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(+)-Catechin Inhibits Intestinal Tumor Formation and Suppresses Focal Adhesion Kinase Activation in the Min/+ Mouse[1]

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ABSTRACT

Colorectal cancer is sensitive to dietary influences. Epidemiological data linking high intake of fruits and vegetables to decreased cancer risk have prompted the search for specific plant constituents implicated in tumor prevention. This task is difficult because of the complex chemical composition of plant foods and the multifactorial nature of carcinogenesis. Researchers are aided in this effort by the C57BL/6J-Min/+ (Min/+) mouse, an animal bearing a germline defect in *Apc* that is similar to the initiating genetic event in the majority of human colorectal cancers. In this study, we treated Min/+ mice with (+)-catechin, a phenolic antioxidant abundant in certain fruits. Administration of (+)-catechin in an AIN-76A diet at doses of 0.1 and 1% decreased the intestinal tumor number by 75 and 71%, respectively. Mechanistic studies linked this effect to (+)-catechin-induced changes in integrin-mediated intestinal cell-survival signaling, including structural alteration of the actin cytoskeleton and decreased focal adhesion kinase (FAK) tyrosine phosphorylation. Immunoblot analysis of small intestine scrapings from Min/+ mice and $Apc^{+/+}$ wild-type C57BL/6J littermates together with excised Min/+ adenomas showed increased expression of phosphorylated FAK in the macroscopically normal enterocytes of untreated Min/+ mice and adenomas. Confirming the relevance of this signaling pathway, treatment of Min/+ mice with (+)-catechin reduced the expression of phosphorylated FAK to a level similar to the wild-type littermate controls. Thus, the natural abundance and favorable bioavailability of (+)-catechin make it a promising addition to the list of potential colorectal cancer chemopreventive agents.

INTRODUCTION

The consumption of fruits and vegetables is inversely related to the risk of colorectal cancer (1) and colorectal adenomas, the precursor lesions of this disease (2). Individuals whose consumption of fruits and vegetables falls within the highest quintile exhibit a 2-fold reduction in adenoma and colon cancer risk (1, 3). The association of decreased adenoma prevalence with fruit consumption is somewhat less consistent than with vegetables; however, the adjusted odds ratio for the highest *versus* the lowest quintile of intake for fruits, including apples, grapes, and raisins, is ~0.65 (95% confidence interval, 0.4–1.05; Ref. 4). Fruits and vegetables contain a number of constituents associated with colorectal cancer prevention (4), such as antioxidant vitamins and numerous micronutrients, including the plant phenolics (5). Fruits and vegetables also contain dietary fiber. The beneficial constituents that prevent colon cancer in plant foods are probably the vitamins and micronutrients and not the fiber, as recent epidemiological data failed to show an association of fiber consumption and reduced colon cancer risk (6, 7). The fact that a diet high in fruits and vegetables is associated with a lower risk of both adenomas and cancer suggests that the responsible agents act early in the adenoma-carcinoma sequence. This observation does not exclude the possibility that these agents have additional tumor-inhibitory activities late in carcinogenesis.

A number of studies in both cell culture and animal cancer models support a role for plant phenolics in colorectal cancer prevention. Plant phenolics are divided into three categories based on their chemical structure: the simple phenols and phenolic acids, hydroxycinnamic acid derivatives, and flavonoids (8). These agents inhibit carcinogenesis in the initiation, promotion, and progression stages. For example, plant phenolics prevent tumor initiation because they reduce levels of carcinogen-DNA adducts in chemically treated cells and they suppress the metabolic activation of carcinogens by inhibiting phase I monooxygenases (8). Examples of inhibitory effects on promotion by plant phenolics include studies showing that they scavenge free radicals, induce the transcription of phase II detoxifying enzymes, and reduce the expression of ornithine decarboxylase (9–11). Finally, plant phenolics inhibit cell proliferation and induce cell death or differentiation in tumor cells, suggesting that they may antagonize later phases of carcinogenesis (8, 12–14).

Flavonoids constitute the most important single group of dietary phenolics and include catechins, proanthocyanins, anthocyanidins, flavones, and flavonols and their glycosides. These compounds are abundant in plant foods, and a typical fruit serving of 200 g contains ~50–500 mg of mixed flavonoids. Catechins are a class of flavonoids with potent antioxidant and cancer chemopreventive properties. These compounds are found in a variety of plants and are present in particularly high amounts in tea leaves, where they may constitute up to 30% of dry leaf weight (6). High levels of monomeric (+)-catechin (Fig. 1) are found in the skins and seeds of fruit such as apples and grapes (15). Red wines and chocolate also are significant sources of (+)-catechin (16, 17). In the case of red wine, the plant phenols, including (+)-catechin, are extracted from the skins of grapes and concentrated during fermentation.

Flavonoids, including (+)-catechin, are potent antioxidants that efficiently scavenge a variety of free radicals (18–20). Studies of the biological effects of (+)-catechin in cell culture and *in vivo* indicate that this compound can also chelate transition metals (21) and inhibit lipid peroxidation (22, 23). The consumption of fruits and vegetables containing (+)-catechin reduced levels of the oxidized base, 8-hydroxydeoxyguanosine, in the DNA of lymphocytes and reduced levels of the oxidized lipid, malondialdehyde, in the urine of humans in an interventional diet trial (24). In keeping with the ability of phenolic antioxidants to induce the expression of phase II detoxifying enzymes in mammalian cells, (+)-catechin produces antimutagenic effects (25). Finally, flavonoids possess anti-inflammatory activity and alter arachidonic acid metabolism through inhibition of both lipoxygenase and cyclooxygenase pathways (26, 27).

One of the most important activities of (+)-catechin may be the modulation of epithelial cell migration (28). (+)-Catechin binds to laminin, an ECM[3] protein, and interferes with cell adhesion, cell

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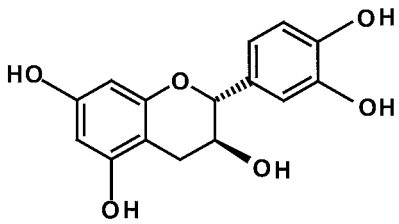
[3] The abbreviations used are: ECM, extracellular matrix; NSAID, nonsteroidal anti-inflammatory drug; CAPE, caffeic acid phenethyl ester; FAK, focal adhesion kinase; DMEM:F-12, DMEM:Ham's F-12; FBS, fetal bovine serum; IP, immunoprecipitation; IB, immunoblot; APC, adenomatous polyposis coli.



(+)-catechin

Fig. 1. Chemical structure of (+)-catechin.

spreading, and protease binding. Pretreatment of a laminin substrate with (+)-catechin led to decreased adhesion and spreading of virus-transformed fetal mouse cells (28). The catechins present in tea, such as epigallocatechin-3-gallate, block tumor cell invasion by inhibiting urokinase and tumor angiogenesis, unfortunately at levels far greater than those achievable by drinking tea (29). Recently, however, epigallocatechin-3-gallate was found to suppress the activity of the matrix metalloproteinases MMP-2 and MMP-9 in HT1080 fibrosarcoma cells with IC_{50}s of 20 and 50 μM, respectively (30). This effect, observed at levels achievable in humans through ingestion of moderate amounts of green tea, was associated with inhibition of invasion through Matrigel (30). Thus, these findings suggest that catechins may prevent cancers by modulating early changes in stromal interactions with initiated cells and, possibly, late changes associated with tumor cell invasion.

Very few data exist on the ability of flavan-3-ols such as (+)-catechin to inhibit carcinogenesis *in vivo*. Although the epicatechins found in tea extracts did not inhibit carcinogen-induced colon tumors in rats (31), tumor formation was delayed in mice fed a diet containing red wine solids (32). Previous studies showed that adenoma formation in Min/+ mice is inhibited by NSAIDs such as sulindac, piroxicam, and aspirin (33–36), and the hydroxycinnamic acid derivatives, CAPE and curcumin (37). This effect may be associated with inhibition of integrin-mediated signaling from the ECM as evidenced by a reduction of the tyrosine phosphorylation state of FAK *in vitro* (38). Administration of the flavonoid, quercetin, or its more readily absorbed glycoside, rutin, did not alter tumor formation in the Min/+ mouse, even when administered at a level of 2% by weight in a standard diet (37). These mixed results suggest that individual compounds of this category may have tissue-specific and/or tumor-specific chemopreventive activities.

Here, we report that treatment of Min/+ mice with diets containing (+)-catechin inhibited intestinal tumor formation with efficacy only moderately lower than that produced by sulindac (36, 37). We also found that *in vitro* exposure of human colon carcinoma cells to (+)-catechin inhibited the tyrosine phosphorylation of FAK and induced changes that alter actin localization and the invasive phenotype. Finally, we found that the relative levels of FAK phosphorylated at Tyr^{397} (FAK-p-Y397) were increased in macroscopically normal enterocytes and adenomas from untreated Min/+ mice when compared with enterocytes from wild-type littermates, and that these defects were corrected in the (+)-catechin-treated Min/+ animals. Our results suggest that (+)-catechin may be a safe, alternative to NSAIDs for the prevention of colorectal adenomas and cancer.

MATERIALS AND METHODS

Materials. (+)-Catechin was purchased from Fluka Chemicals (Ronkonkama, NY). DMEM:F-12 medium (1:1, v/v), FBS, and calf serum were purchased from Life Technologies (Gaithersburg, MD). Rhodamine-conjugated phalloidin was purchased from Molecular Probes (Eugene, OR). Antibody against FAK (clone 77, mouse IgG1) was purchased from Transduction Laboratories (San Diego, CA). The phosphospecific rabbit antibody for FAK-p-Y397 was obtained from Biosource International (Camarillo, CA). Antiphosphotyrosine antibody (clone 4G10, mouse $IgG_{2b\kappa}$) was from Upstate Biotechnology (Lake Placid, NY). Anti-β-actin (clone AC-40; mouse IgG_{2a}) was from Sigma (St. Louis, MO). IPs were performed using the Boehringer Mannheim Protein A kit (Roche Molecular Biochemicals, Indianapolis, IN). Biotinylated antimouse and antirabbit antibodies and Vectashield mounting solution were from Vector Laboratories, Inc. (Burlingame, CA). Streptavidin-horseradish peroxidase was obtained from PharMingen. The micro BCA protein assay reagent kit was purchased from Pierce (Rockford, IL). IB analyses used Optitran nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Electrotransfer of proteins used the electroblot buffers of Owl Separation Systems (Woburn, MA). Matrigel invasion chambers were purchased from Becton Dickinson Labware (Bedford, MA), and hematoxylin 2 was obtained from Richard-Allan Scientific (Kalamazoo, MI). Western blotting used the ECL detection reagents of Amersham Pharmacia Biotech (Piscataway, NJ).

Animal Treatments and Tissue Harvesting. Five-week-old female Min/+ mice and their wild-type littermates were obtained from The Jackson Laboratory (Bar Harbor, ME). After arrival, the littermates were evenly distributed among the treatment groups, and treatment with study diets was started immediately thereafter. Wild-type C57BL/6J and untreated Min/+ control groups were fed AIN-76A chow and given tap water to drink *ad libitum*. Treatment groups were fed AIN-76A pelleted with either 0.1% or 1.0% (+)-catechin (Research Diets, New Brunswick, NJ). The animals were routinely checked for signs of anemia or bowel obstruction and weighed weekly to assure similar growth and food intake among groups.

At 110 days of age, all mice were again weighed, rendered moribund by CO_2 inhalation, and euthanized by cervical dislocation. The intestine of each animal was removed from stomach to distal rectum. Each intestine was opened along its entire length, flushed with cold PBS, and examined under a direct bright light and by transillumination. The intestinal tumors were then counted by an individual blinded to the animal's genetic or treatment status. Several tumors from all regions of the small intestine (duodenum, jejunum, and ileum) were excised from each animal, as well as segments of tumor-free duodenum, small bowel, and colon. These tissues were snap frozen and stored separately in liquid nitrogen. Enterocyte samples for immunoblotting were obtained by mechanical dissociation, using the edge of a glass microscope slide (39), and washed twice in cold PBS prior to storage at −70°C. Tissues used for enterocyte collections were macroscopically free of tumors. However, the possibility of some microadenoma contamination cannot be excluded in the case of samples obtained from Min/+ mice. Enterocytes prepared in this manner also contain lamina propria and associated fibroblasts. This method, however, is best suited for studying stromal-epithelial cell interactions (40). For consistency, all enterocyte samples were collected from 4-cm segments of the proximal small intestines.

Cell Culture and Treatment Conditions. The sources for human colon carcinoma cell lines DLD-1 and HT-29 and rodent NIH3T3 cells were as described (38). Cultures were maintained in DMEM:F-12 medium supplemented with 10% FBS and cultured at 37°C in a humidified 5% CO_2 incubator. Cells in log-phase growth (~70% confluent) were treated with (+)-catechin in fresh medium containing 0.1% FBS. Unless otherwise indicated, the treatment time was 24 h. Growth curves were prepared by plating 10^5 cells and allowing them to attach and expand for 48 h prior to treatment with (+)-catechin (125 μM). Cells were washed and trypsinized, and the number of viable, attached cells was counted using a hemocytometer at timed intervals.

IB Analysis and IP. Procedures and buffers for cell lysis, protein determination, IP, and IB analysis were exactly as described previously (38). All IP and IB experiments were repeated separately at least twice. Samples from two mice of the same treatment group were pooled to prepare the protein extracts.

Fluorescent Histochemistry. DLD-1 and HT-29 colon cancer cells (5×10^3) were seeded into 4-well Lab-Tek chamber slides (Nunc, Inc., Naperville, IL) and incubated for 48 h prior to treatments. The medium was aspirated and replaced with DMEM:F-12 containing 0.1% FBS with or without (+)-catechin (125 μM) or genistein (100 μM). Treated cultures were incubated an additional 24 h and then fixed in 4% PBS-buffered formaldehyde for 20 min. After repeated washings in PBS, slides were placed in 50 mM NH_4Cl for 15 min and in 0.2% Triton X-100 for 10 min. Blocking was for 30 min in PBS

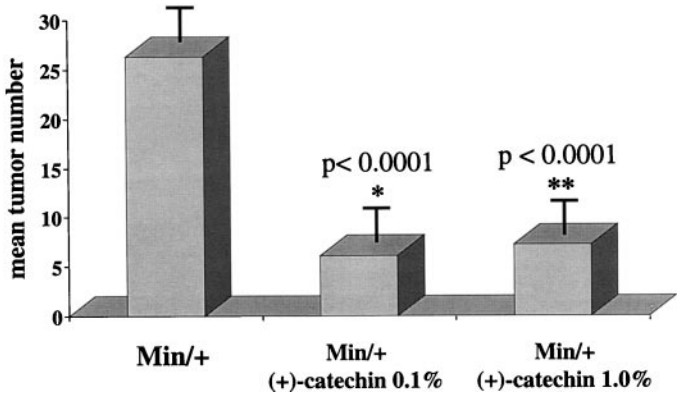


Fig. 2. Treatment with (+)-catechin prevents tumor formation in the Min/+ mouse. Values represent mean ± SD (*bars*) with $n = 10$ in each treatment group.

containing 3% BSA. Rhodamine-conjugated phalloidin in PBS containing 1% BSA was applied, and the slides were placed in a humidified chamber at room temperature for 1 h. After additional washing, slides were mounted with coverslips, and imaging was performed using the Zeiss LSM510 confocal microscope of the Core Imaging Facility at the Weill Medical College of Cornell University.

Invasion Assay. The invasion assay was performed as detailed previously (38). Briefly, HT-29 cells were subjected to the standard treatment conditions (see above), and then trypsinized. The number of viable cells was determined by trypan blue exclusion and counted as above. Cells (5×10^4) in low-serum medium were placed on a Matrigel-coated polycarbonate filter in each well of the invasion assay plates (8-mm wells; 8-μm pores). Conditioned medium from NIH3T3 cells, supplemented with 10% calf serum, was placed in the bottom chambers as a chemoattractant. Plates were incubated at 37°C for 24 h. Cells that migrated to the bottom compartment were stained with hematoxylin. A blinded viewer counted the number of cells present in four fields per chamber, using a light microscope, and the experiment was repeated separately. Statistical analysis of the data from this assay was computed by Student's *t* test.

RESULTS

(+)-Catechin Inhibits Tumor Formation in Min/+ Mice. Beginning at 5 weeks of age, Min/+ mice were fed an AIN-76A diet supplemented with either 0.1% or 1.0% (+)-catechin by weight. The rationale for selecting these concentrations was based on the work of Arii *et al.* (41), and on estimation of the maximal (+)-catechin concentration achievable in a nonsupplemented human diet (15). Control animals received AIN-76A diet without additions. All animals remained healthy and active with stable weight during the 10-week treatment time. At 110 days of age, the animals were euthanized, and the number of tumors in their intestines was counted. The untreated control group mice contained 26 ± 11.1 adenomas, a finding consistent with that of previous studies (35, 37). As shown in Fig. 2, the treated groups had a significantly lower tumor number, with a mean of 6.4 ± 3.7 adenomas in the 0.1% (+)-catechin group, and 7.6 ± 4.0 in the 1.0% (+)-catechin group, corresponding to reductions of 75 and 71%, respectively. The overall distribution of tumors throughout the intestinal tract was unchanged by (+)-catechin administration because uniform decreases in tumor number at all locations were observed in the treated mice (Table 1).

(+)-Catechin Inhibits Cell Proliferation and Alters Cytoskeleton Structure in Colon Cancer Cell Lines. Stringent regulation of the growth, survival, and migration of normal epithelial cells involves the coordination of signals delivered through cell-cell contacts, cell-ECM interactions, and cell surface growth factor receptor-ligand associations (42). Dysregulation of any of these processes may be responsible for the abnormalities observed in tumors. Previous work in our laboratory showed that effective agents in the suppression of intestinal tumors, including both NSAIDs and phenolic antioxidants, alter the cytoskeletal structure of colon epithelial cells (38). This inhibition is likely to reflect changes in cellular adhesion, which is dependent on the integrity of the actin cytoskeleton. In view of the important role of the actin cytoskeleton in cell proliferation and migration, we next examined the effect of (+)-catechin on the proliferation and the cytoskeletal structure of two different human colon cancer cell lines, HT-29 and DLD-1. The cells both contain wild-type β-catenin protein and are APC null. Unlike HT-29, DLD-1 cells are well differentiated and have a flat, nonrefractile appearance. The cells were each cultured to achieve log-phase growth, and then four replicate cultures per time point were treated with 125 μM (+)-catechin in low-serum medium. Untreated control cells were grown under identical conditions without drug addition. At 24-h intervals, sets of cultures of the two cell lines were washed with PBS and trypsinized, and the attached cells on each dish were stained with trypan blue and counted. As shown in Fig. 3, treatment with (+)-catechin inhibited the growth of both cell lines by >50% compared with untreated control cultures. However, this dose of (+)-catechin was not significantly cytotoxic because >90% of cells excluded trypan blue at the conclusion of each treatment interval up to 72 h, and the total number of cells in the treated groups increased relative to the starting point.

To determine the effect of (+)-catechin on cytoskeletal structure, the same conditions were used to treat HT-29 and DLD-1 cells that were grown on coverslips. After incubation of cultures treated with and without the flavonoid, rhodamine-labeled phalloidin was applied to the fixed cells to allow visualization of the actin fiber network. Confocal microscopic imaging of the untreated control cells revealed organized actin stress fibers anchored to focal adhesions in a fan-shaped array (Fig. 4). This structure is characteristic of adherent cells with intact cell-ECM contacts. Treatment with 125 μM (+)-catechin in low-serum medium produced a reorganization of the actin cytoskeleton. Characteristics of the response to drug treatment included retraction and loss of the stress fibers, the circumferential accumulation of actin at the cell periphery, and punctate actin staining. Reorganization of the actin cytoskeleton was more clearly illustrated in DLD-1 cells, but the increased peripheral staining of treated HT-29 cells suggested that a similar response was induced in them as well. Control cells were also cultured with genistein, a specific inhibitor of protein tyrosine kinases that blocks the tyrosine phosphorylation of FAK and the assembly of focal adhesions and actin stress fibers in epithelial cells (38, 43). Exposure to 100 μM genistein under the same treatment conditions also affected the actin cytoskeleton of these cell lines.

(+)-Catechin Reduces the Invasive Capacity of HT-29 Human Colon Cancer Cells. Changes in epithelial cell-ECM adhesion can modulate cell migration and invasion. In the gut, enterocytes produced from stem cells must migrate from the proliferative zone of the intestinal crypt to the luminal surface, where senescent cells are extruded. Normally, the very rapid rate of enterocyte proliferation and migration in the intestines protects this tissue from tumorigenesis. The germline *Apc* mutation present in Min/+ mice is associated with

Table 1 *Distribution of tumors in Min/+ mice*

Values represent mean ± SD tumor number per mouse in each group ($n = 10$). Statistical analysis of variance (ANOVA) showed that the distribution of tumors along the intestinal tract did not change significantly in any group.

	Duodenum	Small intestine	Colon
Min/+ control	4.5 ± 2.5	20.7 ± 8.8	0.6 ± 0.8
(+)-Catechin, 0.1%	1.5 ± 1.2[a]	4.8 ± 2.7[b]	0.1 ± 0.3
(+)-Catechin, 1.0%	2.0 ± 1.5[c]	5.5 ± 2.8[b]	0.1 ± 0.3

[a–c] Significant reduction in tumor number occurred in the duodenum and small intestine of both treatment groups when compared with untreated Min/+ mice: [a] $P < 0.005$; [b] $P < 0.0001$; [c] $P < 0.05$ (all compared to Min/+).

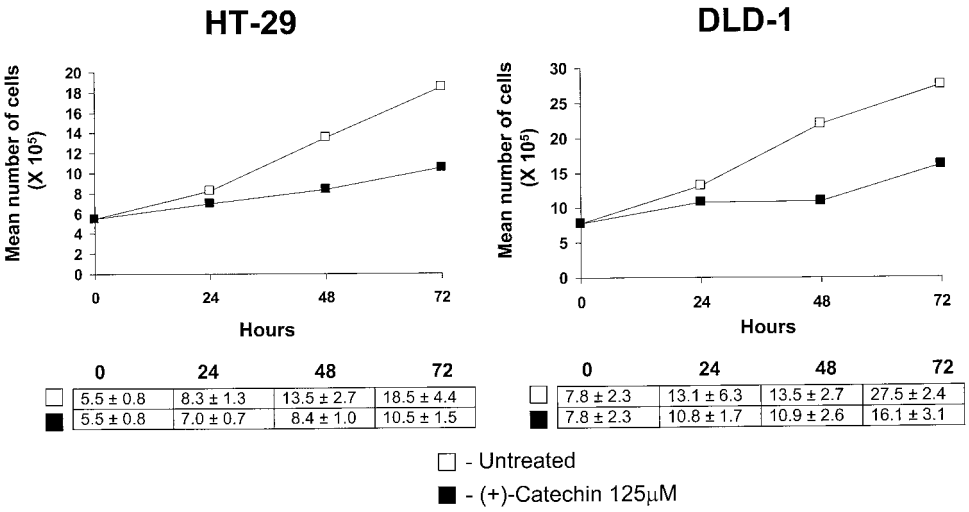


Fig. 3. Treatment with 125 μM (+)-catechin suppresses the growth of HT-29 (*left*) and DLD-1 (*right*) human colon cancer cells. Cells were counted using a hemocytometer after staining with trypan blue. More than 90% of cells excluded trypan blue, indicating that (+)-catechin was not cytotoxic at this concentration. All samples for each time point were counted in quadruplicate and represent the mean number of cells ± SD.

abnormally slow migration of enterocytes along the crypt-villus axis (44), an alteration that may contribute to tumor formation in these animals. Even more clinically significant is the abnormal migration that is characteristic of later stages of carcinogenesis when cancer cells traverse the basement membrane and invade the submucosa. The cytoskeletal changes induced by (+)-catechin in intestinal tumor cells suggested that this agent might alter tumorigenesis by modulating cancer cell migration.

To examine the effect of (+)-catechin on the invasive cancer phenotype, HT-29 cells were seeded into Boyden chambers and allowed to attach for 48 h. These cells were exposed to 50 or 125 μM (+)-catechin under standard treatment conditions. The invasive activ-

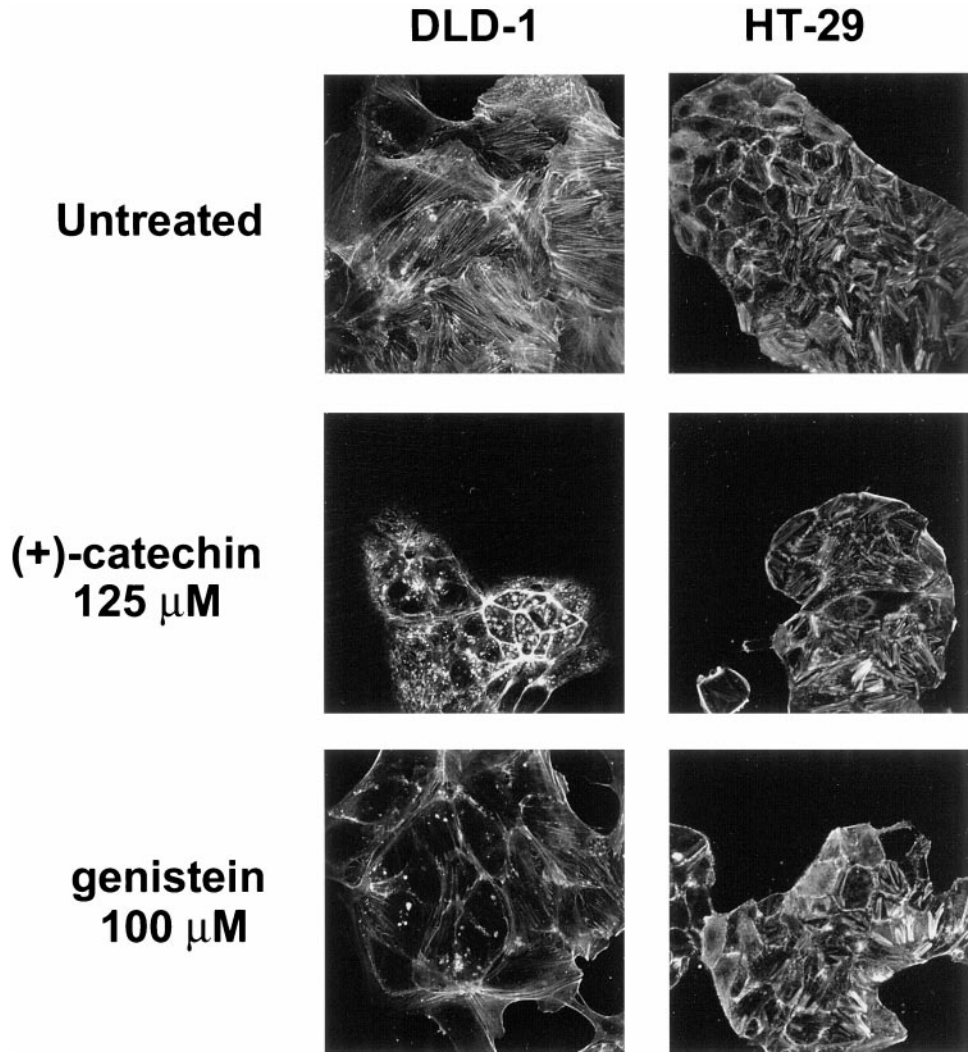


Fig. 4. The actin cytoskeleton is altered by treatment with (+)-catechin (*middle*) and genistein (*bottom*). DLD-1 (*left panels*) and HT-29 (*right panels*) human colon cancer cells were stained with rhodamine phalloidin and imaged using a Zeiss LSM510 confocal microscope at ×63 power.

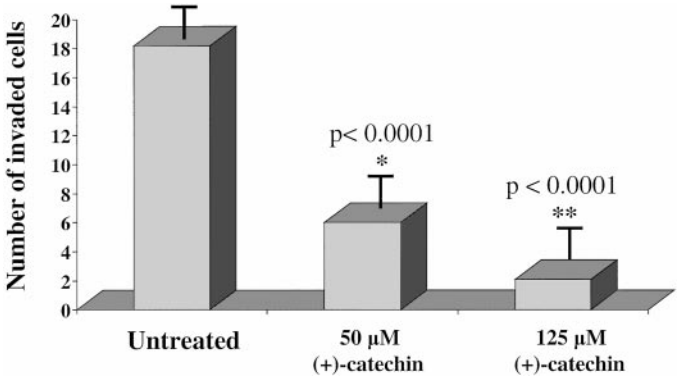


Fig. 5. Treatment of HT-29 with (+)-catechin causes a dose-dependent reduction of colon cancer cell invasion. The cells were treated for 24 h with two concentrations (+)-catechin and placed in modified Boyden chambers. Cells migrating through the membrane were stained with hematoxylin and counted by a viewer blinded to treatment status. Studies were performed in duplicate, with eight total fields counted per experiment. Values are expressed as mean number of cells per field ± SD (*bars*).

ity of HT-29 was assessed by counting the number of cells that migrated through the Matrigel-coated polycarbonate filters. The results in Fig. 5 show a dose-dependent inhibition of tumor cell migration after treatment with (+)-catechin, with a 90% reduction in cell invasion at the highest drug dose. This inhibition of invasion was not attributable to toxicity, as indicated by the results shown above in Fig. 3.

(+)-Catechin Modulates Integrin-mediated Signaling in Colorectal Cancer Cell Lines and in Enterocytes and Tumors from Min/+ Mice. In normal epithelial cells, cell-ECM signaling originates in the region of the integrin cytoplasmic domain and focal adhesions, structures that link the actin filaments of the cytoskeleton and the ECM. FAK is a cytoplasmic tyrosine kinase associated with the focal adhesions and the major protein to become tyrosine phosphorylated after integrin activation. FAK phosphorylation regulates cell survival (45–48), proliferation (49–51), and migration (52–54).

The activation of FAK downstream signaling depends on the phosphorylation of Tyr^{397}. When this residue is phosphorylated, a SH2 domain is created that allows the binding of c-Src and the further tyrosine phosphorylation of other secondary FAK residues (54, 55). Tyr^{397} is the primary site of FAK autophosphorylation *in vitro* and the main phosphorylation target in response to integrin activation (56). To investigate the effect of (+)-catechin on focal adhesion-associated signaling, we examined the steady-state levels of FAK expression and relative levels of FAK tyrosine phosphorylation after treatment of HT-29 and DLD-1 cells. This experiment assessed overall levels of tyrosine phosphorylation by IP of total cell lysates using an antiphosphotyrosine antibody followed by Western blot analysis for FAK. As shown in Fig. 6, treatment of colorectal cancer cells with (+)-catechin decreased the levels of overall tyrosine-phosphorylated FAK (Fig. 6, *left panel*), although steady-state FAK expression remained unchanged (Fig. 6, *right panel*). The sensitivity of DLD-1 to (+)-catechin appears to be greater than that of HT-29 cells because levels of overall tyrosine-phosphorylated FAK were reduced by 25 μM (+)-catechin in the former, whereas this effect was observed only at a (+)-catechin concentration of 125 μM in the latter. Similar results were obtained when these cells were treated with the tyrosine kinase inhibitor, genistein (100 μM). Again, DLD-1 cells showed a marked reduction of overall levels of tyrosine-phosphorylated FAK expression, and HT-29 cells showed relative insensitivity. These differences in sensitivity are consistent with the fact that DLD-1 cells have a more differentiated phenotype and yielded more pronounced cytoskeletal changes when treated with these compounds (Fig. 4).

We next studied FAK expression and tyrosine phosphorylation in enterocytes from Min/+ mice treated with tumor-inhibiting doses of (+)-catechin. After 10 weeks of treatment with 0.1% (+)-catechin or control diet, enterocytes were harvested from macroscopically normal 4-cm segments of proximal small bowel from Min/+ mice and their wild-type littermates. Adenomas in the small intestines of treated and control Min/+ mice were also removed for analysis. Lysates were prepared separately from these enterocyte and adenoma cells and normalized for the amount of protein; aliquots were resolved by SDS-PAGE. The relative levels of FAK-p-Y397 were assessed using a phosphospecific rabbit polyclonal antibody in parallel with the detection of overall FAK expression using a monoclonal anti-FAK antibody. By this means, IPs were obviated. After electrotransfer of the resolved proteins onto nitrocellulose, the membrane was cut horizontally, and the bottom portion was probed with a monoclonal antibody directed against β-actin to provide an internal control for any sample loading variability. As shown in Fig. 7 *A*, there was no difference in relative levels of FAK protein between normal-appearing mucosa from Min/+ mice and that of their wild-type C57BL/6J littermates. However, FAK protein is frankly overexpressed in the adenomas isolated from Min/+ in comparison to normal-appearing intestinal mucosa from this animal. In examining the relative level of

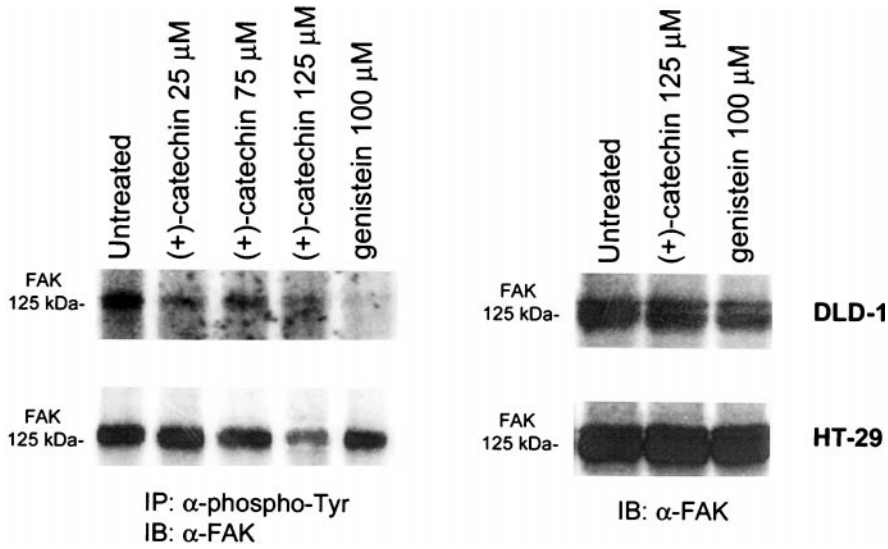


Fig. 6. (+)-Catechin reduces the overall tyrosine phosphorylation of FAK in colon cancer cells. Whole cell lysates were precleared on agarose A beads for 4 h at 4°C and standardized for protein content (700 μg) prior to IP with the antiphosphotyrosine antibody, clone 4G10, followed by IB with a FAK-specific antibody (clone 77). Control cells were treated with genistein at levels sufficient to inhibit tyrosine phosphorylation without inducing significant cytotoxicity.

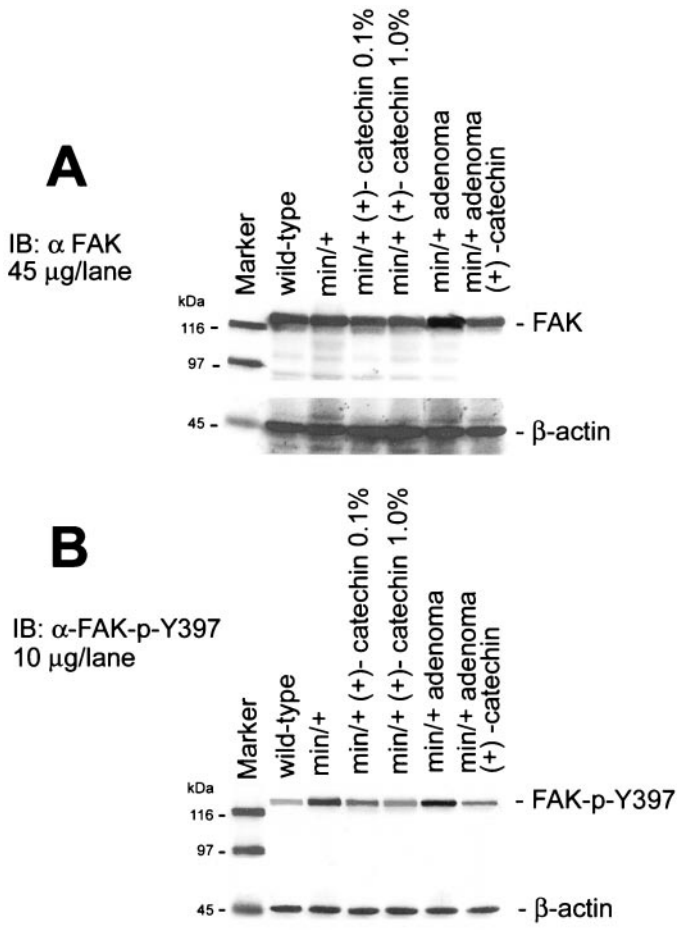


Fig. 7. (+)-Catechin alters FAK expression and relative levels of the signaling active form, FAK-p-Y397, *in vivo*. Whole cell lysates, pooled from specimens of two mice from the same treatment group, were precleared and standardized for protein content as detailed in the legend of Fig. 6. Equivalent amounts of protein were then resolved by SDS-PAGE for IB analyses using FAK-specific antibody (45 μg of protein per lane; *A*) or phosphospecific antibody recognizing FAK-p-Y397 (10 μg of protein per lane; *B*). Both membranes were cut horizontally after electrotransfer; portions shown at the *bottom of each panel* were reacted with antibody to β-actin as an internal loading control.

FAK-p-Y397, noted above to initiate FAK downstream signaling, we found that this form of the kinase was elevated in normal-appearing Min/+ enterocytes, as well as in Min/+ adenomas (Fig. 7*B*). Treatment with (+)-catechin reduced the overall expression of FAK protein in the residual adenomas of treated Min/+ mice (Fig. 7*A*, *extreme right-hand lane*), and also lowered the expression of FAK-p-Y397 in the macroscopically normal mucosa of Min/+ mice (Fig. 7*B*, *middle lanes*). Moreover, expression of FAK-p-Y397 in the adenomas was also inhibited in treated Min/+ mice (Fig. 7*B*, *extreme right-hand lane*). Taken together, these changes in the relative expression and activation of FAK appear to be very early events in *Apc*-mediated tumorigenesis *in vivo*. Furthermore, these results suggest that inhibition of the expression and phosphorylation of FAK is associated with the chemopreventive activity of (+)-catechin.

DISCUSSION

The data presented in this study show that intestinal tumors in Min/+ mice are prevented by dietary administration of the flavonoid, (+)-catechin. The level of tumor prevention achieved at the lowest dietary concentration (0.1%) was not improved with a 10-fold increase in dose. This response level (70–75%) is comparable to that observed with similar doses of other phenolic antioxidants, such as curcumin and CAPE, and somewhat less than that produced by the potent NSAID, sulindac, at a dose of 0.02% (Table 2). This finding is significant because, unlike sulindac and curcumin, which exhibit toxicity at chemopreventive doses, and CAPE, which is not yet readily available by synthetic means, (+)-catechin is nontoxic and widely available.

The intestinal adenoma-carcinoma sequence can be thought of as progressive dysregulation of enterocyte survival signaling pathways. Cells at the earliest stage of carcinogenesis, exemplified by the initiated but normal-appearing enterocytes of Min/+ mice, display phenotypic differences when compared with those of the more advanced adenomas (57–59). One of the earliest changes in adenoma development illustrated by these studies may be altered cell migration (44), a process that is regulated by tyrosine kinases, including the nonreceptor Src family of kinases and FAK. Because FAK can transmit signals that enhance cell growth and survival, it is reasonable to expect that inhibiting the activation and/or downstream signaling of this kinase will prevent or impede the progression of initiated enterocytes to the adenoma stage. This hypothesis is supported by the data presented here because treatment of Min/+ mice with (+)-catechin inhibited the occurrence of visible tumors and lowered the relative expression of FAK-p-Y397, a surrogate of FAK activity. Our data also suggest that by the time intestinal cells lose function of the second *Apc* allele and advance to the adenoma stage, further loss of growth control is achieved by the elevation of both FAK protein expression and tyrosine phosphorylation. More persistent and severe alterations in cell growth resulting from mutations in oncogenes (Ki-*ras*, c-*MYC*) and/or tumor suppressor genes (*p53*, *p16*^{INK4a}, *DCC1*) may also occur (57–59). Presumably, these additional derangements permit adenoma progression despite the inhibition of FAK expression effected by chemopreventive drug treatment. Finally, these studies of colorectal cancer cell lines provide insight into the effect of (+)-catechin on a more advanced stage of carcinogenesis. In these cells, (+)-catechin in relatively noncytotoxic concentrations altered the structure of the actin cytoskeleton and inhibited cell invasion (Figs. 4 and 5), suggesting that this compound may also prolong tumor latency or inhibit progression to an invasive phenotype.

It is possible that Min/+ enterocytes are selected phenotypically *in vivo* for increased integrin-mediated ECM adhesion signaling. A consequence of this selection would be the outgrowth of cells with improved migration and survival. Fig. 7 shows that FAK protein expression is the same in Min/+ and wild-type enterocytes but increased in tumors. It also shows that the phosphorylation of FAK Tyr^{397} is increased in apparently normal Min/+ enterocytes compared with the wild-type cells. Therefore, the altered phosphorylation status of FAK is likely to represent an early event resulting from the *Apc*$^{+/-}$ genotype, and the up-regulation of FAK protein levels in the tumors constitutes a later change resulting from the conversion to *Apc*$^{-/-}$. The results shown in Fig. 7 also suggest that the alterations in integrin-mediated survival signaling are not restricted to the replicative cells of the intestinal crypts. There is a >10-fold difference in the number of cells per crypt (~250) *versus* those per villus (~3500; Ref. 60). Hence, the alterations in expression of FAK-p-Y397 demonstrated by Western blotting most likely reflect the phenotype of the more numerous postmitotic cells of the villi. It will be instructive for

Table 2 *Reduction of tumors in Min/+ mice by chemopreventive compounds*

Agent and dose	Adenoma reduction	Reference
(+)-Catechin, 1.0%	75%	Current report
(+)-Catechin, 0.1%	71%	Current report
CAPE, 0.15%	63%	35
Curcumin, 0.1%	64%	35
Sulindac, 0.02%	95–98%	33, 34

future studies to investigate the expression and distribution of proteins relevant to FAK-mediated signal transduction (*e.g.*, proteins of the phosphoinositide-3-OH kinase-PKB pathway) in Min/+ *versus* C57BL/6J enterocytes and to compare these results with those from Min/+ tumors.

The compound studied here, (+)-catechin, is a flavonoid constituent of fruits and vegetables. The total plant-derived phenol content of an average adult Western diet is 0.17%, or ~1.0 g per day of the usual dietary intake (8). This amount varies greatly depending on the type and proportion of fruits and vegetables ingested. The most common biologically active flavonoids are quercetin and its glycoside, rutin. Although both of these compounds inhibit neoplasia in carcinogen-induced skin and colon cancer models (61–63), recent work showed that addition of quercetin and rutin to the diet at levels as high as 2.0% failed to inhibit tumor formation in Min/+ mice (37). This may be attributable to poor bioavailability of these compounds because they are rapidly conjugated for elimination *in vivo* (8).

When human subjects ingest (+)-catechin, the compound is readily absorbed. Maximum plasma concentrations are reached in ~1 h, and the half-life of elimination is ~3 h. (+)-Catechin undergoes extensive metabolism to sulfates, glucuronides, and *O*-methylated catechin conjugates, and these active derivatives persist in tissues and may undergo reabsorption in the intestine after enterohepatic circulation (64–67). Certain plant foods and beverages are good dietary sources of (+)-catechin. For example, the concentration of (+)-catechin and related phenols in red wine is at least 1.0 ± 0.01 g/liter (67, 68). This high level occurs because the fermentation process used in the production of red wine allows optimal extraction of catechin and other polyphenols from the fruit. In a common genus of wine and table grapes, *Vitis vinifera*, for example, the levels of (+)-catechin are 14–560 mg/kg in the seeds and 14–42 mg/kg in the skins (15), both of which are exposed to the solvent, alcohol, during fermentation. Recent work showed that dietary supplementation with a 1% grape seed extract reduced tumor formation in Min/+ mice by 44% (41). Our data suggest that this result may have been attributable to the high (+)-catechin content of the grape seed extract. Epidemiological studies of colorectal cancer provide conflicting data concerning the relationship between wine intake and cancer (69). Most case-control and cohort studies suggest that high alcohol intake increases cancer risk; however, when the type of alcohol was examined, the significant elevations of risk were found more often for beer, with less risk for spirits and the least risk for wine (70, 71). No study has examined the effect in humans of modest red wine intake alone on colon cancer risk.

By nutrient density estimation, a 0.1% catechin diet, such as shown here to be effective in the Min/+ mouse, can be achieved in humans by an average consumption of 560 mg/day. This amount is contained in ~500 ml of red wine or a lesser volume of wine if foods containing (+)-catechin are also regularly consumed (*e.g.*, grapes, apples, and chocolate; Ref. 70). Our failure to see a dose response together with the considerable efficacy of the (+)-catechin diets suggests that the actual concentration required for tumor prevention may be <0.1%, making it feasible for dietary modification alone to achieve tumor prevention. Even if regular dietary ingestion of this compound is not possible, the (+)-catechin concentration of 0.1% used in this study corresponds to a human dose of only 8 mg/kg/day, and (+)-catechin has been administered safely to humans in oral doses of 8–80 mg/kg (71).

In summary, these studies add (+)-catenin, a naturally occurring and minimally toxic compound, to a growing list of agents that suppress *Apc*-associated intestinal tumor formation. Because of its wide bioavailability, this agent may prove effective against a variety of human epithelial tumors. Further study is needed to understand the effects of this agent upon all stages of the adenoma-carcinoma sequence.

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