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The Polyphenol Epigallocatechin-3-Gallate Affects Lipid Rafts to Block Activation of the c-Met Receptor in Prostate Cancer Cells

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The HGF/c-Met pathway is an important regulator of signaling pathways responsible for invasion and metastasis of most human cancers, including prostate cancer. Exposure of DU145 prostate tumor cells to HGF stimulates the PI3-kinase and MAPK pathways, leading to increased scattering, motility, and invasion, which was prevented by the addition of EGCG. EGCG acted at the level of preventing phosphorylation of tyrosines 1234/1235 in the kinase domain of the c-Met receptor without effecting dimerization. HGF-induced changes were independent of the formation of reactive oxygen species, suggesting that EGCG functioned independent of its antioxidant ability. ECG, another tea polyphenol, was as effective as EGCG, while EGC and EC were less effective. EGCG added up to 4 h after the addition of HGF still blocked cell scattering and reduced the HGF-induced phosphorylation of c-Met, Akt, and Erk, suggesting that EGCG could act both by preventing activation of c-Met by HGF and by attenuating the activity of pathways already induced by HGF. HGF did not activate the MAPK and PI3-K pathways in cells treated with methyl-β-cyclodextrin (mCD) to remove cholesterol. Furthermore, subcellular fractionation approaches demonstrated that only phosphorylated c-Met accumulated in Triton X-100 membrane insoluble fractions, supporting a role for lipid rafts in regulating c-Met signaling. Finally, EGCG treatment inhibited $DiIC_{16}$ incorporation into membrane lipid ordered domains, and cholesterol partially inhibited the EGCG effects on signaling. Together, these results suggest that green tea polyphenols with the R1 galloyl group prevent activation of the c-Met receptor by altering the structure or function of lipid rafts.

Key words: c-Met; EGCG; lipid rafts; prostate cancer; signaling

INTRODUCTION

The c-Met/HGF (hepatocyte growth factor) pathway is deregulated in numerous malignancies, including prostate cancer, and overexpression of both c-Met and HGF is associated with poor prognosis [1]. Increased expression of c-Met directly correlates with Gleason scores and is associated with poorly differentiated tumors [2,3]. HGF, likewise, is elevated in the serum of patients with prostate cancer and is associated with metastatic disease independent of PSA levels and age, and patients have a decreased overall survival [4,5]. It is believed that the overexpression of c-Met and/or HGF in prostate cancer leads to the paracrine stimulation of epithelial cells, resulting in uncontrolled growth, motility, and invasion. In vitro studies have additionally suggested that overexpression of c-Met itself can lead to ligand-independent stimulation of cell signaling [1]. HGF, normally secreted by cells in the surrounding stroma [3,6], binds to its receptor, c-Met, on neighboring tumor cells, stimulating receptor activation and phosphorylation of residues on the c-Met cytoplasmic tail. These residues serve as sites for docking proteins, such as GAB1, which relay the signal and activate transduction pathways including the MAPK, p38, PI3-K, and JNK pathways, resulting in stimulation of tumor cell proliferation, morphogenesis, motility, invasion, and metastasis.

Numerous approaches are being employed to target c-Met signaling, using both low-molecular weight compounds or antibodies which can act in a variety of ways to block the HGF/c-Met interaction, including interfering with c-Met catalytic activity or inhibiting c-Met binding to downstream adapter

Abbreviations: HGF, hepatocyte growth factor; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; EC, epicatechin; ECG, epicatechin gallate; NAC, *N*-acetyl-L-cysteine; mCD, methyl-β-cyclodextrin; ROS, reactive oxygen species; DCFH, dichlorofluorescein diacetate; H_2O_2, hydrogen peroxide; GEE, glutathione ethyl ester.

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molecules [7]. However, it is becoming apparent that simply targeting one pathway will not completely inhibit a multifaceted disease such as cancer. Therefore, within the past decade numerous studies have focused on the use of plant-derived natural compounds to treat cancer, since many of these compounds act in a multimodality fashion.

Plant-derived polyphenols have shown promise as chemopreventive/chemotherapeutic agents against a variety of cancers, including breast and prostate cancer. Tea (−)-epigallocatechin-3-gallate (EGCG) inhibits numerous signaling pathways contributing to cancer, including the EGF, PDGF, and FGF pathways, as well as downstream kinases such as Akt and Erk [8,9]. Since the effects of EGCG treatment are so wide ranging this impacts proliferation, cell survival, migration, invasion, and metastasis [8]. In vivo mouse studies have also demonstrated a reduction in tumor growth and burden, increased lifespan, decreased proliferation, and increased apoptosis in tumor xenografts [10]. Additionally, EGCG has successfully passed through Phase I clinical trials with limited toxicity and is currently in a number of Phase II trials [11–15]. For instance, the Bettuzzi group demonstrated that green tea polyphenols significantly reduced the progression of high-grade PIN to prostate cancer [14], while our Phase II study demonstrated that consumption of green tea capsules (Polyphenon E) lowered serum levels of PSA, HGF, and VEGF in men with prostate cancer [15].

We have demonstrated that EGCG inhibited HGF-induced c-Met signaling, motility, and invasion in the nontumorigenic breast cell line, MCF10A, and the highly tumorigenic cell line MDA-MB-231 [16]. Inhibition occurred at the level of c-Met receptor [16]. Our recent publication also demonstrated that EGCG also blocked HGF-induced activation of c-Met in three different lung cancer cell-lines [17]. However, the mechanism by which EGCG and ECG function to block HGF induced c-Met activation is unclear. In the current study, we demonstrate that HGF stimulated c-Met activity in the DU145 prostate carcinoma cell line localizes to lipid rafts and this can be blocked by EGCG which disrupts lipid rafts.

MATERIALS AND METHODS

Cell Lines and Reagents

The human prostate cancer cell line DU145 was obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI-1640 medium (Mediatech, Inc., Manassas, VA) supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA) and 1% penicillin and streptomycin (Mediatech, Inc.) and maintained in a 5% CO_2 incubator at 37°C. Cells were subcultured once reaching 80–90% confluency. HGF, LY249002, U0126, and PD98059 were purchased from Calbiochem, San Diego, CA. The green polyphenols, epicatechin (EC), epicatechin gallate (ECG), EGC, and EGCG were purchased from Sigma, St. Louis, MO with a purity of >90%, >98%, >95%, and >95%, respectively. The following antibodies were purchased from Cell Signaling Technology (Beverly, MA) and were used at the following dilutions: phospho-Erk1/2 (Thr202/Tyr204) 1:1000; phospho-Akt (Ser473) 1:1000; phospho-FAK (Tyr576/577) 1:1000; phospho-Paxillin (Tyr118) 1:1000; phospho-Jnk (Thr183/Tyr185) 1:500; phospho-p38 MAPK (Thr180/Tyr182) 1:500; phospho-Met (Tyr1234/1235) 1:500. Phospho-FAK (Tyr 397) was purchased from BD Transduction Laboratories (San Jose, CA) and used at 1:1000. Antibodies specific for c-Met and E-cadherin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and were used at 1:1000. Glutathione ethyl ester, *N*-acetyl-L-cysteine (NAC), ammonium pyrrolidinedithiocarbamate, diphenyleneiodonium chloride, methyl-β-cyclodextrin (mCD), hydrogen peroxide, and simvastatin were purchased from Sigma.

Protein Isolation and Western Blotting

Protein was typically isolated from cells approximately 80% confluent in six-well tissue culture dishes by adding 500 μL of 100°C SDS sample buffer. Samples were processed for electrophoresis and Western blotting as described [16].

Densitometry

Densitometry was performed on appropriate phosphorylated antibody blots using ImageJ software to compare pixel intensity. All values are shown as fold change of control compared to protein load control bands (total-Erk, total-Akt, or tubulin).

Invasion Assay

Costar transwell inserts were coated with 50 μL of a 1:25 dilution of Matrigel® and invasion assays were performed as previously described [16].

Immunofluorescence Staining

Immunofluorescence (IF) microscopy was performed as previously described [16].

Lipid Raft Separation

Cells were seeded the night before for 80% confluency in a 15 mm dish. The following day cells were either treated with serum-free media in the presence or absence of HGF (33 ng/mL). The cells were incubated for 30 min and then the isolation of Triton-soluble and -insoluble fractions was performed as described [18].

Sucrose Density Gradient Untracentrifugation

DU145 prostate cells in 10 cm^2 plates at 75% confluency were treated in serum-free conditions with 15 mM mCD for 45 min or 10 μM EGCG for 75 min. The cells were placed on ice and washed

with ice-cold PBS. The cells were scraped into 750 μL of 500 mM sodium carbonate, pH 11.0 and homogenized by three, 20-s bursts of an ultrasonic disintegrator. The homogenates were adjusted to 40% sucrose by adding an equal volume of 80% sucrose in 25 mM 2-(*N*-morpholino) ethanesulfonic acid, pH 6.5, 0.15 M NaCl (MBS) and placed at the bottom of an ultracentrifuge tube. A volume of 1.5 mL of 30% sucrose in MBS was overlaid upon the 40% sucrose, followed by 2 mL of 5% sucrose in MBS. The samples were centrifuged for 20 h at 160 000*g*. Ten 0.5 mL fractions were collected from the top of the tube. Three hundred microliters of 4× Laemmli buffer containing 2-mercaptoethanol was immediately added and each sample boiled for 5 min. Thirty microliters of fractions 2–10 were run by SDS–PAGE, transferred to PVDF, and probed with tubulin, caveolin-1, and flotillin-2.

$DiIC_{16}$ Staining

DU145 cells were plated at 5×10^4 in four-well chamber slides. The following day, the cells were treated with 5 μM EGCG or EC. $DiIC_{16}$ (Invitrogen, Carlsbad, CA) was added directly to the media at 1 μM and incubated at room temperature for 2 min. The media were aspirated and the cells washed 1× with 1× PBS. The cells were fixed with 4% paraformaldehyde, washed and nuclei stained with DAPI. Cells were visualized by immunofluorescence microscopy as described above.

Measurement of ROS Formation

Cells were seeded into 24-well plates at approximately 80% confluency and incubated overnight. Cells were washed three times with PBS to remove all media and serum and placed in M199 media. Cells were then incubated with 5 μM dichlorofluorescein diacetate (DCFH) (Molecular Probes) and inhibitors as indicated for 45 min, washed three times with PBS, and then treated with HGF or H_2O_2 in serum/phenol-free M-199. Fluorescence intensity was measured on a TECAN Genios plate reader using FITC excitation and emission wavelengths.

RESULTS

EGCG Blocks HGF-Induced Scattering, E-Cadherin Internalization, Motility, and Invasion of DU145 Prostate Cancer Cells

Fluorescence microscopy revealed that a 12 h incubation of DU145 prostate cancer cells with HGF at 33 ng/mL resulted in the loss of cell–cell association and a profound scattering of elongated cells (Figure 1A). The addition of EGCG at a concentration of 5 μM completely blocked the effect of HGF (Figure 1A); concentrations as low as 1 μM were equally effective (results not shown). IF microscopy indicated that HGF treatment of prostate tumor cells also induced the internalization of E-cadherin, a cell surface adhesion protein important in regulating cell–cell interactions and EGCG prevented this (Figure 1A).

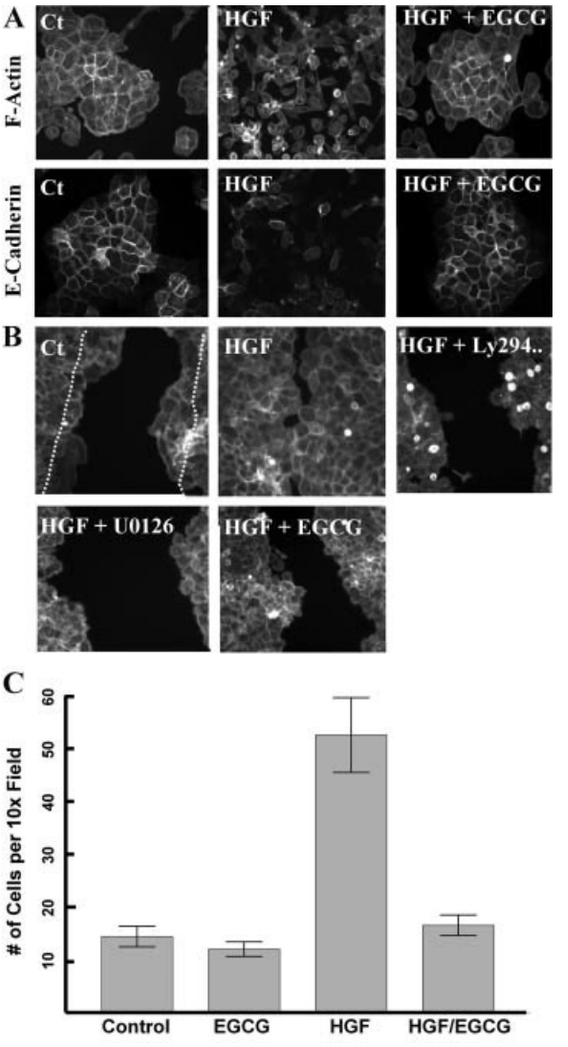

Figure 1. EGCG prevents HGF-mediated scattering, motility, and invasion. (A) DU145 cells grown overnight were treated with HGF (33 ng/mL) in the absence or presence of 5 μM EGCG for 16 h, fixed and stained for F-actin (top three panels) or E-cadherin (bottom three panels). (B) Cells were grown overnight, a wound was formed in the confluent monolayer, and cells were left untreated or treated with HGF for 16 h in the presence of U0126, LY294002, or 5 μM EGCG. The dotted lines in the control panel represent the edges of the wound at $T = 0$ min, thus indicating the amount the wound closed in nontreated cells. (C) Invasion assays were employed as described in the Materials and Methods Section.

A wound-healing assay was employed to determine if EGCG prevented HGF-stimulated increases in cell motility. A 15 h incubation of confluent cultures of DU145 cells in the absence of HGF resulted in minimal closure of a scrape wound, while in contrast, the addition of HGF resulted in an almost complete closure of the wound (Figure 1B). The HGF-

induced closure was not the result of proliferation, since HGF did not alter growth kinetics of DU145 cultures under these conditions (results not shown). Preincubation of cells with 5 μM EGCG, 25 μM LY294002 (PI3-kinase inhibitor), or 10 μM U0126 (MAPK inhibitor) largely prevented the HGF-induced closure of the wound (Figure 1B).

Finally, invasion assays employing well inserts layered with Matrigel were performed on control cells, cells exposed to HGF, cells exposed to 5 μM EGCG, and cells exposed to HGF preincubated for 30 min with 5 μM EGCG. HGF induced a four- to fivefold increase in the number of cells invading through the artificial basement membrane (Figure 1C). EGCG-treated cells were statistically no less invasive than control cells; however, EGCG completely blocked the HGF-induced increase in invasion (Figure 1C).

EGCG Prevents Activation of the c-Met Receptor

We next determined if EGCG prevented the activation of cellular pathways normally induced by the addition of HGF. Western blot analysis indicated that exposure of cells to HGF resulted in the rapid increase in the levels of Akt-P and Erk-P, indicating rapid activation of the PI3-kinase and MAPK pathways, respectively (Figure 2A). Preincubation of cells with the inhibitors U0126 or LY294002 largely prevented the HGF-induced increase in levels of Erk-P and Akt-P, respectively (Figure 2A). EGCG concentrations as low as 1.2 μM reduced HGF-induced increases in Akt-P and Erk-P (Figure 2B), while concentrations greater than 2.5 μM, almost completely blocked the activation of these pathways by HGF (Figure 2A and B). Simultaneous addition of EGCG with HGF was less effective suggesting a minimum preincubation time is required.

Activation of the c-Met receptor did not lead to increased levels of FAK-397-p, FAK-576-p, or paxillin-118-p, proteins that can interact with integrins to regulate motility (Figure 2C). However, pretreatment of cells with EGCG reduced the levels of FAK-576-p and paxillin-118-p by over 50% (Figure 2C), perhaps accounting in part for the reduction in motility observed in the presence of HGF and consistent with c-Met-independent effects of EGCG.

To determine if EGCG inhibits the HGF-mediated activation of the c-Met receptor, cells were preincubated with EGCG for 1 h followed by the addition of HGF. Western blot analysis indicated that HGF induced a fivefold increase in the level of Met-P (1234/1235) as early as 5 min after addition (Figure 2D), and this level was sustained over a 60-min period. Levels of Met-P began to drop by 2 h after the addition of HGF (results not shown). Preincubation of cells with EGCG largely prevented the HGF-mediated increase in Met-P (Figure 2D), consistent with the effect of EGCG on downstream signaling.

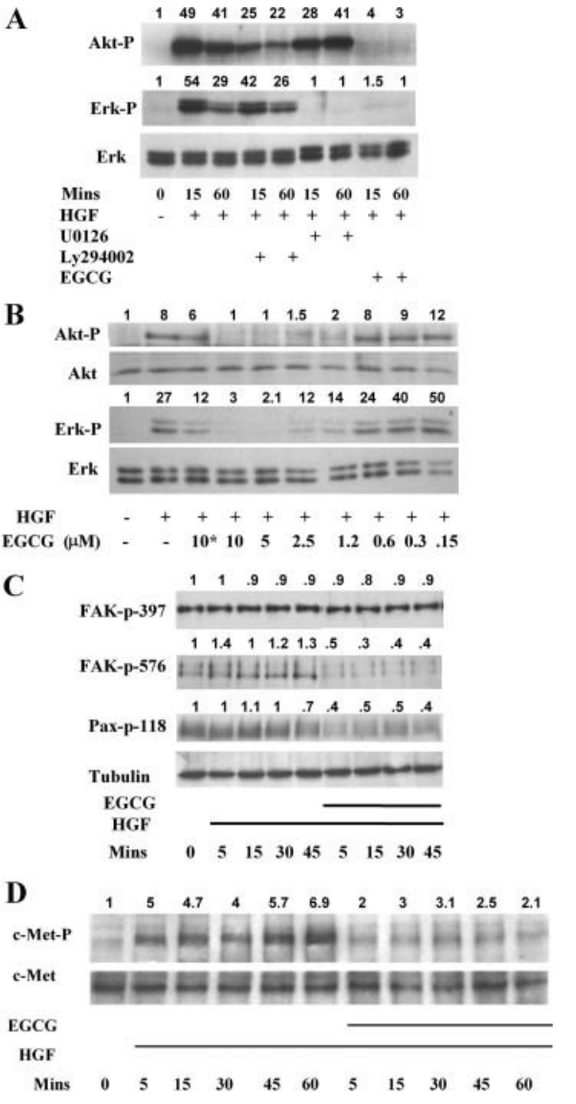

Figure 2. Western blot analysis indicates that EGCG blocks HGF-induced signaling at the level of the c-Met receptor. (A) Cells were treated with HGF for 15–60 min in the presence of 25 μM LY294002, 10 μM U0126, or 5 μM EGCG. Small values above the phosphorylated bands indicate fold-change densitometry values compared to control using total Erk as a protein load control. (B) Cells treated were indicated with HGF (30 min) and/or EGCG at the indicated concentrations. EGCG was added 30 min prior to the addition of HGF except for the lane noted with an asterisk, in which case the cells were not pretreated for 30 min prior to addition of HGF. Densitometry values indicate fold-change of the phosphorylated protein compared to total Akt or Erk, which were used as protein load controls. (C) Cells were treated with HGF for periods of 5–45 min in the absence or presence of 5 μM EGCG. Densitometry was performed using tubulin as a protein load control. Values indicated fold-change compared to control. (D) Cells were treated with HGF for 5–60 min in the presence or absence of 5 μM EGCG (added 30 min prior to the addition of HGF). Densitometry of phosphorylated c-Met was compared to the levels of total c-Met as a protein load control. Values indicate fold-change compared to control.

HGF-Induced Scattering of Prostate Tumor Cells Does Not Require Reactive Oxygen Species Formation

EGCG is a potent antioxidant [10] and may act to prevent the formation of reactive oxygen species (ROS) in the presence of HGF. ROS can activate a number of intracellular signaling pathways that contribute to increased motility and invasion. To determine if the addition of HGF stimulated the formation of ROS, cellular fluorescence-based assays were employed. Cells were loaded with DCFH and treated with hydrogen peroxide (H_2O_2), HGF or were left untreated. Fluorescent intensities were measured over time and are plotted in Figure 3A. The rate of DCF formation was not increased in cells exposed to HGF as compared to control cells, while hydrogen peroxide stimulated the formation of DCF (oxidized DCFH) by fivefold as compared to control, which could be inhibited by co-treatment with the antioxidant, NAC.

HGF-mediated scattering assays were performed to determine if the addition of inhibitors of the ROS pathway prevented response to the growth factor. Cells were preincubated with glutathione ethyl ester (GEE), diphenyleneiodonium chloride (DPI), ammonium pyrrolidinedithiocarbamate (PDTC) or NAC for 1 h, followed by the addition of HGF. FITC-phalloidin staining was used to visualize cells after an overnight incubation with the ROS inhibitors in combination with HGF. Figure 3C demonstrates that none of the

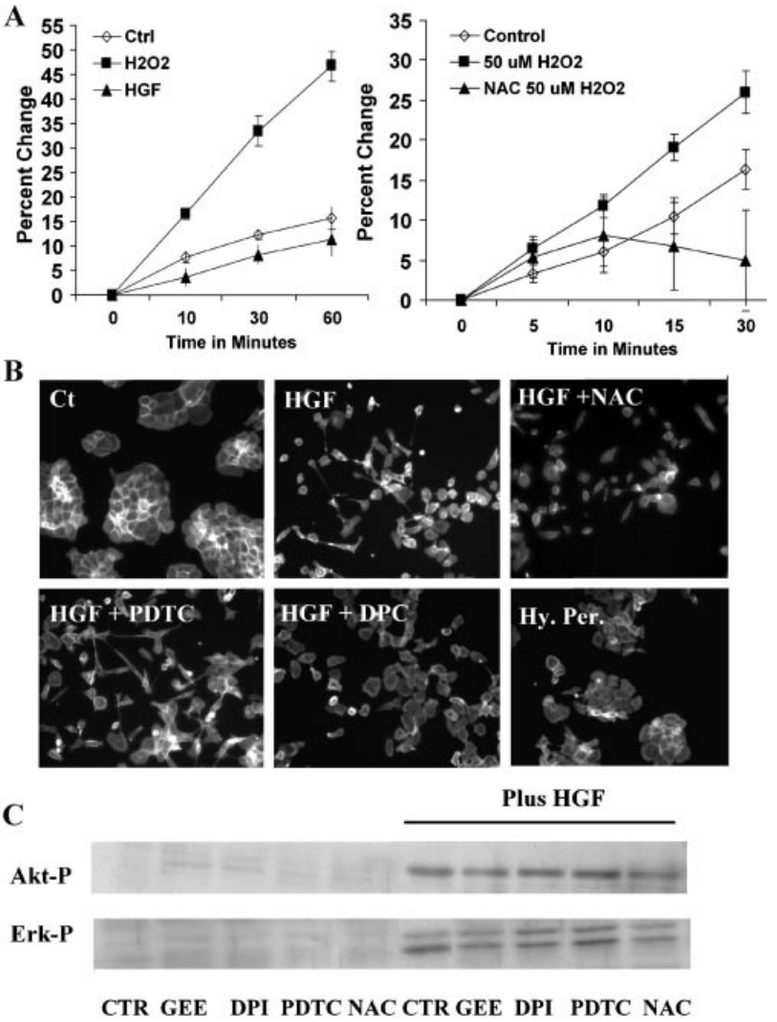

Figure 3. EGCG blocks HGF-induced cell scattering and signaling independent of ROS activity. (A) Cells were loaded with DCFH and treated with reagents as indicated in the figure for up to 30 min. Fluorescence readings were plotted as percent change over time. (B) Cells in colonies were not treated or treated with HGF alone or HGF with NAC at 5 mM, PDTC at 100 μM, or DPC at 10 μM overnight. The remaining panel represents cells treated overnight with 200 μM hydrogen peroxide, indicated that ROS production does not induce cell scattering. Cells were fixed and stained for F-actin. (C) Cells were pretreated for 30 min with the indicated drugs and either left untreated or treated with HGF for 30 min. Western blot analysis was performed following electrophoresis of 20 μg of protein per well (BCA protein assay).

inhibitors blocked the HGF-induced scattering of cells, although NAC did prevent the elongation of cells characteristic of exposure to HGF. NAC also effectively prevented the formation of ROS induced by hydrogen peroxide (Figure 3B). The addition of hydrogen peroxide at a concentration demonstrated to induce ROS formation (Figure 3A) did not induce the scattering of DU145 tumor cells (Figure 3C). Consistent with the results presented above, none of the inhibitors prevented the HGF-induced increase in levels of Akt-P or Erk-P (Figure 3C).

EGCG and ECG Are More Effective at Inhibiting c-Met Activation Than EGC or EC

To determine if other green tea catechins also blocked HGF-mediated signaling, DU145 cells were treated with HGF alone or in combination with 10 μM EGCG, EGC, EC, or ECG and incubated overnight. Phase-contrast microscopy indicated that only ECG was as effective as EGCG in blocking HGF-induced cell scattering (Figure 4A), supporting our earlier work in breast tumor cells [16], concluding that the gallate group plays an important functional role in the activity of the catechins (Figure 4C). In addition, Western blot analysis revealed that ECG, like EGCG, prevented HGF-stimulated Akt-P and Erk-P at concentrations as low as 1 μM, while EC and EGC were ineffective in inhibiting activation of Erk-P at concentrations as high as 10 μM (Figure 4B). Interestingly, levels of Akt-P were lower in cells treated with EC or EGC as compared to HGF alone; although this reduction did not prevent cellular scattering.

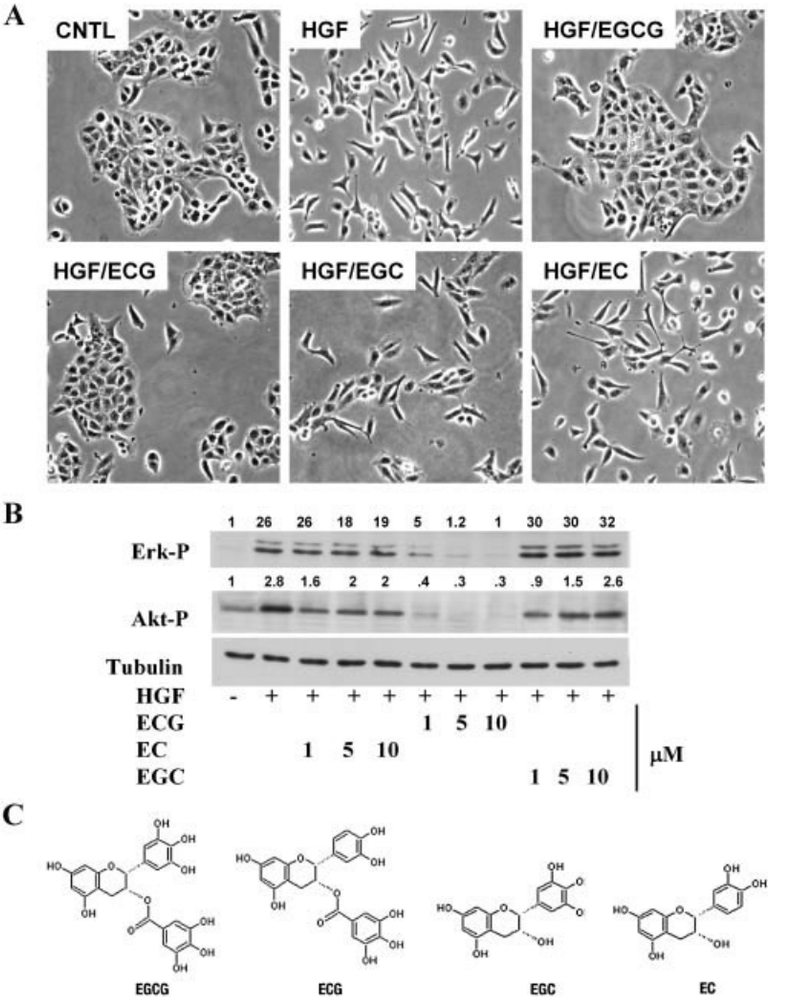

Figure 4. EGCG and ECG are more active c-Met inhibitors than EGC and EC. (A) Cells were left untreated or treated with HGF overnight in the presence of 10 μM EGCG, ECG, EGC, or EC to analyze HGF-induced cell scattering. Phase-contrast images were taken using a 10× objective on a Nikon Eclipse TE300 inverted microscope. (B) Cells pulsed with HGF for 30 min were indicated following pretreatment with the indicated concentrations of ECG, EC, and EGC. Western blot analysis was performed using the indicated antibodies. Densitometry was performed and the small values above the phosphorylated bands indicate fold-change compared to control using tubulin as a protein load control. (C) The chemical structures for EGCG, ECG, EGC, and EC are shown. It appears that the gallate structure found in EGCG and ECG is important to prevent HGF-induced signaling.

Delayed Addition of EGCG Attenuates HGF-Induced Levels of Phosphorylated c-Met, Akt, and Erk

If EGCG only prevented the initiation of signaling stimulated by HGF, then adding it after the addition of this growth factor should prove less effective. To examine this, HGF was added to cultures of DU145 cells, followed by the addition of EGCG at $\frac{1}{2}$, 1, 2, and 4 h post-HGF treatment. Following an overnight incubation, cells were fixed and stained with FITC-phalloidin and visualized using a fluorescence microscope. HGF stimulated scattering in DU145 cells but was ineffective in promoting scattering of cells also treated with EGCG added up to 4 h after the addition of HGF (Figure 5A).

To determine if the delayed addition of EGCG altered levels of phosphorylated c-Met, Akt, and Erk, cells were treated with HGF, and at 30 or 60 min after the addition of HGF, EGCG was added. Cells were harvested at the time points indicated in Figure 5B and Western blot analysis was performed. Levels of phosphorylated c-Met, Akt, and Erk peaked at 60 min following the addition of HGF and slowly decreased over a 4-h period, with levels of Met-P showing the largest decrease (>50%) by 4 h. The delayed addition of EGCG first resulted in a decrease in levels of Met-P followed by inhibition of Akt-P and Erk-P. For instance, 30 min after the addition of EGCG to cells already exposed to HGF for 30 min (60 min total incubation time), levels of Met-P decreased by 50%, while levels of Akt-P and Erk-P remained unchanged as compared to cells exposed to only HGF for 60 min. By 120–240 min after the delayed addition of EGCG, Met-P was no longer detectable, while levels of Akt-P and Erk-P were reduced by 50% as compared to cells only treated with HGF for 240 min.

Activation of c-Met Depends on Cholesterol-Rich Organized Regions of Membrane That Are Disrupted by EGCG

It has been suggested that EGCG may affect lipid raft membrane domains [19,20]. To determine the importance of cholesterol, a component of lipid rafts, in c-Met signaling, cells were treated for 30 min with increasing concentrations of mCD, followed by the addition of HGF for 1 h. Western blot analysis indicated that 15 mM mCD reduced the HGF-mediated increase in Met-P, Erk-P, and Akt-P by greater than 50%, whereas 30 mM mCD

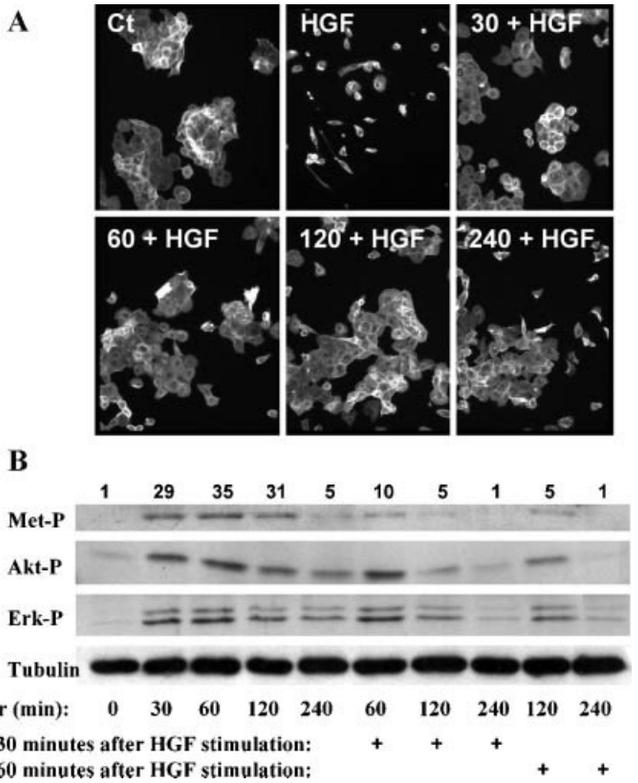

Figure 5. EGCG attenuates active c-Met signaling. (A) Cells were treated with HGF at 0 min followed by the addition of EGCG to a final concentration of 5 μM at 30, 60, 120, or 240 min after the addition of HGF. After an overnight incubation, cells were fixed, stained for F-actin, and visualized using a fluorescence microscope. (B) Cells were stimulated with HGF for the times indicated (min). At 30 or 60 min after the addition of HGF, EGCG (final concentration of 5 μM) was added and cells continued incubating for the indicated times. Cells were harvested at the indicated time points and Western blot analysis was performed using antibodies to the proteins indicated in the figure. Densitometry was performed on the Met-P band and is shown as fold-change compared to control using tubulin as a protein load control.

largely prevented the increased phosphorylation (Figure 6A). Comparable results were seen with the WPMY fibroblast cell-line (Figure 6B) and two lung cancer cell-lines (data not shown), although much lower concentrations of mCD were needed to inhibit HGF-mediated signaling in the WPMY cells.

Next, cells were treated overnight with a range of concentrations of simvastatin to lower cellular levels of cholesterol. Cells were pulsed for 30 min with HGF the next day. As indicated in Figure 6C, simvastatin treatment greater than 2.5 μM lowered Met-P and Akt-P induction by 50% with a smaller effect on Erk-P. This further supports a role for lipid rafts in HGF signaling.

Next, control cells and cells treated with HGF for 30 min were subjected to the cold Triton-X-100 extraction protocol, followed by centrifugation to separate detergent soluble from lipid raft-rich insoluble fractions. Western blot analysis of these fractions indicated that >90% of total c-Met, tubulin, and a lysosome-associated membrane protein, LAMP1, were distributed in the detergent soluble fraction, while in contrast >90% of Met-P distributed in the insoluble fraction with the lipid raft protein flotillin (Figure 6D).

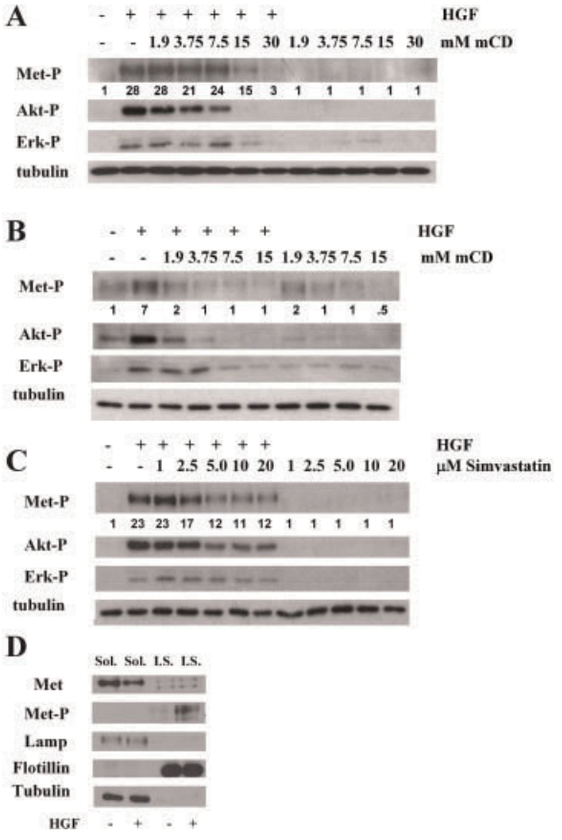

Figure 6. Active c-Met resides in lipid rafts. (A and B) DU145 and WPMY cells were pretreated for 20 min with mCD and left untreated or treated with HGF for 30 min. Western blot analysis was performed. (C) DU145 cells were treated with the indicated concentrations of simvastatin overnight in serum-free medium before being exposed to HGF for 30 min. Western blot analysis was performed to detect Met-P, Akt-P, and Erk-P. (D) Triton X-100 soluble and insoluble fractions were prepared from untreated cells or cells treated for 30 min with HGF. Western blot analysis was performed and equal protein was loaded (BCA protein assay). Densitometry was performed on the Met-P bands (except in Panel D). The values under Met-P bands indicate fold-change compared control. Tubulin was used as a protein load control.

To determine if EGCG modified the composition or functional integrity of lipid rafts, cells were preincubated with EGCG or EC for 1 h, and then incubated for 30 min with the fluorescent dye $DiIC_{16}$, which accumulates in organized lipids found in rafts. Fluorescence microscopy indicated that >90% of the cells detected by phase-contrast microscopy were also stained primarily in the plasma membrane with $DiIC_{16}$ in control cells and cells exposed to EC (inactive in inhibiting c-Met activation), while, in contrast, pretreatment with EGCG reduced the intensity of staining with $DiIC_{16}$ by greater than 80% and the percentage of cells that were stained with this agent by 90% (Figure 7A).

To determine if the effects of EGCG on rafts is cholesterol dependent, DU145 cells were incubated in serum-free medium ± cholesterol for 1 h, followed by the addition of EGCG for 30 min. HGF was added for 30 min and Western blot analysis was performed. As indicated in Figure 7B, preincubation with cholesterol partially prevented the EGCG-mediated inhibition of phosphorylation of Met and Akt, similar results were also observed in a fibroblast cell-line (Figure 7C). Although EGCG inhibition of Met signaling is partially reversed by the addition of exogenous cholesterol, this polyphenol does not act by extracting cholesterol-like mCD. Figure 7D indicates that pretreatment of cells with mCD, but not EGCG, shifted the density of the lipid raft markers flotillin and caveolin-1 on sucrose gradients to a higher density, suggesting EGCG does not act by a mechanism identical to mCD. Finally, contrary to what the Weinstein group observed [20], we found that pretreatment with EGCG did not alter receptor dimerization in the presence of HGF (results not shown).

DISCUSSION

In this report, we demonstrate that EGCG inhibits HGF-induced activation of c-Met and downstream signaling pathways in the prostate carcinoma cell line DU145 at physiologically relevant concentrations. EGCG did not act by blocking ROS accumulation or preventing receptor dimerization. Interestingly, delayed addition of EGCG inhibited c-Met activation to the same extent as pretreatment with EGCG suggesting that EGCG can antagonize or reverse cell signaling which has already been stimulated. We assessed if additional green tea catechins functioned similar to EGCG. EC and EGC were unable to inhibit HGF-induced signaling and

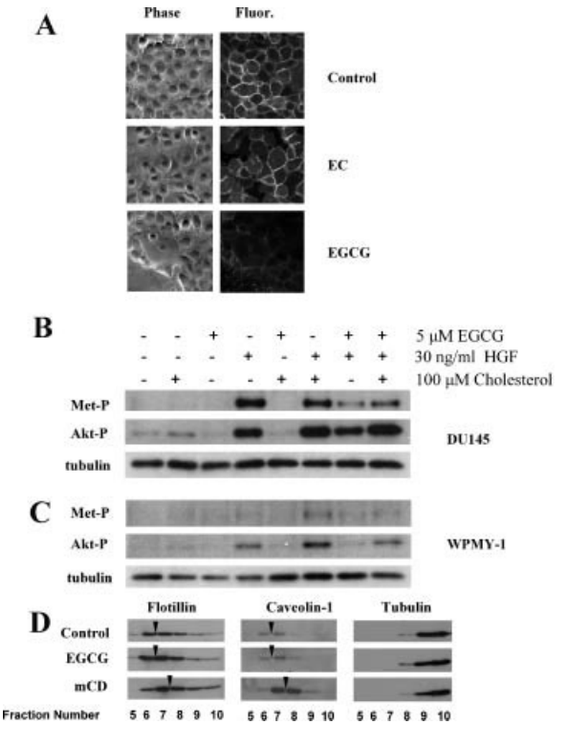

Figure 7. EGCG acts via a different mechanism than mCD to prevent HGF-induced signaling. (A) DU145 cells were exposed to $DiIC_{16}$ for 2 min in the presence or absence of 5 μM EC or EGCG. Cells were fixed and visualized using a fluorescence microscope. (B and C) DU145 or WPMY cells were treated ± cholesterol for 1 h followed by the addition of EGCG for 30 min and a final pulse with HGF for 30 min. Western blot analysis was performed to detect Met-P and Akt-P before being treated for 20 min. Tubulin was used as a protein load control. (D) DU145 prostate cancer cells at 75% confluency were treated in serum-free media with 15 mM methyl-β cyclodextrin (mCD) for 45 min or 10 μM EGCG for 75 min. Raft and nonraft fractions were separated by ultracentrifugation in a discontinuous sucrose gradient. An equal volume of each fraction was run by Western blot analysis and probed with the indicated antibodies.

scattering, whereas ECG functioned similar to EGCG suggesting the importance of the gallate group. Finally, we demonstrate that the phosphorylated, active form of c-Met resides in lipid raft domains and that EGCG exerts its inhibitory effects on c-Met receptor activation by disrupting cholesterol-rich membrane microdomains.

EGCG can antagonize numerous cell signaling receptors, including PDGF-R, VEGF-R, EGF-R, Her-2/Neu, and FceR [8,9,21–23]. Various mechanisms have been proposed to explain how EGCG is capable of inhibiting such a wide variety of signaling pathways. EGCG has been suggested to function as an antioxidant in several publications [24]. EGCG was shown to inhibit UVB induced H_2O_2 production in normal human keratinocytes and inhibit lipid peroxidation [25]. Alternatively, EGCG was also demonstrated to function as an oxidant [26,27], and in fact the addition of EGCG to 20 μM in tissue culture media results in the rapid production of H_2O_2 [27]. As EGCG has been shown to stimulate apoptosis in numerous cell models, these observations suggest that the induction of apoptosis by EGCG is dependent on the production of H_2O_2. This was exemplified by the Yang Laboratory when they demonstrated that apoptosis stimulated by EGCG in H661 lung cancer cells and Ras-transformed human bronchial cells could be blocked by the addition of catalase [26]. In our DU145 model, HGF addition did not result in detectable production of ROS, and co-treatment with antioxidants did not inhibit HGF-induced scattering or motility suggesting that EGCG is not affecting ROS levels. In addition, the Weinstein group concluded that EGCG inhibited EGF-R activation by disrupting receptor dimerization [20], a result we did not observe for c-Met activation.

Lipid raft microdomains are higher ordered membrane structures, rich in sphingolipids and cholesterol, and they are involved in the regulation of cellular signaling through compartmentalization and organization of signaling complexes [28]. There is an increasing interest in the role that lipid rafts may play in intracellular signaling and cell survival pathways in prostate cancer. The Freeman Laboratory has demonstrated that regulation of cholesterol levels, an important component of lipid rafts, can effect cell survival in vitro and in vivo [29]. Therefore, agents that can target lipid raft composition or function might be useful in the treatment of prostate cancer.

We have demonstrated that extraction of cholesterol with mCD inhibited HGF signaling in DU145 cells, a fibroblast cell-line and two lung cancer cell-lines. Additionally, the phosphorylated, active form of c-Met was localized in the Triton-X-100 insoluble fraction and simvastatin, a drug that lowers cholesterol, also reduced HGF signaling. These observations support the hypothesis that active c-Met resides in lipid raft domains and suggest that disruption of lipid raft microdomains could potentially inhibit c-Met signaling and cell invasion. In support of this, we have shown that EGCG may function to inhibit c-Met activity through altering lipid raft structures as observed by the loss of $DiIC_{16}$ staining, and the effect excess exogenous cholesterol has on lowering the potency of EGCG. EGCG does not appear to work like mCD to extract cholesterol, but we propose instead that it acts to alter the organization of raft lipids, perhaps by sequestering cholesterol without changing the detergent insoluble of these membrane domains. The exact molecular mechanisms of action remain to be determined.

These data, in combination with previously published observations, suggest that EGCG can affect such a wide variety of signaling pathways through disruption of high-order membrane structures. Many growth factor receptors, which are deregulated

in cancer, are also localized in lipid rafts, such as EGFR, PDGF, IGF-1, as well as c-Met [20,21,30], and activation of these growth factors can also be inhibited by EGCG [20,31,32]. The Weinstein Laboratory has recently demonstrated that EGCG can block the EGF receptor perhaps via disruption of lipid rafts [20]. Furthermore, they have shown that it is the active form of EGFR that is localized in lipid raft domains [20], similar to our observation that the active, phosphorylated form of c-Met resides in lipid rafts. Consistent with these findings, Tachibana et al. [33] 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. They have also recently found through a subtraction cloning strategy that EGCG binds to the 67-kDa laminin receptor (67 LR) located in lipid rafts [33]. Cells which 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 [33]. In further support of the suggestion that EGCG affects lipid raft domains, Ermakova et al. [34] demonstrated that EGCG binds to the intermediate filament protein, vimentin, and inhibited proliferation in vimentin-positive cells, but failed to inhibit the growth of cells lacking vimentin expression. Interestingly, vimentin has also been proposed to localize in detergent-resistant membrane domains [35].

Green tea is composed of the additional catechins, (−)-EC, (−)-EGC, and (−)-ECG. In our studies, ECG was capable of inhibiting HGF-induced c-Met activation and downstream signaling at similar concentrations compared to EGCG. Treatment with EC, on the other hand, had no effect on lipid organization as observed by $DiIC_{16}$ staining. Therefore, structure function analysis with EC and EGC suggests that the presence of the gallate group on EGCG and ECG is important for mediating inhibition of c-Met activation and signaling. These results agree with our previously published studies in MCF10A and MDA-MB-231 breast cell lines [16].

We have demonstrated that EGCG is capable of inhibiting c-Met activation and downstream effects at physiologically relevant concentrations as low as 1 μM. One of the primary concerns regarding the use of natural products is their low bioavailability and their rapid metabolism. Many published observations report effective doses of EGCG at levels significantly higher than what can be achieved physiologically, including the majority of apoptosis studies. The fact that we were able to observe an inhibitory effect on c-Met activation and signaling at concentrations as low as 1 μM suggests that EGCG may show promise as an antagonist of c-Met signaling in vivo. In fact, tissue data from our phase II trial [15] indicated a reduction in c-Met-P prostate tumor tissue from men who consumed tea polyphenols (data not shown). Furthermore, in our current study, we demonstrated that EGCG was capable of antagonizing HGF-induced c-Met activation, signaling and scattering up to 4 h post-HGF addition. These observations suggest that EGCG may be able to attenuate c-Met signaling which has already been initiated. This is potentially relevant since levels of active c-Met-P may be high in patients prior to consumption of polyphenols.

Phase I trials with EGCG have already been performed with no to limited toxicity [11–13]. EGCG is currently in several additional Phase II clinical trials, several of which have been published previously [13]. Bettuzzi et al. [14] have reported that men with PIN who were administered four green tea catechins over a period of 1 yr were less likely to develop prostate cancer compared to men who were administered placebo control. We have completed a Phase II clinical trial assessing the effect of EGCG in the form of Polyphenon E (contains the four main tea catechins) on biomarkers associated with cancer progression in men with prostate cancer [15]. Men self-administered Polyphenon E over a median period of 40 d and serum and tissue were harvested before trial commencement and at the time of radical prostatectomy. Results indicated that EGCG significantly decreased serum levels of HGF, VEGF, and PSA and tissue levels of phosphorylated Akt and c-Met [15]. These combined observations suggest that EGCG may be efficacious in chemopreventive, adjuvant, or combinatorial therapy approaches to prevent or slow prostate cancer progression.

In summary, we have demonstrated that the active form of c-Met resides in lipid rafts in prostate tumor cells and a variety of other cells. We hypothesize that EGCG inhibits HGF-induced c-Met activation and downstream effects, including E-cadherin internalization, and cell motility and invasion by altering the functional activity of lipid rafts. Furthermore, structure function analysis revealed that the gallate group of EGCG and ECG is important for mediating these inhibitory effects. Further analysis would be necessary to determine whether EGCG mediates its wide range of inhibitory effects via disruption of lipid raft domains in vivo, and if EGCG alters the cholesterol level or location in rafts.

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