

# New Role of (–)-Epicatechin in Enhancing the Induction of Growth Inhibition and Apoptosis in Human Lung Cancer Cells by Curcumin

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## Abstract

Curcumin, a phenolic compound isolated from the plant *Curcuma longa* (Linn), is ingested every day in the Indian subcontinent and is well reported to possess cancer-preventive activity. To achieve effective cancer prevention with curcumin, we need to find a new method to enhance the effects of curcumin in the diet. Based on our evidence that (–)-epicatechin (EC), an inert catechin, enhances the cancer-preventive activity of green tea catechins, we studied the enhancing effects of EC on inductions of growth inhibition and apoptosis in human lung cancer cell lines PC-9 and A549 with curcumin. The combination of curcumin with EC significantly increased the inhibition of cell growth compared with curcumin or EC alone. The combination similarly increased both apoptosis and expression of *GADD153* and *GADD45* genes, associated with their enhanced protein production. Knockdown of *GADD153* or *GADD45* by small interfering RNA abrogated the apoptosis induction and growth inhibition induced by the combination, indicating the crucial role of their upregulation. Treatments of PC-9 cells with c-Jun-NH<sub>2</sub>-kinase inhibitor SP600125, with p38 mitogen-activated protein kinase inhibitor SB202190 and with PD98059 (extracellular signal-regulated kinase 1/2 inhibitor) all increased the upregulation of *GADD153* and *GADD45* genes by the combination. Because EC was previously shown to enhance the incorporation of EGCG into PC-9 cells, we think that EC has similar effects on curcumin. This report is the first report on the enhancing effects of EC on curcumin, and the data suggest that EC plays a significant role in the enhancement of the cancer-preventive activity of curcumin in the diet. *Cancer Prev Res*; 3(8); 953–62. ©2010 AACR.

## Introduction

Lung cancer is the leading cause of cancer-related death in the world, with 1.3 million deaths per year (1–3). In an effort to combat this, lung cancer prevention trials were conducted in some countries but were not successful for various reasons (4). Lung cancer is also one of the main causes of death in the Indian subcontinent (3), with the mortality rate is first in Bangladesh for males. In those areas, people ingest turmeric containing curcumin in every day meals; curcumin is a phenolic compound present in the plant *Curcuma longa* (Linn) consisting up to 2% to 5% of total spices in turmeric. Because curcumin is traditionally well known to have therapeutic effects on various types of diseases, the cancer-preventive activity of curcumin has been intensively studied all over the world and

is indicated effective on various types of cancer in animal experiments (5). We also confirmed that curcumin inhibited the growth of human lung cancer cell lines PC-9 and A549, and induction of apoptosis (6). However, the low bioavailability of curcumin might be a drawback for human prevention study with curcumin. Based on our successful prevention study on the recurrence of colorectal adenomas in Japanese patients as a result of daily consumption of green tea beverage supplemented with tablets of green tea extract containing (–)-epicatechin (EC; ref. 7), we think that the cancer-preventive activity of curcumin could be enhanced using some components of the diet in daily consumption. To achieve this, we first looked at the effects of EC, which is an inert tea catechin (8), without a galloyl group, in the above-mentioned cancer-preventive activities. The combination of curcumin with EC enhanced the growth inhibition of human lung cancer cell line PC-9 and induction of apoptosis.

In the light of the significant enhancing effects of EC on the apoptosis induction of curcumin, we studied the mechanism of enhancement with the combination, focusing on expression of *GADD153*, *GADD45*, and *p21* genes determined by quantitative real-time PCR, compared with those of a single agent alone. In this report, we discuss the results with signal transduction using kinase inhibitors. Although the combination effects of curcumin with EC are anticipated

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in animal experiments, this is the first report that an inert green tea catechin enhances the activity of curcumin.

## Materials and Methods

### Cell lines and chemicals

Human non-small cell lung carcinoma cell line PC-9 was obtained from the National Cancer Center Research Institute, Tokyo, Japan, and A549 was purchased from the American Type Culture Collection. Cells were cultured in RPMI 1640 (Nissui Pharmaceutical Co.) supplemented with 10% fetal bovine serum in 5% CO<sub>2</sub> incubator at 37 °C. Curcumin, EC, MTT, SP600125 [c-Jun-NH<sub>2</sub>-kinase (JNK) inhibitor], SB202190 [inhibitor of p38 mitogen-activated protein kinase (MAPK)], and PD98059 [inhibitor of extracellular signal-regulated kinase (ERK1/2)] were purchased from Sigma. Antibodies of GADD153, GADD45, and β-actin were purchased from Santa Cruz Biotechnology.

### Determination of cell growth inhibition

PC-9 cells were treated with curcumin, with EC alone, and with their combination for 72 hours. The cell viability was measured by trypan blue dye exclusion test (8). The growth inhibition in A549 cells were also measured by MTT assay (9). Briefly, cells (5 × 10<sup>4</sup>/mL) in 96-well plate were treated with various concentrations of curcumin, with EC, and with their combination for 72 hours. After incubation, the cells were washed with fresh medium, treated with MTT solution, and incubated for an additional 3 hours. The formazan crystal was dissolved in 100 μL SDS solution, and the absorbance was measured at 570 nm using a microplate reader (SPECTRAMax 340PC, Molecular devices). The results are based on at least three independent experiments.

### Determination of apoptosis

The proportion of apoptotic cells was determined by 4',6-diamidino-2-phenylindole (DAPI) staining, as reported previously (10). In brief, PC-9 cells were treated with various concentrations of curcumin, EC, and with their combination for 72 hours. Cells were fixed in ice-cold methanol for 10 minutes and then stained with the DAPI reagent (1 μg/mL). The percent of apoptotic cells was calculated by fluorescence microscopy (Bioevo BZ900, Keyence) from at least 200 randomly selected cells. Apoptotic cells were recognized by condensed, fragmented, or degraded nuclei, and the results are based on at least three independent experiments.

### Determination of intracellular curcumin

The intracellular curcumin level was determined by spectrophotometric method (11). Briefly, cells were treated with curcumin, with EC, or with their combination. Cells were collected after 1 hour, and curcumin was removed by washing with cold HBSS. The cells were resuspended in cold lysis buffer (0.1% Triton X-100 and 0.1% NP40 in HBSS), and disrupted by sonication and centri-

fuged for 10 minutes at 14,000 rpm. The supernatant fractions were diluted with HBSS, and curcumin level in the fractions was determined by measuring the absorbance at 427 nm. The results were confirmed by two independent experiments done in triplicate.

### Gene expression determined by quantitative real-time PCR

Cells were treated with curcumin, with EC, and with their combination for 24 hours. Total RNA was isolated by RNAiso reagent (TaKaRa) and then subjected to reverse-transcription, as previously described (12). Expression levels of *GADD153*, *GADD45*, and *p21* genes were quantitatively determined by real-time PCR using the 7300 Real Time PCR System (Applied Biosystems), with SYBR Green Real-time PCR Master Mix (TOYOBO CO). The critical threshold was determined according to the manufacturer's instruction. The relative abundance of the above-mentioned mRNA was normalized against *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) mRNA for quantitative evaluation. The sequence of the primers used was as follows: *GADD153* sense primer: 5'-AGAACCAGGAAACGGAAACAGA-3'; antisense primer: 5'-TCTCCTTCATGCGCTGCTTT-3'; *GADD45* sense primer: 5'-GCCTGTGAGTGAGTGCAGAA-3'; antisense primer: 5'-CCCCACCTTATCCATC-CITT-3'; *p21* sense primer: 5'-TGGAGACTCTCAGGGTCGAAA-3'; antisense primer: 5'-GGCGTTTGGAGTGGTAGAAATC-3'; *GAPDH* sense primer: 5'-TGGTATCGT-GGAAGGACTCATGAC-3'; and antisense primer: 5'-ATGCCAGTGAGCTTCCCGTTC-AGC-3'. The results were confirmed by at least two independent experiments.

### Transfection with small interfering RNA

The small interfering RNA (siRNA) duplexes corresponding to *GADD153* and *GADD45* genes were manufactured by Invitrogen. The siRNA-targeting *GADD153* gene corresponds to the sequence GAGAAUGAACGGCU-CAAGCAGGAAA, and the siRNA-targeting *GADD45* gene corresponds to the sequence GAGCAGAAGACCGAAAG-GAUG-GAUA. A Blast search against the human reference mRNA sequences confirmed that the sequences were not targeted to any other gene transcripts. Stealth RNAi negative control duplex (Invitrogen) was used as a control. PC-9 cells were incubated in antibiotic-free RPMI. The siRNA duplexes were added to the solution after 24 hours in the presence of Lipofectamine RNAi max (Invitrogen), according to the manufacturer's instruction, and the final concentration of siRNA was 10 nmol/L. Cells were treated with vehicle or a combination of 20 μmol/L curcumin and 200 μmol/L EC, 24 hours after transfection, and then cells were collected for quantitative real-time PCR, Western blotting, DAPI staining for apoptosis assay, and trypan blue dye exclusion test after another 24 hours. The experiments were repeated at least twice.

### Western blotting

Cells were treated with curcumin, with EC, and with their combination for 24 hours. After incubation, cells

were washed with ice-cold PBS and directly lysed in SDS sample buffer (10). Proteins were separated by SDS-PAGE, blotted, probed with specific antibodies, and visualized using commercial chemiluminescent detection kit (Pierce Biotechnology). The results were confirmed by at least two independent experiments.

### Statistical analysis

Statistical analyses were conducted by Student's *t* test. The results were considered to be significant when *P* value was <0.05.

## Results

### Enhancement of cell growth inhibition with the combination

We previously reported that EC is an inactive compound. In the beginning, we confirmed that EC does not induce any inhibitory activity on the growth of human lung cancer cell lines PC-9 and A549, even at concentrations of 200  $\mu\text{mol/L}$  (Supplementary Table S1). In contrast, curcumin from 5 to 100  $\mu\text{mol/L}$  inhibited the growth of PC-9 and A549 cells dose dependently and reached ~90% inhibition at concentration of 100  $\mu\text{mol/L}$  (Fig. 1A). Curcumin with 10 to 25  $\mu\text{mol/L}$  concentrations showed 39% to 64% growth inhibition in PC-9 cells and 16% to 63% in A549 cells (Fig. 1A). So we studied whether EC might enhance the effects on growth inhibition of PC-9 and A549 cells by 10 to 25  $\mu\text{mol/L}$  curcumin. Treatment of PC-9 cells with 10  $\mu\text{mol/L}$  curcumin alone reduced the number of viable cells to 61.7% of control, whereas the combination of 10  $\mu\text{mol/L}$  curcumin with 200  $\mu\text{mol/L}$  EC reduced the number to 46.9%, after 72 hours (Fig. 1A). The combination of 20  $\mu\text{mol/L}$  curcumin with 200  $\mu\text{mol/L}$  EC produced growth inhibition as much as a 3.75-fold higher concentration of curcumin (75  $\mu\text{mol/L}$ ) alone did (Fig. 1A). A similar type of enhancement by the combination of curcumin with EC was observed in A549 cells (Supplementary Table S1; Fig. 1A), and the combination of 20  $\mu\text{mol/L}$  curcumin with 200  $\mu\text{mol/L}$  EC produced much higher growth inhibition of both cell lines than did curcumin alone. The results encouraged us to examine the new role of EC in enhancing apoptosis induction with curcumin.

### Enhancement of apoptosis induction with the combination

Numerous *in vitro* and *in vivo* studies revealed the correlation between cell growth inhibition and induction of apoptosis by cancer-preventive agents. Two doses of EC alone (100 and 200  $\mu\text{mol/L}$ ) showed marginal effects, and treatment with 20  $\mu\text{mol/L}$  curcumin alone produced 42.3% apoptosis of PC-9 cells as measured by DAPI staining in Table 1. Next, we studied whether EC could enhance the apoptotic induction of curcumin. The combination of 20  $\mu\text{mol/L}$  curcumin with 200  $\mu\text{mol/L}$  EC increased apoptosis to 59.0% after 72 hours. The combinations with 15 or 25  $\mu\text{mol/L}$  curcumin with 200  $\mu\text{mol/L}$  EC produced apoptosis of 42.3% and 60.0%, respectively (Table 1). As

with growth inhibition, the combination of curcumin with EC resulted in the significant enhancement of apoptosis, and we think that the enhancement of growth inhibition of curcumin by EC is associated, in part, with the induction of apoptosis.

### Enhanced incorporation of curcumin with the combination

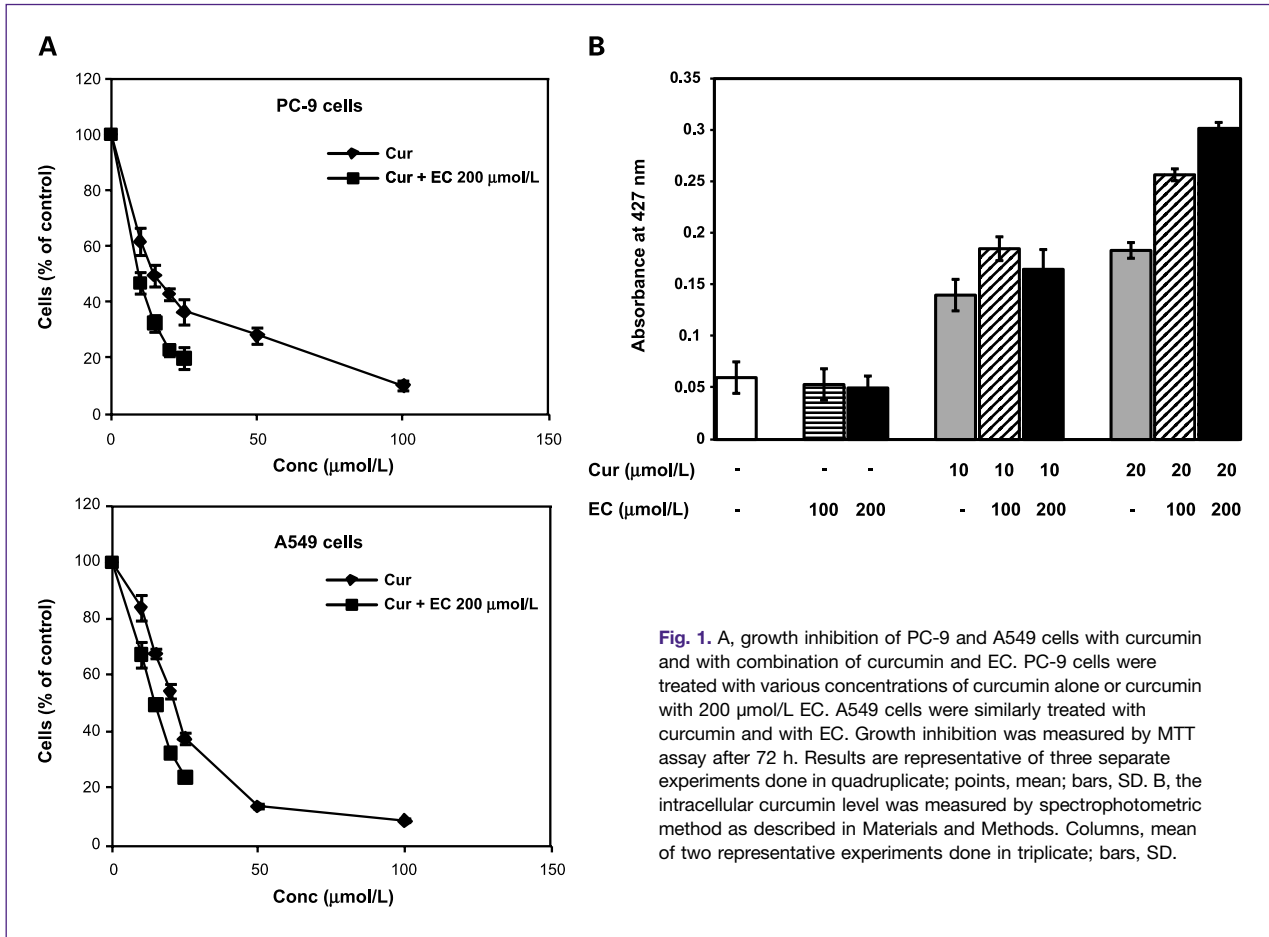
Because EC has enhanced the growth-inhibitory and apoptosis-inducing effects of curcumin, we then examined whether EC enhances the incorporation of curcumin in PC-9 cells. As shown in Fig. 1B, the combination of 200  $\mu\text{mol/L}$  EC with 20  $\mu\text{mol/L}$  curcumin significantly increased the amounts of intracellular curcumin ~1.7-fold compared with curcumin alone. This indicates that EC enhances the uptake of curcumin.

### Enhancement of GADD153 and GADD45 gene expressions with the combination

We previously reported that the combination of EGCG with cancer-preventive agents such as celecoxib and sulindac synergistically enhanced the expression of *GADD153* gene in PC-9 cells (12), so the effects of the combination of curcumin and EC on the gene expression levels were studied to try to grasp the molecular mechanism of the enhancement. Treatment of PC-9 cells with EC enhanced the upregulation of *GADD153* gene by curcumin in a dose-dependent manner (Fig. 2A). The combinations of 20  $\mu\text{mol/L}$  curcumin with 100 or 200  $\mu\text{mol/L}$  EC induced 1.7- and 2.1-fold enhancement in *GADD153* gene expression compared with 20  $\mu\text{mol/L}$  curcumin alone, after 24 hours. The combination effects of 10 and 15  $\mu\text{mol/L}$  curcumin with EC on *GADD153* gene expression were less strong than those produced by the combination of 20  $\mu\text{mol/L}$  curcumin and EC. The expression of *GADD45* gene was induced only in PC-9 cells (Fig. 2A). A combination of 20  $\mu\text{mol/L}$  curcumin with 100 or 200  $\mu\text{mol/L}$  EC induced 1.5- and 3.5-fold enhancement in *GADD45* gene expression compared with 20  $\mu\text{mol/L}$  curcumin alone, after 24 hours. Concomitant with the gene expressions, the *GADD45* protein was also found in PC-9 cells but not in A549 cells (Fig. 2B). Treatment of A549 cells with the combination of 20  $\mu\text{mol/L}$  curcumin and 200  $\mu\text{mol/L}$  EC induced 1.9-fold enhancements in *GADD153* gene expression (Fig. 2C). The combination also increased the level of *GADD153* protein in both cell lines, as shown in Fig. 2B. We previously reported that the expression of *p21* gene was also induced by the combination of EGCG and sulindac (13). However, the combination for the upregulation of *p21* gene was found in PC-9 cells, but was not significant in A549 cells (Fig. 2D).

### Knockdown of GADD153 and GADD45 genes by siRNAs

Because GADD proteins belong to the proteins of *growth arrest and DNA damage-inducible gene* family (14–18), we think that this upregulation of the gene expression by the combination reflects the increase of apoptotic induction



**Fig. 1.** A, growth inhibition of PC-9 and A549 cells with curcumin and with combination of curcumin and EC. PC-9 cells were treated with various concentrations of curcumin alone or curcumin with 200 µmol/L EC. A549 cells were similarly treated with curcumin and with EC. Growth inhibition was measured by MTT assay after 72 h. Results are representative of three separate experiments done in quadruplicate; points, mean; bars, SD. B, the intracellular curcumin level was measured by spectrophotometric method as described in Materials and Methods. Columns, mean of two representative experiments done in triplicate; bars, SD.

(12). To characterize the molecular nature of the enhancement and the functional significance of the *GADD153* and *GADD45* mRNAs on cell growth inhibition and apoptosis, siRNA duplexes were used to knock down the expression of *GADD153* and *GADD45* genes in PC-9 cells. The treat-

ment of PC-9 cells with either *GADD153* siRNA or *GADD45* siRNA inhibited the enhanced expressions of *GADD153* and *GADD45* mRNA by the combination of 20 µmol/L curcumin and 200 µmol/L EC, compared with cells transfected with control siRNA only at 24 hours

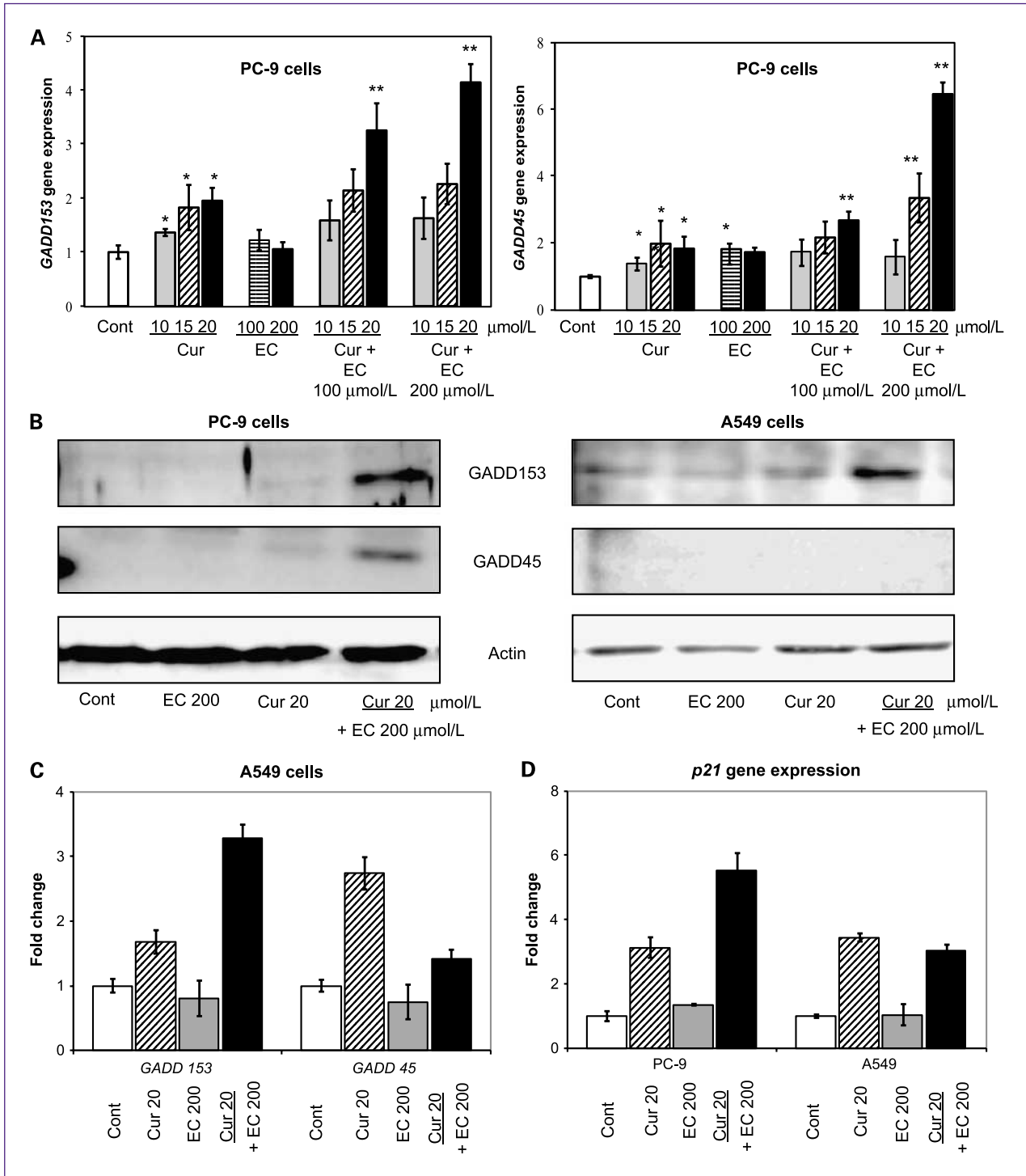
**Table 1.** EC enhances the apoptotic effect of curcumin

Curcumin (µmol/L)	Induction of apoptosis (% of apoptotic cells)			Enhancement with 200 µmol/L EC (%)
	Without EC	With EC (100 µmol/L)	With EC (200 µmol/L)	
PC-9				
0	4.9 ± 0.4	6.2 ± 0.3*	5.8 ± 0.9	
10	17.3 ± 4.4*	18.5 ± 1.1	22.3 ± 2.6	28.9
15	28.9 ± 4.6*	31.1 ± 2.2	42.3 ± 0.6†	46.3
20	42.3 ± 2.1*	50.0 ± 2.6†	59.0 ± 3.6†	39.5
25	45.9 ± 0.4*	50.6 ± 2.1†	60.0 ± 5.2†	30.7

NOTE: Apoptosis was measured by DAPI staining. Values represent mean ± SD of three experiments done in triplicate. Enhancement was calculated as [(apoptosis with EC – apoptosis without EC)/apoptosis without EC] × 100.

\*Significantly different from control, *P* < 0.05.

†The effects of the combination are significantly different from those with curcumin alone, *P* < 0.05.



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**Fig. 2.** Enhancement of *GADD153* and *GADD45* gene expression with the combination. A, the expression of *GADD153* and *GADD45* genes were induced with various concentrations of curcumin and EC alone, or in combination for 24 h. Columns, mean of two representative experiments done in triplicate; bars, SD. \*, significantly different from control,  $P < 0.05$ . \*\*, significantly different from curcumin alone,  $P < 0.05$ . Expression level of genes normalized to GAPDH of control cells was calculated as 1. B, the proteins *GADD153* and *GADD45* were produced in PC-9 cells (left) and A549 cells (right). Actin was used as loading control. Two independent experiments produced similar results. C, A549 cells were treated with 20 μmol/L curcumin and 200 μmol/L EC alone or in combination for 24 h. Columns, mean of two representative experiments done in duplicate. □, control; ▨, curcumin alone; ▩, EC alone; ■, curcumin + EC. Expression level of genes normalized to GAPDH of control cells was calculated as 1. D, the expression of *p21* gene was induced with 20 μmol/L curcumin and 200 μmol/L EC alone, or in combination for 24 h. Columns, mean of two representative experiments done in duplicate; bars, SD. □, control; ▨, curcumin alone; ▩, EC alone; ■, curcumin + EC. Expression level of genes normalized to GAPDH of control cells was calculated as 1.

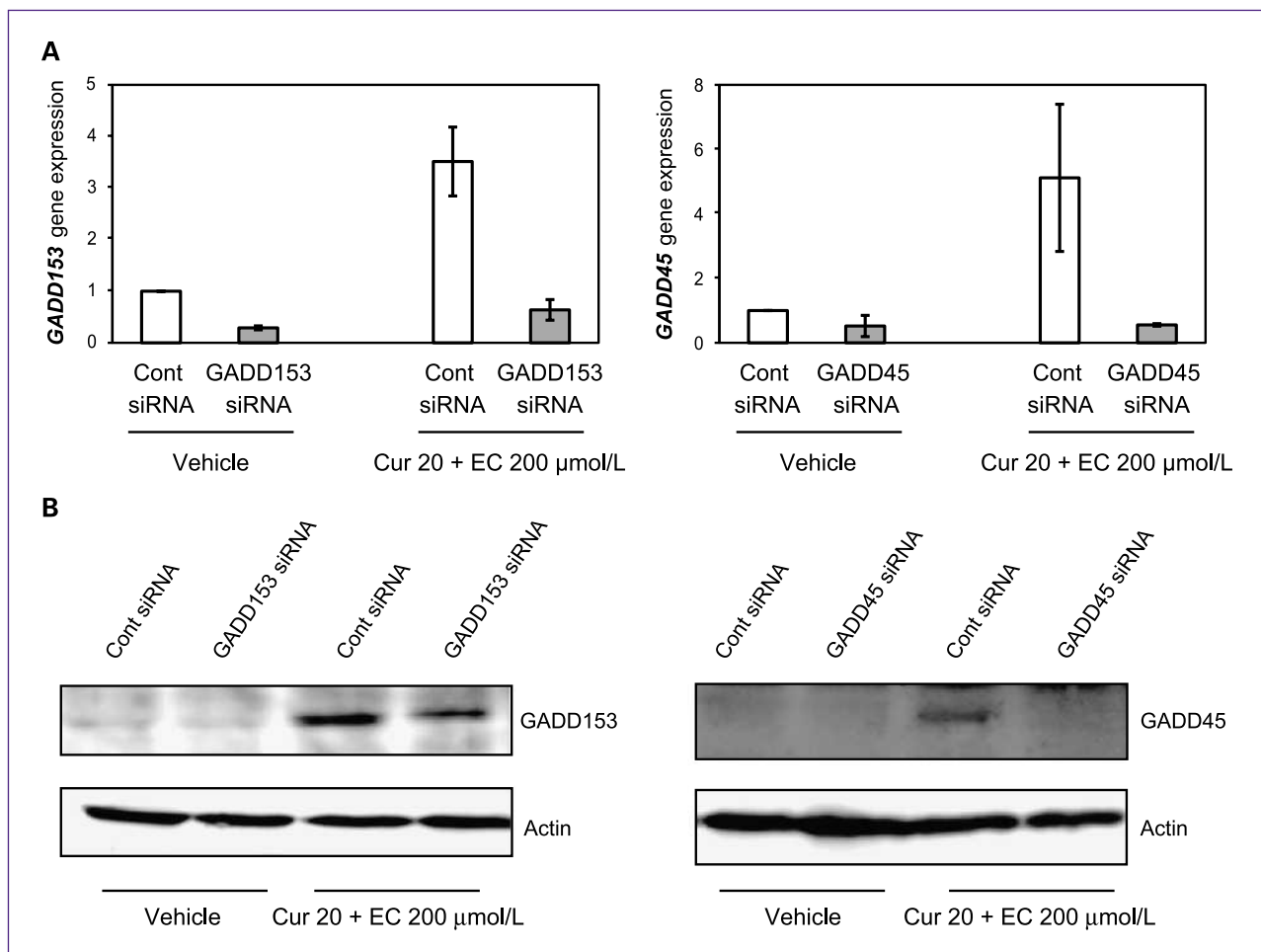
(Fig. 3A). Consistent with the inhibition of gene expression, administration of GADD153 siRNA or GADD45 siRNA also decreased the GADD153 and GADD45 protein level in PC-9 cells at 24 hours (Fig. 3B).

Next, we studied whether the inhibition of GADD153 or GADD45 by siRNA would have any effects on the apoptosis and cell growth inhibition produced by the combination in PC-9 cells. The knockdown of these two genes decreased the apoptotic effects of the combination. The combination of 20  $\mu\text{mol/L}$  curcumin and 200  $\mu\text{mol/L}$  EC induced apoptosis in 47.2% of PC-9 cells in the presence of negative control siRNA (Table 2), but PC-9 cells treated with GADD153 siRNA or GADD45 siRNA showed reduced apoptosis in response to the combination. The inhibition of cell proliferation induced by the combination was also attenuated in GADD153- and GADD45 siRNA-

treated cells, compared with cells treated with control siRNA (Table 2). These results indicate that GADD153 and GADD45 also play a role in the induction of apoptosis by the combination of curcumin and EC.

#### Effects of MAPK inhibitors on GADD153 and GADD45 gene expressions with the combination

The regulations of various cellular events in response to the extracellular stimuli are closely associated with the MAPK family (19). It is also known that MAPK signaling pathways are associated with the regulation of expression of GADD153 and GADD45 genes (16, 17, 20). We therefore next studied whether MAPK might be involved in the upregulation of GADD153 and GADD45 gene expressions with the combination of curcumin and EC, by addition of MAPK inhibitors SB202190 (p38 MAPK inhibitor),



**Fig. 3.** Knockdown of GADD153 and GADD45 genes by siRNAs. A, PC-9 cells were transfected with siRNA duplexes against GADD153, GADD45, or control siRNA. Twenty-four hours after transfection, cells were treated with vehicle or combination of 20  $\mu\text{mol/L}$  curcumin and 200  $\mu\text{mol/L}$  EC for 24 h. Columns, the amounts of GADD153 and GADD45 mRNA with the mean of three separate experiments done in duplicate; bars, SD. Expression level of genes normalized to GAPDH of control siRNA-treated cells was considered as 1. B, PC-9 cells were transfected with siRNA duplexes against GADD153, GADD45, or control siRNA. Twenty-four hours after transfection, cells were similarly treated with vehicle or combination for 24 h and then subjected to Western blotting. Actin was used as loading control. Two independent experiments produced similar results.

**Table 2.** Effects of GADD153 and GADD45 siRNAs on apoptosis and growth inhibition produced by combination

Treatment	% of apoptosis		Viable cells (% of control)	
	Vehicle	Cur 20 $\mu$ mol/L + EC 200 $\mu$ mol/L	Vehicle	Cur 20 $\mu$ mol/L + EC 200 $\mu$ mol/L
Control siRNA	4.8 $\pm$ 0.6	47.2 $\pm$ 2.9	100.0	44.2 $\pm$ 6.0
GADD153 siRNA	5.3 $\pm$ 1.2	28.8 $\pm$ 1.6	101.0 $\pm$ 11.9	62.2 $\pm$ 6.2
GADD45 siRNA	6.2 $\pm$ 1.4	27.8 $\pm$ 4.5	101.8 $\pm$ 4.4	61.2 $\pm$ 6.1

NOTE: PC-9 cells were transfected with siRNA duplexes against GADD153, GADD45, or control siRNA. Twenty-four hours after transfection, cells were treated with vehicle or combination of 20  $\mu$ mol/L curcumin and 200  $\mu$ mol/L EC for 24 h and then subjected to DAPI staining for apoptotic cell death and trypan blue dye exclusion test. Results are representative of two separate experiments done in quadruplicate; values represent mean  $\pm$  SD.

SP600125 (inhibitor of JNK), and PD98059 (ERK1/2 inhibitor), in PC-9 cells. Pretreatments of PC-9 cells with 20  $\mu$ mol/L SB202190 and 30  $\mu$ mol/L SP600125 increased the levels of GADD153 gene expression by 4.2- and 2.3-fold, respectively, compared with enhancement produced by the combination (Fig. 4A). These two inhibitors also increased GADD45 gene expression in a dose-dependent manner (Fig. 4A). However, pretreatment with 30  $\mu$ mol/L PD98059 increased the expression of GADD153 and GADD45 genes by about 2.6- and 3.1-fold, respectively, compared with enhancement produced by the combination (Fig. 4A). Treatments with all three MAPK inhibitors showed different effects on GADD153 gene expression in PC-9 cells: PD98059 had no effect, whereas SB202190 at 20  $\mu$ mol/L and SP600125 at 30  $\mu$ mol/L increased GADD153 gene expression by 3.3- and 2.8-fold, respectively, over control. They did not induce any enhancement on the GADD45 gene expression (Fig. 4B). In addition, three inhibitors did not have any effect on the expression of GADD153 and GADD45 genes induced by 20  $\mu$ mol/L curcumin. As for the phosphorylations of p38, JNK, and ERK, we confirmed by Western blotting that EC did not cause any enhancement in the protein phosphorylation (data not shown). The above data indicate that MAPK signaling pathways are correlated with the enhancement of GADD153 and GADD45 gene expressions by the combination of curcumin and EC.

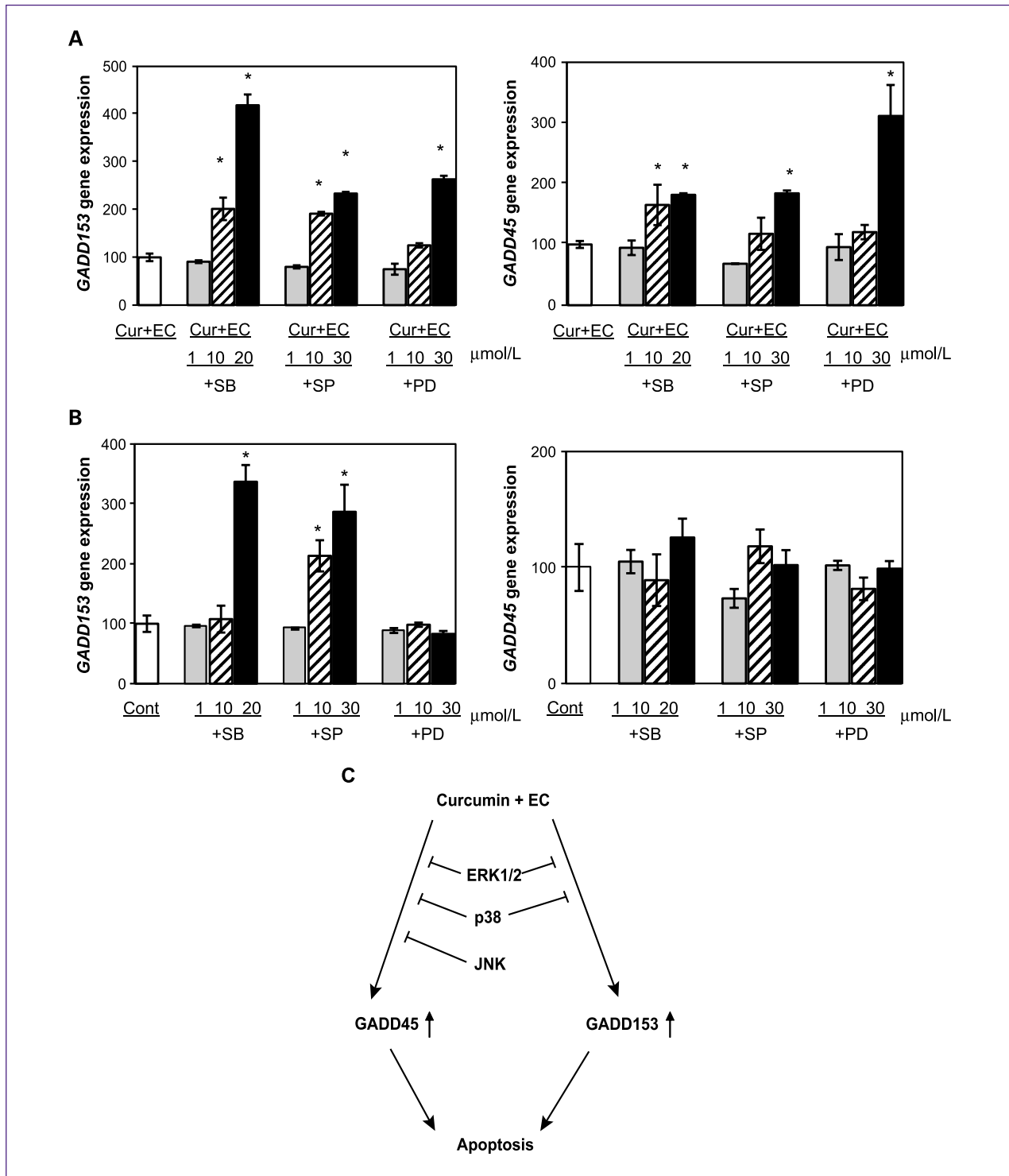
## Discussion

In our current study, we found that the activity of curcumin is significantly enhanced by the presence of the inactive green tea catechin EC in human lung cancer cell lines PC-9 and A549. The presence of EC increased the cell growth inhibition by curcumin. The combination also induced apoptosis that might be associated with the growth inhibition. Our results well supported previous evidence that the cell growth inhibition produced by curcumin is mediated through the induction of apoptosis (21–25). So the enhancement of cell growth inhibition by the combination of curcumin and EC is likely to use the same

mechanism for apoptotic induction. We also found that the presence of EC significantly increased the incorporation of curcumin into the cells, and the results are consistent with our previously reported results that EC enhanced apoptosis, growth inhibition, and inhibition of tumor necrosis factor- $\alpha$  release from BALB/3T3 cells by increasing the cellular incorporation of EGCG (8).

The combination of curcumin and EC also enhanced the expression of GADD153 and GADD45 genes compared with the expression produced by curcumin alone. Numerous studies have presented the correlation between the upregulation of GADD153 and GADD45, and induction of apoptosis (26–28). In human colon cancer cell lines, curcumin induces DNA damage, apoptosis, and GADD153 gene upregulation (26). The induction of apoptosis in mice exposed to hyperoxia was associated with the upregulation of GADD153 and GADD45 gene expressions (29). So the above-mentioned upregulation of GADD153 and GADD45 genes by the combination of curcumin and EC might be the result of increased transcription after DNA damage by curcumin (26). In our present study, specific inhibition of GADD153 and GADD45 in PC-9 cells by treatment with siRNAs reduced both the enhancement of apoptosis and cell growth inhibition by the curcumin-EC combination, indicating the crucial role of these two genes in enhancement of apoptosis induction in PC-9 cells by the combination of curcumin and EC.

Numerous studies revealed that MAPK pathways namely ERK1/2, p38 MAPK, and JNK are related to the functions of the GADD gene family. We previously reported that ERK1/2 and p38 MAPK were active in PC-9 cells, and inhibition of ERK by PD98059 reduces the upregulation of GADD153 gene expression in PC-9 cells, induced by the combination of EGCG with celecoxib, whereas SB203580 (p38 inhibitor) did not show any reduction (12). In human melanoma cells, however, p38 MAPK inhibitor SB203580 attenuated the enhanced expression of GADD153 and GADD45 genes induced by interleukin 24 (30). Although MAPK pathways are associated with GADD153 and GADD45 gene expression (28, 31), their effects vary depending on the cell type and individual agent used. In



**Fig. 4.** Effects of MAPK inhibitors on *GADD153* and *GADD45* gene expression with the combination. PC-9 cells were pretreated with various concentrations of SB202190, SP600125, and PD98059 for 1 h followed by (A) 20  $\mu\text{mol/L}$  curcumin and 200  $\mu\text{mol/L}$  EC in combination. Columns, the amounts of mRNA with the mean of two separate experiments done in triplicate; bars, SD. Expression level of genes normalized to *GAPDH* of cells treated with the combination was considered as 100%. \*, significantly different from combination of curcumin and EC,  $P < 0.05$ . B, vehicle. Columns, the amounts of mRNA with the mean of two separate experiments done in triplicate; bars, SD. Expression level of genes normalized to *GAPDH* of control cells was considered as 100%. \*, significantly different from control,  $P < 0.05$ . C, schematic illustration of signaling pathways for induction of *GADD* gene expression and apoptosis by curcumin and EC in PC-9 cells. Combination of curcumin and EC increases *GADD153* and *GADD45* gene expression, but does not activate MAPK. ERK1/2 and p38 negatively regulate *GADD153* and *GADD45* gene expression by curcumin and EC. JNK similarly regulates *GADD45* gene expression.



our present experiments, we found that inhibition of JNK, p38 MAPK, and ERK1/2 further increases the expression of *GADD153* and *GADD45* gene expression induced by the combination of 20  $\mu\text{mol/L}$  curcumin and 200  $\mu\text{mol/L}$  EC in PC-9 cells. Our results strongly supported previously reported evidence that the hyperosmolality-induced expression of *GADD153* and *GADD45* genes was increased by inhibition of ERK pathway in murine kidney cells (32). Furthermore, *GADD45* gene expression induced by troglitazone in MCF-7 cells was further increased by both ERK and p38 MAPK inhibitors (20). Thus, the induction of *GADD153* and *GADD45* genes by the combination of curcumin and EC is negatively regulated through MAPK in PC-9 cells. Because MAPK inhibitors increase the levels of *GADD153* and *GADD45* genes, we think that *GADD153* and *GADD45* gene expression play significant roles in the inductions of growth inhibition and apoptosis in human lung cancer cells PC-9 by the combination. A working model describing the relationship between MAPK and the induction of *GADD153* and 45 by the combination of curcumin and EC was presented in Fig. 4C.

Several studies reported that the chemopreventive agents such as EGCG, *N*-(4-hydroxyphenyl)retinamide, and curcumin are not genotoxic (33, 34) and selectively inhibit cancer cells, but not normal cell lines (35–37). For example, EGCG inhibited the growth of A-375 and Hs-294T melanoma cells, but did not affect the growth of normal human epidermal melanocytes (37). Curcumin and sulindac sulfide also selectively inhibited numerous tumor cells (35, 38). So these studies indicate that these chemopreventive agents are not genotoxic. Although the specificity to inhibit cancer cells is not fully understood, the specificity of curcumin towards cancer cells is probably due to an increased cellular uptake and a reduced level of glutathione, and activation of NF- $\kappa$ B in cancer cells (35).

Turmeric is widely consumed in different parts of the world and is common in the Indian subcontinent as a dietary spice and pigment. The most active component of the turmeric is curcumin. In one phase I clinical trial, it was found that curcumin is not toxic for humans even at a dose of 8 g/d (39). The average daily intake of turmeric is approximately 2.0 to 2.5 g in some countries as a dietary spice, which gives up to 100 mg of curcumin ingestion on a regular basis without any side effects (40). The major problem of curcumin for cancer prevention in humans is its poor bioavailability due to its low absorption, and rapid metabolism in the liver and the intestinal wall, resulting in low serum levels (41, 42).

Recently, it was reported that human ingestion of EC was directly linked to improved circulation and other hall-

marks of cardiovascular health, and that the pure EC consumed by humans produced an effect similar to consumption of flavanol-rich cocoa (43). In our current study, we found that 20  $\mu\text{mol/L}$  curcumin in combination with 200  $\mu\text{mol/L}$  EC produced growth inhibition similar to that achieved with 75  $\mu\text{mol/L}$  of curcumin when used alone in PC-9 cells. Based on the above results, we can assume that the combined administration of 100 mg of curcumin with 1 g of EC will give much greater activity than that produced by curcumin alone. In addition, this combination will give the extra benefit of EC for patients with cardiovascular diseases in areas where people ingest turmeric containing curcumin.

This report presents a new possibility for curcumin in combination with EC, and this combination will provide significant enhancement in growth inhibition and apoptosis of lung cancer cells in humans, more than the effects produced by curcumin alone. It might be interesting to study whether the presence of EC can increase the serum level of curcumin by raising its bioavailability in some animal experiments. Our current results for the first time showed that EC, an inactive component of green tea, significantly enhances the growth inhibition, apoptosis, and expression of *GADD* gene family members, *GADD153* and *GADD45* genes, in human lung cancer cell PC-9 induced by treatment with curcumin. The results with this practical combination of curcumin and EC encourage us to move on to a promising strategy for successful human lung cancer prevention.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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