Epicatechin gallate-induced expression of NAG-1 is associated with growth inhibition and apoptosis in colon cancer cells

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There is persuasive epidemiological and experimental evidence that dietary polyphenolic plant-derived compounds have anticancer activity. Many laboratories, including ours, have reported such an effect in cancers of the gastrointestinal tract, lung, skin, prostate and breast. The catechins are a group of polyphenols found in green tea, which is one of the most commonly consumed beverages in the world. While the preponderance of the data strongly indicates significant antitumorigenic benefits from the green tea catechins, the potential molecular mechanisms involved remain obscure. We found that green tea components induce apoptosis via a TGF-β superfamily protein, NAG-1 (Non-steroidal anti-inflammatory drug Activated Gene). In this report, we show that ECG is the strongest NAG-1 inducer among the tested catechins and that treatment of HCT-116 cells results in an increasing G1 sub-population, and cleavage of poly (ADP-ribose) polymerase (PARP), consistent with apoptosis. In contrast, other catechins do not significantly induce NAG-1 expression, PARP cleavage or morphological changes at up to a 50-μM concentration. Furthermore, we provide evidence that ECG induces the ATF3 transcription factor, followed by NAG-1 induction at the transcriptional level in a p53-independent manner. The data generated by this study will help elucidate mechanisms of action for components in green tea and this information may lead to the design of more effective anticancer agents and informed clinical trials.

Introduction

Cancer is second only to heart disease as the leading cause of death in the United States and colorectal cancer is one of the most prevalent causes of cancer-related mortality in the western world (1). Further development of therapeutic and preventative means of controlling this disease is clearly needed, particularly as they pertain to gastrointestinal cancer. Epidemiological studies strongly suggest that nutrition plays an important role in carcinogenesis. Dietary factors have been estimated to account for up to 80% of cancers of the gastrointestinal tract and ~30% of cancer morbidity and mortality might be prevented with proper adjustment of diets (2–4). Multiple components in fruits, vegetables, herbs and spices have been found to inhibit tumor formation in experimental animals; many studies have focused on polyphenolic compounds in this regard because of their potent biological properties (5).

Tea (Camellia sinensis) is the second most commonly consumed beverage in the world (an estimated 18–20 billion cups daily) and represents a particularly important source of antitumorigenic polyphenols: it is clearly palatable, inexpensive and widely available, and tea exhibits little or no toxic effects in normal, healthy cells. There are three major types of tea: unfermented green tea, semi-fermented oolong tea and fermented black and red tea. While the chemical composition of teas is complex, the polyphenolic compounds (catechins) constitute the most interesting because of their potent beneficial activity in vivo and in vitro. The major catechins in green tea are (−)-epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epicatechin (EC). Green tea contains more catechins than other types of tea and EGCG has been studied more often for its biological activity because it is the most abundant catechin in this beverage. However, many researchers have also studied the other catechins, including ECG, for their biological activities (6,7).

The antitumor effects of these green tea catechins have been studied at the cell biological level, and the major cellular phenomena induced by the catechins were found to be apoptosis and cell cycle arrest. Potential mechanisms have also been suggested to include anti-oxidative activity (8), inhibition of enzymes related to tumor promotion such as cyclooxygenase and lipoxygenase (9), inhibition of activator protein-1 (10), inhibition of angiogenesis (11), inhibition of vascular endothelial growth factor receptor phosphorylation (12), activation of p53 tumor suppressor protein (13) and inhibition of telomerase activity (14). However, the high concentration of catechins used in most studies may not be relevant to those obtained by tea drinkers and the effects and precise molecular mechanisms by which catechins other than EGCG induce apoptotic cell death are still largely unknown.

Non-steroidal anti-inflammatory drug (NSAID) activated gene (NAG-1) was identified by PCR-based subtractive hybridization as a pro-apoptotic and antitumorigenic protein (15). The human NAG-1 cDNA encodes a secreted protein with homology to members of the TGF-β superfamily and has been identified previously as macrophage inhibitory cytokine-1 (MIC-1) (16), placentally transforming growth factor-β (17), prostate-derived factor (18), growth differentiation factor 15 (19) and placental bone morphogenetic protein (20). It is highly expressed in mature intestinal epithelial cells, but is significantly reduced in human colorectal carcinoma samples and neoplastic intestinal polyps of Min mice (21). In addition,
it has been reported that treatment of prostate cancer cells with recombinant NAG-1 induces apoptosis (22). The data support the link between NAG-1 and apoptosis with increased expression favoring antitumorigenesis. NAG-1 is up-regulated in a prostaglandin-independent manner in human colorectal cancer cells by several NSAIDs (23), as well as by antitumorigenic compounds such as resveratrol (24), genistein (25), diallyl disulfide (26), PPARγ ligands (27), 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (28) and retinoid 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid (29). While some of these compounds induce NAG-1 expression via the p53 tumor suppressor protein, NSAIDs induce NAG-1 in a p53-independent manner (24), suggesting that several pathways are involved in the regulation of NAG-1 expression.

In this report, we present NAG-1 as a novel target protein for catechins, particularly ECG, which can induce its expression in a p53-independent manner. In addition, induction of NAG-1 can promote apoptosis and mediate antitumorigenic activity. ECG is the most potent NAG-1 inducer tested in this study and the ATF3 transcription factor may be involved in the ECG-induced NAG-1 expression. In addition, we have also investigated signaling pathways affected by ECG and propose herein an overview of how ECG induces apoptosis in human colorectal cancer cells.

Materials and methods

Cell lines, reagents and plasmids

Cell lines were purchased from ATCC (Rockville, MD). HCT-116 human colorectal carcinoma cells were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum and gentamicin (10 μg/ml). Catechins including EGCG, EGC, ECG and EC were purchased from Sigma (St Louis, MO), and dissolved in DMSO. HCT-116 p53+/- cells were generously provided by Dr Bert Vogelstein (John’s Hopkins University, Baltimore, MD). The pCG-ATF3 construct was kindly provided by Dr T.Hai (Ohio State University). NAG-1, p53 and actin antibodies were described previously (15,24,27). TSPl (thrombospondin-1), ATF3 and poly (ADP-ribose) polymerase (PARP) antibodies were purchased from NeoMarker, Santa Cruz and Cell Signalling, respectively. The pNAG1086/LUC and pNAG1/LUC constructs were prepared as described previously (27,30).

RNA isolation, northern blot analysis and reverse-transcription (RT)–PCR

Cells at 60–80% confluence in 10-cm plates were treated at indicated concentrations with EGCG or DMSO in the absence of serum. Total RNAs were isolated using TRIzol reagents (Life Technologies, MD) according to the manufacturer’s protocol. After 48 h of transfection, the cells were harvested, washed with PBS, fixed by the slow addition of cold 70% ethanol to a total of 1 ml, and stored at 4°C overnight. The fixed cells were pelleted, washed with 50%, 30% ethanol, and stained in 1 ml of 20 μg/ml propidium iodide, 1 mg/ml RNase in PBS for 20 min. 7500 cells were examined by flow cytometry using a Becton Dickinson Fluorescence-activated cell sorter (FACS) equipped with CellQuest software by gating on an area versus width dot plot to exclude cell debris and cell aggregates. Apoptosis was measured by the level of sub-diploid DNA contained in cells following treatment with compounds using CellQuest software.

Transfection using the luciferase reporter system

HCT-116 cells were plated in 6-well plates at 2 × 10^5 cells/well in McCoy’s 5A or DMEM media, respectively, supplemented with 10% fetal bovine serum. For the co-transfection experiment, 0.5 μg of reporter vector and 0.5 μg of expression vector were transfected with 0.05 μg of pRL-null vector according to the manufacturer’s protocol. After 48 h of transfection, the cells were harvested in 1 × luciferase lysis buffer, and luciferase activity was determined and normalized to the pRL-null luciferase activity using the Dual Luciferase Assay Kit (Promega, WI).

Superarray assay

HCT-116 p53+/- cells were seeded into plates 100-mm in diameter. Once they reached 60–80% of confluency, vehicle or 50 μM ECG was treated for 24 h. Total RNA was extracted using Trizol and cDNA was labeled from 5 μg of total RNA in a RT reaction with biotin-16-dUTP (Roche Diagnostics GmbH). The RT reaction was performed using MMLV Reverse Transcriptase (Promega, WI). The labeled cDNA was hybridized to GEArray Q Series Human Cancer PathwayFinder Gene Array (HS-006, SuperArray Bioscience) and normalized to the pRL-null vector according to the manufacturer’s protocol. After 48 h of transfection, the cells were harvested in 1 × luciferase lysis buffer, and luciferase activity was determined and normalized to the pRL-null luciferase activity using the Dual Luciferase Assay Kit (Promega, WI).

Results

The green tea catechin, ECG induces NAG-1 expression in HCT-116 cells

Green tea contains more catechins than other types of tea (32) and EGCG has been studied most often for its antitumorigenic activity because it is the most abundant catechin in this beverage. Therefore, we performed northern analysis to see if EGCG induces NAG-1 transcripts in HCT-116 cells. As shown in Figure 1A, NAG-1 is induced by EGCG, with a higher expression at 100 μM. This result may indicate that EGCG induces NAG-1 expression at only high concentrations. Next, we asked if other green tea catechins induce NAG-1 expression. While EGCG has been well studied and is known to have pro-apoptotic activity in several cancer cells, other catechins including EGC, EGC and EC have not been examined.
investigated in as much detail. Therefore, HCT-116 cells were treated with 50 \( \mu \)M of EGCG, EGC, ECG or EC for 24 h and western analysis was carried out. As shown in Figure 1B, NAG-1 expression was highly induced with ECG treatment, but not with EGCG, EGC or EC compounds. We also treated with various combinations of two catechins and found that NAG-1 was only expressed when ECG was present (data not shown). We selected two catechins for further analysis, EGCG (a well-known antitumorigenic catechin) and EGC (the most significant NAG-1 inducer), and treated HCT-116 cells with different concentrations for 24 h, and then looked for NAG-1 protein expression using western analysis. The tumor suppressor protein p53 was also measured because it is known that the antitumorigenic activity of EGCG is mediated by p53 (13,33); other polyphenolic compounds including resveratrol and genistein induced NAG-1 expression via p53 (24,25). Both compounds increased NAG-1 expression, with the highest NAG-1 induction at 50 \( \mu \)M ECG and 100 \( \mu \)M EGCG (Figure 1C). The same membrane was stripped and re-probed with p53 antibody, which showed that the p53 protein was induced by EGCG, but not by ECG. This indicates an obvious link between NAG-1 expression and p53 induction by EGCG, whereas ECG-induced NAG-1 expression seems to be unrelated to p53 expression. Overall, these results suggest that the two catechins may affect different molecular mechanisms to induce NAG-1 expression in HCT-116 cells.

Examination of catechin compounds on cell growth rate and apoptosis

NAG-1 expression is linked to apoptosis (15,17,23,34). To investigate the effects of catechins on the growth of colorectal cancer cells in culture different catechins were added to the culture medium for 48 h, at 50 \( \mu \)M concentrations. Sulindac sulfide was used as a positive control for apoptosis in HCT-116 cells (15). A significant induction of apoptosis was observed when cultures were treated with ECG, whereas other catechins failed to have this effect (Figure 2A). Sulindac sulfide was used as a positive control for apoptosis in HCT-116 cells (15). Next, we examined the ability of different catechins to inhibit cell growth. HCT-116 cells were treated with different catechins for 7 days and the cell growth rate was measured. As shown in Figure 2B, ECG treatment resulted in the complete inhibition of cell growth, whereas other catechins did not affect any significant inhibition at 50 \( \mu \)M, suggesting that ECG is more potent than any of the other catechins with respect to cell growth arrest. Interestingly, EGCG and EGC treatment slightly promoted cell growth during the time course. This effect has been described previously in human colon cancer cells treated with low concentrations of EGCG (35). Taken together with previous reports, 50 \( \mu \)M ECG results in growth arrest and enhanced apoptosis in HCT-116 cells, whereas other catechins do not induce apoptosis at the same concentration.

**PARP cleavage by ECG**

As ECG induces cell growth arrest and apoptosis in HCT-116 cells, we examined the effect of ECG treatment on cell morphology and cell death pathways. HCT-116 cells were treated with different catechins and western analysis was performed to see if PARP produces two fragments of ~116 and ~85 kDa. As shown in Figure 3, only ECG treatment results in PARP cleavage in HCT-116 cells. The observed cleavage of PARP, a DNA repair enzyme cleaved by caspases during apoptosis, was consistent with previous data showing that ECG induces apoptosis and cell growth arrest.

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**Fig. 1.** Green tea catechins induce NAG-1 expression in HCT-116 cells. (A) HCT-116 cells were treated with indicated concentrations of EGCG for 24 h. Ten micrograms of total RNA was loaded in each lane and transferred onto a Nylon membrane. The blot was hybridized with a NAG-1 probe and re-probed with \( \beta \)-actin cDNA. The hybridization signals were quantified using Scion Image software (Scion), and values for the 1.3 \( \mathrm{kb} \) NAG-1 transcript were normalized to \( \beta \)-actin transcript levels. The ratio of NAG-1 to \( \beta \)-actin is recorded beneath each band. (B) HCT-116 cells were treated with 50 \( \mu \)M of different catechins, EGCG, EGC, ECG and EC, for 24 h. DMSO (0.2\%) was used as a vehicle treatment. Thirty micrograms of total protein was subjected to 14\% SDS-PAGE. The antibodies for NAG-1 and \( \beta \)-actin (Santa Cruz) were applied as described in the Materials and methods. The ratio of NAG-1 to actin is recorded beneath each band. (C) HCT-116 cells were treated with either EGCG or ECG at the indicated concentration for 24 h. Subsequently, 30 \( \mu \)g of total cell lysates were subjected to 14\% SDS-PAGE. NAG-1, p53 and actin antibodies were probed. The blots are representative of two independent experiments.
The sub-G1 population was measured and represented as apoptotic cells. Fifty analyzed for apoptosis as described in the Materials and methods. The plates and incubated with vehicle or indicated compounds for 48 h and

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that the differences in the number of ECG treated and vehicle treated cells

Proliferation Assay (Promega, WI). Values are expressed as mean
growth was measured using CellTiter 96
96-well plate and incubated with vehicle (DMSO) or various catechins. Cell

Fig. 2. Growth retardation and apoptosis of HCT-116 cells treated with different catechins. (A) Flow cytometric analysis of catechin-treated HCT-116 cells. HCT-116 cells were plated at $3 \times 10^5$ cells/well in 6-well plates and incubated with vehicle or indicated compounds for 48 h and
analyzed for apoptosis as described in the Materials and methods. The sub-G$_1$ population was measured and represented as apoptotic cells. Fifty micromoles of each catechin was used, whereas 30 $\mu$M sulindac sulfide was used as a positive control. Apoptosis is represented by the fold increase in the sub-G$_1$ population over vehicle treatment. The data represent mean $\pm$ SD.

"P < 0.05 compared with vehicle treated sample. (B) Effect of catechins on HCT-116 cell growth. HCT-116 cells were plated at 1000 cells/well in a 96-well plate and incubated with vehicle (DMSO) or various catechins. Cell growth was measured using CellTiter 96$^\text{TM}$ AQueous One Solution Cell Proliferation Assay (Promega, WI). Values are expressed as mean $\pm$ SD of 4-5 replicate experiments. Statistical analysis was performed and * indicates that the differences in the number of ECG treated and vehicle treated cells are significant at P < 0.001.

Fig. 3. ECG induces apoptosis in HCT-116 cells. HCT-116 cells were treated with 50 $\mu$M of different catechins for 48 h. Cells and media were harvested and 30 $\mu$g of total proteins were loaded onto SDS-PAGE. Western analysis was performed using PARP antibody (Cell Signaling). Cleaved PARP was shown at $\sim$85 kDa, whereas intact PARP bands showed at $\sim$120 kDa. The data are representative of two independent experiments.

Promoter activity of NAG-1 in the presence of ATF3

To investigate the molecular mechanism at the transcriptional level by which ECG induces NAG-1 expression, the expression of two transcription factors, EGR-1 and ATF3, were examined. EGR-1 has been shown to bind the NAG-1 promoter and transactivate troglitazone-induced NAG-1 expression (27,30), whereas ATF3 has been shown to be induced by several compounds. Interestingly, ATF3 was induced 12 h after ECG treatment (Figure 4A). In contrast, EGR-1 was not

altered by ECG treatment. To see if ATF3 mediates NAG-1 expression at the transcriptional level, we examined the sequences for ATF3 transcription factor binding sites through the 3.5 kb NAG-1 promoter sequences (30), and found one at the $\sim$948 to $\sim$955 bp region of the NAG-1 promoter (Figure 4B, left panel). The pNAG1086/LUC or pNAG41/LUC promoter constructs were co-transfected with either empty vector or a pEG-ATF3 expression vector. Both NAG-1 constructs do not contain p53 binding sites. As shown in Figure 4B, ATF3 expression induced NAG-1 promoter activity in pNAG1086/ LUC transfection, suggesting that ATF3 mediates NAG-1 expression through the putative ATF3 binding site located at $\sim$955 bp promoter region.

Other potential pathways affected by ECG

Previous results suggest that p53 is not involved in ECG-induced NAG-1 expression. To confirm the p53-independent mechanism of ECG, we used p53 null HCT-116 cells (HCT-116 p53$^-/-$) and examined NAG-1 expression in the presence of different catechins. As expected, NAG-1 was only induced in ECG-treated cells (Figure 5A), further providing evidence that ECG-induced NAG-1 expression is p53 independent. These data are consistent with Figure 1C, indicating that ECG-induced NAG-1 expression is p53 independent. To examine other potential pathways by which ECG induces antitumorigenic activity in a p53-independent manner, we performed superarray analysis to characterize the mRNA expression profile of cancer-related genes in HCT-116 p53$^-/-$ cells. After incubation with or without 50 $\mu$M ECG, cellular RNA was extracted, and cancer-related gene expression was analyzed using Superarray$^\text{TM}$ membranes. Genes that were up-regulated $>$3.0-fold by ECG were Bcl-x, CDC25A, PAI-1, DNA-PK, E-cadherin and TSP1, whereas significantly (<0.5 of control) down-regulated genes were APAF-1 and RB1 (data not shown). Among these, we further analyzed TSP1 (Figure 5B), because its induction can contribute to inhibition of angiogenesis (36). Oligonucleotide primers were designed for TSP1 as described in the Materials and methods and RT-PCR was performed. TSP1 and NAG-1 transcription were markedly up-regulated in HCT-116 p53$^-/-$ cells with ECG treatment, whereas the levels of transcription of GAPDH were not affected (Figure 5C). We then performed western analysis of HCT-116 p53$^-/-$ cells to confirm that ECG induces TSP1 protein expression. As shown in Figure 5D, TSP1 is induced by ECG treatment in HCT-116 p53$^-/-$ cells as early as 1 h after treatment with increased expression following 24-48 h of treatment. Taken together, ECG may alter several genes, including TSP1, in HCT-116 p53$^-/-$ cells, which probably account for its apoptotic and/or antitumorigenic activities.

Discussion

Some of the most promising pharmaceutical agents described to date for the prevention of colorectal cancer are the NSAIDs. NSAID activated gene (NAG-1) has been identified in our laboratory by PCR-based subtractive hybridization as a pro-apoptotic and antitumorigenic protein induced by NSAIDs (15). The human NAG-1 cDNA encodes a secreted protein with homology to members of the TGF-$\beta$ superfamily and it has been reported that NAG-1 over-expression from a recombinant adenoviral vector results in up to an 80% reduction of MDA-MB-468 and MCF-7 breast cancer cell viability (17). The data support the evidence that NAG-1 is linked to
Fig. 4. ATF3 may be involved in ECG-induced NAG-1 expression. (A) HCT-116 cells were treated with 50 µM ECG at different time points and the expression of NAG-1, ATF3, EGR-1 and actin was measured. (B) Schematic diagram of pNAG1086/LUC and pNAG41/LUC constructs. Both constructs do not contain p53-binding sites; the ATF3 binding site is shown in the left panel. The pNAG1086/LUC construct or pNAG41/LUC was transiently co-transfected with either empty vector (control) or ATF3 expression vector, pCG-ATF3 into HCT-116 cells. The cells were grown for 2 days and harvested for luciferase activity. The y-axis shows fold induction (over relative luciferase activity of empty vector transfected cells as 1.0). Values are mean ± SD of three independent transfections.

Fig. 5. Alteration in gene expression by ECG in HCT-116 p53−/− cells. (A) HCT-116 p53−/− cells were obtained from Dr Vogelstein at Johns Hopkins University and treated with different catechins (50 µM each). Thirty micrograms of total protein were loaded and western analysis was performed using NAG-1 and actin antibodies. The data are representative of two independent experiments. (B) HCT-116 p53−/− cells were treated with either vehicle or 50 µM ECG for 24 h and then superarray analysis was performed according to the manufacturer’s protocol. GEArray Series Human Cancer PathwayFinder Gene Array (HS-006) was used and thrombospondin was indicated as position number 87. (C) Electrophoretic analysis of RNAs from vehicle or ECG-treated HCT-116 p53−/− cells, RT-PCR was performed as described in the Materials and methods. Amplified PCR products were run on a 1.2% agarose gel. (D) Western analysis of TSP1 from HCT-116 p53−/− cells treated with 50 µM ECG at different time points. Identical results were obtained in two replicate experiments.
apoptosis and that its reduced expression may enhance tumorigenesis. Although highly expressed in mature intestinal epithelial cells and significantly reduced in neoplastic intestinal epithelium (21), there are conflicting reports of NAG-1 expression in prostate cancer (37-40). Thus, more detailed studies are required to elucidate NAG-1 function in prostate carcinogenesis. In contrast, the function of NAG-1 in human colorectal cancer has been well established. NAG-1 overexpression causes suppression of tumor growth in a xenograft animal model and inhibits cell growth in vitro (15). NAG-1 is induced by several anticancer compounds (24,25,29,31). In this report, we provide further evidence that ECG, an inducer of apoptosis in colorectal cancer cells, induces NAG-1 expression mediated by the ATF3 transcription factor.

Catechins, which are water-soluble substances with limited ability to pass through the plasma membrane of cells, may bind to certain cell surface receptors, thereby provoking receptor-like intracellular signals. In addition, catechins might utilize the kinase cascade in a similar manner to toxic substances or cellular stress. Overall, our understanding of the molecular mechanisms by which catechins exert their antitumorigenic effect is not clear, but induction of apoptosis by catechins is one observable fact to explain their antitumorigenic activity. Although it is unclear from epidemiologic studies whether green tea has any effect on human colorectal cancer risk (41), experimental evidence indicates clearly that green tea components induce antitumorigenic activity by apoptosis in colorectal cancer cells (5,35). In this report, we provide NAG-1 induction as a novel mechanism of catechin-induced apoptosis, and show that ECG, EGCG, EC and EGC individually have a variable affect on expression of NAG-1 protein, apoptosis induction and cell growth rate in human colorectal cancer cells. Interestingly, ECG has a distinct mechanism to induce NAG-1 expression in HCT-116 cells. HCT-116 cells contain intact p53 alleles and EGCG (100 μM)-induced NAG-1 expression is mediated by p53 protein induction at the transcriptional level. Since EGCG treatment in HCT-116 cells induces the p53 protein and a high concentration of EGCG (100 μM) is known to induce oxidative DNA damage (42), it is likely that DNA damage causes p53 induction followed by NAG-1 induction in HCT-116 cells. The data also support previous reports that NAG-1 is regulated by p53 (17,24,34) and EGCG activates the p53 protein (13).

ECG induces NAG-1 expression in a p53-independent manner as shown in Figures 1 and 5. ECG induces NAG-1 expression in cells lacking p53 (Figure 5) and a NAG-1 promoter that lacks the p53-binding site is activated in the presence of ECG (data not shown). Furthermore, p53 protein expression is not induced by ECG (Figure 1C). Taken together, ECG induced NAG-1 expression occurs in a p53-independent manner. We therefore investigated other pathways by which ECG might induce NAG-1 expression in HCT-116 cells. As shown in Figure 4, the ATF3 transcription factor may play an important role in ECG-induced NAG-1 expression. ATF3 is induced by camptothecin and etoposide: agents known to induce apoptosis (43,44). Furthermore, ATF3 is highly expressed after sulindac sulfide treatment and may also play an important role in sulindac sulfide-induced apoptosis and antitumorigenic activity (45,46). Tetracycline-inducible over-expression of ATF3 suppresses cell growth and slows cell cycle progression from G2 to S phase (47). In this report, we show that ECG-induced NAG-1 expression is mediated by ATF3 in a p53-independent manner (Figure 6). This is interesting because p53 tumor suppressor proteins are often mutated in cancer, suggesting that ECG might be valuable as a therapeutic agent. Indeed, many researchers have investigated gene alteration profiles to search for p53-independent pathways affected by green tea components in neuron (48) and prostate (49) cancer cells. In this study, we also found several genes that were changed by ECG treatment in human colorectal cancer cells. One of the ECG-induced genes is Thrombospondin-1 (TSP1), which can inhibit angiogenesis and modulate the activity of TGF-B1. It is notable that TSP1 expression was seen earlier than NAG-1 expression following ECG treatment of HCT-116 cells, suggesting that ECG could induce TSP1 expression with subsequent modulation of NAG-1. We are currently investigating the relationship between TSP1 and NAG-1 expression.

It has been reported that ECG and EGCG exhibit different biological activity compared with other catechins in many aspects: EGCG and ECG are more potent free radical scavengers than other catechins (50); EGCG and ECG might be potent neuroprotective agents for Parkinson’s disease (51); and EGCG and ECG are potent inhibitors of fatty-acid synthesis with resultant selective cytotoxic effects on human cancer cells (52). Thus, EGCG and ECG may be more biologically active than other catechins in green tea. In this report, we further show that EGCG and ECG may affect different mechanisms regarding NAG-1 induction. Although ECG differs from EGCG only by the lack of one hydroxyl group on the B-ring, ECG is more biologically active than EGCG, consistent with previously reported specificity of NAG-1 regulation by other compounds. Many NSAIDs are structurally related to each other but have distinct effects on NAG-1 expression. For instance, sulindac sulfide induces NAG-1 expression whereas sulindac sulfone does not (15). Another example would be the differential effects of resveratrol and resveratrol derivatives on NAG-1 expression (24). Changing the hydroxyl group of resveratrol to a methoxy group diminishes NAG-1 induction, compared with resveratrol, indicating that the hydroxyl group may play a pivotal role in the induction of NAG-1. Therefore,
it is not surprising that a relatively minute difference in catechin structure greatly affects NAG-1 expression but the molecular basis for this distinction remains to be determined. The concentration of green tea catechins in human plasma has been reported to reach no higher than 1 μM even with consumption of large amounts of the beverage (53). Higher levels are expected to be present in the lumen of the gastrointestinal tract. Absorption, metabolism and/or degradation would result in lower concentrations in the colonic luminal fluids and, therefore, lower tissue concentrations than we could expect were present in our treated cells. Yang et al. reported that the efficacy of 50% inhibition in cell culture varied, but generally ranged between 22 and 130 μM for EGCg (54). Taken together with our study, a 50 μM catechin in cell culture reflects a higher range of plasma concentration; however, to know the exact effective concentration in the cell culture, the bioactivity, degradation, as well as metabolite effects of catechins in the cell culture should be considered.

In summary, the present study demonstrates that green tea catechins (EGCG, ECG, EGC and EC) exhibit a variety of actions on human colorectal cancer cells, including apoptosis, cell growth and NAG-1 expression. Many mechanisms may simultaneously contribute to the beneficial effects of catechins in human colorectal cancer. However, p53-independent induction of NAG-1 by green tea catechins, particularly ECG, is one solid molecular mechanism to explain their beneficial effects. Nevertheless, more work is required to elucidate the molecular and clinical chemopreventive effects of green tea on human colorectal cancer.

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