Published in final edited form as:

Gastroenterology. 2008 June ; 134(7): 1972–1980. doi:10.1053/j.gastro.2008.02.095.

A Green Tea Component Suppresses Posttranslational Expression of Basic Fibroblast Growth Factor in Colorectal Cancer

MUGDHA SUKHTHANKAR^{*}, KIYOSHI YAMAGUCHI^{*}, SEONG-HO LEE^{*}, MICHAEL F. MCENTEE^{*}, THOMAS E. ELING[‡], YUKIHIKO HARA[§], and SEUNG JOON BAEK^{*}

^{*}Laboratory of Environmental Carcinogenesis, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee

[‡]Laboratory of Molecular Carcinogenesis, NIEHS, Research Triangle Park, North Carolina

[§]Mitsui Norin Co., Ltd, Food Research Lab, Miyabara, Fujieda City, Japan

Abstract

Background & Aims—Green tea catechins are known to have anticarcinogenic effects. Epigallocatechin-3-gallate (EGCG) accounts for almost 50% of the total catechin content in green tea extract and has very potent antioxidant effects. EGCG also inhibits angiogenesis, possibly through the inhibition of proangiogenic factors including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which in turn, inhibits tumor growth and metastasis. However, the exact molecular mechanism by which EGCG suppresses bFGF expression is not known. Our objective was to elucidate the molecular mechanisms by which EGCG inhibits bFGF expression in colorectal cancer.

Methods—We examined posttranslational regulation of bFGF by EGCG in human colorectal cancer cells. We also examined bFGF in intestinal tumor formation of APC^{Min/+} mice with and without catechin treatment.

Results—The bFGF protein was quickly degraded in the presence of EGCG, but a proteasome inhibitor suppressed this degradation. EGCG was also found to increase ubiquitination of bFGF and trypsin-like activity of the 20S proteasome, thereby resulting in the degradation of bFGF protein. Furthermore, EGCG suppressed tumor formation in APC^{Min/+} mice, compared with vehicle-treated mice, in association with reduced bFGF expression.

Conclusions—The ubiquitin-proteasome degradation pathway contributes significantly to down-regulation of bFGF expression by EGCG. Catechin compounds have fewer adverse effects than chemotherapeutic agents and hence can be used as proof-of-concept in cancer therapeutics to suppress growth and metastasis by targeting proteins such as bFGF.

Colorectal cancer is a common cause of morbidity and mortality in men and women throughout developed countries. It is the third most common cancer and a leading cause of cancer-related deaths in the United States.¹ Dietary components play an important role in the pathogenesis and prevention of this cancer.² Therefore, the use of dietary compounds for the prevention and therapy of this cancer would be of major importance with potentially fewer adverse effects than therapeutic drugs. Green tea has received much attention as a suitable dietary agent

Address requests for reprints to: Seung Joon Baek, PhD, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, 2407 River Dr, Knoxville, Tennessee 37996. e-mail: sbaek2@utk.edu; fax: (865) 974-5616..

Conflicts of interest: No conflicts of interest exist.

because of its antitumorigenic activity.^{3,4} The most active constituents of green tea are polyphenols (catechins), including epigallocatechin 3-gallate (EGCG), epigallocatechin, epicatechin-3-gallate (ECG), and epicatechin.⁵ The antitumor effects of these green tea catechins (particularly EGCG) have been studied at the cellular level, and the catechins are reported to induce apoptosis and cell cycle arrest in cancer cells.^{6,7} Other potential mechanisms include antioxidative activity, inhibition of cyclooxygenase and lipoxygenase, inhibition of activator protein-1, activation of p53 tumor suppressor, and inhibition of telomerase activity.⁸ EGCG also potentially acts as an antiangiogenic agent by suppressing the expression of proangiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).⁹

Angiogenesis is a normal physiologic process in growth, development, and wound healing as well as in the pathology of tumors transitioning from a benign to malignant state. One of the more potent angiogenic molecules is bFGF (also known as FGF-2), which is synthesized in tumor cells and secreted into surrounding tissue. The bFGF protein is a pleiotropic cytokine present in basement membranes and the subendothelial extracellular matrix of blood vessels. The multiple molecular forms of coexpressed bFGF (17.8, 22.5, 23.1, and 24.2 kilodaltons) result from a canonical AUG and 3 unusual CUG start codons.¹⁰ In this study, we investigated the molecular mechanisms involved in the suppression of tumor growth by EGCG, which suppresses angiogenesis by down-regulating bFGF. We demonstrate that EGCG affects bFGF protein at the posttranslation level by modulating protein degradation. In addition, we also demonstrate that EGCG suppressed polyps in APC^{Min/+} mice with down-regulation of bFGF.

Materials and Methods

Cell Culture and Reagents

All cell lines were purchased from the American Type Culture Collection (Manassas, VA), except the head and neck cancer cell line, SPCCY-1, which was obtained from Dr Dong M. Shin (Emory University, Atlanta, GA). All chemicals were purchased from Fischer Scientific (Pittsburgh, PA), unless otherwise specified. Primary antibodies for bFGF and β -catenin were purchased from BD Transduction Laboratories (San Jose, CA). VEGF and actin primary antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and cyclin D1 antibody was from Cell Signaling Technology (Beverly, MA). V5 antibody was obtained from Invitrogen (Carlsbad, CA).

Reverse-Transcriptase Polymerase Chain Reaction and Western Blot Analysis

Reverse-transcriptase polymerase chain reaction (RT-PCR) was performed as previously described.¹¹ The PCR primers were as follows: human *bFGF* forward, 5'-agagcgaccctcatcaag-3' and human *bFGF* reverse, 5'-actgcccagtcgttcagt-3'; human *GAPDH* forward, 5'-gggctgctttaactctgtt-3' and human *GAPDH* reverse, 5'-tggcaggttttctagacgc-3'. The thermal cycling conditions were as follows: initial denaturation at 94°C for 2 minutes, followed by 25-29 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute, and final extension for 10 minutes at 72°C. Western blot analysis was performed as previously described.¹²

Cloning of bFGF Expression Vector, Transfection, and Histidine Pull-Down Experiments

The full-length human *bFGF* complementary DNA (cDNA) (18 kilodaltons) was isolated by RT-PCR from LoVo cells using forward (5'-atggcagccgggagcatcac-3') and reverse (5'-gctcttagcagacattggaagaaa-3') primers, obtained from the reported human *bFGF* cDNA sequence (GenBank No. NM_002006). Amplified PCR products were then cloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen). LoVo cells were transiently transfected with either bFGF expression vector (pcDNA3.1/V5-His-TOPO/bFGF) or control vector

(pcDNA3.1/V5-His-TOPO/LacZ) using Lipofectamin 2000 (Invitrogen), according to the manufacturer's protocol. The bFGF proteins containing 6 histidine residues at their C-termini were purified using ProBond nickel-chelating resin (Invitrogen), according to the manufacturer's protocol, and subjected to Western blot.

Enzyme-Linked Immunosorbent Assay for bFGF

Enzyme-linked immunosorbent assay (ELISA) was done using tissue lysates from the green tea-fed APC^{Min/+} mice and lysates from HCT-116 cells. Tissue samples and cells were homogenized in radio immunoprecipitation assay buffer and then sonicated at 4°C, followed by centrifugation for 20 minutes at 14,000 rpm. The supernatant was collected and subjected to ELISA. Quantikine FGF basic Immunoassay kit (R&D Systems, Minneapolis, MN) was used for mouse tissue samples, and the Raybiotech kit (Norcross, GA) was used for cell lysates. All tests were carried out in triplicate. The microplate was gently tapped, and optical densities were read immediately using a microtiter plate reader (Bio-Tek Instruments, Winooski, VT) at 450 nm. The amount of bFGF protein was normalized to nanograms of bFGF per milligram total protein.

Ubiquitin and 20S Proteasome Assay

An ubiquitin enrichment kit (Pierce, Rockford, IL) was used according to the manufacturer's protocol. Ubiquitin-enriched fractions of cell lysates were subjected to Western analysis. A Proteasome-Glo Assay Systems Kit from Promega (Madison, WI) was used to detect the 20S proteasomal activity. This kit allows 3 separate assays that differ in their ability to detect different protease activities based on their substrate components. The LoVo cells were treated with vehicle (dimethyl sulfoxide [DMSO]) and EGCG (50 μ mol/L). Samples were prepared in triplicate, and 30 μ g of each sample was loaded in a white-walled 96-well plate, to which equal amounts of chymotrypsin-like, trypsin-like, and caspase-like substrates were added. The plate was incubated for 1 hour at room temperature in the dark, and the luminescence for the 3 activities was recorded on a multidetection microplate reader (BioTek Instruments, Winooski, VT). Relative luminescence units (RLU) were analyzed.

Animal Study

The APC^{Min/+} mice were randomly divided into 3 groups of 9 mice, each to receive vehicle, EGCG, or ECG. APC^{Min/+} mice were provided EGCG or ECG (0.01%) in their drinking water for 2 months starting at 6-7 weeks of age. Sucrose (3%) was added to the drinking water of all groups, including controls, to increase palatability. Twenty-four hours after final treatment, the mice were killed, and the intestinal tract was isolated and washed with phosphate-buffered saline (PBS). Tumor numbers and sizes in the small intestine were assessed with a stereoscopic microscope as previously described.¹³ All animal research procedures were approved by the University of Tennessee Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines.

Immunostaining for Factor VIII

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated. Antigen retrieval was done with EDTA buffer (10 mmol/L Tris/1 mmol/L EDTA/0.05% Tween 20, pH 9.0) at 95°C for 20 minutes in a steamer. The slides were then soaked in Tris-buffered saline/pH 7.6 for 5 minutes, and loaded onto a Dako Autostainer (Dako, Carpinteria, CA). Samples were blocked with 5.3% hydrogen peroxide and serum-free protein for 5 minutes, respectively. Factor VIII polyclonal antibody (1:4000; Dako) was then applied, followed by Envision Polyclonal horseradish peroxidase (HRP) (Dako), both for 30 minutes. DAB/ chromogen was applied for 10 minutes, and counterstaining was carried out with hematoxylin followed by dehydration and coverslipping.

Statistical Analysis

The Student *t* test was used to analyze the differences between samples. $P < .05$ was considered statistically significant and represented with 1 asterisk. Similarly, 2 and 3 asterisks were used to show $P < .01$ and $P < .001$, respectively. Tumor load, tumor polyps, and ELISA for in vivo mice experiments were analyzed using ANOVA with the Dunnett test, where $P < .05$ was considered significant.

Results

Basic FGF Suppression by Green Tea Catechins

Although extensive research has focused on the mechanisms involved in how EGCG affects VEGF suppression,^{14,15} little attention has been given to the suppression mechanism of EGCG on bFGF expression. To address this, we examined 4 human colorectal cell lines and found that only 2 cell lines, HCT-116 and LoVo, expressed bFGF. LoVo cells highly expressed bFGF protein, whereas HCT-116 cells marginally expressed bFGF. Notable bFGF was detected as 2 bands (18 and 24 kilodaltons), and EGCG treatment reduced both bands. On the other hand, VEGF was expressed in all 4 cell lines and was also suppressed by 50 $\mu\text{mol/L}$ EGCG (Figure 1A). We focused on LoVo cells for our initial bFGF studies and treated them with different doses of 2 catechins, EGCG and ECG, to determine the effect of these compounds on the expression of bFGF. We first carried out RT-PCR and found that there was no change in the expression of *bFGF* messenger RNA (mRNA) by 50 $\mu\text{mol/L}$ of EGCG or ECG (Figure 1B, *left panel*). Next, we treated LoVo cells with different doses of EGCG and ECG. Both EGCG and ECG at 50 $\mu\text{mol/L}$ completely suppressed bFGF protein expression; similar results were obtained by immunocyto-chemistry using catechin-treated LoVo cells (data not shown). Furthermore, EGCG suppressed bFGF expression in LoVo cells in a time-dependent manner, starting at 5 minutes (Figure 1C). To examine the effects of catechins on bFGF protein expression in HCT-116 cells, in which bFGF levels were inherently lower, the ELISA assay was performed. As shown in Figure 1D, both ECG and EGCG significantly suppressed intracellular bFGF ($P < .05$) at 50 $\mu\text{mol/L}$ concentration. Finally, we investigated noncolorectal cancer cells to see whether EGCG affected bFGF expression in other cell types. All cells showed a response to EGCG, except MCF-7 breast cancer cells (Figure 1E), suggesting that bFGF expression occurred in most of the cancer cells and that EGCG may reduce bFGF expression.

Proteasome Inhibitor Restores the Suppression of bFGF by EGCG

EGCG can function as both an antioxidant as well as a prooxidant.¹⁶ To investigate whether the suppression of bFGF is related to oxidative stress after EGCG treatment in a cell culture system, LoVo cells were pretreated with glutathione (GSH), which is a major intracellular antioxidant. As shown in Figure 2A, there is partial recovery of bFGF expression in the presence of EGCG and GSH, suggesting that EGCG-generated oxidative stress may contribute to the suppression of bFGF but that effects are largely unrelated to any prooxidative property of EGCG. Because bFGF protein levels began to decrease within 5 minutes of EGCG treatment (Figure 1C), we postulated that a signaling cascade(s) is involved in this quick response. As shown in Figure 2B, there was no restoration of bFGF expression in the presence of U0126 (extracellular signal-regulated kinase inhibitor), SP600125 (c-Jun N-terminal kinase inhibitor), MG132 (nuclear factor- κB inhibitor), or LY294002 (phosphoinositide-3 kinase inhibitor). We also tested other signaling pathway inhibitors for EGFR, protein kinase C, glycogen synthase kinase 3- β , casein kinase II, mTOR, and p38 mitogenactivated protein kinase, but none of these altered the expression of bFGF (data not shown). Interestingly, 5 $\mu\text{mol/L}$ lactacystin reversed the suppression of bFGF by EGCG, suggesting that the proteasomal-dependent degradation pathway may be involved in EGCG-induced bFGF suppression. Finally, we examined other proteasome inhibitors, epoxomicin and AW9155, and found that they did not reverse EGCG-

mediated bFGF suppression (Figure 2B, *right panel*), indicating that an inhibitory effect of trypsin-like activity of lactacystin, in comparison with other inhibitors, may contribute to the EGCG-mediated bFGF suppression.

To examine further whether protein degradation of bFGF was involved in the EGCG-induced bFGF suppression, LoVo cells were pretreated with DMSO (vehicle) or EGCG for 1 hour and then cotreated with cycloheximide (10 $\mu\text{mol/L}$) for 1, 3, 6, 12, and 24 hours. EGCG caused rapid degradation of bFGF protein in the presence of cycloheximide, first observed less than an hour after treatment (Figure 2C, *left panel*) in comparison with vehicle-treated samples. To confirm the efficacy of cycloheximide, we also examined levels of cyclin D1 and found that it was degraded as early as 1 hour following treatment with cycloheximide in both EGCG-treated and untreated samples (Figure 2C, *left panel*), consistent with a previous report.¹⁷ Treatment with cycloheximide for longer periods of time (48 and 72 hours) showed similar results (Figure 2C, *right panel*), indicating that bFGF is very stable under cycloheximide treatment but is readily degradable in the presence of EGCG in LoVo cells. Although β -catenin has been ubiquitously expressed in colon adenomas and cancers and is regulated by ubiquitin-proteasomal degradation,¹⁸ β -catenin was not degraded within 24 hours but slightly degraded with longer incubation periods (Figure 2C, *right panel*).

Mechanism of bFGF Protein Degradation by EGCG

Ubiquitination targets proteins involved in a large number of different biologic processes for proteasome degradation.¹⁹ Lactacystin, a proteasome inhibitor, blocked the suppression of bFGF by EGCG, leading us to hypothesize that the ubiquitin-proteasome pathway plays an important role in suppression of bFGF by EGCG. Hence, ubiquitin-enriched samples of LoVo cell lysates were incubated with different doses of EGCG (1, 10, and 50 $\mu\text{mol/L}$). When these samples were examined by immunoblot with antiubiquitin antibody, the samples treated with 10 and 50 $\mu\text{mol/L}$ EGCG contained increased amounts of ubiquitination, seen as a ladder of the ubiquitin protein (Figure 3A). Subsequently, when the same samples were subjected to immunoblot using bFGF antibody, bFGF was suppressed in a dose-dependent manner (Figure 3A). We then examined the effect of EGCG on the 20S proteasome activity as described in the Materials and Methods section. The catalytic core of the 20S proteasome possesses 3 different proteolytic activities: chymotrypsin like, trypsin like, and caspase like.²⁰ As shown in Figure 3B, EGCG significantly decreased chymotrypsin-like and caspase-like activities, but increased the trypsin-like activity of 20S proteasome, as measured by a specific substrate. This result is consistent with previous data, showing that only lactacystin containing trypsin-like inhibitory property affects bFGF suppression by EGCG (Figure 2B).

Exogenous bFGF Proteins Responding to Cycloheximide

The expression vector containing the full-length of *bFGF* and *LacZ* (control) were cloned and transfected into the LoVo cells as described in the Materials and Methods section. The LoVo cells were allowed to grow for 24 hours and then subjected to cycloheximide treatment. The *LacZ* protein was not degraded in the presence of vehicle; however, it began to degrade 6 hours after EGCG treatment (Figure 4A, *top panel*). In contrast, there was drastic degradation of bFGF protein within 1 hour of EGCG treatment (Figure 4A, *bottom panel*), whereas bFGF levels in vehicle-treated samples only started to decrease between 1-6 hours after treatment, indicating that EGCG facilitates the degradation of recombinant bFGF protein. We also carried out the same experiment in HCT-116 cells, where bFGF is marginally expressed, and the results obtained were similar to those in LoVo cells (data not shown). To elucidate further whether bFGF has the ability to interact specifically with ubiquitin, we used histidine tag pull-down assays followed by Western blot analysis after transient transfections of bFGF and *LacZ* (control) expression vectors. Cell lysates were pulled down using nickel resin, and copurified proteins were analyzed by immunoblot using specific antibodies for ubiquitin and V5. As

shown in Figure 4B, V5-His-tagged bFGF and LacZ were efficiently pulled down by nickel resin, and the resin-purified bFGF complex contained strong ubiquitin-protein binding compared with LacZ. The purified proteins were confirmed by V5 Western blot, as shown at the bottom of Figure 4B. Taken together with the previous data, these results suggest that the bFGF protein specifically binds to the ubiquitin complex, and EGCG enhances ubiquitination of bFGF and trypsin-like activity of the 20S proteasome, resulting in the degradation of bFGF protein.

Effects of bFGF on Intestinal Tumorigenesis in APC^{Min/+} Mice

Finally, we evaluated tumor formation in APC^{Min/+} mice fed green tea catechins, comparing 3 groups: untreated controls (Veh) and mice treated with either EGCG or ECG, both at a dose of 0.01% in their drinking water. The gross morphology of the small intestinal tumors revealed a striking reduction in the size and number of tumors in EGCG-treated APC^{Min/+} mice, compared with the untreated group (Figure 5A). The EGCG-treated group also showed a statistically significant reduction in the total number of polyps and tumor load ($P < .05$) (Figure 5B and 5C) compared with controls. Although ECG-treated mice did not show significant reductions, we saw a trend suggesting that ECG may also reduce polyp numbers. Adenomas were examined histologically in the small intestine (Figure 5D, top). Factor VIII is a marker for endothelial cells used to evaluate angiogenesis,²¹ and bFGF is a proangiogenic factor in adenomas as well as adenocarcinomas.²² Immunostaining for Factor VIII on control and EGCG-treated small intestinal tumors suggested that endothelial-lined capillaries were more prevalent in the control group, compared with EGCG-treated tumors (Figure 5D, bottom). Finally, ELISA assay was performed using the small intestinal tissue samples from the respective 3 groups, and results showed significant suppression of bFGF by EGCG ($P < .05$) compared with controls using the Dunnett test (Figure 5E). However, there was no significant effect in the ECG-treated small intestinal tissues, concordant with the tumor data shown in Figure 5A-C. These data suggest that EGCG may suppress intestinal tumorigenesis in vivo by reducing bFGF expression and angiogenesis.

Discussion

Green tea acts as an anticancer agent, especially in colon cancer. Although the molecular mechanism of this anticancer effect is still not entirely understood, most green tea effects are believed to result from EGCG.²³ In addition to its chemopreventive activity, EGCG is known to possess antiangiogenic properties through inhibition of proangiogenic factors including VEGF and bFGF.^{24,25} Our results show that EGCG suppresses the protein levels of bFGF and VEGF in colorectal cancer cells, which could account for reduced angiogenesis and hence hamper tumor growth and metastasis. It has been previously reported that EGCG down-regulates bFGF expression,²⁵ but the exact mechanism had not been determined. In this study, we investigated the molecular mechanism involved in the suppression of bFGF by EGCG and found that this occurred specifically through posttranslational modification. We also examined the transcriptional regulation of bFGF by EGCG, but we could not identify any changes in mRNA level (Figure 1B) or mRNA stability in the presence of EGCG (data not shown) in LoVo cells. Thus, posttranslational regulation of bFGF by EGCG may fully account for the EGCG-induced bFGF suppression.

EGCG possesses a strong antioxidant activity²⁶ as well as prooxidant activity in cell culture systems, producing H₂O₂ in the media.²⁷ Therefore, we investigated whether the prooxidative activity may cause bFGF suppression in our cell culture. GSH is one of the major intracellular antioxidants. When GSH and EGCG are added to LoVo cells, bFGF protein is only partially recovered, which indicates that oxidation, presumably H₂O₂ generation by EGCG, contributes to EGCG-induced bFGF suppression but not to a major extent (Figure 2A). To elucidate further

the molecular mechanism of EGCG in signaling pathways, we tested for involvement of different pathways using kinase inhibitors. We found that lactacystin, an inhibitor of proteasomal degradation, inhibited EGCG-induced bFGF suppression (Figure 2B). These results were also confirmed using the recombinant bFGF proteins (Figure 4). LoVo cells were transfected with the expression vectors containing either *bFGF* or *LacZ* and then treated with EGCG for 1 hour, followed by cycloheximide treatment for 0, 1, 6, and 24 hours. There was no significant change in vehicle-treated *LacZ* transfected samples, whereas the recombinant bFGF proteins were rapidly degraded in the presence of vehicle and EGCG. These results highlight the involvement of the ubiquitin-proteasome pathway as a novel mechanism facilitated by EGCG. There was, however, no change in β -catenin levels, even though it is known to be degraded by the ubiquitin-proteasome pathway (Figure 2C, left panel). One consideration is that LoVo cells contain a mutant adenomatous polyposis coli tumor suppressor protein that would not bind to β -catenin in mediating ubiquitination. However, after 24 hours, β -catenin seems to be degraded, indicating that other pathways may be involved in β -catenin degradation in the presence of EGCG.

The ubiquitin-enriched fractions were also obtained from EGCG-treated samples, and we found that EGCG increased the ubiquitin activity in a dose-dependent manner (Figure 3A). This result was confirmed by immunoprecipitation of bFGF and *LacZ* with ubiquitin and V5 antibodies (Figure 4B). Ubiquitin is one of the most conserved eukaryotic proteins, and it conjugates other proteins through a well-defined enzymatic pathway.²⁸ The ubiquitin-proteasome pathway is now being recognized as an important regulatory system in cancer pathways and, in fact, in many cellular processes.²⁹ Proteins are conjugated with ubiquitin and subsequently transferred to the 26S proteasome, which is a multicatalytic subunit complex consisting of a 20S barrel-shaped proteolytic core and a 19S cap-like regulatory complex.³⁰ Our data support this mechanism of degradation, showing that EGCG facilitates ubiquitination of bFGF and specifically triggers trypsin-like activity of the 20S proteasome, leading to degradation of bFGF. In contrast, chymotrypsin-like activity of the proteasome is associated with tumor cell survival, and EGCG has been known to decrease proteasome activity via chymotrypsin-like activity of the 20S proteasome.³¹ It is therefore very surprising that EGCG increases only trypsin-like activity of the 20S proteasome. However, our data strongly support the hypothesis that EGCG increases trypsin-like activity of the 20S proteasome. First of all, proteasome inhibitors (Epoxomicin and AW9155) other than lactacystin did not show any effect on EGCG-induced bFGF suppression (Figure 2B). When these inhibitors were compared in their activity, only lactacystin possesses the inhibitory effect on trypsin-like activity. Another explanation of lactacystin specificity in EGCG-mediated bFGF degradation is that, unlike other proteasome inhibitors, lactacystin has reactive hydroxyl groups that might compete with EGCG for activation of 20S proteasomal activities. To our knowledge, this is the first report that EGCG specifically enhances trypsin-like activity of the 20S proteasome. In addition, our data provide a rationale to develop anticancer drugs that increase ubiquitination of angiogenic protein and also increase trypsin-like activity of the 20S proteasome.

An in vivo study using *APC^{Min/+}* mice treated with EGCG or ECG indicated that intestinal tumor load and polyp numbers were significantly reduced only in the EGCG-treated mice; there were no significant changes in the mice provided ECG. Although it is possible that the concentration and solubility of ECG reduced its effectiveness, EGCG was clearly shown to suppress bFGF to a greater extent (Figure 1B). EGCG-mediated reductions in bFGF levels in *APC^{Min/+}* mouse tumors, by ELISA assay, correlated with apparently fewer Factor VIII-labeled blood vessels in the tumors, suggesting an inhibitory effect on angiogenesis in the EGCG-treated mice. Thus, EGCG indeed suppressed bFGF expression associated with a reduction in tumorigenesis in *APC^{Min/+}* mice.

The concentration of green tea catechins in human plasma has been reported to reach no higher than 1 $\mu\text{mol/L}$ even with consumption of large amounts of the beverage.³² However, higher levels are expected to be present in lumen of the gastrointestinal tract. Although the 50 $\mu\text{mol/L}$ EGCG dose in cell culture reflects a higher range of plasma concentration, it is possible that this concentration can be reached in the intestinal tract. The exact effective concentration remains to be determined; however, the bioavailability and degradation as well as metabolic effects of catechins in the cell culture should be considered.

Our study raised many intriguing and important questions that require clarification in the future. In particular, the molecular feature of EGCG that serves as a signal trigger for ubiquitination and the sites of ubiquitination of bFGF in the presence of EGCG should be examined. In addition, the specific enhancing activity of EGCG on trypsin-like activity of the 20S proteasome remains to be elucidated. Overexpression of bFGF is very common in human colorectal cancer cells and in adenomas derived from mouse models, and these findings provide a molecular mechanism that details regulation of bFGF by EGCG.

Acknowledgements

Supported primarily by NIH grant R21CA109423 (to S.J.B.) and K26RR016645 (to M.F.M.).

The authors thank Misty Bailey and Nichelle Whitlock for their critical review.

Abbreviations used in this paper

bFGF, basic fibroblast growth factor; ECG, epicatechin-3-gallate; EGCG, epigallocatechin 3-gallate; VEGF, vascular endothelial growth factor.

References

1. Parkin DM, Bray F, Ferlay J, et al. Global cancer statistics, 2002. *CA Cancer J Clin* 2005;55:74–108. [PubMed: 15761078]
2. Singh PN, Fraser GE. Dietary risk factors for colon cancer in a low-risk population. *Am J Epidemiol* 1998;148:761–774. [PubMed: 9786231]
3. Sun CL, Yuan JM, Koh WP, et al. Green tea, black tea and colorectal cancer risk: a meta-analysis of epidemiologic studies. *Carcinogenesis* 2006;27:1301–1309. [PubMed: 16638787]
4. Chung FL, Schwartz J, Herzog CR, et al. Tea and cancer prevention: studies in animals and humans. *J Nutr* 2003;133:S3268S–S3274.
5. Anderson RF, Fisher LJ, Hara Y, et al. Green tea catechins partially protect DNA from (.)OH radical-induced strand breaks and base damage through fast chemical repair of DNA radicals. *Carcinogenesis* 2001;22:1189–1193. [PubMed: 11470748]
6. Nakazato T, Ito K, Ikeda Y, et al. Green tea component, catechin, induces apoptosis of human malignant B cells via production of reactive oxygen species. *Clin Cancer Res* 2005;11:6040–6049. [PubMed: 16115949]
7. Lim YC, Lee SH, Song MH, et al. Growth inhibition and apoptosis by (-)-epicatechin gallate are mediated by cyclin D1 suppression in head and neck squamous carcinoma cells. *Eur J Cancer* 2006;42:3260–3266. [PubMed: 17045795]
8. Yang CS, Lambert JD, Ju J, et al. Tea and cancer prevention: molecular mechanisms and human relevance. *Toxicol Appl Pharmacol* 2007;224:265–273. [PubMed: 17234229]
9. Jung YD, Ellis LM. Inhibition of tumour invasion and angiogenesis by epigallocatechin gallate (EGCG), a major component of green tea. *Int J Exp Pathol* 2001;82:309–316. [PubMed: 11846837]
10. Florkiewicz RZ, Sommer A. Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from non-AUG codons. *Proc Natl Acad Sci U S A* 1989;86:3978–3981. [PubMed: 2726761]

11. Cho KN, Sukhthankar M, Lee SH, et al. Green tea catechin (-)-epicatechin gallate induces tumour suppressor protein ATF3 via EGR-1 activation. *Eur J Cancer* 2007;43:2404–2412. [PubMed: 17764926]
12. Baek SJ, Kim JS, Jackson FR, et al. Epicatechin gallate-induced expression of NAG-1 is associated with growth inhibition and apoptosis in colon cancer cells. *Carcinogenesis* 2004;25:2425–2432. [PubMed: 15308587]
13. Baek SJ, Okazaki R, Lee S-H, et al. Nonsteroidal anti-inflammatory drug-activated gene-1 over expression in transgenic mice suppresses intestinal neoplasia. *Gastroenterology* 2006;131:1553–1560. [PubMed: 17101328]
14. Lee YK, Bone ND, Strega AK, et al. VEGF receptor phosphorylation status and apoptosis is modulated by a green tea component, epigallocatechin-3-gallate (EGCG), in B-cell chronic lymphocytic leukemia. *Blood* 2004;104:788–794. [PubMed: 14996703]
15. Sartippour MR, Shao ZM, Heber D, et al. Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *J Nutr* 2002;132:2307–2311. [PubMed: 12163680]
16. Chen C, Shen G, Hebbar V, et al. Epigallocatechin-3-gallate-induced stress signals in HT-29 human colon adenocarcinoma cells. *Carcinogenesis* 2003;24:1369–1378. [PubMed: 12819184]
17. Dufourny B, van Teeffelen HA, Hamelers IH, et al. Stabilization of cyclin D1 mRNA via the phosphatidylinositol 3-kinase pathway in MCF-7 human breast cancer cells. *J Endocrinol* 2000;166:329–338. [PubMed: 10927622]
18. Rice PL, Kelloff J, Sullivan H, et al. Sulindac metabolites induce caspase- and proteasome-dependent degradation of β -catenin protein in human colon cancer cells. *Mol Cancer Ther* 2003;2:885–892. [PubMed: 14555707]
19. Fisher RD, Wang B, Alam SL, et al. Structure and ubiquitin binding of the ubiquitin-interacting motif. *J Biol Chem* 2003;278:28976–28984. [PubMed: 12750381]
20. Naujokat C, Fuchs D, Berges C. Adaptive modification and flexibility of the proteasome system in response to proteasome inhibition. *Biochim Biophys Acta* 2007;1773:1389–1397. [PubMed: 17582523]
21. Takahashi Y, Bucana CD, Liu W, et al. Platelet-derived endothelial cell growth factor in human colon cancer angiogenesis: role of infiltrating cells. *J Natl Cancer Inst* 1996;88:1146–1151. [PubMed: 8757194]
22. Kusafuka K, Hiraki Y, Shukunami C, et al. Cartilage-specific matrix protein chondromodulin-I is associated with chondroid formation in salivary pleomorphic adenomas: immunohistochemical analysis. *Am J Pathol* 2001;158:1465–1472. [PubMed: 11290564]
23. Chung FL. The prevention of lung cancer induced by a tobacco-specific carcinogen in rodents by green and black tea. *Proc Soc Exp Biol Med* 1999;220:244–248. [PubMed: 10202397]
24. Cao Y, Cao R, Brakenhielm E. Antiangiogenic mechanisms of diet-derived polyphenols. *J Nutr Biochem* 2002;13:380–390. [PubMed: 12121824]
25. Sartippour MR, Heber D, Zhang L, et al. Inhibition of fibroblast growth factors by green tea. *Int J Oncol* 2002;21:487–491. [PubMed: 12168090]
26. Stoner GD, Mukhtar H. Polyphenols as cancer chemopreventive agents. *J Cell Biochem Suppl* 1995;22:169–180. [PubMed: 8538195]
27. Tian B, Sun Z, Xu Z, et al. Chemiluminescence analysis of the prooxidant and antioxidant effects of epigallocatechin-3-gallate. *Asia Pac J Clin Nutr* 2007;16(Suppl 1):153–157. [PubMed: 17392095]
28. Kamitani T, Kito K, Nguyen HP, et al. Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein. *J Biol Chem* 1997;272:28557–28562. [PubMed: 9353319]
29. Harada T, Harada C, Wang YL, et al. Role of ubiquitin carboxy terminal hydrolase-L1 in neural cell apoptosis induced by ischemic retinal injury in vivo. *Am J Pathol* 2004;164:59–64. [PubMed: 14695319]
30. Parcellier A, Schmitt E, Gurbuxani S, et al. HSP27 is a ubiquitin-binding protein involved in I- κ B α proteasomal degradation. *Mol Cell Biol* 2003;23:5790–5802. [PubMed: 12897149]
31. Nam S, Smith DM, Dou QP. Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo. *J Biol Chem* 2001;276:13322–13330. [PubMed: 11278274]

32. Yang CS, Chen L, Lee MJ, et al. Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiol Biomarkers Prev* 1998;7:351–354. [PubMed: 9568793]

Figure 1.

Green tea catechins suppress bFGF expression in different cell lines. (A) Western blot analysis of bFGF using 4 different colon cancer cell lines. The cells were treated with 50 μ mol/L of EGCG for 24 hours. Equal amounts of total proteins (30 μ g) were separated by SDS-PAGE and transferred to nitrocellulose membranes, and bands corresponding to bFGF were detected by enhanced chemiluminescence. (B) *Left panel*: RT-PCR for LoVo cells treated with 50 μ mol/L of ECG and EGCG. Number of cycles used was 29 for *bFGF* and 25 cycles for *GAPDH*. Reaction products were analyzed on 1.2% agarose gel. *Right panel*: Western blot analysis of bFGF using LoVo cell lysates treated with indicated doses of EGCG and ECG for 24 hours. (C) LoVo cells were treated with 50 μ mol/L of EGCG, and samples were harvested at different time points. Cell lysates were then subjected to Western blot analysis for bFGF and actin. (D) HCT-116 cells were treated with DMSO, ECG, or EGCG (10 and 50 μ mol/L) for 24 hours. The bFGF levels were determined by ELISA and expressed as nanograms of bFGF per milligram of total cell protein content. Data represent the mean $\pm$ SD of 3 independent experiments. * P < .05, compared with control (*Veh*). (E) Western blot analysis of bFGF for 2 lung (A549 and H292), breast (MCF-7), prostate (PC-3), head and neck (SPCCY-1), and brain (T98G) cancer cell lines. Cells were treated with 50 μ mol/L of EGCG for 24 hours and subjected to Western analysis.

Figure 2.

EGCG effects of signaling pathway inhibition on bFGF expression. (A) Prooxidative effect of EGCG. LoVo cell lysates were preincubated with GSH (5 μ mol/L) for 1 hour then treated with EGCG (50 μ mol/L) for 24 hours and subjected to Western analysis. (B) The cells were serum starved, and the following inhibitors were added 30 minutes before EGCG treatment: extracellular signal-regulated kinase inhibitor U0126 (6 μ mol/L), c-Jun N-terminal kinase inhibitor SP600125 (50 μ mol/L), nuclear factor- κ B inhibitor MG132 (25 μ mol/L), lactacystin (5 μ mol/L), phosphoinositide-3 kinase inhibitor LY294002 (10 μ mol/L), Epoxomicin (1 μ mol/L), and AW9155 (1 μ mol/L). The lysates were obtained after 24-hour treatment and subjected to immunoblot. An equal amount of loading proteins was estimated by actin probing. (C) Effect of EGCG on protein degradation of bFGF. LoVo cells were treated with either vehicle (Veh) or EGCG for 1 hour, followed by cycloheximide (10 μ mol/L) treatment, and cell lysates were isolated at different time points. Immunoblot was carried out for bFGF, cyclin D1, β -catenin, and actin. The *bottom panel* shows a quantitative analysis of 3 independent experiments. Asterisks (** and ***) were used to show $P < .01$ and $P < .001$, respectively, using Student t test.

Figure 3.

EGCG enhances ubiquitination and increases trypsin-like activity of the 20S proteasome. (A) Ubiquitinated enriched cell lysates treated with EGCG 1, 10, and 50 $\mu\text{mol/L}$ were obtained using an ubiquitin enrichment kit and subjected to Western blot using an antiubiquitin antibody. The same cell lysates were also subjected to bFGF Western blot analysis, and data were normalized using actin. (B) 20S proteasome activity. LoVo cells were plated in triplicate and treated with EGCG (50 $\mu\text{mol/L}$) and vehicle (DMSO). The triplicate cell lysates were measured for chymotrypsin-like, trypsin-like, or caspase-like activity of the 20S proteasome. The luminescence was recorded on a microplate reader, and a graph was plotted using mean readings of 3 independent values. Error bars are SD. *** $P < .001$. The y-axis was presented as the relative luminescence unit (RLU), as a vehicle-treated sample is 1.

Figure 4.

EGCG causes rapid degradation of bFGF. (A) LoVo cells were transfected with LacZ (*top panel*) and bFGF (*bottom panel*) expression vectors tagged with histidine and V5, using Lipofectamin 2000, and were allowed to stabilize overnight. They were subsequently treated with either vehicle (*Veh*) or EGCG (50 μ mol/L) for 1 hour followed by cycloheximide (*CHX*, 10 μ mol/L) treatment. The cell lysates were harvested after 0-, 1-, 6- and 24-hour treatment, and Western blot analysis was carried out for V5 and actin. (B) LoVo cells were transfected with LacZ and bFGF expression vectors using Lipofectamine 2000. The LacZ and bFGF (containing 6 histidine residues at their C-termini) were purified using ProBond nickel-chelating resin. The Western blot analysis was performed to measure ubiquitin and V5-His-tagged recombinants from copurified proteins.

Figure 5.

(A) Gross morphology of small intestinal tumors (*arrows*) from Veh-, EGCG-, and ECG-treated groups. (B and C) Effects of oral administration of EGCG or ECG (0.01%) on tumor number and tumor load (mean $\pm$ SE) in comparison with Veh (n = 9/group). Tumor load was calculated by number of tumors $\times$ average diameter. The *asterisk* denotes *P* value < .05 using the Dunnett test to compare treated with control groups. (D) *Top*: Histopathologic representation of small intestinal tumors from control and EGCG-treated group (original magnification, $\times 100$). *Bottom*: Immunohistochemical staining for Factor VIII (*brown*) on control and EGCG-treated small intestinal tumors as an indication of vascular density and, therefore, angiogenesis (original magnification, $\times 200$ magnification). (E) Mean levels of bFGF (nanogram/milligram total protein by ELISA assay) in lysates of small intestinal tumors from control (Veh) and EGCG- and ECG-treated mice. An *asterisk* indicates a statistically significant difference compared with vehicle-treated sample. *P* < .05 using the Dunnett test.