

Synergistic Effects of (–)-Epigallocatechin Gallate with (–)-Epicatechin, Sulindac, or Tamoxifen on Cancer-preventive Activity in the Human Lung Cancer Cell Line PC-9¹

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Abstract

The study on incorporation of [³H](–)-epigallocatechin gallate (EGCG) into human lung cancer cell line PC-9 indicated that the [³H]EGCG incorporation was significantly enhanced by (–)-epicatechin, an inert tea polyphenol without a galloyl moiety. (–)-Epicatechin enhanced apoptosis, growth inhibition of PC-9 cells, and inhibition of tumor necrosis factor- α release from BALB/c-3T3 cells by EGCG and other tea polyphenols with a galloyl moiety in a dose-dependent manner. Moreover, the effects of EGCG on induction of apoptosis were also synergistically enhanced by other cancer-preventive agents, such as sulindac and tamoxifen. This paper reports significant evidence that whole green tea is a more reasonable mixture of tea polyphenols for cancer prevention in humans than EGCG alone and that it is even more effective when it is used in combination with other cancer preventives.

Introduction

Intense interest in green tea as a cancer preventive in humans has increased for six main reasons: (a) wide range of target organs in rodent carcinogenesis experiments (1, 2); (b) growth inhibition of various human cancer cell lines (3–5); (c) inhibition of lung metastasis in mice (1); (d) wide distribution of [³H]EGCG⁴ in various organs of mice (6); (e) cancer-preventive results with humans from a prospective cohort study (7); and (f) no severe adverse effects with green tea tablets in humans (8).

Green tea contains several tea polyphenols, including EGCG, EGC, ECG, and EC. Their composition varies depending on the location of cultivation of the tea plant (*Camellia sinensis*), variety of the plant, season of harvest, and manufacturing process. The usual composition is 10–15% EGCG, 6–10% EGC, 2–3% ECG, and 2% EC, with EGCG being the main constituent by our analysis. As we reported previously (4), tea polyphenols inhibit growth of human lung cancer cell line PC-9, with the order of potency being EGCG = ECG > EGC >> EC (4). From these results, EGCG would appear to be the most important compound of the four because of its activity and its high

content, but we found that green tea extract, *i.e.*, tea itself, had stronger effects than the polyphenol content would have indicated (3). This allows us to think that the constituents of green tea extract together have synergistic or additive effects on cancer-preventive activity. Support for this idea was obtained from our discovery that [³H]EGCG incorporation was significantly enhanced by EC, suggesting that EGCG has synergistic potential for cancer-preventive activity with EC. Moreover, synergistic possibilities of other tea polyphenols with EC or those of EGCG with other cancer-preventive agents could be anticipated. To test our hypothesis, we chose induction of apoptosis as a parameter of synergistic effects because apoptosis is commonly induced by tea polyphenols, sulindac, and tamoxifen (5, 9, 11).

Here, we present the first evidence that cotreatment with EGCG and EC, ECG and EC, and EGC and EC synergistically induced apoptosis of PC-9 cells, mediated through enhanced incorporation of tea polyphenols into the cells. Furthermore, cotreatment with EGCG and sulindac or EGCG and tamoxifen significantly enhanced induction of apoptosis by EGCG. These results strongly indicate that green tea itself is a more effective and practical cancer preventive than EGCG alone and that drinking green tea enhances the cancer-preventive activity of sulindac and tamoxifen, resulting in smaller doses of these drugs and fewer adverse effects.

Materials and Methods

Chemicals. EGCG was purified from Japanese green tea leaves. EGC, ECG, and EC were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). All tea polyphenols used in the experiments were >98% pure. [³H]EGCG (48.1 GBq/mmol) was labeled at the 2',6'-positions of the EGC moiety and 2,6-positions of the galloyl moiety with tritium gas (Amersham, Buckinghamshire, United Kingdom; Ref. 6). The stability of ³H labeling in EGCG was confirmed by high-performance liquid chromatography analysis (6). Sulindac and tamoxifen were purchased from Sigma Chemical Co. (St. Louis, MO).

[³H]EGCG Incorporation into PC-9 Cells. PC-9 cells were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) containing phenol red, kanamycin, and 10% fetal bovine serum. PC-9 cells (1×10^6) were incubated with 100 μ M [³H]EGCG (4×10^7 dpm) with or without various concentrations of each tea polyphenol for 1 h at 37°C. After incubation, cell-associated radioactivity was measured by scintillation counter, as described previously (4). ³H radioactivity incorporated into PC-9 cells without tea polyphenols was taken to be 100%. Results are expressed as the means of two independent experiments performed in duplicate.

DNA Fragmentation. PC-9 cells (2×10^5 cells/ml) in 24-well plates were incubated with each compound at various concentrations in the absence or presence of EC for 2 days. DNA fragmentation was measured by quantitation of cytosolic oligonucleosome-bound DNA by using an ELISA kit (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's instructions. Briefly, the cytosolic fraction corresponding to $\sim 2 \times 10^4$ – 10^5 cells/ml was used as the source in a sandwich ELISA with a primary antihistone antibody coated on the microtiter plate and a secondary anti-DNA antibody coupled to peroxidase. Amounts of oligonucleosome released into cytoplasm were detected by their absorbance at 415 nm. The results were confirmed by two independent experiments performed in duplicate.

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⁴ The abbreviations used are: EGCG, (–)-epigallocatechin gallate; EGC, (–)-epigallocatechin; ECG, (–)-epicatechin gallate; EC, (–)-epicatechin; TNF- α , tumor necrosis factor- α .

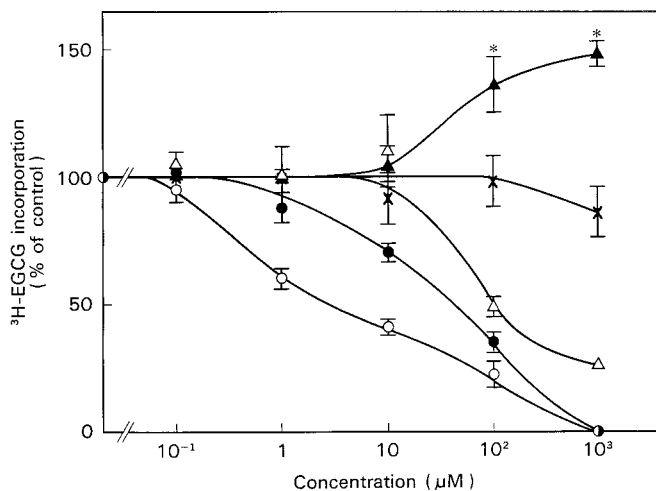


Fig. 1. Enhancement of [^3H]EGCG incorporation into PC-9 cells by EC but not by the other tea polyphenols. PC-9 cells were incubated with 100 μM [^3H]EGCG in the presence of various concentrations of EGCG (\circ), ECG (\bullet), EGC (Δ), EC (\blacktriangle), and caffeine (\times). Incorporation of [^3H]radioactivity into the cells in the absence of nonradioactive tea polyphenols expressed as 100%. Data points, means of two separate experiments performed in duplicate; bars, SD. *, $P < 0.01$.

Growth Inhibition of PC-9 Cells. PC-9 cells (4×10^5 cells/ml) were incubated with EGCG, ECG, or EGC in the absence or presence of EC for 2 days. Numbers of viable cells were counted by the trypan blue dye exclusion test (4). Percentage of control was calculated as follows: [number of viable cells in a treated group/number of viable cells in a nontreated group ($\sim 14 \times 10^5$ cells/ml)] $\times 100\%$. The results were confirmed by two independent experiments performed in duplicate.

Inhibition of TNF- α Release from BALB/c-3T3 Cells. BALB/c-3T3 cells were maintained in MEM (Nissui Pharmaceutical Co.) containing 10% fetal bovine serum. BALB/c-3T3 cells (2×10^5 cells per 0.5 ml) in a 12-well plate were preincubated with each of the tea polyphenols for 1 h. Then, 0.2 μM okadaic acid was added to the medium. Twenty-four h after incubation, concentration of TNF- α in the medium was determined by ELISA (Genzyme, Cambridge, MA), as described previously (12). The results were confirmed by two independent experiments performed in duplicate.

Statistical Analysis. The Student's t test was performed to compare [^3H]EGCG incorporation and induction of apoptosis. Statistical significance levels were $P \leq 0.05$ and $P \leq 0.01$.

Results and Discussion

Enhancement of [^3H]EGCG Incorporation by EC. The incorporated radioactivity of [^3H]EGCG was $\sim 120,000$ dpm per 10^6 PC-9 cells 1 h after incubation with 100 μM [^3H]EGCG (4×10^7 dpm). Incubation of [^3H]EGCG with nonradioactive EGCG decreased the incorporated radioactivity in a dose-dependent manner (Fig. 1). In this assay, the effects of ECG, EGC, EC, and caffeine were also studied. ECG and EGC inhibited [^3H]EGCG incorporation in a dose-dependent manner, but their inhibitions were slightly weaker than that of EGCG (Fig. 1). The concentrations inducing 50% inhibition (IC_{50} s) were 2.5 μM for EGCG, 40 μM for ECG, 100 μM for EGC, and >1 mM for caffeine. These results indicated that ECG and EGC incorporate into the cells in a manner similar to EGCG and that their incorporation is dependent on the galloyl moiety in tea polyphenols. In addition, we found that EC at concentrations of >100 μM significantly increased [^3H]EGCG incorporation rather than inhibiting it: the increase with 1 mM EC was a significant 1.5-fold. The results indicated that EC, which has no galloyl moiety, enhances incorporation of EGCG and other tea polyphenols that do have galloyl moiety.

Enhancement of Apoptosis by EC with EGCG, ECG, and EGC. It has been reported that green tea polyphenols stimulated apoptosis of various cancer cell lines, such as prostate, lymphoma, colon, and lung,

in vitro (5, 9). EGCG and ECG strongly induced DNA fragmentation in PC-9 cells after 2 days of incubation at concentrations of over 50 and 100 μM , respectively. The effects of EGC were much weaker than those of EGCG and ECG (data not shown). EC alone, even at a concentration of 500 μM , did not induce DNA fragmentation. These results confirmed that EC is an inert compound for induction of apoptosis in PC-9 cells, which correlated well with previously reported results (Fig. 2; Ref. 5).

Next, we sought to find out whether EC enhances induction of apoptosis with EGCG. We selected an assay condition in which 75 μM EGCG alone induced apoptosis marginally and 100 μM EGCG induced it significantly (Fig. 2). The cells were treated with four different concentrations of EC in the absence or presence of either 75 μM or 100 μM EGCG. Fig. 2 clearly shows enhanced DNA fragmentation by EC with two different concentrations of EGCG. EGCG at a concentration of 75 μM with various concentrations of EC synergistically induced apoptosis: 50 μM EC enhanced apoptosis 3.4-fold; 100 μM EC enhanced it 4.5-fold; and 200 μM EC enhanced it 7.2-fold. At 100 μM EGCG, treatment with 100 μM EC also enhanced apoptosis ~ 2.1 -fold. Moreover, EC at a concentration of 200 μM stimulated induction of apoptosis by ECG and EGC ~ 2 -fold and ~ 3 -fold, respectively (Table 1).

Enhancement of Cell Growth Inhibition by EC with EGCG, ECG, and EGC. Along with its induction of apoptosis, EC also enhanced growth inhibition of the cells in a dose-dependent manner. Because we have reported that EGCG induced accumulation of G_2 -M cells, probably G_2 -M arrest (4), we think growth inhibition by tea polyphenols is caused by apoptosis and G_2 -M arrest. After 2 days of incubation with 100 μM EGCG alone, the number of viable cells was 73.3% of control, whereas that with 200 μM EC alone was 97.8%. Cotreatment with 100 μM EGCG and 200 μM EC reduced the number to 27.8% (Table 1). Specifically, 200 μM EC enhanced growth inhibition ~ 2.7 -fold. These results correlated well with the results of enhanced [^3H]EGCG incorporation and enhanced apoptosis (Fig. 2 and Table 1). EC also enhanced growth inhibition of PC-9 cells by ECG and EGC (Table 1). From these results, we concluded that EC, an inert tea polyphenol, enhanced growth inhibition as well as apoptosis in combination with other tea polyphenols. Green tea extract at a concentration of 500 $\mu\text{g}/\text{ml}$ completely inhibited growth of PC-9

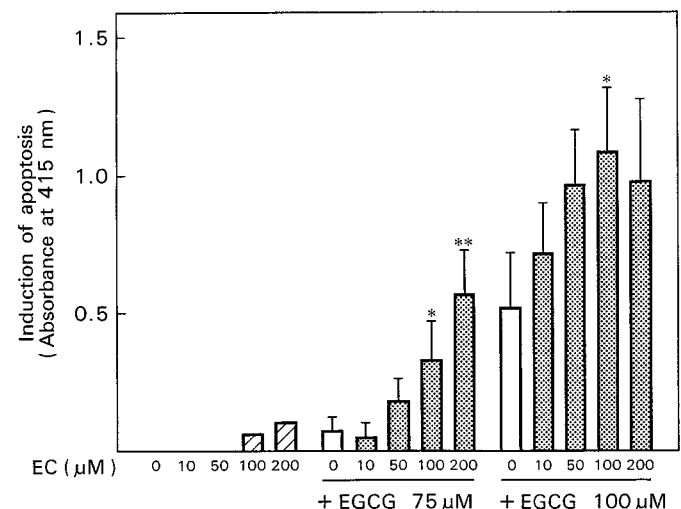


Fig. 2. Synergistic induction of apoptosis with EC and EGCG. PC-9 cells (2×10^5 cells/ml) were incubated with 75 or 100 μM EGCG in conjunction with four different doses of EC. Cytosolic oligonucleosome-bound DNA was measured by ELISA, as described in "Materials and Methods." Columns, means of two separate experiments performed in duplicate; bars, SD. \square , EC alone; \square , EGCG alone; \square , EGCG + EC. *, $P < 0.05$; **, $P < 0.01$.

Table 1 Synergistic effects by cotreatment with EC and other tea polyphenols on apoptosis, growth inhibition, and TNF- α release

	Induction of apoptosis ^a (A _{415 nm})		Growth inhibition ^b (% of control)		Inhibition of TNF- α release ^b IC ₅₀ (μ M)	
	Without EC	With EC (200 μ M)	Without EC	With EC (200 μ M)	Without EC	With EC (100 μ M)
EGCG (100 μ M) ^d	0.52 \pm 0.22	0.10 \pm 0.03		97.8		>500 ^c
ECG (50 μ M) ^d	0.27 \pm 0.13	0.98 \pm 0.28	73.3	27.8	60	15 ^e
EGC (200 μ M) ^d	0.14 \pm 0.03	0.53 \pm 0.18	62.2	43.3	30	7
		0.43 \pm 0.08 ^e	100.0	72.2	ND	ND

^a Values represent the mean \pm SD of two separate experiments performed in duplicate.

^b Values are representative of two separate experiments performed in duplicate.

^c IC₅₀ of EC.

^d Concentration for experiment on apoptosis and growth inhibition.

^e $P < 0.01$.

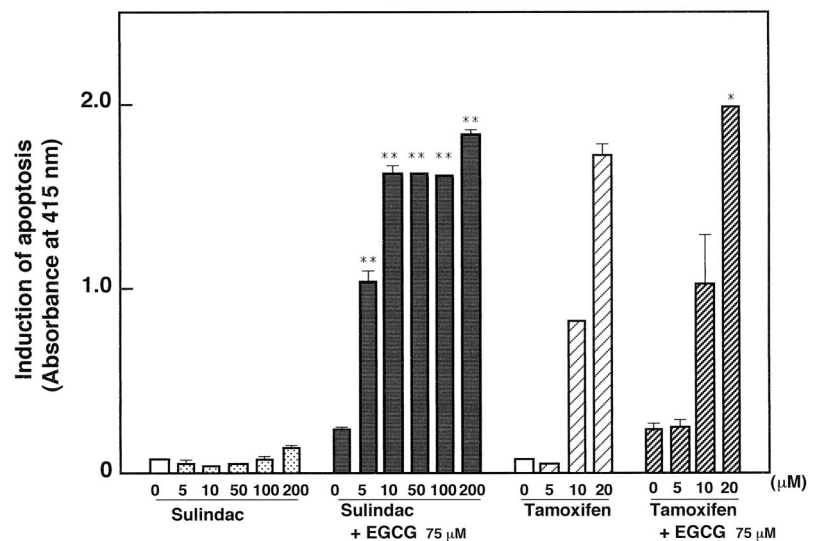
cells (3). In this concentration of green tea, the molar concentrations of each tea polyphenol were 100 μ M (45.8 μ g/ml) EGCG, 100 μ M (30.6 μ g/ml) EGC, 25 μ M (7.7 μ g/ml) ECG, and 30 μ M (8.7 μ g/ml) EC.

Synergistic Effects of EC with EGCG and ECG on Inhibition of TNF- α Release. On the basis of our finding that TNF- α is an endogenous tumor promoter, we think increase of TNF- α in organs, including lung, may promote carcinogenesis (13). We also found that various cancer inhibitors including EGCG commonly inhibited TNF- α gene expression and its release from BALB/c-3T3 cells. Therefore, we think reduction of TNF- α level is a key criterion of cancer-preventive agents (14). Therefore, we next examined whether EC enhances inhibition of TNF- α release. Table 1 shows that EC alone did not inhibit TNF- α release significantly up to concentrations of 500 μ M, whereas EGCG inhibited it with an IC₅₀ of 60 μ M. Treatment with EGCG and 100 μ M EC reduced the IC₅₀ from 60 to 15 μ M (*i.e.*, 4-fold enhancement). Similarly, EC also stimulated the inhibitory effects of ECG on TNF- α release, reducing IC₅₀ from 30 to 7 μ M. These results clearly demonstrated that EC stimulates the cancer-preventive activity of EGCG and other tea polyphenols mediated through inhibition of TNF- α release and induction of apoptosis (Table 1).

Enhancing Effects of EGCG with Sulindac or Tamoxifen. To extend the study of synergistic effects, we examined whether other cancer-preventive agents can enhance anticancer activity by EGCG in a manner similar to that of EC. We chose two preventive agents, sulindac and tamoxifen, because these two agents induce apoptosis of human cancer cells and inhibit TNF- α release from BALB/c-

3T3 cells (10, 11, 14). As we expected, both sulindac and tamoxifen significantly and synergistically enhanced apoptosis induced in PC-9 cells by EGCG. Specifically, Fig. 3 shows that sulindac at concentrations up to 100 μ M did not induce apoptosis of PC-9 cells, whereas 10 μ M sulindac with 75 μ M EGCG induced apoptosis, an 8-fold enhancement (Fig. 3). Sulindac is a popular agent used for suppression of colon adenoma formation in familial adenomatous polyposis patients (15), but its usage is restricted because of its side effects (16). We also found that cotreatment with sulindac and EGCG synergistically inhibited cell growth of mouse colon adenocarcinoma cell line, Colon 26, more strongly than sulindac alone or EGCG alone (data not shown). Our results described here provide a new application method, whereby drinking green tea enhances the preventive effects of sulindac while reducing its toxicity when both are given together to humans. Whether cotreatment with green tea extract and sulindac synergistically inhibits development of colon adenoma in C57BL/6J-Min/+ (containing dominant mutation in the *APC* gene) is under investigation. Fig. 3 also shows the effect of tamoxifen on apoptosis of PC-9 cells at 10 and 20 μ M. Cotreatment with tamoxifen and EGCG induced apoptosis more strongly than tamoxifen alone or EGCG alone (Fig. 3). The enhanced effects of tamoxifen and EGCG were also observed in inhibition of TNF- α release from BALB/c-3T3 cells, as well as in inhibition of cell growth of human breast cancer cell line, MCF-7 (data not shown). A combination of green tea extract and tamoxifen synergistically reduced development of spontaneous mammary tumors in C3H/OuJ mice (17) and

Fig. 3. Synergistic effect with EGCG and sulindac and additive effect with EGCG and tamoxifen on induction of apoptosis. PC-9 cells (2×10^5 cells/ml) were incubated with different doses of sulindac or tamoxifen in the presence or absence of 75 μ M EGCG. Cytosolic oligonucleosome-bound DNA was measured by ELISA, as described in "Materials and Methods." Columns, means of two separate experiments performed in duplicate; bars, SD. □, nontreated; ▤, sulindac alone; ■, sulindac + EGCG; ▨, tamoxifen alone; ▩, tamoxifen + EGCG. *, $P < 0.05$; **, $P < 0.01$.



of carcinogen-induced mammary tumors in rodents.⁵ A recent epidemiological study revealed that increased consumption of green tea resulted in decreased recurrence of breast cancer in Japanese patients (18). Thus, there is a significant possibility that high consumption of green tea (usually >10 cups per day) will enhance the preventive activity of tamoxifen against breast cancer development.

The mechanisms of action of sulindac, tamoxifen, and EC are thought to be different, because sulindac and tamoxifen did not stimulate [³H]EGCG incorporation into PC-9 cells, whereas EC did (data not shown), and the synergistic potential of sulindac was much stronger than that of tamoxifen or EC. EGCG induces accumulation of G₂-M cells of PC-9 cells (4), whereas sulindac and tamoxifen do not have any effect on cell cycle regulation of cancer cell lines (10, 11). Looking at other results with EGCG, recently, the synergistic effect of EGCG and curcumin was reported on growth inhibition of oral epithelial cells (19). On the other hand, treatment with catalase and EGCG was also reported to inhibit apoptosis induced by EGCG (5). Although cotreatment with two preventive agents has produced interesting results, mechanisms of action have not been well identified. The cancer-preventive activity of sulindac is assumed to inhibit cyclooxygenase and induce apoptosis. Because EGCG inhibits cyclooxygenase (2), a combination of EGCG and sulindac should strongly inhibit cyclooxygenase. Recent study shows us that one of the molecular targets for colon cancer is cyclooxygenase-2 (20). Therefore, whether cotreatment with EGCG and sulindac would similarly enhance inhibition of cyclooxygenase-2 by sulindac should be further investigated. Here, all our results point to green tea as a suitable candidate for use in combination with cancer-preventive agents such as sulindac and tamoxifen to reduce their adverse effects.

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