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ORIGINAL ARTICLE

The green tea catechins, (−)-Epigallocatechin-3-gallate (EGCG) and (−)-Epicatechin-3-gallate (ECG), inhibit HGF/Met signaling in immortalized and tumorigenic breast epithelial cells

RLH Bigelow and JA Cardelli

Department of Microbiology and Immunology, Feist-Weiller Cancer Center, Louisiana State University-Health Sciences Center, Shreveport, LA, USA

The hepatocyte growth factor (HGF) receptor, Met, is a strong prognostic indicator of breast cancer patient outcome and survival, suggesting that therapies targeting Met may have beneficial outcomes in the clinic. (−)-Epigallocatechin-3-gallate (EGCG), a catechin found in green tea, has been recognized as a potential therapeutic agent. We assessed the ability of EGCG to inhibit HGF signaling in the immortalized, nontumorigenic breast cell line, MCF10A, and the invasive breast carcinoma cell line, MDA-MB-231. HGF treatment in both cell lines induced rapid, sustained activation of Met, ERK and AKT. Pretreatment of cells with concentrations of EGCG as low as 0.3 μM inhibited HGF-induced Met phosphorylation and downstream activation of AKT and ERK. Treatment with 5.0 μM EGCG blocked the ability of HGF to induce cell motility and invasion. We assessed the ability of alternative green tea catechins to inhibit HGF-induced signaling and motility. (−)-Epicatechin-3-gallate (ECG) functioned similar to EGCG by completely blocking HGF-induced signaling as low as 0.6 μM and motility at 5 μM in MCF10A cells; whereas, (−)-epicatechin (EC) was unable to inhibit HGF-induced events at any concentration tested. (−)-Epigallocatechin (EGC), however, completely repressed HGF-induced AKT and ERK phosphorylation at concentrations of 10 and 20 μM, but was incapable of blocking Met activation. Despite these observations, EGC did inhibit HGF-induced motility in MCF10A cells at 10 μM. These observations suggest that the R1 galloyl and the R2 hydroxyl groups are important in mediating the green tea catechins' inhibitory effect towards HGF/Met signaling. These combined *in vitro* studies reveal the possible benefits of green tea polyphenols as cancer therapeutic agents to inhibit Met signaling and potentially block invasive cancer growth.
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Correspondence: Dr JA Cardelli, Department of Microbiology and Immunology, Louisiana State University-Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130, USA.
E-mail: jcarde@lsuhsc.edu
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Introduction

Breast cancer is the second most diagnosed form of cancer in women, affecting more than 200 000 patients a year and resulting in almost 40 000 deaths per year, according to the American Cancer Society. Recent work has revealed that tissue expression of the Met receptor is a strong prognostic indicator of patient outcome and survival. The survival rate of patients with high Met expression was found to be significantly lower to that of patients with normal expression patterns (Ghoussoub *et al*., 1998; Camp *et al*., 1999). Met levels were associated with high nuclear and histological grade, and interestingly Met expression was found to be a stronger prognostic factor than the traditional markers such as Her2/neu and EGFR (Edakuni *et al*., 2001; Tolgay Ocal *et al*., 2003). Currently, no activating mutations have been found in Met from breast cancer specimens. Instead, wild-type Met or hepatocyte growth factor (HGF) are overexpressed, leading to the constitutive activation of a number of signaling pathways contributing to invasion and metastasis (Trusolino and Comoglio, 2002). These observations suggest the importance of gaining a better insight into the role of Met in tumorigenesis and the potential of targeting the Met/HGF pathway in therapeutic strategies.

Met was originally discovered as a chromosomal translocation in an osteosarcoma cell line that was treated with a chemical carcinogen (Park *et al*., 1986). Met is a transmembrane receptor for HGF/scatter factor. Ligand binding results in autophosphorylation of the receptor on cytoplasmic tyrosine residues. These sites serve as docking sequences for several SH2/SH3 containing molecules including Gab1, Src, Grb2, and PI3K, which in turn, are capable of activating a number of downstream signaling components including AKT, Ras/MAPK and the JAK/STAT pathway (Trusolino and Comoglio, 2002; Birchmeier *et al*., 2003). Activation of these pathways is associated with increased scattering/motility, invasion, proliferation, survival and morphogenesis (Trusolino and Comoglio, 2002; Birchmeier *et al*., 2003). Numerous laboratories have demonstrated that deregulation of the Met/HGF pathway can lead to increased tumorigenesis and metastasis in nude mice models (Trusolino and Comoglio, 2002). Additionally,

studies have also shown that expression of the chimeric protein, TRP-MET, in transgenic mice leads to the development of mammary carcinomas (Liang *et al*., 1996).

Given the high cost of cancer therapy, significant emphasis has recently been placed on chemoprevention, especially with naturally occurring products. Epidemiological and preclinical studies have demonstrated that polyphenols derived from green tea have profound chemopreventative and antitumor effects (Lambert and Yang, 2003; Crespy and Williamson, 2004). (−)-Epigallocatechin-3-gallate (EGCG) is the major bioactive polyphenol present in green tea. Additional catechins found in green tea include (−)-epicatechin (EC), (−)-epigallocatechin (EGC) and (−)-epicatechin-3-gallate (ECG) (Figure 1). Studies have shown that EGCG can induce apoptosis, inhibit proliferation and function as an antioxidant in a wide range of tumor cell-lines (Lambert and Yang, 2003). The mechanisms by which EGCG is able to produce these effects appear wide-ranging. EGCG has been shown to inhibit numerous signaling pathways and kinases, including JNK, AP-1, p44/p42 MAPK, Erk1/2, EGF-R, PDGF-R and FGF-R (Lambert and Yang, 2003). EGCG is also able to inhibit other proteins implicated in cancer, including DNA methyltransferase, topoisomerase I, MMPs and the chymotryptic activity of the 20s proteasome (Berger *et al*., 2001; Nam *et al*., 2001; Fang *et al*., 2003).

In this report, we provide evidence to demonstrate that EGCG can also inhibit Met/HGF signaling in both the immortalized, nontumorigenic, MCF10A, and the highly metastatic, MDA-MB-231, breast cell lines. EGCG can block HGF signaling at the level of the Met receptor in both cell lines and results in inhibition of Met-induced scattering and invasion. In addition, we have demonstrated that the alternative green tea catechins, ECG and EGC also repress HGF-mediated downstream events.

Results

HGF activates the Ras and AKT signaling pathways in MCF10A cells

The Met/HGF signaling pathway is deregulated in numerous types of cancer, including breast cancer; however, the mechanisms underlying HGF/Met signaling in breast epithelial and tumor cells remains to be determined. In order to assess the downstream signaling events induced by HGF in a premalignant model, the nontumorigenic, immortalized breast cell line, MCF10A, was treated with 30 ng/ml HGF for 24 h and cell lysates were prepared at regular intervals. Western blot analysis, using an antibody recognizing the phosphorylated and activated form of Met, indicated that HGF treatment-induced Met phosphorylation as early as 5 min, and high levels of the phosphorylated form were sustained for 2 h before beginning to decrease (Figure 2). The addition of HGF also resulted in the activation of both ERK1/2 and AKT pathways (Figure 2). The phosphorylation status of both ERK1/2 and AKT followed the same pattern as Met, with strong activation by 5 min and a decrease in phosphorylation observed by 2–4 h post-treatment (Figure 2). The downstream kinases p38 and JNK were not activated by HGF in MCF10A cells under the tested conditions (data not shown).

One of the hallmarks of HGF/Met signaling is increased cell motility, scattering and invasion. MCF10A cells are not highly invasive cells, and scatter poorly in response to HGF. Therefore, a scratch/wound assay was performed in order to determine which signaling pathways were important for HGF/Met-induced motility. MCF10A cells were grown to confluency in a six-well plate, and pretreated with LY294002 and U0126, PI3K and ERK inhibitors, respectively, in serum-free media for half an hour. A wound was created in the MCF10A monolayer with a pipette tip, and the cells were then treated

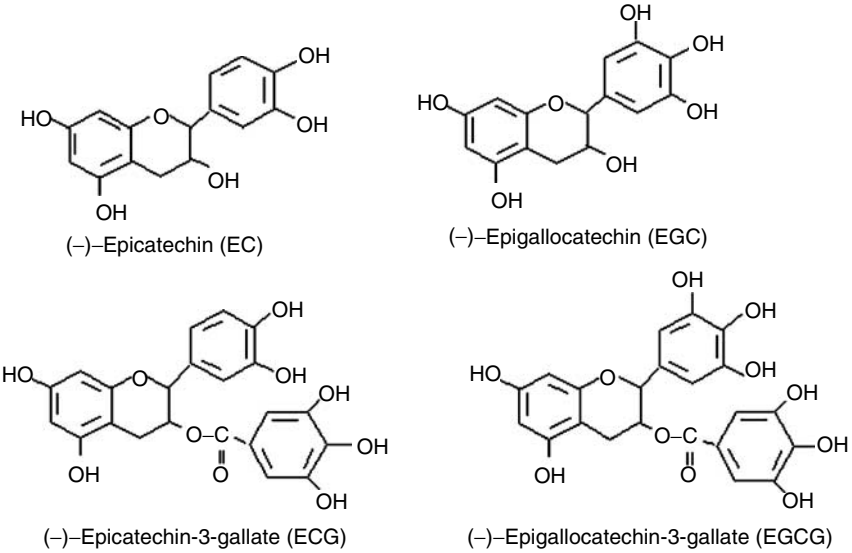

Figure 1 The structure of the green tea catechins.

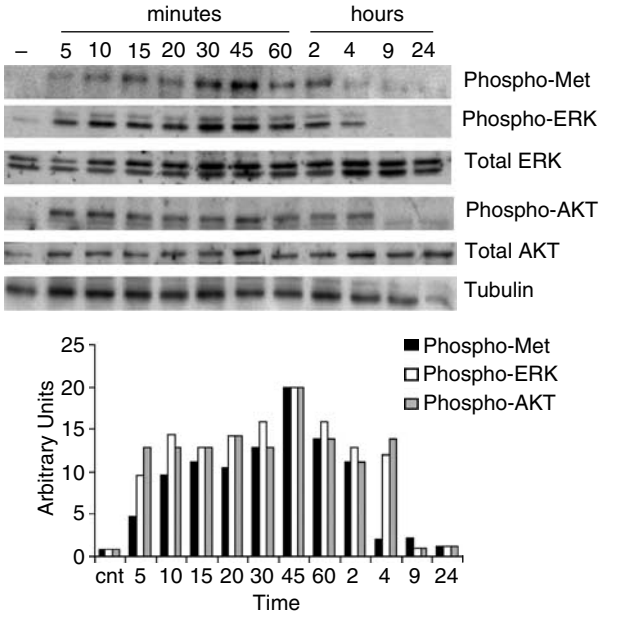

Figure 2 MCF10A breast epithelial cells are responsive to HGF-induced Met activation. 3×10^4 MCF10A cells were serum starved for 4–5 h and treated with or without (−) 30 ng/ml HGF for the indicated times in serum-free media. Protein lysates were taken with boiling laemmli buffer. Protein (10 μl) was loaded per well for Western blot analysis and probed with the indicated antibodies. HGF treatment induces rapid activation of the HGF receptor, Met, and the downstream kinases ERK and AKT, which is sustained up to approximately 4 h. Tubulin was used as a load control. The graph represents quantification of Met, ERK and AKT activation when normalized to tubulin using densitometry. (−) Untreated controls were given a value of 1. The experiment was performed three times and a representative experiment is shown.

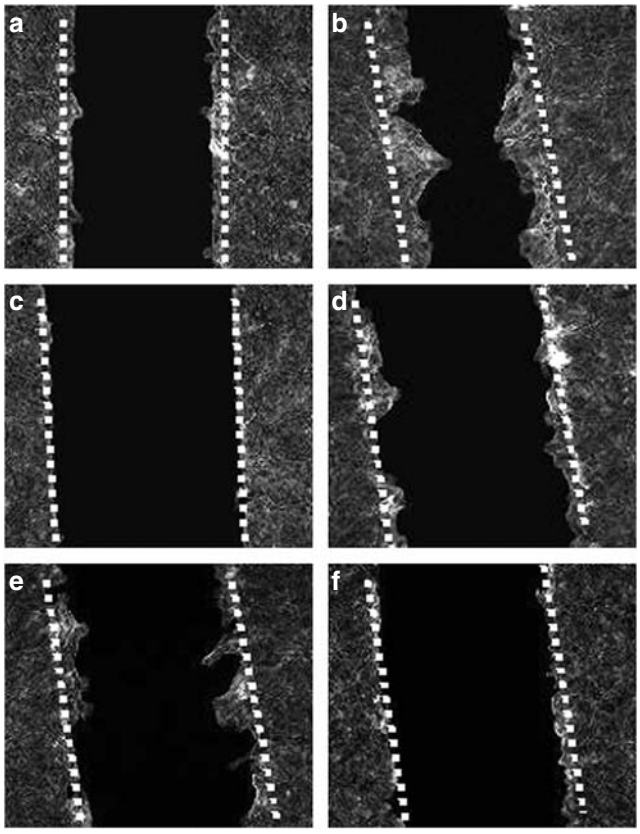

Figure 3 EGCG blocks HGF-induced motility in MCF10A breast epithelial cells. 5×10^5 MCF10A cells were plated in a six-well plate and grown overnight to confluency in serum containing media. The cells were serum starved for 4–5 h. The cells were pretreated with or without 50 μM LY294002, 10 μM U0126 or increasing concentrations of EGCG (0.07–20 μM) for half an hour in serum-free media. The monolayer was scratched with a pipette tip and washed with 1 × PBS to remove floating cells. HGF (30 ng/ml) +/− the indicated inhibitors or EGCG was added and the cells were incubated overnight. The cells were fixed with paraformaldehyde and stained for actin. Representative fields are shown. Untreated cells (**a**), were not highly motile, whereas HGF treatment (**b**) significantly increased MCF10A motility. LY294002 and U0126 abrogated HGF-induced movement (**c** and **d**, respectively). Treatment with 0.6 μM EGCG (**e**) partially blocked HGF-induced motility, while 5.0 μM EGCG (**f**) completely blocked HGF-induced motility.

with or without 30 ng/ml HGF and the inhibitors overnight.

The MCF10A cells under control conditions did not migrate into the wound (Figure 3a), while HGF treatment induced significant migration into the wound compared to controls (Figure 3b). Pretreatment with LY294002 or U0126 were both capable of blocking HGF stimulated cell motility, suggesting the necessity of these signaling pathways for HGF-induced movement (Figure 3c and d, respectively).

EGCG inhibits HGF/Met motility and signaling in MCF10A cells

EGCG has been shown to inhibit signaling by several growth factor receptors, including EGF-R, HER-2/neu, PDGF-R and FGF-R (Liang *et al*., 1997; Sachinidis *et al*., 2000; Masuda *et al*., 2003; Sah *et al*., 2004). In order to determine if EGCG can also inhibit HGF-induced motility, the wound/scratch assay was performed as stated above and cells were pretreated with and without increasing concentrations of EGCG (0.07–20 μM). EGCG was able to partially block HGF-induced motility at concentrations as low as 0.6 μM (Figure 3e) and was able to completely block MCF10A motility at 5 μM (Figure 3f). Concentrations of EGCG lower than 0.6 μM were not capable of abrogating cell movement (data not shown). EGCG at these low concentrations did not induce cell death under the conditions used in these experiments (data not shown). However, concentrations greater than 40 μM have been demonstrated by others to affect proliferation and viability of breast epithelial cells and similar results have been obtained in our laboratory (data not shown) (Kavanagh *et al*., 2001).

The mechanism by which EGCG inhibits growth factor signaling is not clear. Several published studies demonstrated that EGCG directly inhibited the enzymatic activity of AKT, ERK and DNA methyltransferase *in vitro* (Fang *et al*., 2003; Sah *et al*., 2004), while other studies indicated that this catechin could block the binding of a growth factor with its receptor. In order to determine if EGCG can block HGF signaling at the

level of Met receptor activation, MCF10A cells were pretreated for half an hour with increasing concentrations of EGCG (0.07–20 μM). HGF (30 ng/ml) was added in the presence of EGCG for 15 min and protein lysates were prepared. Western blot analysis revealed that concentrations of EGCG as low as 0.07 μM EGCG partially blocked Met activation, while concentrations of 0.3 μM and above completely blocked phosphorylation of Met, Erk and Akt (Figure 4). EGCG was capable of blocking HGF-induced Met signaling at all timepoints following HGF addition (data not shown).

In vitro kinase assays were performed to assess if EGCG was capable of directly inhibiting the Met receptor. Met was immunoprecipitated from MCF10A cells and incubated with and without increasing concentrations of EGCG. Results revealed that EGCG did not repress the autocatalytic phosphorylation of Met, suggesting that this catechin is not directly inhibiting the kinase active site (data not shown). It remains to be determined how EGCG blocks Met activation. Since the tea catechins can block the activation of a growing number of growth factor receptors, it seems unlikely that the polyphenols are directly preventing binding of growth factors to their cognate receptor. It is possible that the catechins are disrupting select plasma membrane domains such as lipid rafts that might be important in regulating receptor signaling.

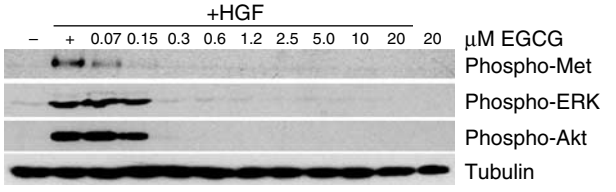

Figure 4 EGCG inhibits HGF-induced Met signaling in MCF10A cells. 3×10^4 MCF10A cells were serum starved for 4–5 h and pretreated with the indicated concentrations of EGCG alone for 0.5 h. The media was removed and the cells were treated with (+) or without 30 ng/ml HGF (–), with and without the presence of the indicated concentrations of EGCG for 15 min. One well was also treated with 20 μM EGCG alone as a control. Protein lysates were taken with boiling laemmli buffer. Protein (10 μl) was loaded per well for Western blot analysis and probed with the indicated antibodies. EGCG, as low as 0.15 μM, partially blocks HGF-induced Met phosphorylation, and treatment with 0.3 μM EGCG inhibits downstream activation of ERK and AKT. Tubulin was used as a load control.

The Green tea polyphenols, ECG and EGC, also block HGF-induced Met signaling and motility in MCF10A cells

Green tea is composed of additional catechins, including EC, ECG and EGC (Lambert and Yang, 2003). In order to determine if these polyphenols were also capable of inhibiting HGF-induced signaling, MCF10A cells were pretreated with and without increasing concentrations of EC or EGC (0.6–20 μM), or ECG (0.07–20 μM) for 0.5 h. The cells were then treated with and without 30 ng/ml HGF for 15 min and protein lysates were prepared and subjected to Western blot analysis. Preincubation of cells with EC at concentrations up to 20 μM did not significantly prevent HGF-induced phosphorylation of Met, AKT or ERK (Figures 5a and 6a). However, pretreatment of MCF10A cells with ECG partially blocked HGF-induced Met activation at concentrations as low as 0.15 μM, and completely inhibited Met, AKT and ERK phosphorylation at 0.6 μM (Figures 5b and 6b). Interestingly, treatment of cells with EGC was not capable of blocking HGF-induced Met phosphorylation, and actually resulted in activation of the Met receptor at 20 μM EGC alone (Figures 5c and 6c). However, EGC did repress HGF-induced AKT and ERK activation at concentrations higher than and equal to 2.5 μM (Figures 5c and 6c).

In order to determine if these green tea catechins were able to inhibit HGF-induced motility, a wound assay was performed with MCF10A cells that were grown to confluency and treated overnight with and without 30 ng/ml HGF $\pm$ 1, 5 or 10 μM of EC, ECG or EGC. Control, untreated MCF10A cells did not significantly move into the wound (Figure 7a), while HGF treatment induced significant cell movement into the scratch (Figure 7b). Similar to the results observed by Western blot, EC was not capable of inhibiting HGF-induced cell motility at any concentration tested (Figure 7c–e). Treatment of cells with 1 μM ECG was capable of

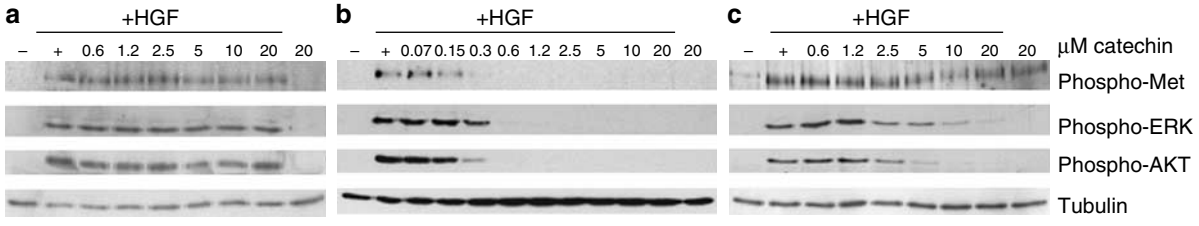

Figure 5 The EGCG analogs, ECG and EGC, block HGF-induced Met signaling in MCF10A cells, while EC does not. 3×10^4 MCF10A cells were serum starved for 4–5 h and pretreated for 0.5 h with the indicated concentrations of EC, ECG or EGC. The media was then replaced with media $\pm$ 30 ng/ml HGF, +/– the indicated concentrations of the phytochemicals or 20 μM EC, ECG or EGC alone. Protein lysates were taken after a 15-min incubation and the samples run by Western blot analysis and probed with the indicated antibodies. HGF treatment (+) induced phosphorylation of Met, AKT and ERK compared to untreated controls (–). (**a**) Treatment with EC, at any concentration, did not inhibit HGF-induced Met signaling. (**b**) Treatment of MCF10A cells with concentrations of ECG, as low as 0.3 μM up to 20 μM was able to completely block HGF-induced activation of Met, ERK and AKT. (**c**) EGC treatment was only capable of completely inhibiting HGF-induced ERK and AKT activation at higher concentrations of 10 and 20 μM. EGC did not inhibit Met phosphorylation.

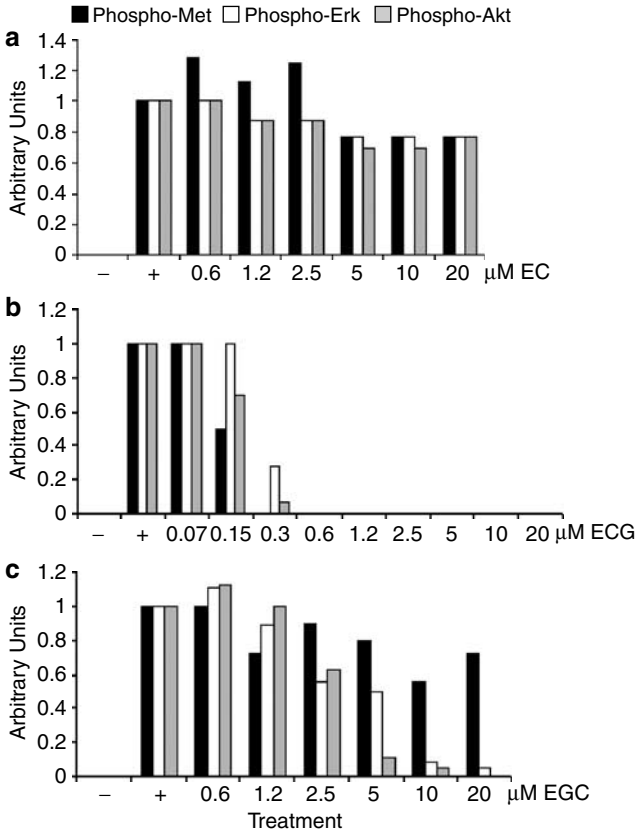

Figure 6 ECG and EGC block HGF-induced signaling. Quantification of Western blot analysis from Figure 5 as normalized to tubulin using densitometry. (+) HGF treated cells were given a value of 1. (**a**) EC did not significantly block HGF-induced Met, ERK or AKT activation. (**b**) ECG inhibits Met, ERK and AKT activation by HGF completely at concentrations equal to or higher than 0.6 μM. (**c**) EGC does not block Met phosphorylation, but does inhibit ERK and AKT activation beginning at 2.5 μM. The experiment was performed in triplicate and a representative experiment is shown.

partially inhibiting HGF-induced motility (Figure 7f), whereas higher concentrations of 5 and 10 μM completely blocked cell movement (Figure 7g and h, respectively). EGC treatment did not block HGF-induced motility at 1 or 5 μM (Figure 7i and j, respectively); however, it was capable of repressing motility at higher concentrations of 10 μM, despite not being able to inhibit Met activation at this concentration (Figure 7k).

EGCG inhibits HGF/Met signaling in MDA-MB-231 cells

MDA-MB-231 cells are a highly invasive metastatic cell line and the role of Met signaling in these cells remains to be defined. Time course and Western blot analysis revealed that HGF significantly increased Met, AKT and ERK phosphorylation in a sustained manner as early as 5 min (Figure 8a).

In order to determine if EGCG can block HGF/Met signaling in a malignant cell line, MDA-MB-231 cells were pretreated for half an hour with increasing concentrations of EGCG (0.6–30 μM) in serum-free media. The cells were then treated for 15 min in the presence of 30 ng/ml of HGF with and without EGCG. Western blot analysis revealed that, similar to the MCF10A cells, EGCG could block HGF-induced Met, AKT and ERK activation in MDA-MB-231 cells (Figure 8b).

A modified Boyden chamber invasion assay was performed with the MDA-MB-231 cell line to determine if EGCG was capable of inhibiting HGF-induced invasion. A total of 20 000 cells were plated on top of Matrigel-coated inserts in the absence or presence of 30 ng/ml HGF $\pm$ 5 μM EGCG and incubated for 16 h. The cells that migrated to the undersurface of the insert were fixed, stained and five random fields were counted per insert. The fold change compared to that of untreated control was determined. HGF treatment increased the number of cells that invaded compared to untreated controls by 6.7-fold (Figure 9). Treatment of MDA-MB-231 cells with 5 μM EGCG significantly decreased the number of HGF induced invasive cells to 2.2-fold compared to control cells (Figure 9). Concentrations as low as 1 μM, close to physiologically obtainable levels, also had an effect on blocking invasion (results not shown). Interestingly, EGCG treatment alone was also able to inhibit the invasive capability of the MDA-MB-231 cells by 50% (Figure 9).

Discussion

The study of plant polyphenols as anticancer agents has increased substantially in recent years, due in part to their profound effects *in vitro* and *in vivo* on tumor cell signaling pathways regulating growth and apoptosis. Additionally, epidemiological studies have revealed promising preventative and therapeutic roles for polyphenols. For instance, published studies have revealed an inverse correlation between urinary tea polyphenols and gastric cancer, a reduction in colon cancer incidence in individuals who consumed tea and an improved prognosis of stage I and II breast cancer patients in those patients who drank five or more cups of green tea (Nakachi *et al*., 1998; Su and Arab, 2002; Sun *et al*., 2002). EGCG, the major catechin found in green tea, has been shown to inhibit proliferation and induce apoptosis in many cell lines (Lambert and Yang, 2003). *In vivo* mouse studies have established that EGCG can function as a strong chemopreventative agent against UV, chemically and genetically induced carcinogenesis (Crespy and Williamson, 2004). Kavanagh *et al*. (2001) demonstrated that green tea extract given postinitiation significantly increases mammary tumor latency and decreases tumor weight and metastases in DMBA-treated rats. One of the accepted mechanisms by which EGCG accomplishes these effects is via inhibition *in vivo* of growth factor signaling (Lambert and Yang, 2003). In support of this, a published study from the Mukhtar laboratory demonstrated that oral administration of green tea catechins blocked IGF signaling and reduced the activity of the PI3K and MAPK

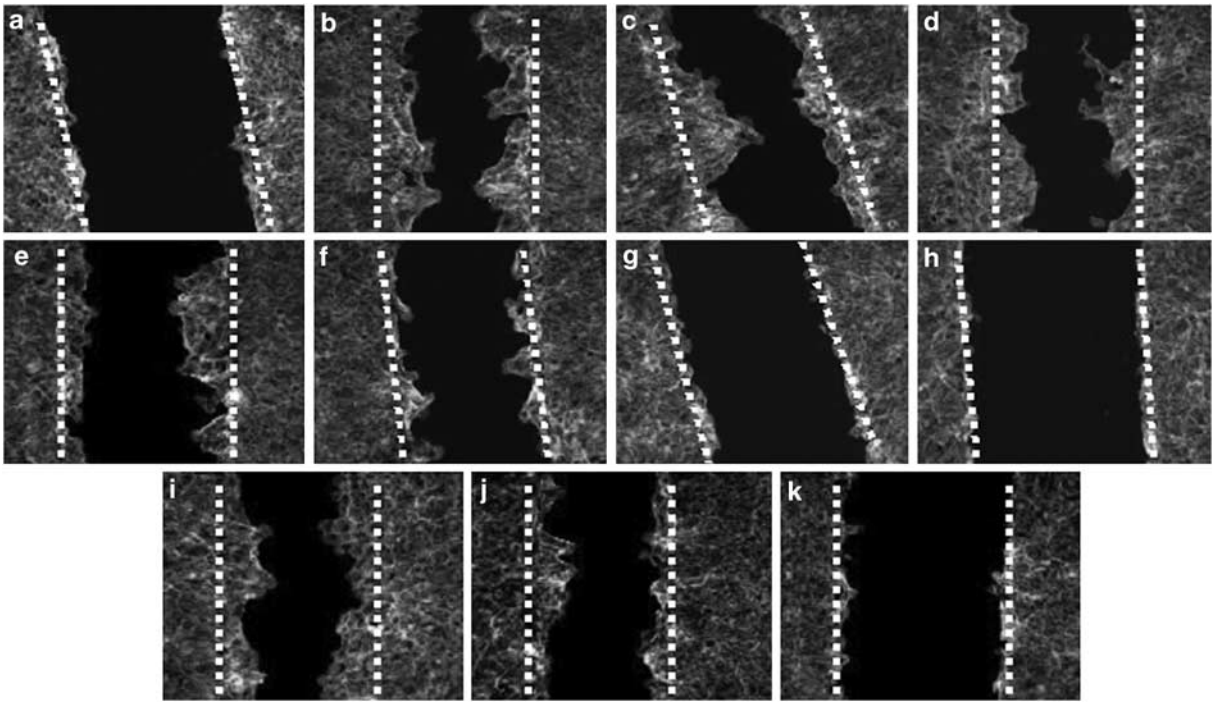

Figure 7 The EGCG analogs, ECG and EGC, block HGF-induced motility in MCF10A cells. A scratch assay was performed and the MCF10A cells were treated similarly as stated in Figure 2, ±30 ng/ml HGF, ±1, 5 or 10 μM EC, ECG or EGC. **(a)** Control, untreated cells did not significantly move into the wound. **(b)** HGF-induced MCF10A cell motility into the wound. **(c–e)** HGF +1, 5 and 10 μM EC, respectively. EC treatment was not capable of blocking HGF-induced motility at any concentration tested. **(f–h)** HGF + 1, 5 and 10 μM ECG, respectively. 1 μM ECG was partially able to block HGF-induced motility, whereas treatment with 5 and 10 μM ECG completely blocked HGF motility. **(i–k)** HGF +1, 5 and 10 μM EGC, respectively. EGC 1 and 5 μM was not capable of repressing HGF-induced MCF10A motility; however, treatment with 10 μM EGC completely blocked HGF-induced motility.

pathways in the TRAMP prostate tumor mouse model (Adhami *et al*., 2004).

The HGF/Met signaling pathway is deregulated in the majority of cancers and is associated with poor prognosis in breast cancer (Ghoussoub *et al*., 1998; Camp *et al*., 1999; Trusolino and Comoglio, 2002). Downstream activation of the PI3-kinase/AKT and the Ras/ERK pathways have both been found to be necessary for HGF-induced motility as well as adherence junction disassembly in the Madin–Darby dog kidney cell model (Royal and Park, 1995; Potempa and Ridley, 1998). Less information is available regarding Met signaling pathways in breast epithelial and cancer cells. We selected two human breast epithelial cell-lines for our studies, MCF10A and MDA-MB-231. MCF10A is an immortalized, nontumorigenic cell line that we believe, appropriately represents a preneoplastic cell, while MDA-MB-231 cells are invasive breast cancer cells. In this report, we demonstrate that HGF induces the sustained activation of the MAPK and PI3-kinase pathways in the immortalized breast cell-line MCF10A and the invasive tumor cell-line MDA-MB-231, and both pathways are necessary for HGF-induced cell motility of MCF10A cells.

We also demonstrate that EGCG at physiologically relevant concentrations is capable of blocking HGF-induced signaling in both the nontumorigenic, immortalized cell line, MCF10A, and the invasive cancer line, MDA-MB-231. EGCG treatment, at concentrations as low as 0.3 μM, completely blocked HGF-induced Met, ERK and AKT phosphorylation and concentrations as low as 0.6 μM inhibited HGF-induced increases in cell motility of MCF10A cells, while 1–5 μM concentrations of EGCG suppressed invasion of MDA-MB-231 cells. EGCG also inhibited basal levels of invasion compared to untreated controls. These combined observations suggest that EGCG may have anti-invasive activity in those cancers that have a deregulated Met signaling pathway, such as breast cancer.

Green tea is also composed of other catechins, including epicatechin (EC), epicatechin-3-gallate (ECG) and epigallocatechin (EGC) (Lambert and Yang, 2003). We assessed the ability of the alternative green tea polyphenols to inhibit HGF-induced signaling and motility in MCF10A cells. EC was not able to block HGF-mediated downstream events. ECG, however, functioned similar to EGCG and was able to inhibit HGF signaling and dramatically inhibited HGF-induced cell motility at low concentrations. Interestingly, EGC treatment was not capable of blocking HGF-induced Met phosphorylation at concentrations as high as 20 μM but inhibited both AKT and ERK activation and HGF-induced cell motility at concentrations higher than 2.5 μM. This suggests that while EGC may not inhibit HGF/Met signaling at the level of the receptor, its ability to block the downstream signaling molecules,

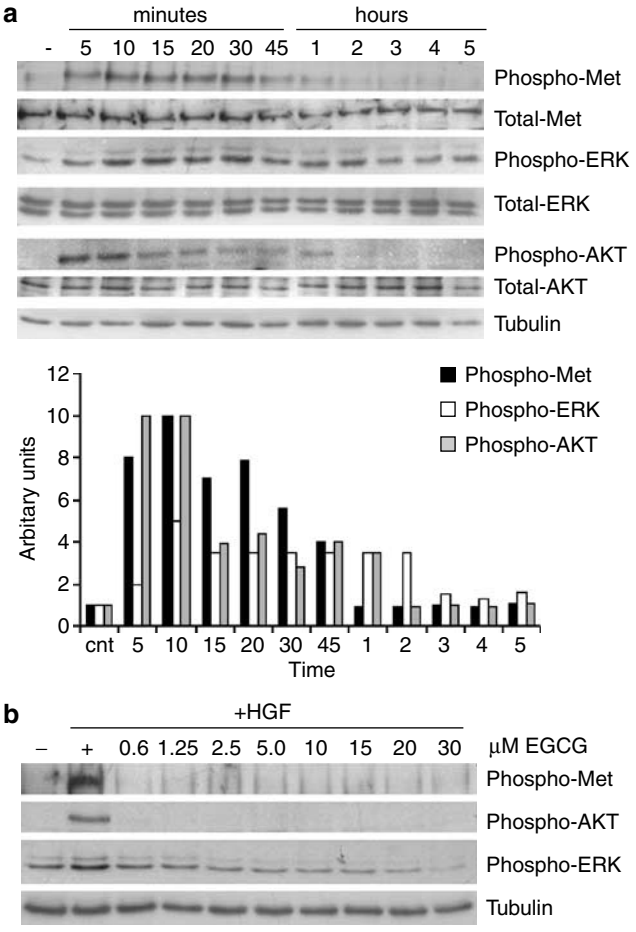

Figure 8 EGCG can also block HGF-induced Met signaling in MDA-MB-231 breast carcinoma cells. (**a**) 3×10^4 MDA-MB-231 cells were treated similar to MCF10A cells in Figure 1. HGF treatment induces rapid activation of the HGF receptor, Met, and the downstream kinase AKT, compared to control (−), which is sustained up to approximately 1–2 h. Phospho-ERK basal levels are higher in MDA-MB-231 cells than in MCF10A cells; however, a slight induction of ERK activation is noted by 5 min through 1 h. Tubulin was used as a load control. The graph represents quantification of Met, ERK and AKT activation when normalized to tubulin levels and is a representative experiment out of triplicates. (−) untreated control cells were given a value of 1. (**b**) 3×10^4 MDA-MB-231 cells were treated similar to MCF10A cells in Figure 2. Concentrations of EGCG, as low as 0.6 μM, are capable of blocking HGF-induced Met phosphorylation and activation of the downstream kinases AKT and ERK. (−) Untreated control, (+) Positive HGF treated control.

AKT and ERK, is sufficient to inhibit HGF-induced motility. When compared to the structure of the green tea catechins, the results of these studies suggest that the R1 galloyl group is necessary for Met inhibition, as both EGCG and ECG are the only catechins to contain this functional group (Figure 1). The R2 hydroxyl group may also play a role in inhibition of downstream signaling proteins as it is present in EGCG and EGC (Figure 1). EC, however, contains neither the R1 galloyl nor the R2 hydroxyl group and, interestingly, was not capable of blocking Met activation and downstream effects, again suggesting the importance of these functional groups (Figure 1).

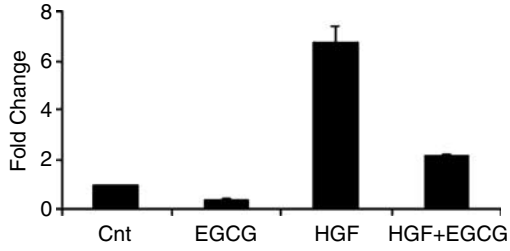

Figure 9 EGCG inhibits HGF-induced invasion in MDA-MB-231 cells. Diluted matrigel 100 μl (1:25 in serum-free media) was plated on top of Costar Transwell Inserts. After rehydration, MDA-MB-231 cells (2×10^4) were added to the top of the transwell inserts $\pm$ 5 μM EGCG. Serum-containing DMEM $\pm$ 30 ng/ml HGF 600 μl was plated in the bottom of the wells. The cells were incubated for 12 h after which time the cells were fixed and those cells remaining on the top surface were removed with a cotton swab. The cells that migrated to the bottom of the insert were stained with crystal violet and counted in five random fields. The experiment was performed in triplicate and repeated three times. HGF treatment induced MDB-MB-231 invasion, which was blocked by treatment with 5 μM EGCG.

Similar to our studies, Liang *et al*. (1997) demonstrate that green tea catechins inhibit EGF signaling in A431 cells with EGCG having the strongest effect, followed by ECG and EGC. Treatment with EC had no effect (Liang *et al*., 1997). Several groups have shown similar results with regard to inhibition of proliferation and induction of apoptosis by green tea catechins. EC was not effective at inhibiting cell growth of H661, H441 or H1299 lung carcinoma or HT-29 colon cancer cell lines. EGCG, ECG and EGC treatment resulted in similar growth inhibition in H441 and HT-29 cells, whereas EGCG and EGC had the strongest effects in H661 and H1299 cells (Yang *et al*., 1998). EC is also ineffective in inducing apoptosis in human bronchial 21BES cells and in blocking tumor growth of HT29 colon cancer cells in nude mice, whereas EGCG treatment was effective in both studies (Yang *et al*., 2000; Jung *et al*., 2001).

EGCG is not a select protein inhibitor and, in fact, is able to block the enzymatic activity of many proteins, including DNA methyltransferase, JNK, AP-1, matrix metalloproteinases and FGF-R (Fang *et al*., 2003; Lambert and Yang, 2003). It has previously been shown that EGCG inhibits the activity of a number of growth factor receptors, including the EGF receptor as well as the downstream kinases, AKT and ERK, following growth factor addition (Sah *et al*., 2004). The authors also found that EGCG directly inhibits the kinase activity of AKT and ERK in cell-free *in vitro* assays after EGF stimulation and suggest that EGCG could function at multiple sites to block signaling pathways (Sah *et al*., 2004). In our studies, EGCG was able to repress the HGF induced increase in Met phosphorylation, as well as blocking activation of the downstream kinases, AKT and ERK. However, *in vitro* kinase assays demonstrated that EGCG did not inhibit Met directly (data not shown). In our system, it is possible that EGCG functions more indirectly at the level of the Met receptor to inhibit the transduction pathway, perhaps by preventing receptor dimerization via alteration in lipid

rafts. Alternatively, EGCG may activate tyrosine phosphatases, thus preventing an increase in phosphorylated Met. In addition, EGCG may block multiple kinases downstream of receptor activation, including AKT and ERK, to antagonize HGF signaling.

The potentially multiple mechanisms by which EGCG is able to accomplish these numerous effects is currently unclear. There are several reports that suggest that EGCG may bind directly to proteins to inhibit their activity, such as DNA methyltransferase (Fang *et al*., 2003). Alternatively, EGCG may produce a wide spectrum of effects via its antioxidant or pro-oxidant activity. EGCG blocked UV-induced H_2O_2 production in keratinocytes, which was associated with an inhibition of ERK1/2, JNK and p38 UV-B-induced phosphorylation (Katiyar *et al*., 2001). Conversely, the apoptotic response of many cells to EGCG may be due to its oxidative activity via production of H_2O_2 as observed in H661 lung cancer cells and Ras-transformed bronchial cells (Yang *et al*., 1998, 2000). Fujimura *et al*. (2004) suggest that EGCG may interact with lipid rafts, which conceivably would result in a variety of downstream effects via alterations of membrane fluidity and enzymatic activity of membrane-anchored proteins. The authors have also recently found a receptor for EGCG (Tachibana *et al*., 2004). A subtraction cloning strategy was used to determine that EGCG binds to the 67-Kd laminin receptor (67 LR). It was additionally found that cells that overexpressed the laminin receptor were capable of binding higher levels of EGCG and an inhibitory antibody to the laminin receptor blocked the ability of EGCG to suppress proliferation (Tachibana *et al*., 2004). Studies in our lab, however, showed no difference in EGCG repression of HGF/Met signaling after treatment with the same laminin receptor inhibitory antibody, suggesting that EGCG activity is not limited to binding to the 67-Kd LR (data not shown).

We have demonstrated that EGCG is also able to inhibit HGF-induced signaling, motility and invasion in both immortalized, nontumorigenic as well as tumorigenic breast epithelial cell lines. Our results combined with other catechin inhibition studies suggest that consumption of green tea or tea polyphenols may have striking chemopreventative and anticancer effects in humans during early as well as later stages of breast cancer development (Lambert and Yang, 2003). Unfortunately, many of these initial *in vitro* studies have used concentrations of EGCG well above the concentration that is systemically available after administration to humans. The maximum obtainable plasma concentration of EGCG is approximately 1 μmol/l in humans (Lambert and Yang, 2003). It is believed that the low bioavailability of EGCG and other polyphenols is most likely due to their high molecular weight and the presence of hydroxyl groups (Lambert and Yang, 2003). Our studies have used concentrations of EGCG that are closer to physiological relevance, thereby suggesting the potential of using these compounds as chemopreventative or therapeutic agents in humans.

However, despite the fact that we and others have observed striking inhibition of Met and other growth factor receptors by green tea catechins *in vitro*, the ability of these compounds to have similar effects in a patient have yet to be determined. Several studies have shown inhibition of growth factor signaling in mouse models (Cao and Cao, 1999; Adhami *et al*., 2004). It is hoped that green tea catechins would have similar results in human patients. In this regard, future phase II studies are aimed at assessing the ability of EGCG to inhibit Met signaling in human breast and prostate cancer patients in pre- and postsurgical biopsies at our institute.

Materials and methods

Cell culture

The cell-line MCF10A was maintained in 1:1 DMEM:F-12 (Cellgro, Herndon, VA, USA), 5% serum (Invitrogen, Carlsbad, CA, USA), 0.5 μg/ml hydrocortisone (Calbiochem, San Diego, CA, USA), 10 μg/ml insulin (Sigma, St Louis, MO, USA), 20 ng/ml EGF (Sigma), 0.1 μg/ml cholera toxin (ICN Biomedicals, Aurora, OH, USA) and penicillin/streptomycin (Cellgro). MDA-MB-231 cells were obtained from ATCC and maintained in DMEM with 10% FBS and penicillin/streptomycin. Cell lines were maintained in a 37°C incubator with 5.0% CO_2.

Western blot

3×10^4 cells were plated in a 24-well plate and grown in serum containing media. For time course analysis, cells were serum starved for 4–5 h and treated with 30 ng/ml HGF for the indicated times. Protein lysates were taken by adding 125 μl boiling laemlli to the cells. In green tea catechin inhibition studies, the cells were pretreated with or without increasing concentrations of EGCG, EC, ECG or EGC for 1/2 h. The media was removed and 30 ng/ml HGF +/− increasing concentrations of the catechins were added, and the cells were incubated for 15 min. Protein lysates were taken by adding 125 μl boiling laemlli to the cells. Lysates (10 μl) were run on a 10% acrylamide gel, transferred onto nitrocellulose, blocked and probed with antibodies to the following proteins: phospho-Met, phospho-AKT, phospho-FAK, phospho-Erk, Total AKT (Cell Signaling Technology, Beverly, MA, USA), Total Met and Total ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Tubulin (Lab Vision, Fremont, CA, USA) was used as a load control.

Scratch motility assay

5×10^5 MCF10A cells were plated in a six-well plate and grown overnight to confluency in serum containing media. The cells were serum starved for 5 h. The monolayer was scratched with a pipette tip and washed with 1 × PBS to remove floating cells. Serum-free media ±30 ng/ml HGF +/− increasing concentrations of the catechins were added and the cells were incubated overnight. The cells were fixed with 4% paraformaldehyde for 10 min and stained with phalloidin for 20 min and representative fields were photographed.

Invasion assay

Matrigel (BD Transduction Laboratories) was diluted 1:25 with serum-free DMEM. Diluted matrigel (100 μl) was plated on top of Costar Transwell Inserts (VWR, West Chester, PA, USA) and allowed to gel for 2 h at 37°C. The matrigel was rehydrated with 100 μl serum-free DMEM for 2 h. MDA-MB-231 cells were trypsinized and diluted to 2×10^5 cells/ml in

serum-free media. The diluted cell suspension 100 μl was added to the top of the transwell inserts $\pm 5\,\mu$M EGCG. In total, 600 μl serum-containing DMEM $\pm$ 30 ng/ml HGF was plated in the bottom of the wells. The cells were incubated for 12 h at 37°C in a 5.0% CO_2 incubator. The media was aspirated and the cells fixed for 20 min with 10% formaldehyde. The matrigel and cells remaining on the top surface were removed with a cotton swab. The cells that migrated to the bottom of the insert were stained with 0.1% crystal violet and counted in five random fields. The experiment was performed in triplicate and repeated three times.

Abbreviations

EGCG, (−)-epigallocatechin-3-gallate; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EC, (−)-epicatechin; HGF, hepatocyte growth factor.

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