Published in final edited form as:

Biochem Biophys Res Commun. 2009 November 20; 389(3): 527–530. doi:10.1016/j.bbrc.2009.09.019.

Suppression of Met activation in human colon cancer cells treated with (–)-epigallocatechin-3-gallate: minor role of hydrogen peroxide

Christine Larsen^a and Roderick H. Dashwood^{a,b,*}

^a Linus Pauling Institute, Oregon State University, Corvallis, OR 97331-6512, USA

^b Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR 97331, USA

Abstract

Colorectal cancer is the second leading cause of cancer-related deaths in the U.S. Met, the receptor for hepatocyte growth factor (HGF), is over-expressed in colon tumors and is associated with poor prognosis. Recently, the green tea polyphenol (–)-epigallocatechin gallate (EGCG) was reported to suppress Met activation in breast cancer cells. However, the possible confounding effect of hydrogen peroxide (H₂O₂), produced when EGCG is added to cell culture media, was not assessed. In the present study, the human colon cancer cell lines HCT116 and HT29 were used to examine the relationships between Met activation, EGCG treatment, and H₂O₂ generation. At concentrations of 0.5, 1 and 5 μM, EGCG suppressed markedly the activation of Met in the presence of HGF. Concentrations of 10 μM EGCG and below generated low amounts of H₂O₂ (<1.5 μM), whereas higher H₂O₂ concentrations (>5 μM) were required to directly increase the phosphorylation of Met. Moreover, suppression of Met activation by EGCG occurred in the presence or absence of catalase, suggesting that such effects were not an ‘artifact’ of H₂O₂ generated from EGCG in cell culture media. We conclude that EGCG might be a beneficial therapeutic agent in the colon, inhibiting Met signaling and helping to attenuate tumor spread/metastasis, independent of H₂O₂-related mechanisms.

Keywords

Tea polyphenols; metastasis; colon cancer; Met signaling

Introduction

According to the American Cancer Society, colorectal cancer is the second most common cause of cancer-related deaths in the US. Disease survival is related to tumor stage. For example, Stage I colorectal cancer, in which the carcinoma remains localized in the sub-mucosa of the colon epithelium, has an overall 5-year survival rate of over 90%. In contrast, the 5-year survival rate for metastatic disease (Stage IV) drops to less than 10%. Since metastasis accounts for >90% of colon cancer deaths [1], therapies that target this process and block disease

*Corresponding author: Fax: 1-541-737-5077, rod.dashwood@oregonstate.edu (R.H. Dashwood).

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progression are of major interest. The receptor tyrosine kinase, Met, has been identified as an important protein that is essential for metastasis in colorectal carcinogenesis [2–6]. When Met is activated by its ligand, hepatocyte growth factor (HGF), it induces proliferation, motility, and invasion; all processes that are required for metastasis to occur.

Green tea and its associated polyphenols have been shown to reduce intestinal tumor formation in the *Apc*^{Min/+} mouse [7,8]. EGCG, which is the most abundant polyphenol in green tea, has been shown to interfere with several cancer-related signaling pathways *in vitro* and *in vivo*. However, the ability of EGCG to suppress Met signaling in colon cancer cells remains to be studied. After oral ingestion, most EGCG accumulates in the intestine [9]. This makes the use of EGCG an attractive therapeutic approach for the possible prevention of colon tumor metastasis.

EGCG has been shown to produce large amounts of hydrogen peroxide (H₂O₂) when added to cell culture media [10]. This has been proposed to represent a possible ‘artifact’ when working with tea polyphenols *in vitro*. Therefore, empirical evidence is needed whenever cell culture studies use EGCG, to verify that effects are due to EGCG rather than H₂O₂ generated in the experiment. The present study provides evidence that EGCG suppresses Met signaling in human colon cancer cells, and that this effect is independent of H₂O₂ generated under the conditions used here.

Materials and methods

Cell culture

HCT116 and HT29 human colon cancer cells were obtained from American Type Culture Collection (Manassas, VA). They were maintained in McCoy’s 5A media (Invitrogen, Carlsbad, CA) with 10% FBS and 1% penicillin/streptomycin. Cells were grown at 37°C with 5% CO₂.

EGCG/HGF cell treatment

Cells (0.8×10^6) were plated in 60 mm dishes and grown in serum-containing media for 48 h. Cells were then incubated in serum-free media for 4 h. After serum starvation, cells were pretreated for 30 min with various concentrations of EGCG (Sigma-Aldrich, St. Louis, MO) followed by treatment with 30 ng/ml HGF (Calbiochem, San Diego, CA). In some experiments, 30 U/ml catalase (Roche Applied Science, Indianapolis, IN) were added to the media 10 min prior to addition of EGCG.

H₂O₂ cell treatment

Cells (0.8×10^6) were plated in 60 mm dishes and grown in serum-containing media for 48 h. Cells were then serum-starved for 4 h. Various concentrations of H₂O₂ (Sigma-Aldrich, MO) were added and cells were incubated for an additional 30 min prior to immunoblot analyses.

Western blotting

Cells were placed in IP lysis buffer, vortexed, and then centrifuged at 10,000 rpm for 5 min. The supernatant was collected and protein concentrations were determined by BCA assay (Pierce, Rockford, IL). Proteins (10–20 mg) were separated by SDS-PAGE on a 4–12% bis-Tris gel (Novex, San Diego, CA) and transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA). Equal protein loading was confirmed with Amido Black staining and β -actin levels. The membrane was blocked for 1 h with 2% bovine serum albumin, followed by overnight incubation with primary antibody at 4°C, and finally incubated for 1 h with secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Antibody dilutions were as follows: Phospho-Met (Tyr1234/1235), 1:1000 (Cell Signaling Technology, Beverly,

MA), total Met, 1:1000, (Cell Signaling Technology), and β -actin, 1:5000 (Sigma). Detection was by Western Lightning Chemiluminescence Reagent Plus (PE Life Sciences, Boston, MA) with image analysis on an AlphaInnotech photodocumentation system.

H₂O₂ measurement

To measure H₂O₂ production, serum-starved cells were incubated with various concentrations of EGCG in phenol red-free McCoy's 5A media (Invitrogen) for 30 min. Aliquots of media were analyzed using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR), as previously reported [11].

Enzyme-linked immunosorbent assay (ELISA)

Cell culture media was pretreated in the presence or absence of 30 U/ml catalase (Roche). EGCG (5 μ M) was then added to the media and cells were incubated for 30 min prior to the addition of 30 ng/ml HGF (Calbiochem). Cells were incubated for an additional 15 min and lysed in RIPA buffer containing phosphatase inhibitors (Pierce). The ELISA was performed according to the instruction manual for STAR phospho-Met (Tyr1230/Tyr1234/Tyr1235) ELISA kit (Upstate).

Cell viability

HCT116 cells were incubated with various concentrations of H₂O₂ for 15 min. The cells were then collected and stained with Guava Viacount® Reagent (Guava Technologies, Hayward, CA) for 5 min. The percentage of viable cells was determined by using the Guava ViaCount Assay on a Guava Personal Cytometer.

Results

Phosphorylation of the Met receptor previously was reported to be inhibited by EGCG in immortalized and tumorigenic breast epithelial cells [12]. To determine if this occurred in human colon cancer cells, HCT116 and HT29 cells were pre-incubated with various concentrations of EGCG for 30 min, followed by the addition of HGF to the media. As expected, in the absence of EGCG pretreatment, HGF alone strongly increased the levels of p-Met in HCT116 cells (Fig. 1A) and HT29 cells (Fig. 1B). However, activation of the Met receptor was suppressed by EGCG concentrations as low as 0.5 μ M. This suppression occurred in both cell lines, and was evident after normalizing p-Met to total Met in the immunoblots (Fig. 1C).

The amount of H₂O₂ produced by EGCG under the current conditions was measured after incubating various concentrations of EGCG in cell culture media for 30 min. With no EGCG present, ~0.1 and 0.25 μ M H₂O₂ was detected in media from HCT116 and HT29 cells, respectively (open bars, Fig. 2A). The concentration of H₂O₂ increased with EGCG added to the media. However, only at the highest concentration of 10 μ M EGCG was H₂O₂ measured at a level above 1 μ M, in each cell line (solid bar, Fig. 2A).

To investigate the direct effects of H₂O₂ on Met activation, HCT116 cells were incubated with various concentrations of H₂O₂ for 30 min, in the absence of HGF, and p-Met was analyzed by immunoblotting. Concentrations at or below 2.5 μ M H₂O₂ had no effect on Met phosphorylation, as indicated by the lack of change in p-Met levels compared with the 0 μ M H₂O₂ control (Fig. 2B). When the concentration of H₂O₂ exceeded 2.5 μ M, however, there was increased phosphorylation of Met, most notably at the highest levels of 10 and 20 μ M H₂O₂ (Fig. 2B).

The direct effects of H₂O₂ were further examined in the presence and absence of HGF (Figs. 2C,D). In the absence of HGF, p-Met/total Met levels were increased by concentrations of H₂O₂ in the range 5–100 μ M H₂O₂ (Fig. 2D, open bars). A similar trend was seen in cells treated with H₂O₂ plus HGF (Fig. 2D, gray bars). However, compared to –HGF controls, receptor activation was enhanced at concentrations below 20 μ M H₂O₂ (compare, for example, white and gray bars at 5 μ M H₂O₂ in Fig. 2D). At concentrations of 20, 50, and 100 μ M H₂O₂, phosphorylation of the Met receptor was decreased slightly when HGF was present, as compared with the corresponding –HGF controls. We also sought to test the upper limit for Met activation in response to H₂O₂; concentrations of 500 and 1000 μ M H₂O₂ strongly reduced p-Met and total Met in the immunoblots, as well as expression of the loading control β -actin (Fig. 2C).

The viability of HCT116 cells treated with these concentrations of H₂O₂ was assessed. There was little or no effect on viability when cells were exposed to H₂O₂ at concentrations up to 100 μ M (Fig. 2E). However, at the highest concentrations of 500 and 1000 μ M H₂O₂ the percentage of viable cells was reduced to less than 45% and 8% viability, respectively (Fig. 2E).

To further assess the role of H₂O₂ and to differentiate its effects from those of EGCG, subsequent experiments included catalase. Immunoblotting revealed that, in the presence or absence of catalase, HGF produced a similar level of Met phosphorylation, and EGCG maintained its inhibitory effects (Fig. 3A). This was confirmed for the same cell lysates using an ELISA kit to detect p-MET, revealing that Met activation was virtually identical in the presence or absence of catalase (Fig. 3B). Thus, inhibition of the Met receptor by EGCG can occur in the absence of H₂O₂.

Discussion

Previous studies have shown that tea polyphenols are capable of suppressing receptor tyrosine kinase activity [13–15]. In particular, the Met receptor was shown to be inhibited in breast cancer cells treated with EGCG, the major polyphenol in green tea [12]. Here, EGCG effectively suppressed the activation of the Met receptor in human colon cancer cells, and at a concentration as low as 0.5 μ M EGCG, which is likely to be within the physiological range [16,17].

EGCG rapidly produces H₂O₂ when added to cell culture media [10]. At a concentration of 25 μ M EGCG, for example, 10–12 μ M H₂O₂ was detected in serum-free MEM media [11]. Here, we investigated the H₂O₂ generated following the addition of EGCG to McCoy's 5A media, used in the current study. As suggested by Long *et al.* [10], excess H₂O₂ generated in cell culture media from the oxidation of polyphenolic compounds could represent an artifact in mechanistic studies of tea catechins. Thus, it is important to distinguish the effects of EGCG from those of H₂O₂. Under the present conditions, 10 μ M EGCG produced less than 1.5 μ M H₂O₂ in both HCT116 and HT29 cell lines. This concentration of H₂O₂ did not alter Met phosphorylation levels following direct addition of H₂O₂ to the experiment. However, the Met receptor was activated when concentrations exceeded 2.5 μ M H₂O₂ in the assay. This might be due to the ability of H₂O₂ to inhibit protein tyrosine phosphatases [18], although this was not tested in the present study.

Others have reported that exposing A549 human lung cancer cells to 500 μ M H₂O₂ in the presence of HGF results in the suppression, rather than the activation, of the Met receptor [19]. In the present study, concentrations of 500 and 1000 μ M EGCG clearly had a deleterious effect on the viability of human colon cancer cells; thus, the apparent loss of Met phosphorylation must be viewed in this context, along with the reduced levels of total Met and

β -actin. Importantly, catalase had no effect in these experiments, either in blocking the HGF-mediated increase in p-MET levels, or diminishing the effectiveness of EGCG to suppress Met activation.

We conclude that, under the present conditions, EGCG can effectively suppress Met signaling in colon cancer cells, at physiologically relevant concentrations. Thus, EGCG may be useful as a therapeutic agent for prevention of metastatic spread of colon tumors. The concern over H_2O_2 as a possible artifact was ruled out through the use of catalase, and by experiments showing that the concentrations of H_2O_2 generated by 1–10 μ M EGCG were too low to influence Met phosphorylation. Further studies are warranted to determine the mechanism by which EGCG suppresses activation of the Met receptor.

Acknowledgments

This work was supported in part by grant CA090890 from the US National Cancer Institute.

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A

EGCG (μ M) 0 0 0.1 0.5 1 5 10

HGF - + + + + + +

p-Met

Total Met

 β -actin

B

EGCG (μ M) 0 0 0.1 0.5 1 5 10

HGF - + + + + + +

p-Met

Total Met

 β -actin

Fig. 1.

EGCG suppresses HGF-induced Met signaling. Human colon cancer cells were serum-starved for 4 h and then treated with various concentrations of EGCG for 30 min. HGF (30 ng/ml) was added to the cell culture media and incubated for an additional 15 min. Immunoblotting was performed on cell lysates obtained from (A) HCT116 cells or (B) HT29 cells, using primary antibodies to phosphorylated-Met (p-Met), total Met, or β -actin (loading control). (C) Quantification of immunoblots by densitometry, with p-Met normalized to total Met. Cells treated with HGF alone (no EGCG) were assigned an arbitrary value of 1.0. The wedge-shaped symbol indicates 0, 0.1, 0.5, 1, 5 and 10 μ M EGCG. Data were from a single experiment, and are representative of the findings from three separate experiments.

C

D

F

Fig. 2.

EGCG-generated hydrogen peroxide, and the increased phosphorylation of Met at high H₂O₂ concentrations. (A) HCT116 and HT29 cells were serum-starved for 4 h in phenol red-free McCoy's 5A media. After 30 min of EGCG treatment, aliquots of media were taken and H₂O₂ was measured using the Amplex Red assay. Data (mean±SD, n=3) are representative of the findings from three separate experiments. (B) Western blot analysis of HCT116 cells serum-starved for 4 h then treated with various concentrations of H₂O₂. (C) Immunoblotting of HCT116 cells serum-starved for 4 h, and treated with a broad range of H₂O₂ concentrations, in the presence and absence of HGF. (D) Quantification of immunoblots in (C) by densitometry, showing p-Met normalized to total Met. (E) Viability of HCT116 cells treated with H₂O₂.

Fig. 3.

Catalase does not interfere with the inhibitory effects of EGCG towards HGF-induced Met activation. HCT116 cells were serum-starved for 4 h. Catalase (30 U/ml) was added to the cell culture media 10 min prior to the addition of 5 μ M EGCG. (A) Western blot analysis of the cell lysates. (B) ELISA using STAR phospho-Met ELISA kit (Upstate), with cell lysates from (A). Data (mean $\pm$ SD, n=3) are representative of findings from three separate experiments.