, HT-29 C4 cells were washed with ice-cold PBS and harvested with 200 μl of a lysis buffer (pH 7.4) containing 10 mM Tris-HCl, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium orthovandate, 2 mM iodoacetic acid, 5 mM $ZnCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton-X 100. Typically, an equal amount of protein (20–30 μg/lane) from each sample, as determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA), was separated using 10% mini SDS-polyacrylamide gel eletrophoresis (PAGE) or Criterion 4-15% Tris-HCl Precast gel (Bio-Rad Laboratories) and transferred onto polyvinylidene difluoride (PVDF) membrane. For detection of the phosphorylation of JNK and cyclin D1, molecules of interest, anti-phospho-JNK polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA) and cyclin D1 (M-20) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used, respectively. After hybridization with primary antibody, the membrane was then incubated with the secondary anti-rabbit antibody with horseradish peroxidase (Santa Cruz Biotechnology). Final detection was performed with enhanced chemiluminescence (ECL) Western blotting reagents (Amersham Biosciences, Buckinghamshire, England).

In vitro Kinase Assay of JNK

To compare the results obtained from Western blotting of anti-phospho-JNK as described above, *in vitro* JNK kinase assay was performed. After treatments for 1 h with various chemopreventive compounds, HT-29 C4 cell lysates were obtained using the same method as described above. Ten micrograms of total protein of each sample was incubated with 30 μl kinase assay buffer containing 10 μg of GST-c-Jun (amino 1-79) fusion protein, 2 μCi [γ-^{32}P] ATP, and 20 μM ATP for 30 min at 37°C. The product was resolved in 10% SDS-PAGE, and the phosphorylation of GST-c-Jun was visualized by autoradiography.

RESULTS

AP-1 Transcription Activation Effects

To investigate and compare the effects of various natural chemopreventive agents on the transcription activation of AP-1, we performed stable transfection of HT-29 human colon cancer cells with AP-1–luciferase reporter gene. TPA and hydrogen peroxide (H_2O_2) were used as positive controls, and treatments with both agents caused a significant increase in AP-1 reporter gene activity in a dose-dependent manner (Fig. 2). Figure 3 shows the kinetics of the activation of AP-1 activity by the isothiocyanates, 10 μM of PEITC or SUL, and the AP-1 luciferase activity was highest at 6 h after treatments, and this time point of 6-h treatments with inducers was chosen thereafter. The modulation of AP-1 activity by various doses of chemopreventive agents was also evaluated in the absence and presence of 100 nM TPA, and the results are shown in Figs. 4, 5, and 6. Using the AP-1-luciferase assay, three categories of compounds were selected based on their structural similarity: group A, isothiocyanates; group B, curcumin and resveratrol; and group C, tea catechins and procyanidins. Of the isothiocyanates (group A compounds), PEITC and SUL dose-dependently increased the AP-1–luciferase activity and decreased the activity at higher doses in the absence or presence of TPA, whereas AITC increased the AP-1-luciferase activity even at the highest dose tested (50 μM) (Fig. 4). PEITC at 50 μM almost completely abolished the AP-1–luciferase activity regardless of TPA cotreatment, and this was due to cytotoxicity as described later. At the dose range between 1 and 10 μM, AITC had little effect on AP-1 activtiy, but increased dramatically at 25 μM (20-fold without TPA and 32-fold with TPA), and the induction was the highest at dose 35 μM and above. In Fig. 5, the effects of CUR and RES on the AP-1-luciferase activity were compared, and the cyclin-dependent kinase inhibitor (olomoucine) (13) and MEK inhibitor (U0126) (14), which have been reported to inhibit AP-1 transcription activation, were used as positive

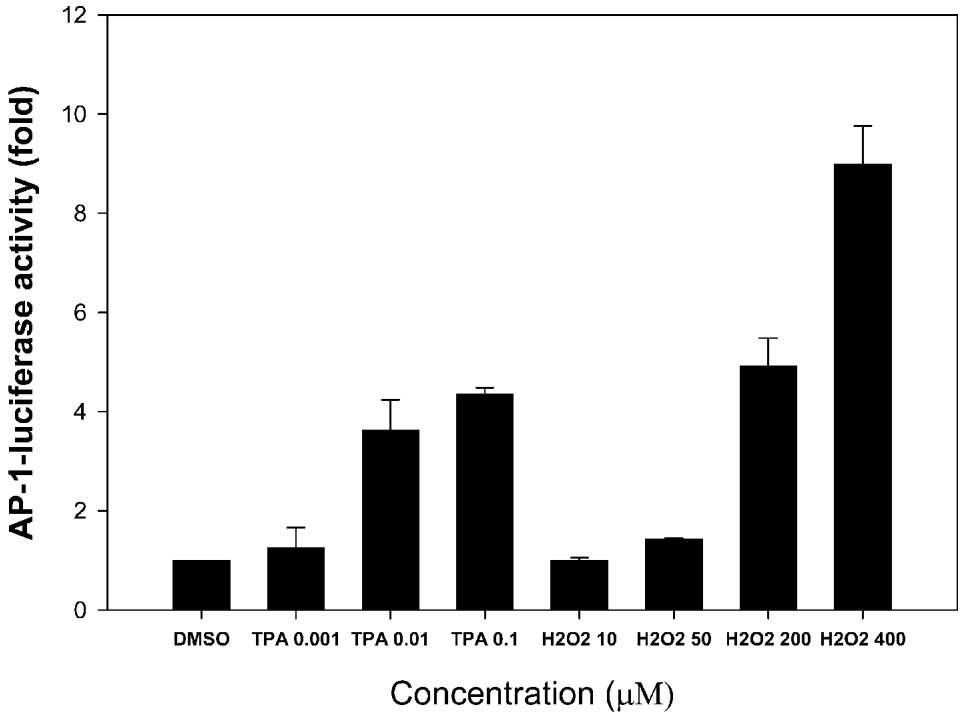

Fig. 2. Effects of TPA and hydrogen peroxide (H_2O_2) on AP-1 reporter gene activity in HT-29 C4 cells. Cells were treated with TPA or for 6 h at the indicated concentration. Values are representative results performed in triplicates with very similar results from three separate independent experiments.

controls. The modulatory effects of CUR and RES on the AP-1–luciferase activity were similar to those of PEITC and SUL. Treatments with CUR increased the AP-1 activity in a dose-dependent manner, and subsequently, AP-1 activity was dramatically attenuated at doses 35 μM and above (Fig. 5). As expected, Olomoucine at 200 μM and U0126 at 10 μM inhibited TPA-induced AP-1–luciferase activity (Fig. 5). Among the tea catechins and the dimers of catechins, procyanidins, EGCG dose-dependently induced the AP-1–luciferase activity, and ECG caused slight induction in the

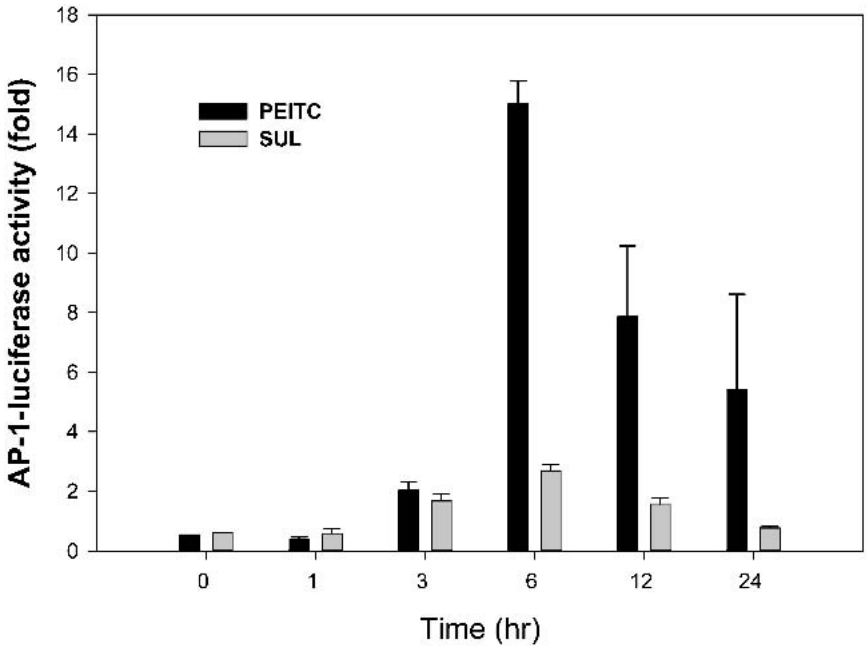

Fig. 3. Effects of 10 μM of PEITC and SUL on AP-1 reporter gene activity in HT-29 C4 cells. Cells were treated with 10 μM of PEITC or SUL for the indicated time periods. Values are representative results performed in triplicates with very similar results from three separate independent experiments.

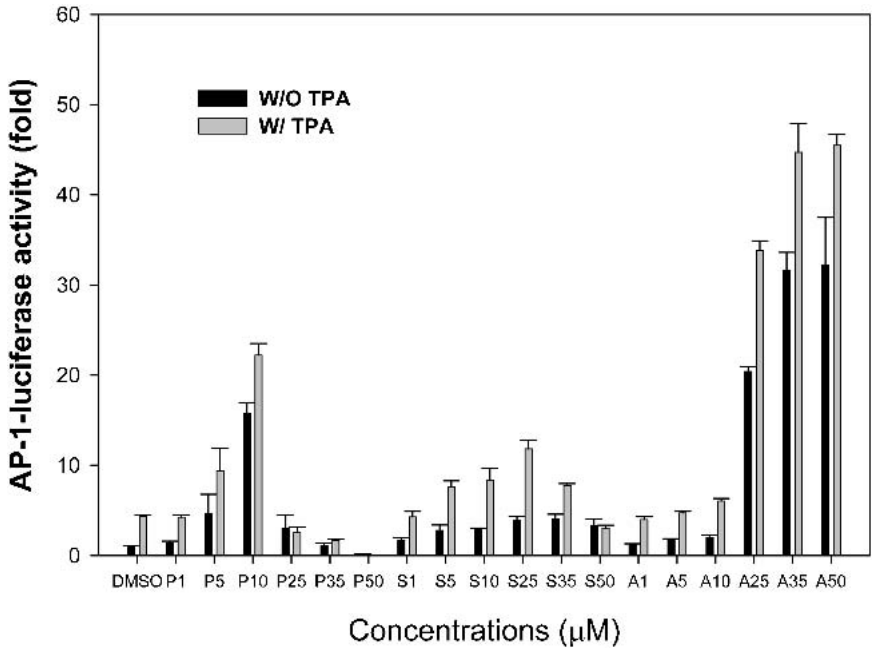

Fig. 4. Effects of various natural chemopreventive compounds on AP-1-reporter gene activity in HT-29 C4 cells in the absence and presence of TPA. Cells were treated with various group A compounds of isothiocyanates [P (phenethyl isothiocyanate), S (sulforaphane), A (allyl isothiocyanate)] at the indicated concentrations for 1 h, followed by 6 h treatment with TPA (100 nM) or DMSO (0.1%). Values are representative results performed in triplicate with very similar results from three separate independent experiments.

AP-1-luciferase activity with increasing doses (Fig. 6). EC and procyanidin dimers (PCB1 and PCB2), however, had little effect on AP-1–luciferase activity (even up to 100 and 50 μM, respectively). Taken together, in the absence of TPA, the highest induction of the AP-1–luciferase activity in HT-29 C4 cells was by AITC at the concentrations above 35 μM (35-fold), followed by CUR at 25 μM (21-fold), PEITC at 10 μM (10-fold), and EGCG at 100 μM (12-fold) (Figs. 4, 5, and 6).

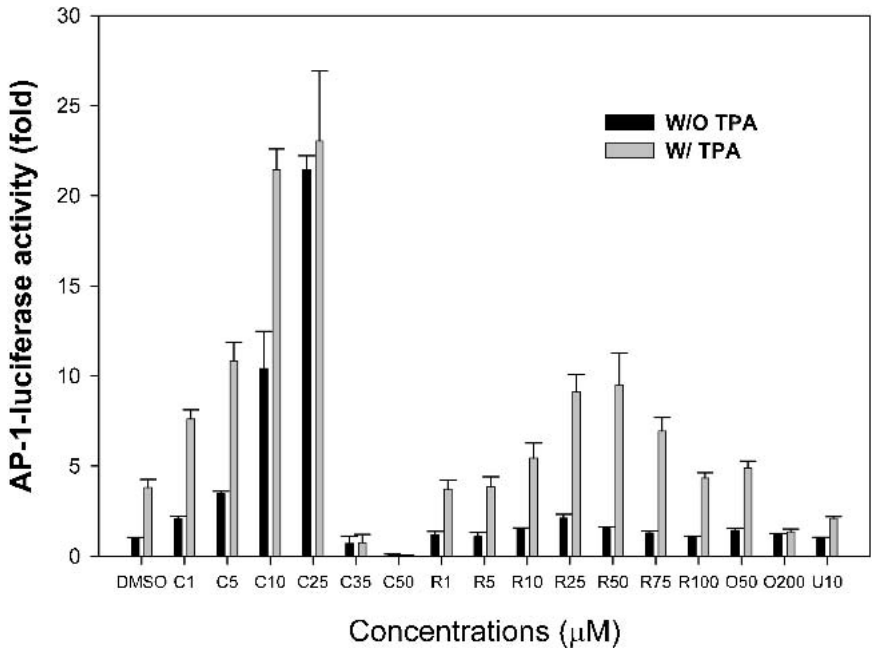

Fig. 5. Effects of various natural chemopreventive compounds on AP-1 reporter gene activity in HT-29 C4 cells in the absence and presence of TPA. Cells were treated with various group B compounds of curcumin and resveratrol [C (curcumin), R (resveratrol), O (olomoucine), U (U0126)] at the indicated concentrations for 1 h, followed by 6 h treatment with TPA (100 nM) or DMSO (0.1%). Values are representative results performed in triplicate with very similar results from three separate independent experiments.

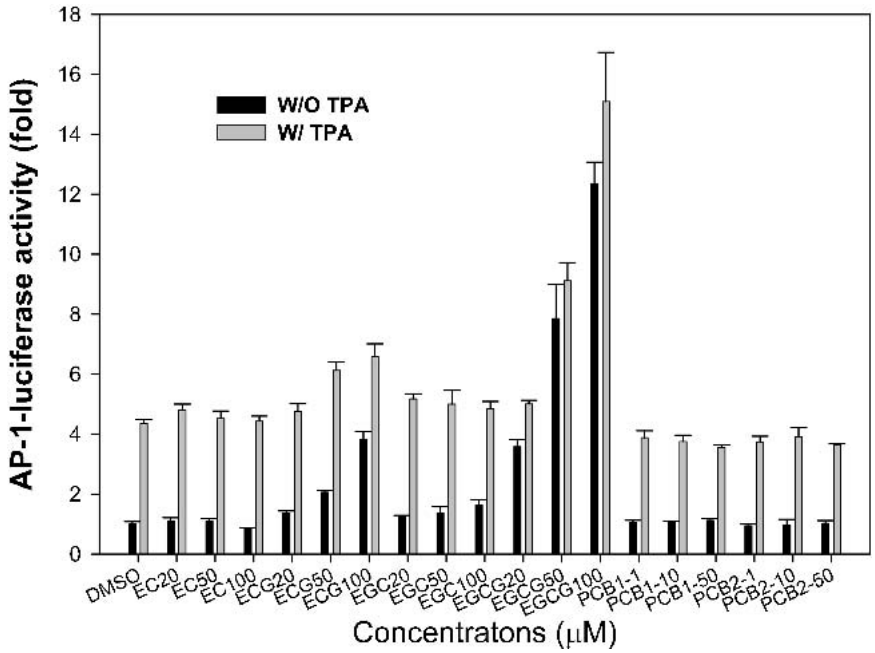

Fig. 6. Effects of various natural chemopreventive compounds on AP-1 reporter gene activity in HT-29 C4 cells in the absence and presence of TPA. Cells were treated with various group C compounds of tea catechins and procyanidins, at the indicated concentrations for 1 h, followed by 6 h treatment with TPA (100 nM) or DMSO (0.1%). Values are representative results performed in triplicate with very similar results from three separate independent experiments.

These compounds displayed a similar pattern in the induction of AP-1–luciferase activity in the presence or absence of the AP-1 stimulator such as TPA. On the other hand, PEITC, SUL, and CUR inhibited the AP-1–luciferase activity only at the higher doses. These results suggest that the modulation of AP-1 transcription activation by chemopreventive agents may be highly dose-dependent and compound specific.

Cell Viability Effects

To investigate whether the AP-1–luciferase activity modulated by some of these compounds was related to cell viability or cell death, we measured the cell viability of HT-29 C4 using the MTS assay, and the results are shown in Table I. The concentrations of the chemopreventive agents studied

Table I. Effects of Various Chemopreventive Compounds on Cell Viability of HT-29 Human Colon Cancer Cells Transfected with AP-1 (HT-29C4)

	Cell viability (%) Concentration (μM)				
	1	10	25	50	100
PEITC	97.7 ± 2.1†	72.6 ± 5.8‡	16.7 ± 3.3‡	13.1 ± 13.1‡	—§
SUL	96.4 ± 1.8	74.9 ± 5.4‡	57.4 ± 11.2‡	15.3 ± 8.8‡	—
AITC	98.4 ± 6.0	—	92.5 ± 3.2	76.7 ± 6.6‡	33.0 ± 2.7‡
CUR	90.4 ± 2.9†	66.1 ± 13.2‡	47.0 ± 7.6‡	14.2 ± 8.8‡	—
RES	101.9 ± 2.5	—	97.9 ± 9.9	87.9 ± 4.5‡	35.4 ± 11.8‡
EC	101.8 ± 16.5	—	102.2 ± 14.6	96.3 ± 12.5	95.5 ± 6.9
ECG	97.6 ± 9.3	—	92.9 ± 6.3	62.0 ± 4.7‡	60.0 ± 1.9‡
EGC	99.8 ± 8.2	—	99.4 ± 10.8	95.0 ± 12.9†	85.8 ± 8.6‡
EGCG	103.4 ± 13.8	—	90.3 ± 2.0‡	66.5 ± 2.5‡	56.4 ± 1.0‡
PCB1	95.6 ± 5.8	105.2 ± 4.0	—	99.9 ± 5.5	98.8 ± 19.2
PCB2	100.5 ± 11.7	98.4 ± 12.8	—	99.4 ± 6.3	98.9 ± 14.3

PEITC, phenethyl isothiocyanate; SUL, sulforaphane; AITC, allyl isothiocyanate; CUR, curcumin; RES, resveratrol; EC, (−)-epicatechin; ECG, (−)-epicatechin-3-gallate; EGC, (−)-epigallocatechin; EGCB, (−)-epigallocatechin-3-gallate; PCB1, procyanidin B1; PCB2, procyanidin B2.
* The values are expressed as means ± SD (n = 4).
† $p < 0.05$, student's *t* test.
‡ $p < 0.01$, student's *t* test.
§ Not determined.

ranged from 1 μM to 100 μM. Of the chemopreventive agents, PEITC, SUL, and CUR exhibited the most potent inhibitory activity against cell viability. The treatment with PEITC caused a dramatic decrease in the cell viability at the concentrations of 25 μM and above. SUL reduced the cell viability to 15% at 50 μM; however, AITC demonstrated relatively weak or little effect on the viability of cells even at 50 μM concentration, although it inhibited the cell viability by 33% at the highest dose of 100 μM. CUR also strongly inhibited the cell viability in a dose-dependent manner. The effects of RES on cell viability were similar to that of AITC. RES had little inhibition on cell viability up to 50 μM but resulted in 35% inhibition at 100 μM. Among the tea catechins, ECG and EGCG showed strong inhibitions on the cell viability in a dose-dependent manner whereas EC and EGC had relatively little effect even at 100 μM. At 100 μM, EGCG reduced more cell viability (56.4%) as compared to ECG (60%). PCB1 and PCB2 were not effective in the inhibition under the current conditions.

Activation of JNK Activity

To investigate whether the increased AP-1–luciferase activity by some of these compounds could be correlated with JNK activity, we examined the phosphorylation of JNK1/2 using immunoblot analysis of HT-29 C4 cells. We tested and compared the effects of the 11 compounds on the phosphorylation of JNK1/2, and the result are shown in Fig. 7A. In the control cells, the 54-kDa isoforms of phospho-JNK1/2 were not detected at the basal level, and only a small amount of 46-kDa isoforms of phospho-JNK1/2 was present. However, both the 54-and 46-kDa forms of phospho-JNK1/2 were induced upon 1-h treatments with 100 nM TPA, 50 μM AITC, and 100 μM EGCG. Interestingly, 50 μM PEITC or SUL strongly increased the 46-kDa isoforms of phospho-JNK, but weakly increased the phosphorylation of the 54-kDa isoform. The effects of the chemopreventive compounds on the protein level of JNK in HT-29 C4 cells are shown in Fig. 7B, and most compounds showed little effects, except with the treatment of CUR, which showed slight decrease in the expression of JNK. In order to corroborate the effects of these compounds on phospho-JNK, we performed *in vitro* kinase assay on the phosphorylation of c-Jun using GST-1-79 c-Jun as a substrate, and the results are presented in Fig 7C. There were significant phosphorylation of c-Jun by TPA, AITC, and EGCG, but surprisingly not with PEITC and SUL, even though these two compounds activated p56-phospho-JNK as shown in Fig. 7A, which suggests that immunoblot using phospho-JNK antibody may not always correspond to the kinase activity of JNK, with the latter supposedly to be more representative of *in vivo* JNK kinase activity.

Cyclin D1 Expression Level

To examine the effects of natural chemopreventive compounds on AP-1–regulated gene, we analyzed the protein expression level of cyclin D1 in HT-29 C4 cells after treatments with the compounds for different times (6, 12, and 24 h). Cyclin D1 has been known to be an endogenous gene that is under the control of AP-1 and plays an important role in cell proliferation (15). As observed in the AP-1–luciferase assay, during 6 h, low concentrations (10 μM) of PEITC, SUL, CUR, and EGCG as well as high concentrations of AITC (50 μM) and EGCG (100 μM) elevated the endogenous cyclin D1 expression compared to vehicle-treated control, whereas high concentrations (50 μM) of PEITC and CUR dramatically reduced the protein level (Fig. 8). The AITC at 50 μM that displayed the highest induction of AP-1–luciferase activity among the tested compounds in 6 h also caused the

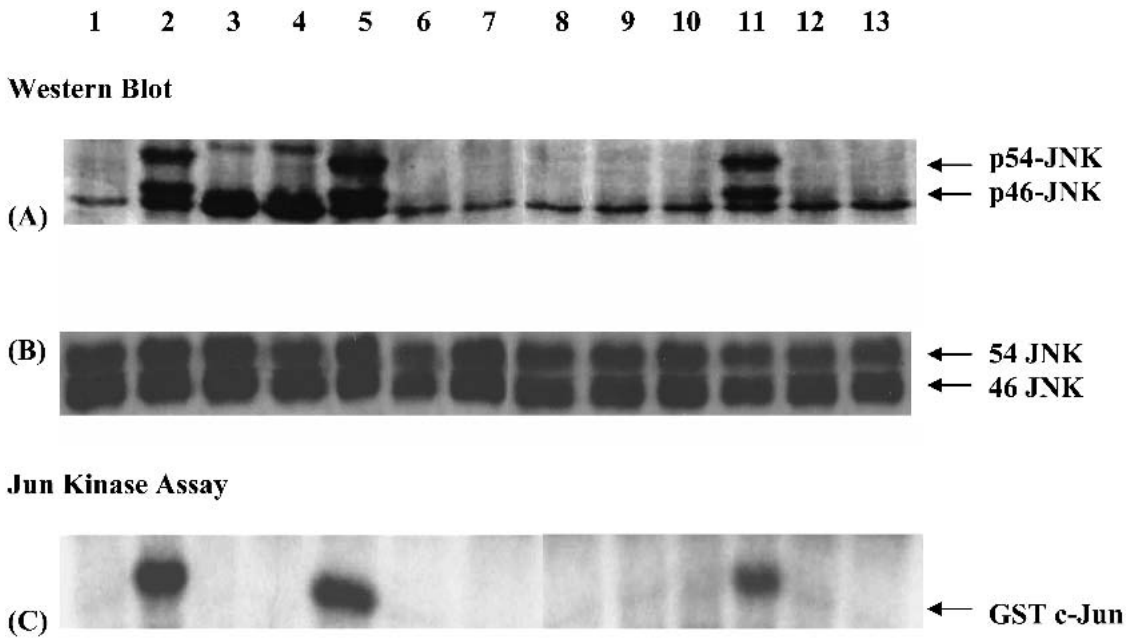

Fig. 7. Effects of various natural chemopreventive compounds on JNK activity in HT-29 C4 cells. (A) Western blot of phosphate-JNK, (B) Western blot of JNK, (C) JNK kinase assay using purified GST c-Jun (1: control; 2: 100nM TPA; 3: 50 μM PEITC; 4: 50 μM SUL; 5: 50 μM AITC; 6: 50 μM CUR; 7: 50 μM RES; 8: 100 μM EC; 9: 100 μM ECG; 10: 100 μM EGC; 11: 100 μM EGCG; 12: 50 μM PCB1; 13: 50 μM PCB2). Data are representative results from three independent experiments with very similar results.

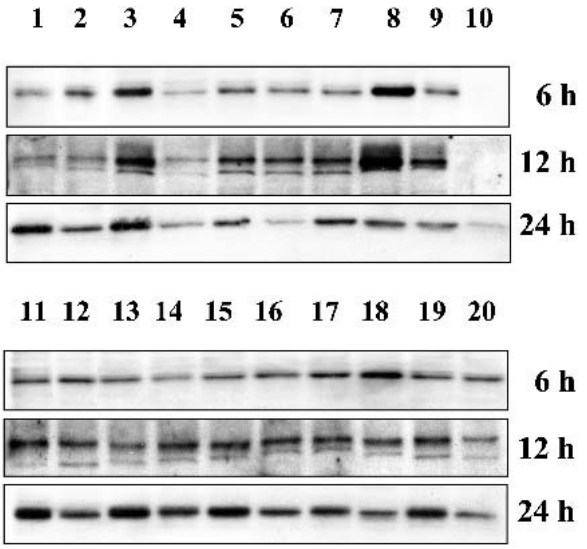

Fig. 8. Effects of various natural chemopreventive compounds on cyclin D1 protein level in HT-29 C4 cells [1 and 11: control; 2 and 12: 10 nM TPA; 3: 10 μM PEITC; 4: 50 μM PEITC (20 μM for 24 h); 5: 10 μM SUL; 6: 50 μM SUL; 7: 10 μM AITC; 8: 50 μM AITC; 9: 10 μM CUR; 10: 50 μM CUR (20 μM for 24 h); 13: 10 μM RES; 14: 100 μM RES; 15: 10 μM ECG; 16: 100 μM ECG; 17: 10 μM EGCG; 18: 100 μM EGCG; 19: 50 μM olomoucine; 20: 200 μM olomoucine]. Data are representative results from three independent experiments with very similar results.

strongest increase in the cyclin D1 protein level. The induction of cyclin D1 protein by low concentrations of PEITC, SUL, and CUR lasted up to 12 h. After 24 h, however, there was a tendency to decrease the cyclin D1 levels in most treatments.

DISCUSSION

Several studies have indicated that colon cancer death can be related to dietary factors (16,17). Recently, there is a considerable interest in using dietary prevention and chemoprevention to decrease the mortality of various cancers (18,19). However, the biological mechanisms of many chemopreventive compounds have not been fully elucidated. In the current study, 11 naturally occurring chemopreventive compounds from different categories of food and edible plant sources were investigated, and comparison was made for their potential chemopreventive biomarkers including cell viability, AP-1-repoter gene activity, and JNK activation in a human colon adenocarcinoma HT-29 cell model. Because the gastrointestinal tract is directly exposed to many dietary components, colon cells may be one of the better organs to be used for the study of the effects of various natural chemopreventive compounds. The effects of various chemopreventive compounds on AP-1 and their biological consequences are quite different with different experimental models or tested compounds, and the underlying reason is not fully understood. We, therefore, established a new model in HT-29 cells, which could potentially be used for high throughput screening of natural chemopreventive compounds based on the AP-1–luciferase reporter activity in these HT-29 C4 cells.

Many isothiocyanates are effective chemopreventive agents against carcinogen-induced cancers in experimental animals (20). The AP-1 binding activity was markedly increased in lung tissue of A/J mice when they were fed with 15 μmol/g of *N*-acetyl-cysteine (NAC) conjugates of benzyl isothiocyanate (BITC) or PEITC (21), and our current results with similar doses (10 μM) (Fig. 4) appear to support these previous observations. Isothiocyanates are produced from glucosinolates by myrosinase when cruciferous vegetables were damaged during processing or by intestinal bacteria during digestion (22), which would potentially make the isothiocyanates directly accessible to the intestinal epithelial cells. Patten and DeLong (23) previously reported on the activation of AP-1 by 25 μM of BITC in HT 29 cells, which is similarly seen in our current study with AITC (Fig. 4). Unlike AITC, however, our study reveals that PEITC and SUL inhibit AP-1–luciferase activity at high doses, suggesting that modulation of AP-1 transcription activation in HT-29 cells by these compounds are highly dose-dependent.

CUR has been shown to suppress the activation of phorbol ester TPA-induced AP-1 in HL-60 cells (1 to 10 μM), and TPA-induced levels of AP-1 in Raji DR-LUC cells (15 μM) (24–26). However, there has been no information on the effects of CUR on AP-1 in colon cancer models such as the HT-29 cells. Here, we show significant induction of AP-1–luciferase activity by CUR itself, as well as synergistic effect rather than inhibitory effect of TPA-induced AP-1–luciferase activity at the concentration range between 1 and 25 μM (Fig. 5) and dramatic decrease in the activity at the doses 35 μM and above. The reason for the difference at lower doses is not clear, but could be due to possible involvement of other mechanisms as well as differences in signaling event between these cell types/lines, and further side-by-side studies would be needed. A recent study has reported that curcumin at 10 μM increases AP-1 and EpRE binding activity up to 3 h in HBE1 cells as well as intracellular glutathione (GSH) content and glutamate-cysteine ligase (GCL), an enzyme for glutathione synthesis (27). In this study, curcumin has also been found to change the nuclear content and/or activation of transcription factors such as Mafs, Nrf2, JunD, and phosphorylated c-Jun. Previously, we have reported that pretreatment of HeLa cells with RES (50 μM) inhibited the transcription of AP-1 reporter gene and activation of ERK2, JNK1, and p38 by UVC and TPA (28). Manna *et al.* (29) also reported that RES inhibited TNF-induced activation of AP-1 in U-937 human histiocytic lymphoma cells. In the current study using the HT-29 C4 cells, however, we did not see any substantial inhibition in AP-1–luciferase activity after treatments with RES at doses 1–50 μM; rather, the AP-1–luciferase activity dose-dependently increased (1 to 50 μM) and decreased at higher doses (75 and 100 μM) (Fig. 5), as seen previously in HeLa cells (28), suggesting that different cell types respond differently.

In a human keratinocyte cell line, HCL14, which contains signature UVB mutations in both p53 alleles, EGCG at 54.5 μM has been shown to inhibit UVB-induced AP-1 transactivation nearly to basal level (30). In normal human keratinocytes, however, EGCG markedly increases AP-1 factor-associated responses through a MAPK signaling mechanism, which is similar to our results (Fig. 6), suggesting that the signaling mechanism of EGCG action could markedly be different between different cell types (31). Currently, there is no report on the effects of procyanidins on AP-1 activity, and here we showed that procyanidines B1 and B2 have no effect on AP-1 under these conditions; hence, it appears that the

potential chemopreventive effects of procyanidines may not be using the AP-1 signaling pathway. However, more studies would be needed in other cell lines/types to understand fully the mechanism of actions of procyanidines.

AP-1 heterodimers are constitutively localized within the nuclei (32). Many kinds of stimuli activate the MAPK cascades that enhance AP-1 activity, leading to proliferation as well as opposite antiproliferation depending on the substrates that are phosphorylated (33). We investigated the effects of these chemopreventive compounds on cell viability of HT-29 C4 cells after 48 h treatments. PEITC, SUL, and CUR, which displayed strong inhibition on AP-1–luciferase activity at 50 μM, exhibit more potent inhibitory effect on cell viability of HT-29 C4, as compared to the rest of the compounds tested at 50 μM (Table I). Previously, in other human cancer cell line studies, the inhibition of cell viability or cell growth by isothiocyanates have been demonstrated in HT-29 colon cancer cells (AITC and SUL) (34,35), leukemia cells (SUL) (36), and cervical HeLa cells (PEITC, BITC) (37). Our current study demonstrates that PEITC and SUL have more potent inhibitory activity on cell viability than AITC (Table I), suggesting that the allyl group may be less effective than other functional groups of isothiocyanate in modulation of AP-1 and inhibition of cell viability inhibition.

Evidence on the inhibitory activity of CUR against cancerous cell viability has previously been reported. CUR has been shown to inhibit cell viability of various cancer cells such as ovarian (38), oral squamous (39), pancreatic (40), androgen-independent and -dependent prostate (41), leukemia (42), colon, kidney, and hepatic cancer cells (43). Liang *et al.* (44) have shown that RES inhibited cell viability in HT-29 cells. Their result showed that RES treatment at 100 μM for 48 h decreased cell viability to about 30%, which is similar to our finding (Table I). When comparing CUR and RES, both have phenolic groups on the two benzene rings; CUR displays much stronger inhibition on cell viability than RES (Table I). The presence of the diketone moiety in CUR has been implicated to be essential for its inhibitory activity against cell proliferation of MCF-7 cells as well as its antioxidant and pro-oxidant activities (45,46).

The effects of tea catechins on cell viability of cancerous cells have widely been studied. EGCG and EGC have been reported to inhibit the cell viability of HCT 116 colorectal carcinoma cells with IC_{50} value of about 46 μM, and in HepG2 hepatocellular carcinoma cells, the IC_{50} value of more than 100 μM after 3 days treatment was reported (47). At the same condition, EC required much higher IC_{50} values for both cell lines. Another comparison study on the inhibitory effects of tea catechins in human cancer cell growth have also demonstrated different inhibitory activities of tea catechins depending on the type of cell lines (48). HT-29 colon cancer cells were more resistant to the tea catechins than lung cancer cells (H661 and H1299), and EC was least effective in the cell viability inhibition as compared to EGC, ECG, and EGCG. In the current study, among the tea cetechins, EGCG and ECG show more induction of AP-1 activity and inhibition on cell viability than EC and EGC (Table I), suggesting that the gallate group at the C-3 position of C ring in the catechin structure may be responsible for the activities in these effects. The galloyl moiety of tea catechins has been appeared to be required for both the antioxidant and the antiproliferative effects in Caco-2 cells (49).

Studies on the effect of procyanidins on cancer cell viability are few. Carnesecchi *et al.* (50) reported that procyanidin-enriched extract (flavanol and procyanidin content: 941 mg/g) from cocoa powder at 50 μg/ml inhibited the growth of Caco-2 cells by 75% after 10 days' incubation. In the current study, however, we did not find any effect of PCB1 and PCB2 on the inhibition of cell viability at the given condition (Table I). In the previous study, the experiment was performed with a mixture of various compounds rather than a pure compound, and the extracts were replaced every 24 h. Consequently, the effects of natural chemopreventive compounds on cell viability may vary with the type of cells and other conditions, such as concentrations and duration of treatments.

Activation of JNKs will lead to translocation of JNKs to the nucleus, where phosphorylation of transcription factors such as c-Jun occurs and thereby enhances its transcriptional activity (6,50). At least three genes, JNK1, JNK2, and JNK3, encoding the proteins JNK1, JNK2, and JNK3 are expressed in the brain, testis, and heart (51). As all of them are able to form both 46 and 54 kDa proteins, a total of 12 different splicing forms will be present (52), although the significance of this heterogenecity is largely unknown (53). From our Western blotting analysis, 50 μM PEITC and SUL strongly activated the 46-kDa isoforms of phospho-JNKs whereas TPA, 50 μM AITC, and 100 μM EGCG, showed activation of both 46- and 56-kDa enzymes (Fig. 7A). Because the AP-1–luciferase activity in the current study was greatly induced by AITC and EGCG at the same concentrations (50 and 100 μM, respectively), the transcription activation of AP-1 might be closely related to the activation of the 54-kDa isoforms of JNK. All other compounds do not seem to have any activation of JNKs at the given condition. Isothiocyanates such as PEITC and AITC activate JNK in human leukemia HL cells, Jurkat T-cells, HeLa, and human embryonic kidney 293 cells (54,55), however, little is known about the effect of isothiocynates on JNK in colon cancer models including HT-29 cells. We have previously shown that PEITC could induce persistent JNK activation and apoptosis in various cell types including HeLa cells and Jurkat cells (56,57). Interestingly, JNK was not activated by PEITC treatment in PC-3 human prostate carcinoma cells possibly because of the lack of p53 in the cells (58).

CUR inhibits JNK activation in human breast cancer cell lines (59), prostate cancer cells (41), and Jurkat T cells (57) in a dose-dependent manner, although the effect of CUR in HT-29 human colon cancer cell line on JNK activity has been not reported. The effect of RES on JNK activity has been varied. It was reported that RES did not alter the phosphorylation of p38 and JNK in the SK-mel28 and A375 human melanoma cells (60), although cotreatments of the cells with 1 μM paclitaxel and 50 μM *trans*-RES were effective in reducing both early and late JNK/SAPK activation in SH-SY5Y human neuroblastoma cells (61). In our current study, RES is effective on the inhibition of cell viability at 50 μM and above, but shows no significant inhibition on JNK or AP-1 activity at the given conditions, suggesting that other signaling mechanism(s) may be involved.

EGCG at 25 to 250 μM activates MAPKs (ERK, JNK, and p38) in a dose- and time-dependent manner, which is similar to our results, whereas EGC activated ERK and p38 (12). Studies on the effect of procyanidins on JNK activity are

little. Tyagi *et al.* (62) reported that grape seed extract, a dietary supplement rich in flavonoid procyanidins, has a strong and sustained increase in phospho-JNK1/2 levels, JNK activity, and phospho-c-Jun levels in human prostate carcinoma DU145 cells. We, however, do not see a significant change on JNK and AP-1 activity as well as cell viability by procyanidins in our system.

Our current results show that the compounds that induce AP-1 activity also activate JNK, with the exception of CUR (Fig. 7B). The mechanism of action of CUR could involve other signal transduction pathways such as NF-κB in HT-29 colon cancer cell, and this remains to be elucidated. Interestingly, in our JNK kinase assay, AITC and ECGC, which activate the 54-kDa isoforms of phospho-JNK, strongly activated c-Jun phophorylation (Fig. 7C). It has been suggested that p54 JNKs are more strongly phosphrylated than are the p46 JNKs, which may be compatible with the study of Dreskin *et al.* (63).

The Western blot analysis of endogenous cyclin D1 in the current study shows that the modulation of the cyclin D1 expression by natural chemopreventive compounds is also dose- and time-dependent and is well correlated with the AP-1–luciferase activity induced by these compounds. At low concentration (10 μM), treatment with PEITC, SUL, and CUR activated cyclin D1 at 6 h, but at high concentration (50 μM) these compounds decreased the protein expression. It can be postulated that high doses of these compounds are strong inducers of cancer cell death through, at least in part, inhibition of AP-1 and cyclin D1 pathway. Higher concentrations of chemopreventive compounds such as PEITC and BHA have been suggested to activate not only the MAPK pathway, but also the caspase pathway that will lead to apoptotic cell death (64). However, the chemopreventive mechanisms of these compounds at low concentrations and other compounds including AITC and EGCG at higher concentrations appear to be more complex. Although low concentrations of PEITC and CUR and higher concentrations of AITC and EGCG induced AP-1–luciferase activity and endogenous cyclin D1 expression in early time courses (6 and 12 h), these inductions decreased after longer treatment (24 h) as seen in cyclin D1 data (Fig. 8). Despite the involvement of survival mechanisms in early time course, suggested by AP-1 activity, a constant exposure to these compounds may result in cell death. The reason for this discrepancy is currently not clear, but other signaling mechanisms may be involved. In addition, the activation of AP-1 by these natural compounds could also lead to the initiation of chemoprevention pathway or cell death. On the other hand, it has been suggested that at low concentrations of some of these chemopreventive agents such as PEITC, BHA, and CUR, they may induce the phase II detoxifying genes including GST and QR through activating MAPKs pathway or changing the nuclear content of transcription factors involved (27,64). Our recent unpublished data also show a strong induction of p21 by some natural compounds while they also induce cyclin D1 (manuscript in preparation).

In summary, cellular signal transduction phenomena that are induced by many chemopreventive agents may be dependent on the kind of the agents, concentrations of the agent, and the length of treatment period, as well as the cell types. In this study, we have demonstrated that the HT-29 C4 cells with stably expressed AP-1–luciferase reporter gene could potentially be a useful model system for studying signal transduction pathways elicited by natural compounds that have chemopreventive effects in colon cancer. Using this model system, we examined various natural compounds that possess chemopreventive activities; their modulatory properties of signal transduction pathways potentially leading to cancer protection as well as cell growth inhibition in colon cancer cells. However, due to the highly complex mechanisms of signal transduction pathways in cancer, with various cross-talks between the pathways, blocking or inducing a single signaling pathway may not account for the overall chemoprevention action elicited by natural compounds. In the current study, we showed that, at low concentrations, PEITC, SUL, and CUR induced not only the expression of AP-1–luciferase reporter gene but also that of endogenous cyclin D1 protein and decreased these expressions at higher concentration. On the other hand, AITC and phenolic compounds such as ECG and EGCG at higher concentration induced the expressions of the AP-1 reporter gene and endogenous cyclin D1 protein, but they had little effects at low concentrations. The activation of AP-1 has been believed to play a positive role in cell proliferation. Here, we show for the first time, however, that the activation of AP-1 by potential natural compounds at certain concentrations could also lead to the initiation of chemopreventive/chemotherapeutic mechanisms or cancer cell death. The studies of AP-1 and JNK as well as cyclin D1 expression as described in our study here, together with other signaling pathways in the future, would begin to address the overall biological mechanisms of these naturally occurring cancer chemopreventive compounds.

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