(-)-Epigallocatechin gallate downregulates EGF receptor via phosphorylation at Ser1046/1047 by p38 MAPK in colon cancer cells

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We previously reported that (-)-epigallocatechin gallate (EGCG) in green tea alters plasma membrane organization and causes internalization of epidermal growth factor receptor (EGFR) resulting in the suppression of colon cancer cell growth. In the present study, we investigated the detailed mechanism underlying EGCG-induced downregulation of EGFR in SW480 colon cancer cells. Prolonged exposure to EGCG caused EGFR degradation. However, EGCG required neither an ubiquitin ligase (c-Cbl) binding to EGFR nor a phosphorylation of EGFR at tyrosine residues, both of which are reported to be necessary for EGFR degradation induced by epidermal growth factor. In addition, EGCG induced phosphorylation of p38 mitogen-activated protein kinase (MAPK), a stress-inducible kinase believed to negatively regulate tumorigenesis, and the inhibition of p38 MAPK by SB203580, a specific p38 MAPK inhibitor, or the gene silencing using p38 MAPK-small interfering RNA (siRNA) suppressed the internalization and subsequent degradation of EGFR induced by EGCG. EGFR underwent a gel mobility shift upon treatment with EGCG and this was canceled by SB203580, indicating that EGCG causes EGFR phosphorylation via p38 MAPK. Moreover, EGCG caused phosphorylation of EGFR at Ser1046/1047, a site that is critical for its downregulation and threonine phosphorylation of the EGFR, it has previously been reported to cause a marked inhibition of the EGF-stimulated endocytosis and downregulation of cell surface receptors (18). However, the regulatory mechanism behind EGFR phosphorylation at Ser1046/7 remains to be clarified.

There is an evidence that cisplatin induces EGFR internalization, which is mediated by p38 mitogen-activated protein kinase (MAPK)-dependent phosphorylation of the receptor on threonine 669 (15). Gemcitabine also induces EGFR internalization and subsequent degradation that may be a novel mechanism for gemcitabine-mediated cell death (5). In addition, recent reports show that the activation of p38 MAPK is necessary for gemcitabine-induced cytotoxicity (20,21). Therefore, it is of interest to investigate the effect of anticancer drug on p38 MAPK since this signaling pathway is implicated in suppression of tumorigenesis (22,23).

(-)-Epigallocatechin gallate (EGCG) is the major biologically active polyphenol in green tea; it has been shown to inhibit the growth of several types of cancer cell lines via suppression of phosphorylation (i.e. activation) of the EGFR and inhibition of several downstream signaling pathways (2,4,6,24). We previously reported that the inhibitory effect of EGCG on EGF binding to the EGFR or EGFR dimerization is associated with alterations in lipid organization in the plasma membrane (lipid rafts) of colon cancer cells (25). Since lipid rafts are thought to play an important role in endocytosis, it is most likely that the alteration of lipid rafts induced by EGCC is associated with the cellular localization of the EGFR. Moreover, we have recently reported that EGCG can induce internalization of EGFR into endosomes, which can recycle back to the cell surface (26).

In the present study, we investigated the mechanism underlying EGCG-induced EGFR internalization in colon cancer cells because we suggest that this internalization of EGFR would explain, at least in part, the mechanism of anticancer effect of EGFR overexpression has been associated with chemoresistance, disease progression and poor survival (9). In clinical trials, increasing evidence shows the efficacy of EGFR-targeted agents, including monoclonal antibodies on the one hand and tyrosine kinase inhibitors on the other (10). In addition, ongoing clinical trials continue to investigate EGFR therapeutics in various treatment settings (7,10).

Receptor downregulation is the most prominent regulator of EGFR signal attenuation and involves the internalization and subsequent degradation of the activated receptor in the lysosomes (8). Some agents that induce ligand-independent internalization and degradation of EGFR, such as the 225 mouse antibody (11) and gemcitabine (5) could have promising potential for cancer therapies (12). In contrast, the other factors or agents, such as oxidative stress (13), ultraviolet (UV) irradiation (14) and cisplatin (15), can induce internalization of the EGFR, but not degradation. They differ in their effects on the fate of the receptors, downstream signaling and cell proliferation.

With the current knowledge of the mechanism underlying EGFR downregulation, this molecular event seems to involve several important phosphorylation sites. One is the phosphorylation at tyrosine (Tyr) 1045, which provides a docking site for the ubiquitin ligase c-Cbl resulting in ubiquitination of the EGFR (16). The others are the phosphorylation at serine or threonine residues, which is thought to represent a mechanism for attenuation of the receptor kinase activity (17–19). Among the major sites of serine and threonine phosphorylation of the EGFR, it has previously been shown that serine 1046/1047 (Ser1046/7) phosphorylation site is required for EGFR desensitization in epidermal growth factor (EGF)-treated cells (19). Moreover, mutations of Ser1046/7 are reported to cause a marked inhibition of the EGF-stimulated endocytosis and downregulation of cell surface receptors (18). However, the regulatory mechanism behind EGFR phosphorylation at Ser1046/7 remains to be clarified.

Introduction

Colon cancer is the second leading cause of cancer-related mortality in Western countries. Members of the epidermal growth factor receptor (EGFR) family, which are frequently overexpressed in several types of human cancers, including cancers of the lung (1), head and neck (2), prostate (3), breast (4), pancreas (5) and colon (6), have been associated with abnormal growth of these tumors. Binding of ligand to EGFR leads to receptor dimerization, autophosphorylation and activation of several downstream signaling pathways, which upon activation lead to cell proliferation, motility and enhanced survival (7). There are several mechanisms by which EGFR becomes oncogenic, including (i) increased EGFR levels; (ii) auto-crine and/or paracrine growth factor loops; (iii) heterodimerization with other EGFR family members and cross talk with heterologous receptor systems; (iv) defective receptor downregulation (described below in detail) and (v) activating mutations (8). In cohort studies,
EGCG and other related compounds (26). Interestingly, we observed that the inhibition of p38 MAPK abolished the EGCG-induced internalization and subsequent degradation of EGFR, suggesting that this MAPK plays an important role in the change of EGFR trafficking. Furthermore, EGCG induced phosphorylation of EGFR at Ser1046/7 via p38 MAPK, indicating that this phosphorylation plays a pivotal role in EGFR downregulation induced by EGCG.

Materials and methods

Cell culture and chemicals
SW480 human colon cancer cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, San Diego, CA) containing 10% fetal calf serum and after 24 h they were incubated in serum-free medium for an additional 24 h as described previously (26). EGCG was kindly provided by Dr Yukihiko Hara (Mitsui Norin Co., Shizuoka, Japan) and was solubilized in dimethyl sulfoxide. SB203580, AG1478 and PD153035 were purchased from Calbiochem–Novabiochem Co. (La Jolla, CA). EGF and cisplatin were from Sigma Chemical Co. (St Louis, MO).

Western blotting and immunoprecipitation assay

The cells were lysed in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 50 mM NaF, 50 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 1 mM Na3VO4 and 2 mM phenylmethylsulfonyl fluoride) and scraped from the Petri dishes. Protein extracts were then examined by western blot analysis as described previously (26). The antibodies used in these studies were anti-EGFR, glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-EGFR (Ser1046/7), anti-phospho-EGFR (Tyr1045), anti-rabbit IgG, anti-rabbit IgG antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK) and antisense secondary antibodies. Each membrane was developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). For immunoprecipitation, EGFR cell lysates (500 μg each) were incubated for 12 h at 4°C with an anti-EGFR antibody (Santa Cruz Biotechnology) precoupled to anti-mouse IgG agarose beads. Protein extracts were then eluted and examined by western blotting using the indicated antibodies.

Gel mobility shift assays

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in 4% polyacrylamide large gel for 6 h at 4°C to separate proteins clearly.

Small interfering RNA protocol

Predesigned small interfering RNAs (siRNAs) targeting p38 MAPK (On- TARGET plus Duplex J-003512-20, Human MAPK14) was purchased from Thermo Fisher Scientific K.K. (Yokohama, Japan). Sequences are as follows: sense, 5’-GGAAUUCAUGAUUGUAAUU-3’ and antisense, 5’-UAACA- CAUAUGAUUGUAUUCUU-3’. Transfection was performed according to the manufacturer’s protocol (Bio-Rad, Tokyo, Japan). In brief, 2.5 μl of siLentFect (Bio-Rad) and finally 100 nM of siRNA were diluted with fetal calf serum-free Dulbecco’s modified Eagle’s medium, preincubated at room temperature for 20 min and then added to the culture medium containing 10% fetal calf serum. Cells were incubated at 37°C for 48 h with siRNA-siLentFect complexes and subsequently harvested for preparation of western blot analysis.

Immunofluorescence microscopy studies

Immunofluorescence microscopy studies were performed as described previously (26). Live cells grown on coverslip-bottom dishes were first exposed to the mouse anti-EGFR antibody that recognizes the extracellular domain of EGFR at Ser1046/7 via p38 MAPK, followed by exposure to Alexa Fluor 488®-conjugated goat anti-mouse IgG antibody for 1 h for EGFR (green signal). In some cases, the fixed cells were exposed to an anti-c-Cbl antibody, followed by exposure to Alexa Fluor 488®-conjugated goat anti-rabbit IgG antibody for c-Cbl (green signal) and Alexa Fluor 546®-conjugated goat anti-IgG antibody for EGFR (red signal) for 1 h. Finally, they were exposed to 4',6-diamidino-2-phenylindole (Wako, Tokyo, Japan) for 20 min and the cells were then examined by fluorescence microscopy, BIOREVO (BZ-9000) (Keyence, Tokyo, Japan) according to the manufacturer’s protocol.

Quantification of cell surface EGFR by enzyme-linked immunosorbent assay

Quantification of cell surface EGFR was performed as described previously (26). In brief, SW480 cells were pretreated with the indicated compounds and then exposed to the mouse anti-EGFR antibody (Santa Cruz Biotechnology) that recognizes the extracellular domain of the EGFR (1:50 dilution), in Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin for 15 min at 37°C. The cells were then treated with EGCG (25 μM) for 30 min at 37°C and then fixed with 4% paraformaldehyde for 10 min on ice. After blocking with 1% bovine serum albumin in phosphate-buffered saline for 1 h, the cells were exposed to an anti-mouse IgG, horseradish peroxidase-linked whole antibody (GE Healthcare, Piscataway, NJ) for 1 h at room temperature, followed by washing four times with phosphate-buffered saline containing 1% bovine serum albumin. Finally, the cells were exposed to 50 μl of 1-step TM-ELISA reagent (Pierce, Rockford, IL) for 5 min at room temperature. Fifty microliters of 2 M sulfuric acid was then added to each well to stop the reaction. The absorbance of each sample at 450 nm was then measured.

Results

Continuous treatment with EGCG caused degradation of the EGFR in SW480 colon cancer cells

Since EGCG induces apoptosis of colon cancer cells (6), it is of interest to examine the effect of longer exposure to EGCG on these cells that highly express EGFR proteins. We first measured the levels of EGFR in the presence or absence of EGCG by western blot analysis (Figure 1). We examined the effect of various doses of EGCG on the levels of EGFR and found that exposure to 25 μM of EGCG for 24 h significantly induced degradation of EGFR (Figure 1A, lane 4) and that 50 μM of EGCG almost completely degraded them (Figure 1A, lane 5). We next performed a time course experiment and found that treatment with EGCG (50 μM) for 8 h caused partial degradation of EGFR (Figure 1B, lane 5) and that treatment with EGCG for 12 h caused marked degradation of EGFR (Figure 1B, lane 6). Although we recently reported that transient exposure to EGCG induced internalization of cell surface-associated EGFR into endosomes, which could recycle back to the cell surface (26), this was not seen in this study because in the previous study we examined the effect of continuous, not transient, treatment with EGCG. We also performed cell counting assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and found that EGCG did not influence cell number of SW480 within 24 h (data not shown), whereas decrease in the level of EGFR were observed within 12 h (Figure 1B).

The degradation of EGFR induced by EGCG is not associated with c-Cbl in SW480 colon cancer cells

It is well known that c-Cbl, an ubiquitin E3 ligase, plays a role in the downregulation of EGFR (16,27,28). Thus, c-Cbl was shown to ubiquitylate and downregulate EGFR (29) and was formally established as a RING finger-type E3 ubiquitin ligase (30,31). Therefore, it seems unlikely that the internalized EGFR induced by EGF were clearly colocalized with c-Cbl (panel 11). Many reports demonstrate that activation of EGFR induces internalization of EGFR (Figure 2A, panels 2 and 3), which is consistent with our previous studies (26). We next performed colocalization assays of these internalized EGFRs (panels 1–3) and c-Cbl (panels 4–6) and found that internalized EGFR induced by EGCG did not colocalize with the c-Cbl protein (panel 12), whereas the internalized EGFR induced by EGFR were clearly colocalized with c-Cbl (panel 11). Many reports demonstrate that activation of EGFR induces its autophosphorylation following by binding of c-Cbl to the Tyr1045 residue of EGFR (29). In addition, we have shown that EGCG does not cause phosphorylation of EGFR at any tyrosine residues and that the internalized EGFRs induced by EGCG are not phosphorylated at Tyr1045 residue (26). Therefore, it seems unlikely that the internalization of EGFR induced by EGCG is associated with c-Cbl.
To further confirm the above results, we next performed binding assays of EGFR and c-Cbl using immunoprecipitation–western blot analysis. As shown in Figure 2B, EGF caused the interaction of EGFR with c-Cbl (lane 3), whereas EGCG failed to induce their interaction (lane 4). Because both EGF and EGCG did not influence the expression level of c-Cbl (Figure 2B, middle panel), it is probable that EGCG induces internalization of EGFR independently of c-Cbl.

Phosphorylation of EGFR at tyrosine residues is not necessary for its internalization or degradation induced by EGCG in SW480 colon cancer cells

As described above, it is well known that EGF causes phosphorylation of EGFR at Tyr1045, the major docking site for the ubiquitin ligase c-Cbl (16). Therefore, we next investigated whether or not this phosphorylation is required for the degradation induced by EGCG. We first examined the effects of AG1478 and PD153035, both of which are EGFR-specific tyrosine kinase inhibitors (32,33), on the internalization of EGFR induced by EGCG in immunofluorescence microscope studies. We first confirmed that either 2 μM of AG1478 or 10 μM of PD153035 significantly blocked EGF-induced phosphorylation of EGFR at tyrosine residues (Figure 3A, lanes 4 and 6 compared with lane 2). In the presence of AG1478 or PD153035, the internalization of EGFR induced by EGCG was not suppressed (Figure 3B, upper panels 4 and 6 compared with panel 2). In addition, quantification analysis by enzyme-linked immunosorbent assay showed no effect in cell surface amount of EGFR by the inhibition of tyrosine kinase on EGFR (Figure 3B, lower bar graphs, lanes 4 and 6 compared with lane 2). Moreover, we found that EGFR was degraded by EGCG even in the presence of either AG1478 or PD153035 (Figure 3C and D, lane 4 compared with lanes 2, respectively). Therefore, it seems unlikely that phosphorylation of EGFR at any tyrosine residues is necessary for the internalization or degradation of EGFR induced by EGCG.

**EGCG induced phosphorylation of p38 MAPK in SW480 colon cancer cells**

It has recently been reported that cisplatin, a widely used anticancer drug, induces EGFR internalization, which is mediated by p38 MAPK-dependent phosphorylation of the receptor on threonine 669 (15). Receptor internalization induced by UV is also caused by p38 MAPK (34). Since it has been shown that p38 MAPK can negatively regulate tumorigenesis (23), we next examined the effect of EGCG on phosphorylation (i.e. activation) of p38 MAPK in SW480 cells. The upper panels in Figure 4 display representative western blotting images and the lower bar graph provides quantification of phosphorylation (activation) of p38 MAPK after normalization with respect to total p38 MAPK, as determined by densitometry. As expected, p38 MAPK was significantly phosphorylated within 30 min by the treatment with EGCG and reached maximum effect at 1 h after the exposure (Figure 4, lower graph). These results led us to further investigate the role of activated p38 MAPK in EGCG-treated cells.

**Inhibition of p38 MAPK activation suppressed downregulation of EGFR induced by EGCG in SW480 colon cancer cells**

We next examined the effect of a specific inhibitor of p38 MAPK, SB203580 (35), on the internalization of EGFR induced by EGCG. We first confirmed that SB203580 truly inhibited phosphorylation (activation) of p38 MAPK in SW480 cells (Figure 5A). As we have shown previously (26), a 30 min exposure to 25 μM of EGCG caused internalization of EGFR (Figure 5B, panel 2). Quantification analysis by enzyme-linked immunosorbent assay showed ~60% decrease in cell surface amount of EGFR (Figure 5B, lower bar graph). When the cells were pretreated with SB203580 (10 μM) for 1 h and then exposed to EGCG (25 μM) for 30 min, internalization of EGFR was significantly blocked (Figure 5B, upper panel 4 and lower graph, lane 4). We next examined the effect of SB203580 on EGFR degradation induced by continuous treatment of the cells with EGCG. Pretreatment with 2.5 μM of SB203580, which alone did not affect the EGFR levels, markedly suppressed, and 5 or 10 μM of SB203580 almost completely suppressed the degradation of EGFR induced by EGCG (Figure 5C). Furthermore, we verified the involvement of p38 MAPK in EGCG-induced degradation of EGFR utilizing the gene silencing using p38 MAPK-siRNA (Figure 5D). Whereas knock down selectively decreased expression of p38 MAPK (Figure 5D, upper panel), the degradation of EGFR induced by EGCG was significantly restored in p38 MAPK-knocked down SW480 cells (Figure 5D, lower panel, lane 4 compared with lane 2). Taken together, these results provide strong evidence that p38 MAPK plays a crucial role in the downregulation of EGFR induced by EGCG.

**EGCG caused phosphorylation of EGFR at Ser1046/7 and this was blocked by SB203580 in SW480 colon cancer cells**

It has previously been reported that phosphorylation of EGFR on serine and threonine residues is thought to represent a mechanism
for attenuation of its receptor kinase activity (17). It has also been reported that EGFR undergoes a gel mobility shift upon cisplatin treatment, which is mediated by p38 MAPK (15,34). In addition, UV induces phosphorylation of EGFR (gel mobility shift of EGFR) that was suppressed by the inhibition of activation of p38 MAPK (34). Moreover, cisplatin induces EGFR internalization that is mediated by p38 MAPK-dependent phosphorylation of the receptor on threonine 669 (15). Therefore, we examined the effect of EGCG on gel mobility shift of EGFR. First, we verified that cisplatin caused a gel mobility shift of EGFR in SW480 cells (Figure 6A, lane 4), as described in a previous study with MDA-MB-468 cells (15). Indeed, we found that EGCG had an effect similar to that of cisplatin on gel mobility shift of EGFR (Figure 6A, lane 7). The gel mobility shift of the EGFR induced by EGCG, and that induced by

Fig. 2. The internalized EGFR induced by EGCG does not associate with c-Cbl. (A) SW480 cells were first labeled for 15 min at 37°C with an anti-EGFR antibody that recognizes the extracellular domain of the EGFR. They were then treated with EGF (100 ng/ml) or EGCG (25 μM) for 30 min at 37°C, followed by fixation with paraformaldehyde. After permeabilization of the cells with 0.1% Triton X-100, the cells were exposed to an anti-c-Cbl antibody (1:100 dilution) for 1 h and then treated with Alexa Fluor 546-conjugated anti-mouse secondary antibody for EGFR (red signal) and Alexa Fluor 488-conjugated anti-mouse secondary antibody for c-Cbl (green signal). After washing, the cells were exposed to 4′,6-diamidino-2-phenylindole (DAPI) (blue signal) for 20 min and then examined by fluorescence microscopy. Representative results from at least three independent experiments are shown. Scale bar indicates 20 μm. (B) EGF, but not EGCG, induced c-Cbl–EGFR binding. The cells were exposed to EGF (100 ng/ml) or EGCG (25 μM) for 30 min at 37°C. Then, cell lysates (500 μg each) were prepared and incubated for 12 h at 4°C with an anti-EGFR antibody precoupled to anti-mouse IgG-agarose beads. The bound protein was then analyzed by western blotting with an anti-c-Cbl antibody. The lower two panels indicate the corresponding whole-cell lysates (WCLs). MW indicates molecular weight.
cisplatin, were canceled when the cells were pretreated with the specific p38 MAPK inhibitor SB203580 (Figure 6A, lanes 5, 6, 8 and 9). We next performed immunoprecipitation assays using an anti-pan-phospho-threonine antibody in order to clarify whether EGCG as well as cisplatin causes phosphorylation of EGFR at threonine residues (Figure 6B). Although we detected phosphorylation of EGFR at threonine residues when the cells were treated with cisplatin (Figure 6B, lane 2), we did not detect phosphorylation of EGFR at threonine residues when the cells were treated with EGCG (Figure 6B, lane 3). These results led us to speculate that EGCG might cause phosphorylation of EGFR at serine, rather than threonine, residues because p38 MAPK is well known to be a serine/threonine kinase (36).

It has been shown that EGFR desensitization is mediated through phosphorylation at Ser1046/7 in EGF-treated cells (19). In addition, we recently found that activation of p38 MAPK by anisomycin causes EGFR phosphorylation at Ser1046/7 (37). Therefore, we next examined the effect of EGCG on this phosphorylation and found that treatment with EGCG induced within 30 min phosphorylation of EGFR at Ser1046/7 (Figure 6C, upper panels) and that this was attenuated by pretreatment of the cells with the specific p38 MAPK inhibitor, SB203580 (Figure 6C, lower panels). Furthermore, utilizing the gene silencing using p38 MAPK-siRNA, we verified the involvement of p38 MAPK in EGCG-induced phosphorylation of EGFR at Ser1046/7 (Figure 6D) and found that the phosphorylation of EGFR at Ser1046/7 induced by EGCG was significantly suppressed in p38 MAPK-knocked down SW480 cells (Figure 6D, upper panel, lane 4 compared with lane 2). Taken together, our findings strongly suggest that EGCG causes phosphorylation of EGFR at Ser1046/7 through activation of p38 MAPK, resulting in its gel mobility shift and subsequent degradation.

Discussion

In the present study, we examined the mechanism underlying EGCG-induced downregulation of cell surface-associated EGFR in SW480 colon cancer cells because we recently reported that...
EGCG induces downregulation of EGFR via p38 MAPK

Fig. 4. EGCG causes phosphorylation of p38 MAPK within 30 min. SW480 cells were treated with 50 μM of EGCG for the indicated times and protein extracts were then harvested and examined by western blotting using anti-phospho p38 MAPK and anti-p38 MAPK antibodies. The lower bar graph shows the quantification data for the relative levels of phospho-p38 MAPK, after normalization with respect to total p38 MAPK, as determined by densitometry. The asterisks indicate significant increase (\*\*\*P \textless 0.05 and **P \textless 0.01, respectively) as compared with the control (lane 1).

It is well known that EGF induces EGFR autophosphorylation including Tyr1045, which is important for the binding of EGFR to the ubiquitin ligase c-Cbl. This phosphorylated EGFR is rapidly internalized and ubiquitinated by c-Cbl and then can be degraded by lysosomes (16). Since we have previously reported that EGCG causes internalization of EGFR, but not phosphorylation of EGFR at tyrosine residues including Tyr1045 (26), EGCG does not induce EGFR–c-Cbl binding (Figure 2). Therefore, the EGCG-induced degradation of EGFR seen in the present study occurs independently of c-Cbl. Furthermore, we found that EGCG induced both internalization and degradation of EGFR, even when the cells were pretreated with AG1478 or PD153035 to block phosphorylation of EGFR at tyrosine residues (Figure 3). Because the concentrations of these inhibitors we used in this study were high, we cannot exclude the possibility of their unspecific effects. However, based on our findings as a whole, it is most likely that EGCG-induced downregulation of EGFR does not require tyrosine phosphorylation. On the other hand, we found that EGCG caused phosphorylation (activation) of p38 MAPK in a time-dependent manner (Figure 4). This activation was seen within 30 min when the cells were treated with 50 μM (~22 μg/ml) of EGCG.

To investigate the involvement of p38 MAPK in internalization or degradation of EGFR induced by EGCG, we next examined the effect of a specific p38 MAPK inhibitor or the gene silencing using p38 MAPK-siRNA and found that the inhibition of p38 MAPK suppressed the internalization and subsequent degradation of EGFR induced by EGCG (Figure 5). These results strongly suggest that p38 MAPK plays a pivotal role in both the internalization and the degradation of EGFR induced by EGCG. We have seen similar effect when SW480 cells were treated with the p38 MAPK activator anisomycin (37). In the latter study, whereas EGF failed to induce activation of p38 MAPK, both p38 MAPK and stress-activated protein kinase/c-Jun N-terminal kinase were activated by anisomycin, and inhibition of p38 MAPK, but not stress-activated protein kinase/c-Jun N-terminal kinase, canceled anisomycin-induced downregulation of EGFR in SW480 colon cancer cells (37). Taken together, this evidence also supports our conclusion that EGCG-induced activation of p38 MAPK plays an important role in downregulation of EGFR.

Interestingly, our present finding that the internalized EGFR induced by EGCC did not colocalize with c-Cbl (Figure 2) is similar to the case in anisomycin-treated cells (37), indicating that downregulation of EGFR induced by EGCG as well as anisomycin does not require c-Cbl binding. In addition, we found that EGCG underwent gel mobility shift of EGFR (Figure 6A) and EGCG did not cause the phosphorylation of EGFR at threonine residues (Figure 6B), even though cisplatin caused its phosphorylation at threonine residues, which is consistent with the previous study (15). Furthermore, we demonstrated that EGCG caused the phosphorylation of EGFR at Ser1046/7, at a site that was reported to be important for degradation of EGFR (17–19,38) and that the inhibition of p38 MAPK blocked this phosphorylation (Figure 6C and D). Because gel mobility shift of EGFR induced by EGCG was also canceled when the cells were pretreated with SB203580 (Figure 6A), it is most likely that EGCG causes phosphorylation of EGFR at Ser1046/7 via activation of p38 MAPK.

It has previously been reported that although serine residues and Tyr1045 are essential for EGF-induced receptor ubiquitination, only threonine residues are critical for EGFR internalization and degradation (38). Taking this finding into account, it is probable that the phosphorylation of EGFR at serine residues is critical for the down-regulation of EGFR induced by EGCG. The present studies utilizing immunofluorescence microscope (Figure 2) and EGFR tyrosine kinase inhibitors (Figure 3) and the results of our previous study (26) provide evidence that the mechanism of EGCG-induced downregulation of EGFR is different from that of EGF-induced downregulation of the EGFR, whereas with EGF this process involves c-Cbl. The present results also provide strong evidence that phosphorylation of EGFR at serine 1046/1047 via activation of p38 MAPK plays a pivotal role in EGCG-induced downregulation of EGFR. These findings may suggest new therapeutic strategies for inhibiting the proliferation of human cancers that are highly dependent on the function of the EGFR.

It is of interest to summarize our recent studies related the various effects of EGCG on the EGFR in colon cancer cells, with respect to both the time course and the sensitivity to EGCG. The most rapid and sensitive response we have observed is disruption of lipid rafts since this occurred within 5–30 min and with as little as 1–5 μg/ml of EGCG [about one-fourth of the IC50 concentration for growth inhibition (25)]. Similar low concentrations of EGCG also induced within 30 min internalization of the EGFR into membrane vesicles (26). Both effects were reversible in washout studies (26). These events were followed by a decrease in phosphorylation of EGFR at ~96 h in cells treated continuously with 1 μg/ml of EGCG or at ~6 h in cells treated with 20 μg/ml of EGCG (6). The present study indicates that 50 μM (~22 μg/ml) of EGCG induces
phosphorylation (activation) of p38 MAPK within 10–30 min (Figure 4) and phosphorylation of EGFR at Ser1046/7 within the same time frame (Figure 6C). Degradation of total cellular EGFR occurred even later at ~12 h in cells treated with 25–50 μM of EGCG. Chen et al. (39) previously reported that 50 μM of EGCG induces phospho-p38 MAPK in HT29 cells within 1 h, but they did not examine the relationship of this effect to the EGFR. It remains to be determined which of the above inactivating effects of EGCG on the EGFR, or possibly the cascade itself, contributes to the ability of EGCG to inhibit the growth of colon cancer cells. This question is compounded by the multiple additional effects on cancer cells that have been reported for EGCG (39,40).

An important question is the biologic relevance of the present studies since the concentrations of EGCG (50 μM) that activate p38 MAPK are very high compared with the peak plasma concentration of 0.2 to 0.4 μg/ml (~0.44 to 0.88 μM) in patients administered EGCG (40). However, in clinical studies, patients would receive repeated doses of EGCG over a prolonged period of time,
so there could be an accumulative effect. Indeed, in this and previous cell culture studies, we have found that prolonged exposure of cells to EGCG reduces the concentration required to exert a given effect (6,25,26). Furthermore, there is evidence that EGCG can be concentrated in gastrointestinal tissues (41). Nevertheless, our findings will require confirmation in rodent models or clinical studies with EGCG.

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