

Inhibition of Intestinal Tumorigenesis in *Apc*^{min/+} Mice by (–)-Epigallocatechin-3-Gallate, the Major Catechin in Green Tea

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Abstract

The present study was designed to investigate the effects of two main constituents of green tea, (–)-epigallocatechin-3-gallate (EGCG) and caffeine, on intestinal tumorigenesis in *Apc*^{min/+} mice, a recognized mouse model for human intestinal cancer, and to elucidate possible mechanisms involved in the inhibitory action of the active constituent. We found that p.o. administration of EGCG at doses of 0.08% or 0.16% in drinking fluid significantly decreased small intestinal tumor formation by 37% or 47%, respectively, whereas caffeine at a dose of 0.044% in drinking fluid had no inhibitory activity against intestinal tumorigenesis. In another experiment, small intestinal tumorigenesis was inhibited in a dose-dependent manner by p.o. administration of EGCG in a dose range of 0.02% to 0.32%. P.o. administration of EGCG resulted in increased levels of E-cadherin and decreased levels of nuclear β -catenin, c-Myc, phospho-Akt, and phospho-extracellular signal-regulated kinase 1/2 (ERK1/2) in small intestinal tumors. Treatment of HT29 human colon cancer cells with EGCG (12.5 or 20 μ mol/L at different times) also increased protein levels of E-cadherin by 27% to 58%, induced the translocation of β -catenin from nucleus to cytoplasm and plasma membrane, and decreased c-Myc and cyclin D1 (20 μ mol/L EGCG for 24 hours). These results indicate that EGCG effectively inhibited intestinal tumorigenesis in *Apc*^{min/+} mice, possibly through the attenuation of the carcinogenic events, which include aberrant nuclear β -catenin and activated Akt and ERK signaling. (Cancer Res 2005; 65(22): 10623-31)

Introduction

Colorectal cancer is the second most common cancer in both incidence and mortality among men and women in more developed countries (1). Interactions between genetic and environmental factors play a critical role in the etiology of this cancer. Diet is a major environmental factor that affects colorectal carcinogenesis; both risk factors and protective factors have been studied exten-

sively (2). The identification of dietary constituents that prevent colorectal cancer is an important area of research.

Tea (*Camellia sinensis*) is the second most popular beverage worldwide, with green tea commonly consumed in Asia, especially in China and Japan. Epidemiologic studies have not shown a clear conclusion on the relationship between green tea consumption and colorectal cancer risk (3–5). Inhibitory activity of green tea against intestinal carcinogenesis was shown in some studies (6–9) but not in others (10–12). In the *Apc*^{min/+} mouse, mild inhibition of intestinal tumorigenesis by green tea and green tea extract was reported (6, 7). As the most active constituents, catechins and caffeine may confer the cancer inhibitory activity of green tea (3). In green tea, catechins are characteristic polyphenolic compounds accounting for 30% to 42% of the solids in hot-water extracts of tea (on a dry weight basis; ref. 3). (–)-Epigallocatechin-3-gallate (EGCG) is the major catechin that has received the most attention and its inhibitory effect on carcinogen-induced intestinal tumorigenesis was shown in some animal models (3, 8). Caffeine, which accounts for 2% to 6% of green tea solids on dry weight basis, was active in the inhibition of skin tumorigenesis induced by UV light in mice and of lung tumorigenesis induced chemically in F344 rats (13, 14). Caffeine was reported, however, to increase both 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine-induced aberrant crypt foci and colon tumor formation in F344 rats (15, 16). Based on various cell culture studies, many mechanisms have been proposed for the action of green tea and its constituents (17). It is not clear, however, which mechanisms are applicable *in vivo*.

Apc^{min/+} mice are recognized as a genetically relevant animal model mimicking human intestinal carcinogenesis and have been used extensively for various chemoprevention studies (18). These mice carry a dominant heterozygous nonsense mutation at codon 850 of the mouse homologue of the human adenomatous polyposis coli (*APC*) gene. They develop more than 50 tumors throughout the entire intestinal tract, mainly in small intestine, until they die of bowel obstruction, intestinal bleeding, and severe anemia at 150 to 170 days of age (19). The *APC* gene is a tumor suppressor gene and its mutation is significantly implicated in both sporadic and inherited human colorectal carcinogenesis (20–22).

The *Apc* protein functions as a negative regulator of β -catenin by binding to β -catenin with axin and glycogen synthase kinase 3 β , leading to the degradation of β -catenin by ubiquitin-dependent proteasomes (23, 24). β -Catenin is an intracellular anchoring protein that, together with E-cadherin, constitutes the adherens junction, which is essential for epithelial cell homeostasis (25, 26). Truncated *Apc* encoded by mutated forms of the *Apc* gene, which is found in human colon tumors as well as enterocytes of *Apc*^{min/+} mice, typically cannot bind to β -catenin. Consequently,

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this oncoprotein is allowed to escape the Apc-mediated degradation pathway, translocate into the nucleus, interact with T-cell factor-4, and then activate the transcription of many oncogenic genes, such as *c-Myc*, *cyclin D1*, and cyclooxygenase-2 (*COX-2*; refs. 27–32). Deregulated β -catenin signaling occurs in almost all colon tumors of humans (33) and frequently in tumors of animal models, including *Apc*^{min/+} mice (34–36).

Phosphoinositide-3-kinase-Akt (protein kinase B) signaling (a survival pathway) and Ras-extracellular signal-regulated kinase 1/2 (ERK1/2)-activator protein (AP)-1 signaling (a growth promoting pathway) are activated in human colorectal cancer and colon tumors in animal models (37–41). Importantly, suppression of these activated pathways by specific inhibitors was shown to induce cell growth inhibition and apoptosis in human colon carcinoma cells (42, 43).

The present study was designed to investigate which green tea constituents confer the inhibitory activity of green tea against intestinal tumorigenesis in *Apc*^{min/+} mice. This was accomplished by evaluating the antitumorigenic effects of two main constituents of green tea, EGCG and caffeine. Possible mechanisms involved in the inhibitory action by the active constituent were investigated in *Apc*^{min/+} mice as well as in HT29 human colon cancer cells.

Materials and Methods

Breeding and genotyping of *Apc*^{min/+} mice. Male *Apc*^{min/+} (C57BL/6J) and female wild-type littermate mice were initially purchased from The Jackson Laboratory (Bar Harbor, ME) as founders, and our own breeding colony was established in the animal facility of the Susan Lehman Cullman Laboratory for Cancer Research (Rutgers, The State University of New Jersey, Piscataway, NJ). Pups were produced from the colony and weaned at 3 weeks of age. Genotyping was done by routine PCR assays on tail DNA using a commercially available master mix (Bio-Rad, Hercules, CA). An *Apc*^{min} nonsense mutation-specific primer (*Apc*-mutant: 5'-TTCTGAGAAAGACAGAAGTTA-3'), together with a complementary 3'-end primer (*Apc*-common: 5'-TTCCACTTTGGCATAAGGC-3'), detected the mutant *Apc* allele (313 bp), which is only present in heterozygous *Apc*^{min/+} mice. The *Apc*⁺ allele-specific primer (*Apc*-wild-type: 5'-GCCATCCCTTCACGTTAG-3') with the *Apc*-common primer detected the wild-type allele (619 bp).

Animal treatment and tissue harvesting. In two initial experiments, 5-week-old male or 7-week-old female *Apc*^{min/+} mice on AIN93G diets (Research Diets, New Brunswick, NJ) were given 0.16% or 0.08% EGCG (0.16 or 0.08 g EGCG dissolved in 100 mL distilled water containing 0.5 g citric acid) as a sole source of drinking fluid for 9 or 8 weeks, respectively, until the experiments were terminated at 14 or 15 weeks of age. In another experiment, female *Apc*^{min/+} mice at 5 weeks of age on AIN93G diet were given 0.32% EGCG or 0.044% caffeine (prepared in 0.5% citric acid solution as above) in drinking fluid until they were sacrificed at 11 weeks of age. In all three experiments, control groups were given the control fluid (0.5% citric acid) throughout the respective experimental periods. In the dose-response study with EGCG, 5-week-old male *Apc*^{min/+} mice on AIN93G diet were randomized into six groups, and 0%, 0.02%, 0.04%, 0.08%, 0.16%, or 0.32% EGCG solution was given in drinking fluid for 6 weeks until the experiment was terminated at 11 weeks of age. Negative control groups (*Apc*^{+/+} mice receiving either control or 0.32% EGCG fluid) were also included to generate normal small intestine samples for mechanistic studies. Food and fluid intakes as well as body weight of mice were monitored on a weekly basis during the experimental periods. Mice were routinely checked for any abnormalities. For the short-term treatment with EGCG, 14-week-old female *Apc*^{min/+} mice were given a single dose of 75 mg EGCG/kg intragastrically 3 hours before they were sacrificed. Pure EGCG was obtained from Mitsui Norin Co. Ltd. (Shizuoka, Japan) and caffeine and citric acid were purchased from Sigma Chemical Co. (St. Louis, MO).

All mice were euthanized by CO₂ asphyxiation. The small intestine and colon were removed, washed with ice-cold PBS, opened longitudinally, and

flattened on filter paper. The number, location, and size of visible tumors in the entire intestine were determined under a lighted magnifier ($\times 2$). Alternatively, the flattened tissues on filter paper were placed on dry ice briefly to score the visible tumors. The frozen tumors displayed a distinct white color because cells in tumors are denser than those in normal tissue. From each mouse, three 1.5- to 2-cm segments from the middle point of the proximal, middle, and distal small intestine were taken, fixed in 10% buffered formalin for 24 hours, and then transferred to 80% ethanol for histopathologic examination of small intestinal tumors. From the remaining small intestine, visible tumors were excised, pooled, and frozen at -80°C . Normal enterocytes were collected from the small intestine by scraping the mucosal side with a glass microscope slide, washed with cold PBS twice, and then subjected to a brief centrifugation. The resulting enterocyte pellet was frozen at -80°C .

Histopathologic analyses. Each of the fixed small intestinal segments was cut into two pieces at the midpoint; one half of the segment was vertically (and the other half horizontally) embedded into a separate paraffin block. Each tissue block was serially sectioned (5 μm) and one slide from every 10 sections (slide numbers 1, 10, 20, and 30) was routinely stained with H&E. Adenomas (defined as those containing more than five crypts) as well as microscopic adenomas (defined as those with less than five crypts) were scored in each section.

Cell treatment. HT29 colon cancer cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in 95% humidity and 5% CO₂. Approximately 1×10^5 HT29 cells in either EGCG-containing (at 12.5 or 20 $\mu\text{mol/L}$) or DMSO-containing medium (with 10% serum) were plated into each well of a six-well plate (2.5×10^6). Superoxide dismutase (5 units/mL) and catalase (30 units/mL) were added in the medium to stabilize EGCG. The cells were harvested at different time points and the confluence of cells did not exceed 70%.

Preparation of tissue extracts and cell lysates. The frozen tumors or enterocytes were placed into ice-cold lysis buffer [2 mmol/L MgCl₂, 150 mmol/L NaCl, 1% Triton X-100, 10% glycerol, and 1 mmol/L DTT in 50 mmol/L Tris-HCl (pH 7.4)] containing protease and phosphatase inhibitor cocktails (Sigma), *N*-acetyl-Leu-Leu-norleucinal (a proteasome inhibitor, Calbiochem, San Diego, CA), indomethacin (a cyclooxygenase inhibitor, Cayman Chemical, Ann Arbor, MI), and nordihydroguaiaretic acid (a lipoxygenase inhibitor, Sigma) and homogenized by 50 or 30 strokes, respectively, in ice-cold Dounce homogenizer (Wheaton, Millville, NJ). After centrifugation for 10 minutes at $12,000 \times g$, the supernatants were retained as a whole-tissue extract. The HT29 cells were washed with ice-cold PBS twice and then lysed in ice-cold cell lysis buffer [10 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, and 1 mmol/L β -glycerophosphate in 20 mmol/L Tris (pH 7.4)]. The cell lysates were sonicated and then centrifuged at $10,000 \times g$ for 15 minutes at 4°C to remove cell debris and the supernatants were referred as whole-cell lysates. Nuclear and postnuclear fractions (fraction without nuclear fraction) from both tissues and cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce Biotechnology, Lockford, IL). Protease and phosphatase inhibitor cocktails as well as *N*-acetyl-Leu-Leu-norleucinal were added to the extraction buffer from the kit. Low levels ($\sim 10\%$) of cross-contamination between nuclear and postnuclear fractions were found (data not shown).

Western blot analyses. The protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce Chemical). Tissue extracts or cell lysates (denatured at 95°C for 5 minutes in Laemmli sample buffer) containing 20 μg protein were subjected to SDS-PAGE (4–15% gradient, Bio-Rad). Gels were transferred onto polyvinylidene difluoride membranes (Bio-Rad) that were incubated with blocking buffer (Li-Cor Biosciences, Lincoln, NE) for 1 hour at room temperature. Membranes were probed with primary antibody in blocking buffer at 4°C overnight. After washing with TBS thrice, the membranes were incubated with secondary antibodies conjugated to IR fluorophore, Alexa Fluor 680 (Molecular Probes, Eugene, OR), or IRDye 800 (Rockland Immunochemicals, Gilbertsville, PA). Fluorescence was detected with Odyssey Infrared Imaging System (Li-Cor Biosciences). β -Catenin and E-cadherin antibodies were purchased from BD

Bioscience (San Jose, CA). c-Myc, cytosolic phospholipase A₂ (cPLA₂), and cyclin D1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Total or phospho-(Ser473)-Akt, total or phospho-ERK1/2, histone 2A, and histone H3 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Confocal laser scanning microscopy analyses. HT29 cells were rinsed four times with PBS, fixed in methanol for 30 seconds at -20°C, rinsed with PBS, and blocked by incubation with PBS containing 1% bovine serum albumin (BSA) for 1 hour at room temperature. Cells were then washed four times with PBS and incubated with the β-catenin or E-cadherin antibody diluted in PBS containing 1% BSA for 1 hour at room temperature. After repeated washing in PBS, cells were incubated with secondary antibodies for 45 minutes at room temperature, washed, and mounted in VectaShield (Vector Laboratories, Burlingame, CA). Confocal microscopic analyses were done at excitation wavelengths of 488 nm (for FITC) and 543 nm (for tetramethylrhodamine isothiocyanate). Each channel was recorded independently.

Enzyme immunoassay. Tissue extracts were acidified with HCl to pH 2.5, vortexed for 1 minute after adding 1 mL of ethyl acetate, and then centrifuged at 3,000 × g for 5 minutes (Sorvall RT 6000B). The organic layer was collected and then evaporated under N₂. The dried samples were stored at -80°C until analysis. Each sample was reconstituted in 1 mL enzyme immunoassay buffer (Cayman Chemical). Levels of prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄) were measured using an enzyme immunoassay kit (Cayman Chemical).

Statistical analyses. SigmaStat software was used for all statistical analyses. For simple comparisons between two groups, two-tailed Student's *t* test was used. One-way ANOVA combined with appropriate post hoc tests was used for comparisons among multiple groups. Dose response for the inhibition of tumorigenesis by EGCG was determined by Poisson linear and quadratic regression analyses.

Results

Effects of p.o. administration of (-)-epigallocatechin-3-gallate and caffeine on intestinal tumorigenesis in *Apc*^{min/+} mice. In three independent experiments, we evaluated the effects of EGCG and caffeine, the two main constituents in green tea, on intestinal tumorigenesis. As shown in Table 1, p.o. administration of 0.16% or 0.08% EGCG in drinking fluid for 9 or 8 weeks to male or female mice significantly reduced small intestinal tumor formation by 47% or 37%, respectively. The EGCG administration resulted in a more prominent decrease in the number of large-size tumors (>2 mm) than the number of small-size tumors (<1 mm) in the small intestine. Only 0.5 to 0.8 colon tumors per mouse (<4% of total intestinal tumors) were found with large SE, and the colon tumor multiplicity was not affected by EGCG treatment. The overall tumor distribution (ratio among proximal small intestine, distal small intestine, and colon) was also unaffected. In another experiment with female mice, a 6-week p.o. administration of 0.32% EGCG decreased small intestinal tumor numbers by 33% whereas administration of caffeine at a dose of 0.044% in drinking fluid did not have any inhibitory effect. Treatment with 0.044% caffeine resulted in a 21% decrease in omental fat pad weight (175.0 ± 31.4 versus 222.5 ± 23.2 mg per mouse in control *Apc*^{min/+} mice) and a 16% decrease in retroperitoneal fat weight (74.2 ± 11.5 versus 88.4 ± 12.0 mg per mouse in control *Apc*^{min/+} mice). EGCG treatment affected neither omental nor retroperitoneal fat pad weight. In all of these experiments, treatment with EGCG or caffeine did not significantly influence the food and fluid intakes or body weight. The data indicate that EGCG is the active constituent of green tea that possesses an inhibitory activity against intestinal tumorigenesis.

We did a dose-response study with EGCG, covering the concentration range of 0.02% to 0.32%, on the inhibition of intestinal tumorigenesis. As shown in Table 2, 11-week-old control male

Apc^{min/+} mice that did not receive EGCG treatment had 33.0 ± 3.4 tumors in the small intestine and 1.3 ± 0.3 tumors in the colon. The majority of small intestinal tumors were ~1 mm in diameter whereas colon tumors were predominantly >1.5 mm in diameter. EGCG-treated *Apc*^{min/+} mice had 35% to 48% fewer total tumors in the small intestine than untreated control *Apc*^{min/+} mice. Poisson linear and quadratic regression analyses showed significant negative linear (*P* < 0.01) and borderline significant positive quadratic (*P* = 0.05) relationships between small intestinal tumor numbers and percent concentration of EGCG. None of the doses of EGCG influenced food or fluid intake or body weight. No noticeable signs of toxicity were observed in any of the groups.

Some of the samples were subjected to histopathologic analysis. Histologically identified adenomas (Fig. 1) were 58% fewer in 0.32% EGCG-treated *Apc*^{min/+} mice compared with untreated control *Apc*^{min/+} mice [1.3 ± 1.2 (*n* = 12) versus 3.2 ± 1.5 (*n* = 15), *P* < 0.05]. In the 0.32% EGCG-treated group, adenomas and microscopic adenomas were 39% (0.5 ± 0.9 versus 0.9 ± 0.7 in control *Apc*^{min/+} mice) and 66% fewer (0.8 ± 1.4 versus 2.3 ± 1.8 in control *Apc*^{min/+} mice, *P* < 0.05), respectively.

Effect of p.o. administration of (-)-epigallocatechin-3-gallate on β-catenin signaling and E-cadherin protein levels in small intestinal tumors of *Apc*^{min/+} mice. Aberrant β-catenin signaling is a key molecular event in the development of intestinal tumors in *Apc*^{min/+} mice and was the first target of our investigation. For all of the analyses, we concentrated on the comparison between samples from control male *Apc*^{min/+} mice and those from mice treated with the highest dose (0.32%) of EGCG, which showed the strongest inhibition of small intestinal tumor formation. By Western blot analyses, we measured nuclear levels of β-catenin and c-Myc protein in small intestinal tumors and normal small intestine. In our Western blot analyses, normal small intestine from wild-type mice was used as control for the comparison with *Apc*^{min/+} small intestinal tumors. As shown in Fig. 2A, small intestinal tumors exhibited significantly higher levels of both nuclear β-catenin and c-Myc protein compared with normal small intestine. The protein levels of both nuclear β-catenin and c-Myc in small intestinal tumors from EGCG-treated *Apc*^{min/+} mice were significantly lower than those in the untreated controls. These data suggest that the aberrant β-catenin signaling in the tumors was suppressed by EGCG administration. One possible upstream event in the suppression of β-catenin signaling is the up-regulation of E-cadherin protein (26). P.o. administration of EGCG resulted in a significant increase of E-cadherin protein levels in small intestinal tumors (Fig. 2B).

Effect of (-)-epigallocatechin-3-gallate treatment on protein levels of E-cadherin and localization of β-catenin in HT29 human colon cancer cells. We examined whether similar effects of EGCG treatment on E-cadherin and nuclear β-catenin levels in small intestinal tumors could be recapitulated in EGCG-treated colon cancer cells in culture. The HT29 cell line was chosen because these colon carcinoma cells are *Apc*-null and contain wild-type β-catenin protein similar to small intestinal tumors of *Apc*^{min/+} mice. As shown in Fig. 3, treatment of HT29 cells with 12.5 or 20 μmol/L EGCG increased E-cadherin protein levels by 27% to 58%. Treatment with 20 μmol/L EGCG for 24 hours decreased nuclear levels of β-catenin but increased postnuclear levels; the ratio of nuclear to postnuclear β-catenin was significantly reduced whereas the sum of nuclear and postnuclear β-catenin levels was unchanged. EGCG treatment (20 μmol/L for 24 hours) also decreased protein levels of c-Myc and cyclin D1 by 70% and 87%, respectively.

Table 1. Effect of EGCG and caffeine on intestinal tumor formation in *Apc*^{min/+} mice

Group	No. of mice	Final body weight (g)	Small intestinal tumors						Colon tumors	
			Region			Size (mm)				Total (% inhibition)
			Proximal	Middle	Distal	≤1	1-2	≥2		
1. Male <i>Apc</i>^{min/+} mice (5-14 wk of age)										
Control	24	23.2 ± 0.5	5.9 ± 0.8	—	13.8 ± 2.3	11.2 ± 2.2	6.9 ± 1.0	1.6 ± 0.3	19.7 ± 2.8	0.8 ± 0.2
0.16% EGCG	23	24.4 ± 0.4	3.5 ± 0.6*	—	7.0 ± 1.5 [†]	7.5 ± 1.4	2.3 ± 0.4 [‡]	0.7 ± 0.2 [‡]	10.5 ± 1.7 [§] (47%)	0.7 ± 0.1
2. Female <i>Apc</i>^{min/+} mice (7-15 wk of age)										
Control	25	19.0 ± 0.5	6.5 ± 0.7	13.2 ± 1.5	20.0 ± 2.4	18.0 ± 1.7	12.9 ± 0.5	8.8 ± 1.7	39.7 ± 4.1	0.5 ± 0.1
0.08% EGCG	23	19.5 ± 0.4	4.7 ± 0.5	9.9 ± 1.8	10.6 ± 2.1 [†]	13.0 ± 0.9	8.3 ± 1.7	3.8 ± 0.8 [†]	25.2 ± 4.1 [†] (37%)	0.5 ± 0.1
3. Female <i>Apc</i>^{min/+} mice (5-11 wk of age)										
Control	19	17.9 ± 0.5	6.9 ± 1.0	—	22.1 ± 3.6	—	—	—	28.9 ± 3.7	0.7 ± 0.2
0.044% caffeine	13	18.0 ± 3.2	8.5 ± 1.2	—	22.4 ± 3.8	—	—	—	30.8 ± 4.7	0.8 ± 0.3
0.32% EGCG	14	18.1 ± 0.5	5.1 ± 1.0	—	14.3 ± 3.5	—	—	—	19.4 ± 4.2 (33%)	0.8 ± 0.3

NOTE: Three independent experiments (1, 2, and 3) were done with an indicated dose of EGCG or caffeine solution given as the sole source of drinking fluid to the mice. Each value represents mean ± SE. In experiments 1 and 3, small intestine was divided into only two segments (proximal and distal). In experiment 3, the tumor size was not scored because the majority of tumors in 11-week-old mice was ~1 mm in diameter.

**P* < 0.05, statistically different from the value of control group in the column (two-tailed *t* test).

[†]*P* < 0.02, statistically different from the value of control group in the column (two-tailed *t* test).

[‡]*P* < 0.0005, statistically different from the value of control group in the column (two-tailed *t* test).

[§]*P* < 0.001, statistically different from the value of control group in the column (two-tailed *t* test).

To determine possible colocalization of β-catenin with E-cadherin in the plasma membrane, we did confocal laser microscopic analyses. As shown in Fig. 4, in control cells, β-catenin (red) was predominantly localized in the nuclear membrane and also found in both the nucleus and cytoplasm whereas the majority

of E-cadherin (green) was located in the plasma membrane. Treatment of cells with 20 μmol/L EGCG for 24 hours reduced nuclear membrane levels of β-catenin but increased plasma membrane levels of β-catenin. Moreover, β-catenin was apparently colocalized in the plasma membrane with E-cadherin. These data

Table 2. Effect of EGCG on intestinal tumor formation in *Apc*^{min/+} mice

Group	No. of mice	Final body weight (g)	The ratio of body fat to final body weight (mg/g)		Small intestinal tumors			Colon tumors
			Omental	Retroperitoneal	Proximal	Distal*	Total* (% inhibition)	
Control	28	21.9 ± 1.8	12.3 ± 1.1	2.4 ± 0.4	8.4 ± 1.1	24.6 ± 3.1	33.0 ± 3.4	1.3 ± 0.3
0.02% EGCG	13	23.4 ± 1.6	13.2 ± 1.5	2.6 ± 0.5	6.8 ± 1.0	14.2 ± 2.3	21.0 ± 2.9 (36%)	0.8 ± 0.2
0.04% EGCG	10	23.2 ± 1.6	14.8 ± 1.6	3.1 ± 0.6	5.4 ± 1.0	16.0 ± 4.4	21.4 ± 5.1 (35%)	0.6 ± 0.3
0.08% EGCG	20	23.3 ± 1.3	16.0 ± 1.0	3.3 ± 0.3	5.9 ± 0.6	14.0 ± 2.8 [†]	19.9 ± 2.9 [†] (40%)	1.0 ± 0.2
0.16% EGCG	10	23.0 ± 3.0	15.6 ± 1.3	3.0 ± 0.5	7.1 ± 2.2	12.0 ± 3.0	19.1 ± 5.0 (41%)	1.4 ± 0.4
0.32% EGCG	25	22.9 ± 1.7	14.9 ± 2.7	2.7 ± 0.3	5.4 ± 0.9	11.7 ± 2.0 [‡]	17.0 ± 2.5 [§] (48%)	0.8 ± 0.2

NOTE: Five-week-old male *Apc*^{min/+} mice on AIN93G diet were randomized into six groups, and 0%, 0.02%, 0.04%, 0.08%, 0.16%, or 0.32% EGCG was given as the sole source of drinking fluid for 6 weeks until the experiment was terminated at 11 weeks of age. Each value represents mean ± SE. The number of mice was accumulated from four different experiments that were done with different groups and different numbers of mice per group (the accumulation was done through a statistical adjustment; the effects of two factors, *treatment* and *experiment*, as well as the interaction of *treatment* and *experiment*, on the response variable, *tumor numbers*, were initially assessed by two-way ANOVA; neither the factor of *experiment* nor the interaction was found to affect the tumor number significantly; they were excluded in the final statistical analyses).

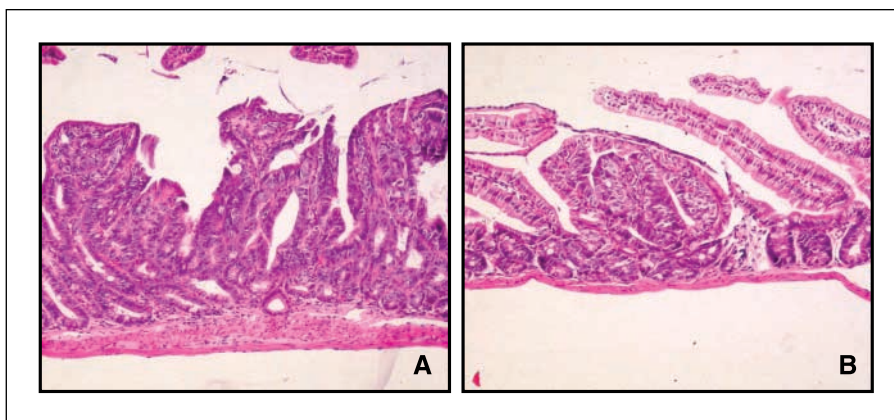
*A significant negative linear relationship (*P* < 0.01) and a positive quadratic relationship (*P* = 0.05) were found between percent EGCG and distal or total small intestinal tumor number (a linear-quadratic Poisson regression model).

[†]*P* < 0.02, statistically different from the value of control group in the column (two-tailed Student's *t* test).

[‡]*P* < 0.001, statistically different from the value of control group in the column (two-tailed Student's *t* test).

[§]*P* < 0.0005, statistically different from the value of control group in the column (two-tailed Student's *t* test).

Figure 1. Histopathologic identification of small intestinal tumors ($\times 10$ magnification). *A*, a tubular adenoma containing 10 crypts. *B*, a microscopic adenoma containing 4 crypts.



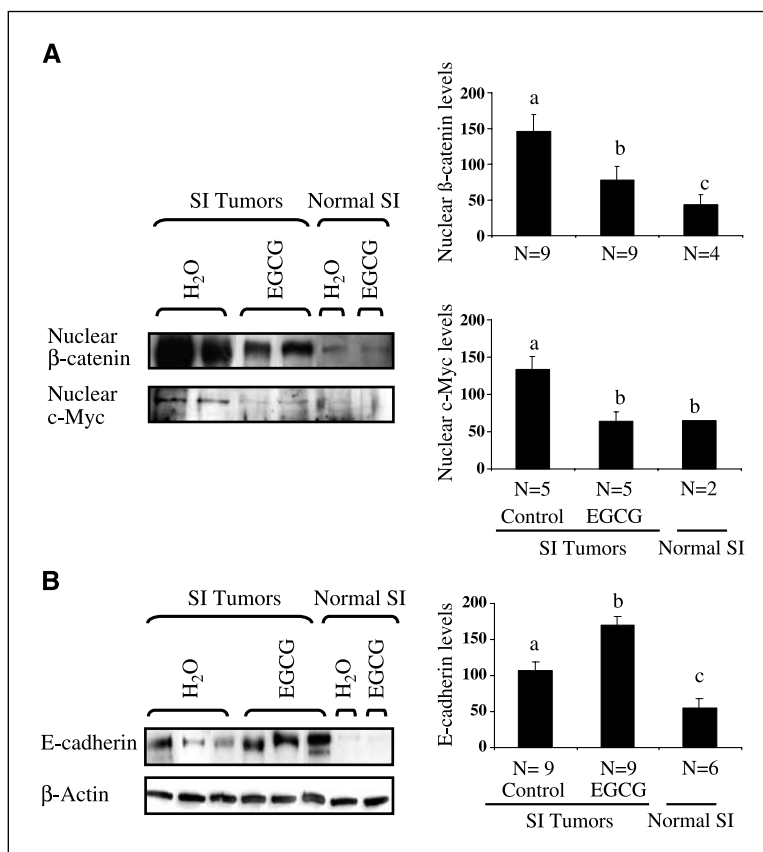
are consistent with the view that EGCG treatment increased E-cadherin protein levels, increased E-cadherin- β -catenin complex formation at the plasma membrane, and prevented β -catenin from localizing into the nucleus.

Effect of p.o. administration of (–)-epigallocatechin-3-gallate on phosphorylation of Akt and extracellular signal-regulated kinase 1/2 in tumors. Total Akt protein levels in small intestinal tumors were significantly higher than those in normal small intestine and levels of Akt phosphorylation (at Ser473) were dramatically elevated (>35 -fold; Fig. 5A). P.o. administration of EGCG markedly suppressed phosphorylation of Akt (Ser473) in small intestinal tumors without significantly altering total levels of Akt protein. Phosphorylation of ERK1/2 was significantly in-

creased in small intestinal tumors as compared with that in normal small intestine although total levels of ERK1/2 protein were not increased in small intestinal tumors (Fig. 5B). P.o. administration of EGCG resulted in significantly decreased phosphorylation of ERK1/2 protein in small intestinal tumors. The data suggest that p.o. administration of EGCG effectively suppressed both Akt and ERK1/2 signaling cascades by inhibiting their phosphorylation.

Effect of p.o. administration of (–)-epigallocatechin-3-gallate on cytosolic phospholipase A₂, prostaglandin E₂, and leukotriene B₄ levels in tumors. As shown in Table 3, protein levels of cPLA₂ were significantly higher in small intestinal tumors than in normal small intestine. PGE₂ and LTB₄ levels in small intestinal tumors were elevated as compared with those in normal

Figure 2. Effects of p.o. administration of EGCG on β -catenin signaling and E-cadherin protein levels in small intestinal tumors and normal small intestine. Western blot analyses of representative samples for each group (*A*, nuclear fraction of cell lysates; *B*, total cell lysates). Protein levels were quantified by using Adobe Photoshop software (normalized by levels of a loading control protein, β -actin). Columns, mean values (in arbitrary units) of the number of mice (*N*) analyzed; bars, SE. Values of columns with different superscripts differ statistically ($P < 0.05$, one-way ANOVA).



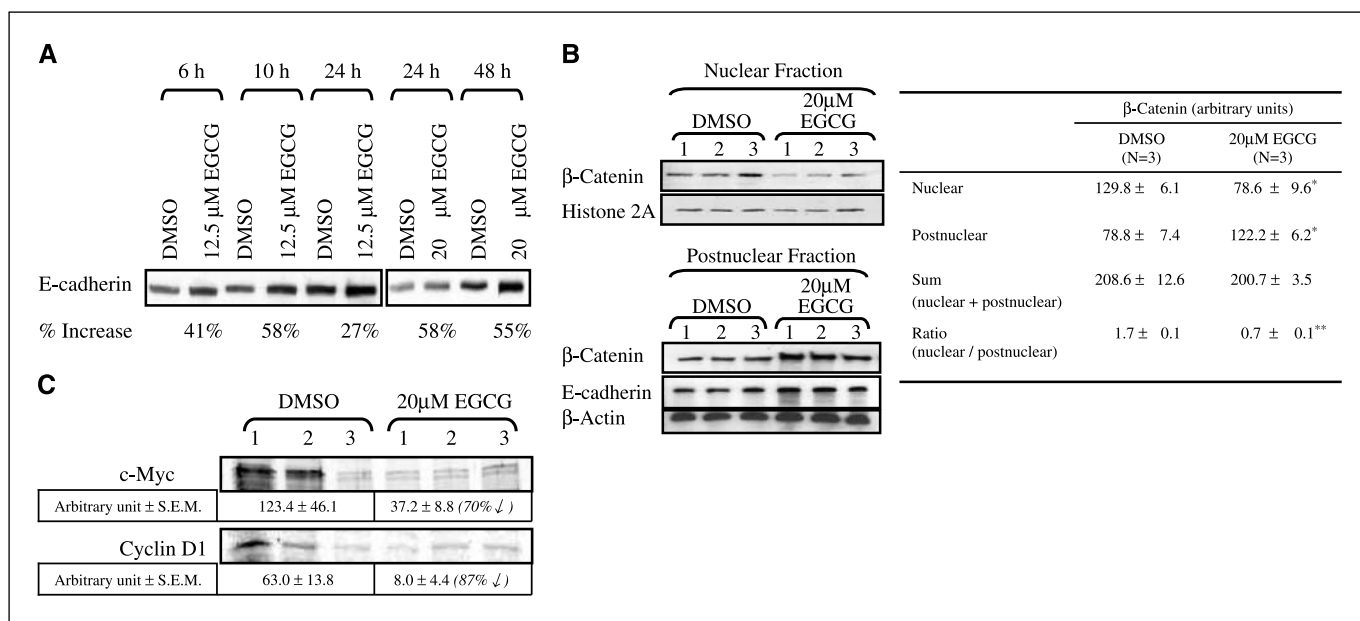


Figure 3. Effects of EGCG treatment on E-cadherin, β -catenin, c-Myc, and cyclin D1 in HT29 human colon cancer cells. Cells were treated with 12.5 or 20 μ mol/L EGCG in the presence of superoxide dismutase (5 units/mL) and catalase (30 units/mL) for different time periods. **A**, Western blot analyses of E-cadherin; percent increase of protein levels in EGCG-treated cells from the corresponding DMSO-treated control cells (after normalization by levels of β -actin). **B**, Western blot analyses of β -catenin and E-cadherin in the nuclear and postnuclear fractions of cell lysates. β -Catenin protein levels (nuclear levels normalized by histone 2A and postnuclear levels normalized by β -actin) were quantified. Values (in arbitrary units) are mean \pm SE of three determinations. Values with * or ** are statistically different from the corresponding values of the DMSO-treated control (two-tailed Student's *t* test; *, $P < 0.02$; **, $P < 0.005$). **C**, Western blot analyses of c-Myc and cyclin D1 using cells treated with DMSO or 20 μ mol/L EGCG in the presence of superoxide dismutase (5 units/mL) and catalase (30 units/mL) for 24 hours. Percent decrease of protein levels in EGCG-treated cells from the corresponding DMSO-treated control cells.

small intestine (16- and 1.6-fold, respectively). P.o. administration of EGCG decreased cPLA₂ protein and PGE₂ levels in small intestinal tumors by 23% and 58%, respectively. Statistical significance, however, was not reached due to large variations among samples within a group. LTB₄ levels in the tumors were not affected by the EGCG administration.

Effects of short-term treatment with (-)-epigallocatechin-3-gallate on phosphorylation of extracellular signal-regulated kinase 1/2 in small intestinal tumors of *Apc*^{min/+} mice. To observe the direct effects or early events after EGCG treatment, 14-week-old female *Apc*^{min/+} mice were given a single dose of 75 mg/kg EGCG intragastrically and were sacrificed 3 hours later. After the administration, levels of EGCG in small intestine

homogenates were measured (44) and found to be \sim 34 μ mol/L. As expected, the treatment did not affect the number and size of tumors in small intestine and colon. Small intestinal tumors from EGCG-treated mice had significantly lower phosphorylation levels of ERK1/2 when compared with control mice (Fig. 6). The treatment, however, did not significantly affect the levels of E-cadherin, nuclear β -catenin, and Akt phosphorylation.

Discussion

Although the effectiveness of green tea and tea polyphenols in inhibiting intestinal/colon tumorigenesis was shown in several animal models (3, 8), it is unclear which constituents of green tea

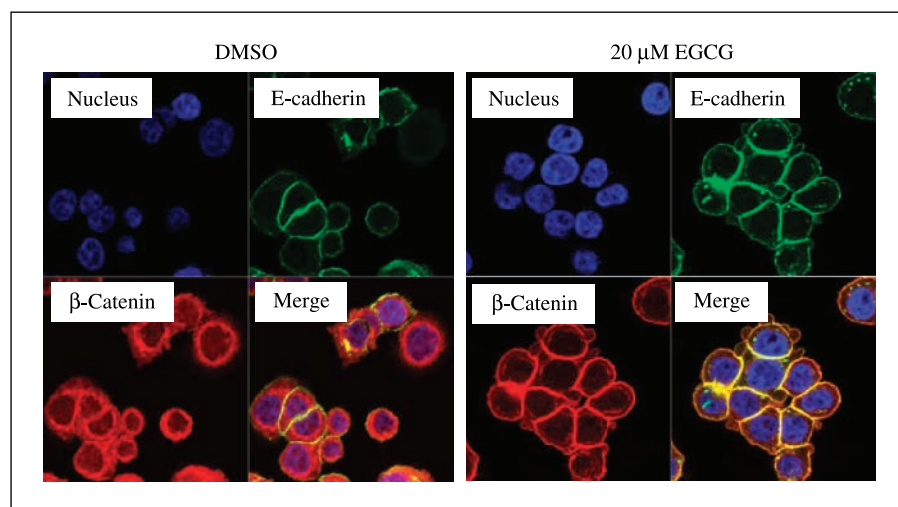
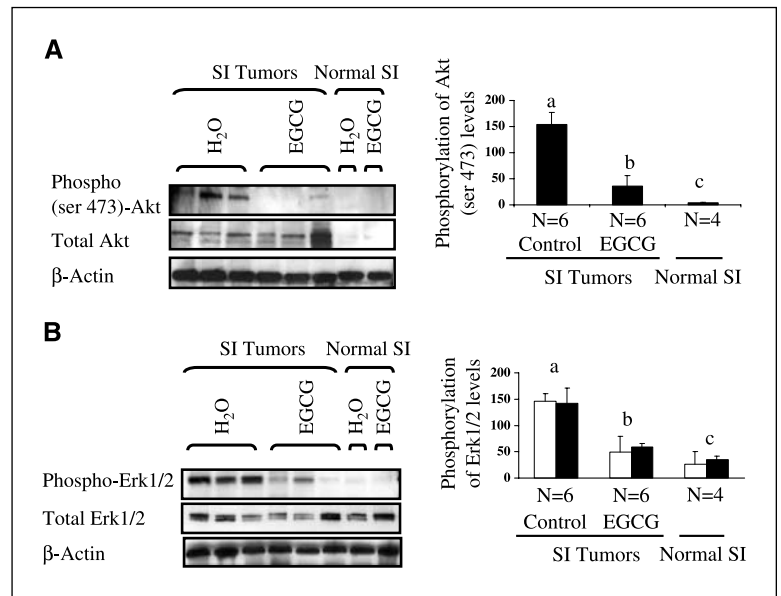


Figure 4. Effect of EGCG treatment on localization of β -catenin in HT29 human colon cancer cells. Confocal laser scanning microscopy analyses of cells treated with DMSO or 20 μ mol/L EGCG in the presence of superoxide dismutase (5 units/mL) and catalase (30 units/mL) for 24 hours. Red, green, or blue immunofluorescence is due to FITC-, tetramethylrhodamine isothiocyanate-, and 4',6'-diamidino-2-phenylindole-conjugated secondary antibodies used for the staining of β -catenin, E-cadherin, and nucleus, respectively.

Figure 5. Effect of p.o. administration of EGCG on levels of phospho-(Ser473)-Akt and phospho-ERK1/2 in small intestinal tumors and normal small intestine. *A* and *B*, Western blot analyses of representative samples for each group. Phosphorylated levels of Akt or ERK1/2 were quantified by using Adobe Photoshop software (normalized by both β -actin and total protein, Akt or ERK1/2, levels). *Columns*, mean values (in arbitrary units) of the number of mice (*N*) analyzed; *bars*, SE. Values of columns with different superscripts differ statistically ($P < 0.05$, one-way ANOVA).



confer the inhibitory activity. In the present study, two main constituents in green tea, EGCG and caffeine, were evaluated for their possible antitumorigenic activities. We found that EGCG in drinking fluid in the range of 0.02% to 0.32% dose-dependently inhibited small intestinal tumorigenesis in *Apc^{min/+}* mice (significant negative linear relationship, $P < 0.01$). The borderline significant ($P = 0.05$) positive quadratic relationship, however, indicates a saturation phenomenon of the inhibition as the concentration of EGCG increases. Caffeine at a dose of 0.044% did not exert any inhibitory effect (Tables 1 and 2). The dose of 0.044% caffeine is in the range of caffeine present in 2% green tea (2 g of tea brewed in 100 mL of hot water), which contains ~0.16% EGCG. At a dose of 0.16%, EGCG exerted 47% inhibition of small intestinal tumor formation (Table 1). It has been reported that 1.5% green tea and 0.1% green tea extract inhibited small intestinal tumor formation in *Apc^{min/+}* mice by 50% and 22%, respectively (6, 7). The amounts of EGCG that were present in these tea extracts were estimated to be 0.3% and 0.01%, respectively, and the extent of inhibition is comparable to what we observed. The activities of other catechins, such as (–)-epicatechin-3-gallate, (–)-epigallocatechin, and (–)-epicatechin, need to be evaluated in the future.

In our animal experiment, we added 0.5% citric acid to EGCG solution, which acidified the fluid to pH 2.7 and enhanced the stability of EGCG at low concentrations (such as 0.08%). Without the addition of citric acid, 0.32% EGCG solution was stable but the mice tended to drink less due to the bitter taste. The addition of 0.5% citric acid masked the bitterness of EGCG at high concentrations (such as 0.32%) and the mice drank the normal volume of fluid.

In our previous studies, administration of green tea was shown to result in body weight and body fat reductions in mice but it is unclear which tea constituents are responsible for this effect (45). The present study indicated that in *Apc^{min/+}* mice, caffeine decreased both omental and retroperitoneal fat pad weights whereas EGCG did not produce such a change. The body fat-lowering effect of caffeine has been reported by Lu et al. (46) in studies on the inhibition of skin carcinogenesis by tea.

Many mechanisms for anticarcinogenic activities of green tea and tea polyphenols have been proposed (reviewed in ref. 17). The activities observed with polyphenols *in vitro*, however, may not be translated to the situation *in vivo* because the polyphenol concentrations used in cell line studies are usually much higher than the achievable levels *in vivo* due to the low bioavailability of tea polyphenols. Therefore, it is important to investigate the

Table 3. Effect of p.o. administration of EGCG on cPLA₂, PGE₂, and LTB₄ levels

	Small intestinal tumors		Normal small intestine
	Control	EGCG	
cPLA ₂ (arbitrary units)	179.1 ± 9.8 ^a (9)	137.5 ± 29.3 ^{ab} (7)	98.7 ± 6.7 ^b (5)
PGE ₂ (ng/mg protein)	9.6 ± 3.3 (9)	4.0 ± 1.4 (8)	0.6 ± 0.1 (6)
LTB ₄ (pg/mg protein)	506.1 ± 178.9 (9)	528.7 ± 187.0 (8)	296.4 ± 171.0 (6)

NOTE: Protein levels of cPLA₂ (by Western blot analyses) were quantified by using Adobe Photoshop software (normalized by β -actin). Both values of cPLA₂ (in arbitrary units) and values of PGE₂ and LTB₄ (by enzyme immunoassay) are mean ± SE of the number of mice (*N*) analyzed. Values with different superscripts differ statistically ($P < 0.05$, one-way ANOVA).

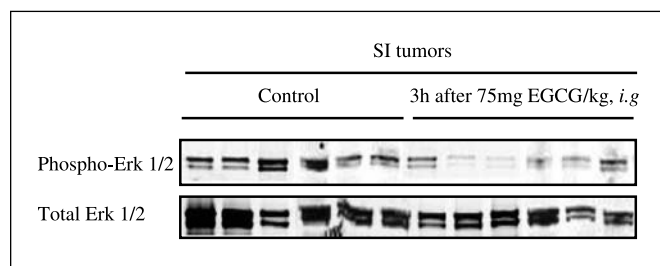


Figure 6. Effect of short-term treatment with EGCG on phosphorylation of ERK1/2. Western blot analyses of six small intestinal tumor samples (six mouse samples) per group for phosphorylated and total ERK1/2.

mechanisms of the inhibitory action of EGCG *in vivo*. The present study is the first report demonstrating that EGCG suppressed the nuclear levels of β -catenin and aberrant β -catenin signaling *in vivo* and this was accompanied by the up-regulation of E-cadherin protein levels (Fig. 2). The adhesion protein E-cadherin is a well-recognized tumor and invasion suppressor that plays a crucial suppressive role in the transition from adenoma to carcinoma in several epithelial cancers, including colorectal cancers (47). A similar increase in E-cadherin protein levels was observed *in vitro* following treatment of HT29 cells with EGCG and this was accompanied by the translocation of β -catenin from the nuclei to the cytoplasm and plasma membrane (Figs. 3 and 4). EGCG treatment of HT29 cells also decreased protein levels of c-Myc and cyclin D1 (Fig. 3). Suppression of β -catenin signaling and an associated increase in E-cadherin levels have been reported to account for the chemopreventive activities of vitamin D, calcium, indole-3-carbinol, and tangeretin (48–51). Green tea, white tea, and EGCG inhibited β -catenin-mediated T-cell factor-4 transcriptional activity in a luciferase reporter assay (52).

The elevation of E-cadherin protein levels by EGCG might be mediated by an attenuation of its transcriptional repression (via decreases in the expression of the slug/snail zinc finger protein family, the transcriptional repressors of E-cadherin) or posttranscriptional modification, including a decrease in the internalization levels (via changes in levels of caveolin-1, a protein that plays an important role in the endocytosis of E-cadherin; refs. 53–56). Further research is required to determine the mechanisms involved in the increase of E-cadherin levels caused by EGCG.

We detected higher protein levels of E-cadherin in small intestinal tumors than in normal small intestine (Fig. 2), which is similar to earlier findings by Carothers et al. (35). The up-regulation of E-cadherin in small intestinal tumors seems to reflect an augmented adherens junction by tight cell-cell contacts in adenomas that were found in *Apc*^{min/+} mice. Although the loss of E-cadherin is a common feature in colorectal carcinomas or invasive colorectal tumors, E-cadherin expression is often high in colorectal adenoma (57).

In our Western blot analyses, normal small intestines from wild-type mice, instead of normal-looking small intestines from *Apc*^{min/+} mice, were used as controls for the comparison with *Apc*^{min/-}

small intestinal tumors to avoid impurity within the control. The normal-looking small intestines from *Apc*^{min/+} mice were found to contain a significant number of microadenomas, which implies that they contain a mixture of tumor cells and normal cells although normal cells may be predominant.

Total levels of Akt protein in small intestinal tumors were significantly up-regulated as compared with those in normal small intestine (Fig. 5A), which was consistent with a previous report by Moran et al. (39). We found that p.o. administration of EGCG dramatically decreased phosphorylation levels of Akt and ERK1/2 (Fig. 5). It needs to be further determined whether these effects were due to the direct inhibition of the kinases responsible for the phosphorylation of Akt and ERK1/2 or the inhibition of upstream events such as the activation of epidermal growth factor receptor, a well-known upstream signal for both phosphoinositide-3-kinase-Akt and Ras-ERK1/2-AP pathways.

cPLA₂ is a key enzyme in releasing the arachidonic acid present in plasma and endoplasmic reticulum membrane. PGE₂ and LTB₄ are two major metabolites in arachidonic acid metabolism, which are produced via COX- and 5-lipoxygenase-dependent pathways, respectively (58). Although cPLA₂ protein levels in small intestinal tumors were higher than those in normal intestine, statistically significant increases in PGE₂ and LTB₄ levels in the tumors were not found (Table 3). PGE₂ production is shown to be accelerated as the tumor size is expanded to larger than 1 mm in diameter (59); the tumor samples we analyzed contained few tumors larger than 1 mm in diameter, which seems to be the reason that significantly elevated levels of PGE₂ were not observed. We found a trend of decreases in cPLA₂ protein and PGE₂ levels due to p.o. administration of EGCG but the results were not statistically significant.

In summary, we found that EGCG inhibited intestinal tumorigenesis in a dose-dependent manner at a dose range of 0.02% to 0.32% in drinking fluid whereas caffeine did not exert any antitumorigenic activity. EGCG effectively inhibited aberrant β -catenin signaling and suppressed activated Akt and ERK signaling cascades in *Apc*^{min/+} mice. These actions may contribute to the cancer chemoprevention efficacy of EGCG. Our data from the short-term treatment with EGCG (Fig. 6) suggest that suppression of activated levels of ERK1/2 is a relatively early event after EGCG administration; however, the results are still preliminary in nature due to large variations among samples. Further studies are needed to better characterize the cancer preventive activity of EGCG.

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Inhibition of Intestinal Tumorigenesis in *Apc^{min/+}* Mice by (–)-Epigallocatechin-3-Gallate, the Major Catechin in Green Tea

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