Epigallocatechin Gallate Suppresses Lung Cancer Cell Growth through Ras–GTPase-Activating Protein SH3 Domain-Binding Protein 1

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Abstract

Green tea is a highly popular beverage globally. Green tea contains a number of polyphenol compounds referred to as catechins, and (−)-epigallocatechin gallate (EGCG) is believed to be the major biologically active compound found in green tea. EGCG has been reported to suppress lung cancer, but the molecular mechanisms of the inhibitory effects of EGCG are not clear. We found that EGCG interacted with the Ras–GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) with high binding affinity \((K_d = 0.4 \mu\text{mol/L})\). We also showed that EGCG suppressed anchorage-independent growth of H1299 and CL13 lung cancer cells, which contain an abundance of the G3BP1 protein. EGCG was much less effective in suppressing anchorage-independent growth of H460 lung cancer cells, which express much lower levels of G3BP1. Knockdown shG3BP1-transfected H1299 cells exhibited substantially decreased proliferation and anchorage-independent growth. shG3BP1 H1299 cells were resistant to the inhibitory effects of EGCG on growth and colony formation compared with shMock-transfected H1299 cells. EGCG interfered with the interaction of G3BP1 and the Ras–GTPase-activating protein. This further suppressed the activation of Ras. Additional results revealed that EGCG effectively attenuated G3BP1 downstream signaling, including extracellular signal-regulated kinase and mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, in wild-type H1299 and shMock H1299 cells but had little effect on H460 or shG3BP1 H1299 cells. Overall, these results strongly indicate that EGCG suppresses lung tumorigenesis through its binding with G3BP1. Cancer Prev Res; 3(5): 670–9. ©2010 AACR.

Introduction

Ras–GTPase-activating protein (GAP) is an important protein in the modulation of the downstream signal transduction of Ras, one of the critical oncoproteins in lung carcinogenesis (1–3). The Ras-GAP SH3 domain-binding protein (G3BP) family was reported to bind the SH3 domain of Ras-GAP (4). The G3BP family of proteins participates in several signaling pathways involved in carcinogenesis, including NF-κB and Ras signaling and the ubiquitin proteasome system (5–8). G3BP1 is one member of G3BP family and exhibits a phosphorylation-dependent endoribonuclease activity on the c-myc 3′-untranslated region in vitro (6, 9). G3BP1 is overexpressed in various human tumors, including lung, breast, head, neck, colon, and thyroid (6). Overexpression of G3BP1 was reported to increase S-phase entry in fibroblasts, and the overexpression was associated with the intact RNA-binding domain (10). Additionally, after binding with p53, G3BP1 expression led to the redistribution of p53 from the nucleus to the cytoplasm (11). The overall evidence suggests that specifically overexpressed G3BP1 in a range of cancers might be a candidate target for anticancer agents.

(−)-Epigallocatechin gallate (EGCG), a major polyphenolic compound in green tea, has numerous alleged bioactivities, including antioxidant, chemopreventive, and anticarcinogenesis activities (12–14). EGCG is reported to affect many different signal transduction pathways, including inhibition of various protein kinases, suppression of the activation of transcription factors such as AP-1 and NF-κB, blockade of growth receptor-mediated pathways, and induction of cell cycle arrest or apoptosis (15–18). EGCG was also found to attenuate growth factor–mediated proliferation, inhibit cell transformation, and repress angiogenesis in various cancer models (19–21).
In the present study, we provide new evidence showing that G3BP1 is a novel target of EGCG. Our results indicated that EGCG might effectively inhibit anchorage-independent growth of H1299 cells because EGCG binds to G3BP1 with high affinity, affecting the Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK)/kinase (MEK)/ERK pathway. Further, short hairpin RNA (shRNA) directed against G3BP1 decreases anchorage-independent growth of H1299 cells. These results indicated that the binding of EGCG with G3BP1 is a novel and important event that provides further clarification of the mechanism of EGCG-mediated inhibition of anchorage-independent cell growth.

Materials and Methods

Materials
EGCG was kindly provided by Dr. Chi-Tang Ho (Rutgers University, Piscataway, NJ), and [3H]EGCG was a gift from Dr. Yukihiko Hara (Food Research Laboratory, Mitsui Norin Co. Ltd., Fujieda, Shizuoka, Japan). The human G3BP1 monoclonal antibody and mouse G3BP1 polyclonal antibody were from BD Biosciences and Abcam, respectively. The Ras-GAP polyclonal antibody was from Cell Signaling Technology. The human lung tumors in A/J mice were grown in RPMI 1640 supplemented with 10% FBS and 0.8 mg/mL G418. The CL13 cells derived from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone–induced lung tumors in A/J mice were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The shMock- and shG3BP1-transfected H1299 cells were produced by Dr. Steven A. Belinsky (University of New Mexico Cancer Center, Albuquerque, NM). The shRNA sequences targeting G3BP1 cloned into the pB8/S-U6 vector (11) were provided by Dr. Y. Shi (Harvard Medical School, Boston, MA).

Cell culture
The H1299 human non–small cell lung cancer, H460 human large cell lung cancer, H520 human lung squamous cell carcinoma, and CL13 cells derived from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol–induced lung tumors in A/J mice were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). The CL13 cells were produced by Dr. Steven A. Belinsky (University of New Mexico Cancer Center, Albuquerque, NM). The HEK293T cells were cultured in DMEM containing 10% FBS. The shMock- and shG3BP1-transfected H1299 cells were maintained in RPMI 1640 supplemented with 10% FBS and 0.8 mg/mL G418.

EGCG pull-down assay
As in our previous study, EGCG was first coupled to the CNBr-activated Sepharose 4B matrix, and the binding between proteins in cell lysates and EGCG was examined by pull-down assay as described previously (22). The proteins bound to the beads were analyzed by immunoblotting with specific antibodies as indicated.

Proteomic analysis
The proteins bound to the EGCG-conjugated beads were analyzed by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF; ref. 23). The silver-stained two-dimensional gels were scanned with an ImageScanner (Amersham) and analyzed with Phoretix Expression software (version 2005; Nonlinear Dynamics). Destaining and in-gel tryptic digestion of the protein spots were done as described (24). The mass spectra were calibrated and processed using Flex Analysis and BioTool 2.2 software (Bruker Daltonics). Peptide mass fingerprinting ion searches were done using Mascot 2.0 software (http://www.matrixscience.com) integrated with BioTool 2.2. The Mass Spectrometry Protein Sequence Database and National Center for Biotechnology Information nonredundant protein databases were searched using the following Mascot settings: taxonomy Mammalia, one incomplete tryptic cleavage allowed, peptide tolerance 50 to 100 ppm, fragment tolerance 0.5 Da, monoisotopic mass, 1 peptide charge state as HCCA protonation, deamidation of lysine and asparagine, oxidation of methionine as a variable modification. For each search, statistically significant (P < 0.05) matches were regarded as correct hits. The threshold score for the Mass Spectrometry Protein Sequence Database was 67 and that for the National Center for Biotechnology Information database was 67 to 78. Additional analysis was done based on two criteria. First, mouse proteins defined as matches were chosen even if another species had higher-ranked hits. Second, matched proteins with isoelectric points (pI) and molecular weights corresponding to those identified in the two-dimensional gel were chosen, although other proteins sometimes had higher-ranked hits.

MALDI-TOF mass spectrometry calibration
The peptides used for the calibration were bradykinin (1-7) _mono (757.399), angiotensin II (M+H)+_mono (1,046.541), angiotensin I (M+H)+_mono (1,296.684), substance P (M+H)+_mono (3,147.471), bombesin (M+H)+_mono (1,347.735), ACTH [1-17] (M+H)+_mono (2,093.086), ACTH [18-39] (M+H)+_mono (2,465.198), and somatostatin (28) (M+H)+_mono (3,147.471).

Construction of G3BP1 plasmids
A G3BP1-encoding cDNA was generated by PCR and subcloned into the BamHI/XhoI sites of the pGEX-5X-1 vector (Amersham Biosciences) to produce the glutathione S-transferase (GST)–G3BP1 fusion protein. Individual serial truncated deletion mutants and full-length G3BP1 were subcloned into the BamHI/XhoI sites of the pcDNA4/His-Max vector (Invitrogen). The pCDNA3/hRas-GAP plasmid was from Dr. Christian Widmann (Lausanne University, Lausanne, Switzerland; ref. 25), and the shRNA sequences targeting G3BP1 cloned into the pB8/S-U6 vector (11) were provided by Dr. Y. Shi (Harvard Medical School, Boston, MA).

Physical binding and Kd measurement
The GST-G3BP1 affinity binding assays were carried out as described (22). For analyzing concentration-dependent uptake, various concentrations of EGCG (0.1-10 μmol/L) were applied. The Kd value was determined through nonlinear regression analysis using the Prism 4.0 software program (GraphPad, Inc.).
Ras activation assay
The active form of Ras in cells was analyzed using a Ras activation assay kit according to the manufacturer's protocol (Upstate Biotechnology, Inc.). In brief, the treated cells were disrupted with Mg2+ lysis/wash buffer (MLB), and then the lysate was incubated at 4°C for 60 min with beads coated with the GST fusion protein, Raf-1 Ras-binding domain, which specifically binds to Ras-GTP. The beads were then washed with MLB thrice, and the washed beads were suspended in 2× Laemmli sample buffer. SDS-PAGE and an immunoblot assay using anti-Ras were used to detect the relative amount of Ras-GTP.

In vitro binding assay
For the GST pull-down assay, the plasmids encoding Ras-GAP were transfected into HEK293 cells. The HEK293-transfected cell lysate supernatant fraction was mixed with GST or GST fusion G3BP1 protein-bound beads, which were incubated with EGCG (0-20 μmol/L). Eluted immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blot using a specific antibody.

Immunoprecipitation assay
The Ras-GAP or G3BP1 plasmid was transfected into H1299 cells, and then cells were treated with EGCG (0-20 μmol/L). Cell lysates were incubated at 4°C overnight in reaction buffer as described for the EGCG pull-down assay but with a His-G antibody and protein G Plus agarose beads. The antibody binding proteins were resolved by SDS-PAGE and analyzed by Western blot.

Western blot analysis
After treating with EGCG (0-20 μmol/L), cells were harvested and disrupted. The protein supernatant fractions were subjected to SDS-PAGE and then transferred to membranes and blocked with 5% skim milk followed by hybridization with the indicated antibodies. Protein bands with the horseradish peroxidase-conjugated secondary antibody were observed with a chemiluminescence detection kit.

MTS assay
The effect of EGCG on viability was estimated using the CellTiter 96 AQueous One Solution Cell Proliferation
Assay kit (Promega) according to the manufacturer's instructions. Cells were seeded in a 96-well plate and then incubated with different concentrations of EGCG.

**Anchorage-independent cell growth assay**
Cells (8 × 10⁴) were treated with various concentrations of EGCG in 1 mL of 0.33% basal medium Eagle's agar over 3 mL of 0.5% basal medium Eagle's agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO₂ incubator for 12 days, and then the cell colonies were automatically counted by a computerized microscope system. Anchorage-independent cell growth is presented as a ratio of colony number per 8,000 seeded cells comparing EGCG-treated cells with untreated control cells in soft agar.

**Statistical analysis**
Data are represented as the mean ± SD of at least three independent experiments done in triplicate. Data were analyzed for statistical significance using one-way ANOVA. The minimum level of significance was set at \( P < 0.05 \).

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**Fig. 2.** Determination of EGCG- and G3BP1-binding affinity. Lung cancer cell lysates, including H460 (A), H1299 (A), and CL13 (B), were precipitated with Sepharose 4B or EGCG-Sepharose 4B affinity beads and then analyzed by Western blot using anti-G3BP1. C, specific assay to determine the binding affinity of G3BP1 and EGCG. The \( K_d \) of the interaction between \( ^3H \)EGCG and G3BP1 was obtained by using a GST-G3BP1 affinity-binding assay as described in Materials and Methods. D, identification of the EGCG-binding site on G3BP1. A series of nucleotide constructs spanning the G3BP1 gene or six deletion mutants were created and expressed. The individual proteins were incubated with EGCG-Sepharose 4B beads for a pull-down assay, and the proteins bound to the beads were analyzed by Western blot using a His-G antibody.
Results

Identification of G3BP1 as an EGCG-binding protein

To identify novel target proteins that interact with EGCG, we conjugated EGCG to Sepharose 4B beads and conducted an affinity chromatography experiment. Proteins bound to EGCG were subjected to two-dimensional electrophoresis and silver stained, and the respective spots were digested with trypsin and identified by MALDI-TOF mass spectrometry. Consequently, G3BP1 (XP_990232) was positively identified by 13 matched peptides and 46.9% sequence coverage using mass spectrometry. Comparison of the experimental (52,148 Da, 5.61) with the theoretical Mr and pI values (51,854 Da, 5.41) of the G3BP1 spot proved to show approximately the same Mr and pI values. G3BP1 was thus identified as a possible molecular target for EGCG (Fig. 1A). We therefore studied the potential interaction of EGCG and G3BP1 in different cell lines, including human H1299 lung cancer cells that have a high abundance of the hG3BP1 protein, human H460 lung cancer cells that express low levels of hG3BP1 (Fig. 1B), and mouse CL13 lung cancer cells (Fig. 2B),

Fig. 3. Inhibitory effects of EGCG on anchorage-independent growth of human lung cancer H1299 (A) and H460 (B) cells and mouse lung cancer CL13 cells (C). Colony formation ability of lung cancer cells treated or not treated with EGCG. A colony formation assay was done as described in Material and Methods, and colonies were counted under a microscope using the Image-Pro Plus software program. Columns, mean of the number of colonies as determined from three independent experiments; bars, SD. *, P < 0.05, significant difference between the group treated with EGCG and the untreated control group.
which also express high levels of mG3BP1. The interaction
was assessed in a pull-down assay using EGCG-Sepharose
4B beads, and G3BP1 was then detected by immunoblot-
ting with anti-G3BP1. The results indicated that EGCG
bound with G3BP1 in cell lysates from human H1299
cells (Fig. 2A, lane 6) and mouse CL13 cells (Fig. 2B,
lane 3) but not with human H460 cells (Fig. 2A, lane 3)
or with Sepharose 4B beads alone (Fig. 2A, lane 5, and B,
lane 2). To characterize the physical binding between
EGCG and G3BP1, the binding affinity ($K_d$) of this com-
plex was assessed using a GST pull-down assay with $^3$H-
labeled EGCG. The $K_d$ value of G3BP1 and EGCG binding
determined to be 0.4 μmol/L (Fig. 2C). The G3BP1 protein
has an NH$_2$-terminal region, a Ras-GAP–binding
region, an RNA recognition motif, a glycine-rich domain,
and a p53-binding region (26). To determine the specific
EGCG-binding region in the G3BP1 protein, we created six
G3BP1 deletion constructs on the basis of the different
specific regions (Fig. 2D, top) and evaluated binding by
EGCG-Sepharose 4B pull-down assay (Fig. 2D, bottom).

Compared with the input control, EGCG binds with full-
length G3BP1 (G3BP1-FL), G3BP1 m1, G3BP1 m2,
G3BP1 m4, and G3BP1 m5 but not with G3BP1 m3 or
G3BP1 m6 (Fig. 2D, bottom). These data indicated that
EGCG interacts with the Ras-GAP–binding region and
the glycine-rich domain of G3BP1.

**EGCG inhibits anchorage-independent growth in lung
cancer cells overexpressing G3BP1**

A previous study suggested that G3BP1 is dramatically
upregulated during cell proliferation and cancer progres-
sion and, in particular, breast cancers and metastatic lung
and prostate cancers (26). We found that G3BP1 was more
abundant in lung cancer cells compared with breast,
colon, or prostate cancer cells (Fig. 1B). To provide addi-
tional supporting evidence for the direct interaction of
EGCG with G3BP1, we chose one human and one mouse
lung cancer cell line each with a high abundance of G3BP1
(H1299 and CL13, respectively) and another with a low
abundance of G3BP1 (H460) for comparing the effects...
of EGCG. Our data indicated that 25 μmol/L EGCG significantly decreased the viability of H1299 but not H460 cells (data not shown). In CL13 cells, as little as 10 μmol/L EGCG dramatically decreased viability (data not shown). These results suggested that lung cancer cells overexpressing G3BP1 are more sensitive to the effects of EGCG. Furthermore, EGCG (10 μmol/L) substantially decreased colony formation of H1299 cells in soft agar (Fig. 3A) but had little effect on colony formation by H460 cells (Fig. 3B). EGCG at 5 or 10 μmol/L also significantly suppressed the colony formation by mouse lung cancer CL13 cells (Fig. 3C). These data indicated that EGCG inhibits anchorage-independent growth much more effectively in cell lines with a high abundance of the G3BP1 protein.

**EGCG affects the activation of Ras**

The first G3BP1 protein was discovered in a screen for proteins that bind the SH3 domain of the Ras-GAP (4). We did a pull-down and immunoprecipitation assay to determine whether EGCG affects the interaction of G3BP1 with Ras-GAP. GST-bound or GST-G3BP1-bound beads were incubated with different concentrations of EGCG-transfected and Ras-GAP-transfected HEK293T cell lysates. The results showed that the binding of Ras-GAP to G3BP1 decreased with increasing concentrations of EGCG.
Additionally, EGCG at concentrations under 20 μmol/L did not affect the levels of G3BP1 or Ras-GAP, but 20 μmol/L EGCG decreased the amount of G3BP1 bound with Ras-GAP in H1299 cells (Fig. 4B). Ras is a small GTPase responsible for transducing molecular signals, and the function of G3BP1 is important in the oncogenic ras signaling pathway (26). H1299 cells were treated with various amounts of EGCG (0, 5, 10, or 20 μmol/L), and the levels of Ras-GTP were measured to examine the activation of Ras in these cells. The results showed that 10 or 20 μmol/L EGCG could decrease the abundance of Ras-GTP compared with untreated cells (Fig. 4C), but EGCG had no effect on the relative abundance of the total Ras protein (Fig. 4C). The results suggested that EGCG binding to G3BP1 might result in suppression of Ras-GAP–bound G3BP1, which catalyzes the formation of inactive Ras-GDP from active Ras-GTP in H1299 cells.

**Knockdown of G3BP1 suppresses anchorage-independent cell growth**

Our data suggest that EGCG directly binds with G3BP1 and is more effective in lung cancer cells that overexpress G3BP1. We therefore next investigated the effects of G3BP1 knockdown on lung cancer cell anchorage-independent growth. Notably, compared with H1299 cells expressing green fluorescent protein–shRNA (H1299-shMock), H1299 cells expressing shG3BP1 exhibited a substantially reduced abundance of endogenous G3BP1 but no change in levels of Ras-GAP (Fig. 5A). Interestingly, the level of Ras-GTP in knockdown H1299-shG3BP1 cells was also much lower than that found in H1299-shMock cells (Fig. 5B). Proliferation of H1299-shG3BP1 cells was slower than that of control cells (Fig. 5C), suggesting that knockdown of G3BP1 might block S-phase entry. Furthermore, H1299 cell viability was inhibited more effectively by EGCG in shMock cells compared with shG3BP1 cells (Fig. 5D). Finally, colony formation in H1299-shG3BP1 cells was significantly less than that found in H1299-shMock cells (Fig. 5E), and 10 μmol/L EGCG decreased the number of colonies formed by shMock cells significantly more than untreated shMock control cells (Fig. 5E). These results suggested that G3BP1 plays a key role in proliferation and anchorage-independent cell growth, and EGCG suppresses anchorage-independent growth of H1299 cells by targeting G3BP1.

**EGCG inhibits phosphorylation of MEK and ERK**

EGCG interacts with G3BP1 and further affects the activation of Ras. We examined the effect of EGCG on MEK and ERK phosphorylation in cell lines that express variable amounts of G3BP1. EGCG inhibited the phosphorylation of MEK and ERK in a dose-dependent manner in H1299 cells compared with H460 cells (Fig. 6A).
H1299-shG3BP1 cells exhibited decreased phosphorylated levels of MEK and ERK1/2 (Tyr202/Tyr204) compared with H1299-shMock cells (Fig. 6B). EGCG suppressed phosphorylation of MEK and ERK in H1299-shMock cells in a dose-dependent manner but had less effect on phosphorylation of these proteins in H1299-shG3BP1 cells (Fig. 6B). These results provide evidence supporting the idea that EGCG inhibits Ras downstream signaling through G3BP1.

Discussion

Tea has been the most popular consumed beverage next to water worldwide for thousands of years. Tea has been alleged to provide various beneficial health effects and medicinal efficacy in the prevention and treatment of many diseases (27). EGCG, the primary active ingredient in green tea, has been suggested to provide cancer-preventive activity by regulating multiple signaling transduction pathways as shown in cancer cell lines and animal models of cancer (28, 29).

Lung cancer is the leading cause of cancer death in both men and women in industrialized countries, and cigarette smoking has been identified as a major cause of lung cancer (30). Mortality from lung cancer could be reduced through early detection and the chemopreventive strategies that can reverse or impede the progression of premalignant disease. Research results suggest that targeted molecular cancer therapies can potentially deliver treatment directly to a specific protein or gene target and optimize efficacy and thus reduce adverse side effects often associated with traditional chemotherapy (22). Lung carcinogenesis is a multistage process in which numerous genes important in the regulation of cellular functions are significant targets for cancer-preventive agents.

The G3BP family of proteins participates in several signaling pathways involved in carcinogenesis. However, the exact function and mechanism of G3BP1 in tumorigenesis is not clear. G3BP1 seems to be overexpressed in several cancer types, but whether this is a cause or a consequence of cancer is not known. We found that cells overexpressing G3BP1 were more sensitive to the anticancer effects of EGCG. Our data support an important functional role for G3BP1 in lung cancer development, and therefore, targeting G3BP1 can be regarded as a useful strategy in chemoprevention and cancer therapy. We showed that EGCG directly interacts with G3BP1 and suppresses G3BP1 binding with Ras-GAP. The biochemical experiments confirmed that EGCG interacted with the Ras-GAP-binding region and glycine-rich domain. Overabundance of the G3BP1 protein induces numerous downstream signaling events, including the activation of Ras signaling, MAPK activation, and phosphorylation of multiple signaling molecules (5–8). The interaction of EGCG and G3BP1 leads to the inhibition of the Ras downstream signaling pathway, including MEK and ERK, resulting in decreased anchorage-independent cell growth. Our data verify that EGCG is more effective in lung cancer cells that overexpress G3BP1 and not as effective in cells with low abundance of the G3BP1 protein. These results indicate that EGCG specifically targets G3BP1 to exert its chemopreventive effects, confirming other reports that EGCG suppresses anchorage-independent lung cancer cell growth (28). Our results provide strong evidence showing that G3BP1 is a direct mediator of EGCG suppression of anchorage-independent lung cancer cell growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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