

Mechanism of Action of (–)-Epigallocatechin-3-Gallate: Auto-oxidation–Dependent Inactivation of Epidermal Growth Factor Receptor and Direct Effects on Growth Inhibition in Human Esophageal Cancer KYSE 150 Cells

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Abstract

(–)-Epigallocatechin-3-gallate (EGCG), the principal polyphenol in green tea, has been shown to inhibit the growth of many cancer cell lines and to suppress the phosphorylation of epidermal growth factor receptor (EGFR). We observed similar effects of EGCG in esophageal squamous cell carcinoma KYSE 150 cells and epidermoid squamous cell carcinoma A431 cells. Pretreatment of KYSE 150 cells with EGCG (20 $\mu\text{mol/L}$) for 0.5 to 24 hours in HAM's F12 and RPMI 1640 mixed medium at 37°C, before the addition of EGF, resulted in a decreased level of phosphorylated EGFR (by 32–85%). Prolonged treatment with EGCG (8 or 24 hours) also decreased EGFR protein level (both by 80%). EGCG treatment for 24 hours also caused decreased signals of HER-2/*neu* in esophageal adenocarcinoma OE19 cells. These effects of EGCG were prevented or diminished by the addition of superoxide dismutase (SOD, 5 units/mL), or SOD plus catalase (30 units/mL), to the cell culture medium. A similar phenomenon on inactivation of EGFR was observed in A431 cells as well. Under culture conditions for KYSE 150 cells, EGCG was unstable, with a half-life of ~ 30 minutes; EGCG dimers and other oxidative products were formed. The presence of SOD in the culture medium stabilized EGCG and increased its half-life to longer than 24 hours and some EGCG epimerized to (+)-gallic catechin-3-gallate. A mechanism of superoxide radical-mediated dimerization of EGCG and H_2O_2 formation is proposed. The stabilization of EGCG by SOD in the culture medium potentiated the activity of EGCG in inhibiting KYSE 150 cell growth. The results suggest that in cell culture conditions, the auto-oxidation of EGCG leads to EGFR inactivation, but the inhibition of cell growth is due to other mechanisms. It remains to be determined whether the presently observed auto-oxidation of EGCG occurs *in vivo*. In future studies of EGCG and other polyphenolic compounds in cell culture, SOD may be added to stabilize EGCG and to avoid possible artifacts. (Cancer Res 2005; 65(17): 8049–56)

Introduction

Green tea has been shown to have cancer preventive activity in a variety of organ sites in animal models (reviewed in refs. 1–5).

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Among the green tea constituents, (–)-epigallocatechin-3-gallate (EGCG) is the most abundant and most active constituent in inhibiting experimental carcinogenesis and related reactions. The chemical structures of EGCG and its derivatives are shown in Fig. 3. Many mechanisms for the anticancer activities of EGCG have been proposed based mainly on studies in cell lines (reviewed in ref. 5). EGCG has been reported to inhibit mitogen activation protein kinases, the activation of activator protein-1, and cell transformation (6–8). EGCG has also been shown to cause inhibition of growth factor receptor phosphorylation, such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and HER-2/*neu* (9, 10). It has also been reported to cause G_0 - G_1 phase arrest (11–13), induce apoptosis (14–16), inhibit DNA methyltransferase activity (17), and inhibit aberrant arachidonic acid metabolism (18–20). Of these activities, the inhibition of EGFR signaling is intriguing and is the subject of the present study.

Liang et al. reported that EGCG inhibited the phosphorylation of EGFR in A431 cells, and the result of a ligand-binding assay suggests that EGCG may block the binding of EGF to EGFR (21). EGCG was also shown to inhibit receptor tyrosine kinase activity with an IC_{50} of 1 to 2 $\mu\text{mol/L}$ in *in vitro* kinase assays. Masuda et al. also showed the inhibitory effect of EGCG on EGFR phosphorylation in two head and neck carcinoma cell lines, YCU-H891 and YCU-N861 (11). It was proposed that the inhibition of the phosphorylation of EGFR by EGCG led to the inhibition of the downstream events, such as the phosphorylation of extracellular signal-regulated kinase, signal transducers and activators of transcription 3, and Akt, resulting in the inhibition of cell growth. Nevertheless, a preincubation of cells with EGCG for at least 30 minutes, or 8 to 16 hours, was employed to show its inhibitory effect on EGFR phosphorylation (11, 21). Similar preincubation periods were used to observe an inhibitory effect of EGCG on PDGFR β phosphorylation (22, 23). The requirement of EGCG preincubation for the inhibition of phosphorylation is rather puzzling. If the inhibitory action is due to the binding of EGCG to EGFR or PDGFR, such a long preincubation time should not be needed. In addition, EGCG is known to be unstable under cell culture conditions (24). In culture medium for HT-29 human colon adenocarcinoma cells, EGCG (50 $\mu\text{mol/L}$) had a half-life of 130 minutes. Theasinensin A (molecular weight, 914) and another dimer (molecular weight, 884) were identified as the major oxidative products. It was previously shown that incubation of EGCG (50 $\mu\text{mol/L}$) in McCoy's 5A culture medium with HT-29 cells generated a maximal level of 25 $\mu\text{mol/L}$ H_2O_2 , and 25 $\mu\text{mol/L}$ EGCG in LHC-9 medium with 21BES cells resulted in a maximum of about 4 $\mu\text{mol/L}$ H_2O_2 (24, 25). In light of this information, it is

mechanistically important to relate the concentrations of EGCG and H_2O_2 in the cell culture system to the observed effects on EGFR phosphorylation and on cell growth.

In air-saturated Tris buffer (0.1 mol/L, pH 9.0), EGCG undergoes auto-oxidation; oxygen is consumed and H_2O_2 is formed (26). The auto-oxidation of EGCG may generate $\cdot O_2^-$ and quinones (27, 28). Superoxide dismutase (SOD), which catalyzes the conversion of $\cdot O_2^-$ to H_2O_2 and O_2 , was shown to stabilize EGCG and decrease the overall H_2O_2 generated (26–28). The effect of SOD indirectly showed the involvement of $\cdot O_2^-$ in the auto-oxidation process.

In the present study with KYSE 150 esophageal squamous cell carcinoma cells and A431 epidermoid carcinoma cells, we observed that preincubation of the cells with EGCG led to decreased level of EGFR and HER-2/*neu* phosphorylation, possibly due to inactivation of the proteins. These effects of EGCG were blocked or delayed by the presence of SOD in the culture medium. On the other hand, the stabilization of EGCG by SOD enhanced its growth inhibitory effect.

Materials and Methods

Cell culture conditions. Human esophageal squamous cell carcinoma cell line KYSE 150 was a gift from Dr. Yutaka Shimada (Kyoto University, Kyoto, Japan). KYSE 150 cells were maintained in 5% CO_2 humidified atmosphere at 37°C in RPMI 1640 and Ham's F12 mixed (1:1, v/v) medium (Mediatech, Inc., Herndon, VA), containing 5% fetal bovine serum (FBS) and 1% streptomycin/penicillin. Human epidermoid squamous cell carcinoma A431 cells and esophageal adenocarcinoma OE19 cells were purchased from American Type Culture Collection (Manassas, VA). A431 cells and OE19 cells were maintained in 10% FBS DMEM or RPMI 1640, respectively, with 1% streptomycin/penicillin.

KYSE 150, A431, or OE19 cells were seeded into 10-cm Petri dishes and cultured until they reached 50% to 60% confluence in full medium. The cells were starved in serum-free medium for 24 hours before they were pretreated with 20 or 50 $\mu\text{mol/L}$ EGCG or other compounds in the presence or absence of SOD (5 or 15 units/mL, Sigma, St. Louis, MO) for 0, 0.5, 2, 8, and 24 hours, respectively. Fifteen minutes before harvesting, 50 ng/mL EGF or 10 ng/mL PDGF-BB (both from Upstate, Lake Placid, NY) was added to activate its receptor. EGCG (100% pure) was a gift from Dr. Y. Hara (Mitsui Morin Co., Shizuoku, Japan). Theasinensin A was produced by oxidation of EGCG and purified; its identity was verified by nuclear magnetic resonance (NMR) and mass spectrometry analysis.¹ Theaflavin digallate (TFdiG) was synthesized previously and the structure was verified by NMR (29).

Western blot. The cells were lysed in lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% TritonX-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β -glycerophosphate, and 1:100 phosphatase inhibitor and protease inhibitor cocktails (Sigma)] and scraped from the Petri dishes. The cell lysate was centrifuged at 14,000 rpm for 10 minutes. The supernatant was removed, and the protein concentration was determined by bicinchoninic acid reagent kit (Pierce, Rockford, IL). The protein samples were loaded into SDS-PAGE gels (4–15% gradient, Bio-Rad, Hercules, CA) and run under 84 V for 2 hours. The proteins in the gels then were electrotransferred onto pure nitrocellulose membrane (Bio-Rad) under 84 V for 1.5 hours. The proteins on the membranes were subsequently blocked with 5% milk blocking buffer (1% bovine serum albumin and 1% milk for phospho-protein detection) for 1 hour at room temperature and blotted with primary antibodies against EGFR and phospho-EGFR at Tyr¹⁰⁶⁸ or HER-2/*neu* and phospho-HER-2/*neu* at Tyr¹²⁴⁸ (Cell Signaling, Beverly, MA) or phospho-PDGFR β at Tyr⁷¹⁶ (Upstate), and β -actin antibody (Sigma). The regular anti-mouse or anti-rabbit IgG (Cell Signaling) or fluorescence-conjugated secondary antibodies

(Molecular Probes, Eugene, OR) were then incubated with the membranes. The bands were illuminated by enhanced chemiluminescence reagents (Amersham, Piscataway, NJ) or detected by Odyssey Infrared Imaging System (Li-Cor Biotechnologies, Lincoln, NE).

Stability of (–)-epigallocatechin-3-gallate and product formation. EGCG (20 $\mu\text{mol/L}$) was incubated in culture medium containing exponentially growing KYSE 150 cells in Petri dishes and as described above, some of the incubation also contained SOD (5 units/mL, Sigma) with or without catalase (30 units/mL, Sigma) for 0, 0.5, 1, 2, 6, and 24 hours. The culture medium was removed from the dish at different time points and mixed immediately with 2% ascorbic acid solution containing 0.01% EDTA to stabilize EGCG (30). The mixture was diluted 10 times, and 50 μL of the resultant solution were injected onto the high-performance liquid chromatographer (HPLC). The conditions for HPLC, using a Supelcosil C₁₈ reversed-phase column, were the same as described previously except that the column elution conditions were modified. The column was eluted at 35°C with 96% A [30 mmol/L NaH_2PO_4 buffer containing 1.8% of acetonitrile and 0.1% of tetrahydrofuran (pH 3.35)] and 4% B [15 mmol/L NaH_2PO_4 buffer containing 58.5% acetonitrile and 12.5% tetrahydrofuran (pH 3.45)] for 5 minutes followed by 82% A and 18% B for 23 minutes at a flow rate of 1 mL/min. In the next 10 minutes, the solvent was changed in a linear gradient to 2% A and 98% B and maintained at 2% A and 98% B for another 8 minutes. After the run, the solvent was changed back to 96% A and 4% B for the analysis of the next sample. The eluent was monitored by the ESA Model 5500 coulometer electrode array system with potential settings at –100, 100, 300, and 500 mV, and four chromatograms were obtained simultaneously. The peak height was used to calculate the concentration of EGCG by comparing with a standard.

To study the formation of H_2O_2 , 20 $\mu\text{mol/L}$ EGCG was added to exponentially growing KYSE 150 cells. At different time points, H_2O_2 was measured in the medium by the Amplex Red Hydrogen Peroxide assay kit (Molecular Probes).

Colony formation assay. Two hundred KYSE 150 cells were seeded into each well of 6-well cell culture plates and cultured overnight in 5% serum medium. Cells were treated with 5, 10, 20, and 50 $\mu\text{mol/L}$ EGCG in the presence or absence of SOD in serum-free medium for 24 hours, then the medium was replaced with fresh 5% serum medium. The culture continued for another 7 days until the cell colonies were of a proper size to be stained. Methylene blue (0.05% in 60% methanol) was used to stain the colonies to blue color. The colony numbers were then counted.

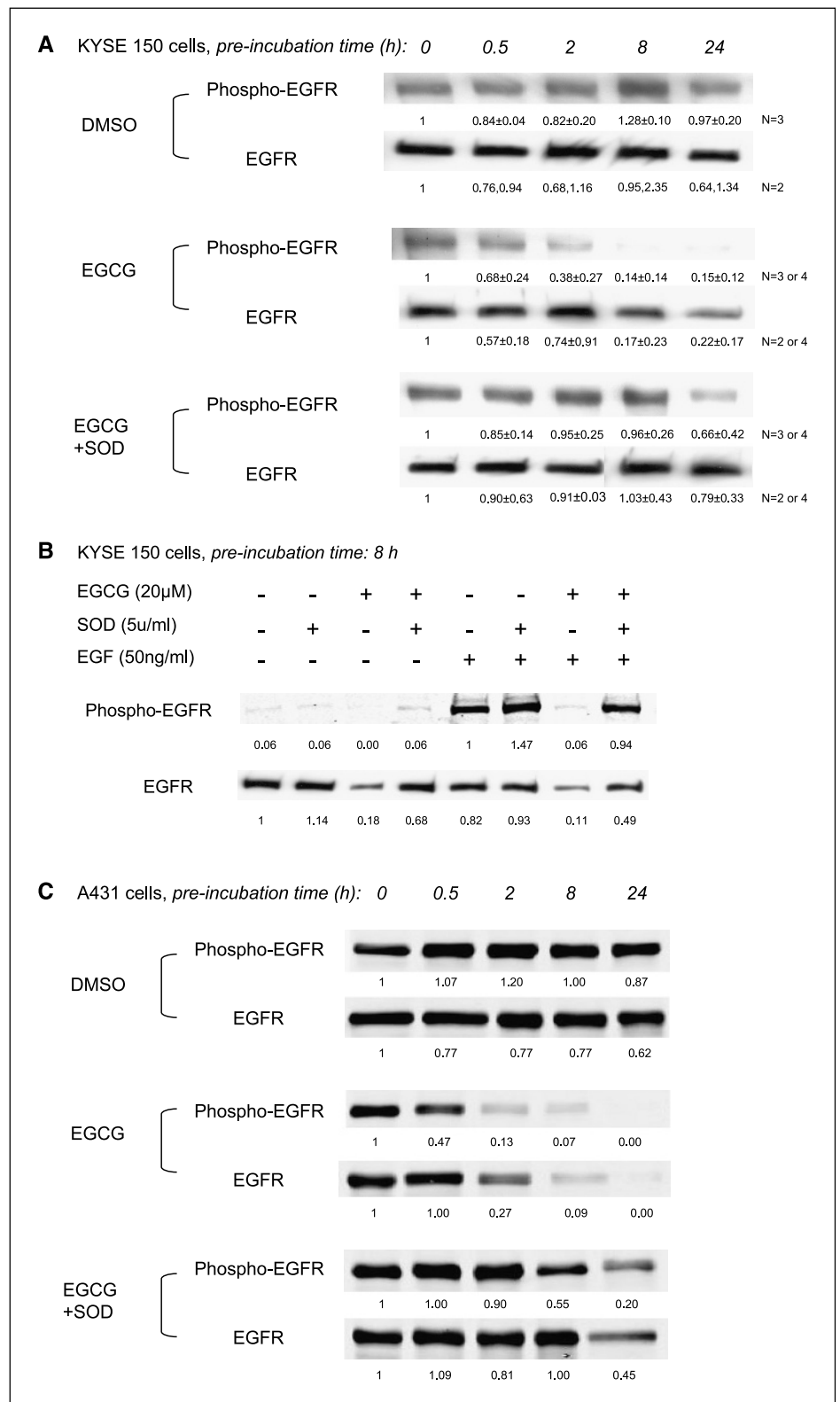
Statistical analysis. Statistical significance was evaluated using the Student's *t* test.

Results

Effects of (–)-epigallocatechin-3-gallate on epidermal growth factor receptor or HER-2/*neu* phosphorylation and protein levels. Phosphorylation of EGFR was previously reported to be inhibited by EGCG in several cancer cell lines (11, 21). We observed a similar effect with EGCG in KYSE 150 (Fig. 1A). Tyr¹⁰⁶⁸ is one of the major autophosphorylation sites of EGFR stimulated by its ligands, and the phosphorylation leads to the docking of signal transduction components growth factor receptor-bound protein 2 and the activation of Ras pathway. Preincubation of cells with EGCG for 0.5 to 24 hours led to a lower level of EGFR phosphorylation (by 32–85%) at site Tyr¹⁰⁶⁸ as determined by Western blot analysis (Fig. 1A). Prolonged treatment (8 or 24 hours) with EGCG also reduced the protein level of EGFR (both by about 80%). These effects by EGCG, on the phosphorylation and protein levels of EGFR, were prevented or markedly delayed when SOD was added to the culture medium (Fig. 1). In another experiment, it was observed that SOD and/or catalase in the absence of EGCG did not have any effect on either phosphorylation or protein levels of EGFR (data not shown). Although the effect of EGCG on the level of phospho-EGFR was only observed after the addition of EGF, the

¹ Unpublished results.

Figure 1. Levels of phospho-EGFR and EGFR protein after pretreatment of KYSE 150 cells with EGCG in the absence or presence of SOD. KYSE 150 cells were seeded into 10-cm Petri dishes and cultured in RPMI 1640 and HAM's F12 mixed medium (1:1, v/v, 5% FBS, 1% streptomycin/penicillin). **A**, cells were then preincubated with EGCG (20 $\mu\text{mol/L}$) in the absence or presence of SOD (5 units/mL) for 0, 0.5, 2, 8, and 24 hours in serum-free medium, following a 24-hour serum starvation. Fifteen minutes before harvesting, EGF (50 ng/mL) was added to activate EGFR. DMSO was used as the solvent and added to the control. Proteins were extracted from the harvested cells and resolved by electrophoresis. The protein levels and phosphorylation of EGFR at Tyr¹⁰⁶⁸ were detected using specific antibodies (Cell Signaling). The results from a typical set of experiments are shown. The band density was normalized by β -actin and is shown below each band as mean \pm SD, or both the two density values are shown when $n = 2$. A large SD was observed on the phospho-EGFR level at 24 hours in the presence of SOD, and this band as shown in the figure was lower than the mean. **B**, cells were preincubated with EGCG (20 $\mu\text{mol/L}$) in the absence or presence of SOD (5 units/mL) for 8 hours in serum-free medium following a 24-hour serum starvation. Fifteen minutes before harvesting, EGF (50 ng/mL) was added to one set of cells to activate EGFR. Proteins were extracted from the harvested cells and analyzed as above. Similar results were also observed in a repeat of the experiment. **C**, levels of phospho-EGFR and EGFR protein after pretreatment of A431 cells with EGCG in the absence or presence of SOD. A431 cells were seeded into 10-cm Petri dishes and cultured in DMEM (10% FBS, 1% streptomycin/penicillin). The treatment and analysis were done in a same manner as in KYSE 150 cells described in (A), except that the doses of EGCG and SOD in this experiment were 50 $\mu\text{mol/L}$ and 15 units/mL, respectively.



EGFR protein level was reduced similarly by EGCG treatment in either EGF stimulated (*right four lanes*) or unstimulated (*left four lanes*) situations (Fig. 1B). These results suggest that in the absence of SOD, the oxidative products formed in the culture medium caused the inactivation of EGFR. Similarly, pretreatment of KYSE

150 cells with EGCG for 24 hours caused a decrease of the phosphorylation of PDGFR β by 60% and this effect was prevented by SOD (data not shown).

Pretreatment of epidermoid squamous cell carcinoma A431 cells with 50 $\mu\text{mol/L}$ EGCG also caused reduction of EGFR protein and

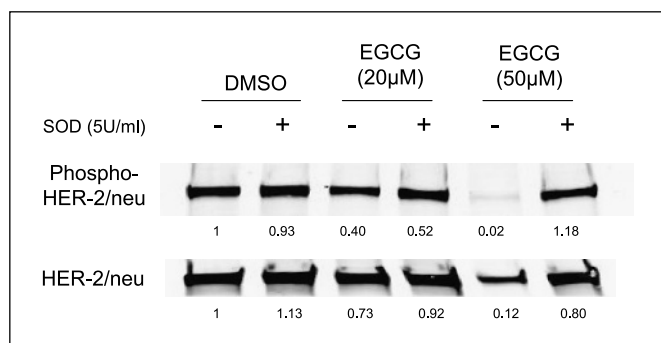


Figure 2. Levels of phospho-HER-2/neu and HER-2/neu protein after pretreatment of OE19 cells with EGCG in the absence or presence of SOD for 24 hours. The treatment with 20 or 50 μmol/L EGCG was done in a similar manner as described in Fig. 1A.

phosphorylation levels (Fig. 1C). The decrease in phosphorylation level was observed after a preincubation of 0.5 hour (by 53%) and was very pronounced by 2 hours. The protein levels showed a 73% decrease after a 2-hour preincubation. Similar to the results in Fig. 1A, the presence of SOD prevented or delayed the decrease in both EGFR phosphorylation and protein levels.

Similar to EGFR, HER-2/neu is a member of the ERBB receptor family and has been shown to be important in cancer development (31). Esophageal adenocarcinoma OE19 cells over-express HER-2/neu by 100-fold (32). Pretreatment of this cell line with EGCG for 24 hours resulted in decrease in both phosphorylation and protein levels of HER-2/neu in a dose-dependent

manner (Fig. 2). These effects can be prevented or delayed by the presence of SOD, whereas SOD itself, as the control, had no effect on either levels.

Stability of (–)-epigallocatechin-3-gallate in cell culture and the effect of superoxide dismutase. EGCG was unstable in culture conditions of KYSE 150 cells or A431 cells, with a half-life of about 30 minutes. After 6 hours of incubation in KYSE 150 culture condition, EGCG was not detectable in the medium (Fig. 3A). During the incubation, an HPLC peak corresponding to the retention time of theasinensin A, an EGCG dimer, appeared. The peak reached a maximum after 1 hour and then decreased (Fig. 3B). From the results in a previous study, it is likely that this peak also contained another EGCG dimer, P₂ (24).

The stability of EGCG was dramatically increased by the presence of SOD (5 units/mL) in the cell culture medium (Fig. 3A), and the half-life of EGCG was increased to longer than 24 hours. The stability of EGCG was not affected by 30 units/mL catalase or bovine serum albumin at 1.1 mg/mL, the amount of protein corresponding to 5 units/mL SOD (data not shown). The presence of 5% serum in the medium, however, prolonged the half-life of EGCG to 1 hour (data not shown). In the presence of SOD, the theasinensin A peak was not detected; instead, GCG, an epimer of EGCG, formed (Fig. 3B). Apparently, with decreased rate of auto-oxidation, EGCG concentration maintained at higher levels and epimerization was observable. In the presence of 20 μmol/L EGCG and absence of SOD under KYSE 150 culture conditions, a maximal concentration of 1.2 μmol/L H₂O₂ was observed after 30 minutes of incubation, but H₂O₂ was not detected in the presence of SOD (Fig. 3B).

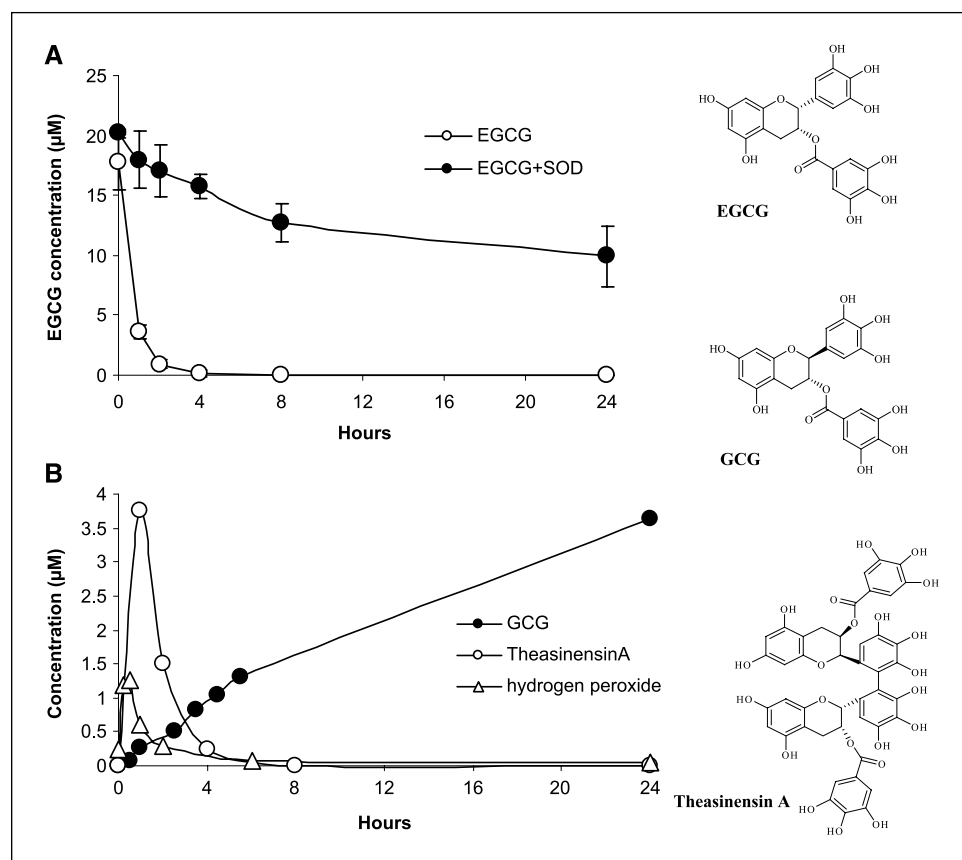


Figure 3. Stability of EGCG and formation of EGCG dimer, GCG, and H₂O₂ in KYSE 150 cell culture in the absence or presence of SOD. A, stability of EGCG (20 μmol/L) in the absence (○) or presence (●) of 5 U/ml SOD. B, generation of dimer, GCG, and H₂O₂ in the absence (○, △) or presence (●) of 5 units/mL SOD. The levels of EGCG dimer and H₂O₂ (in the presence of SOD) and GCG (in the absence of SOD) were at the baseline and were not shown. Structures of EGCG, GCG, and theasinensin A.

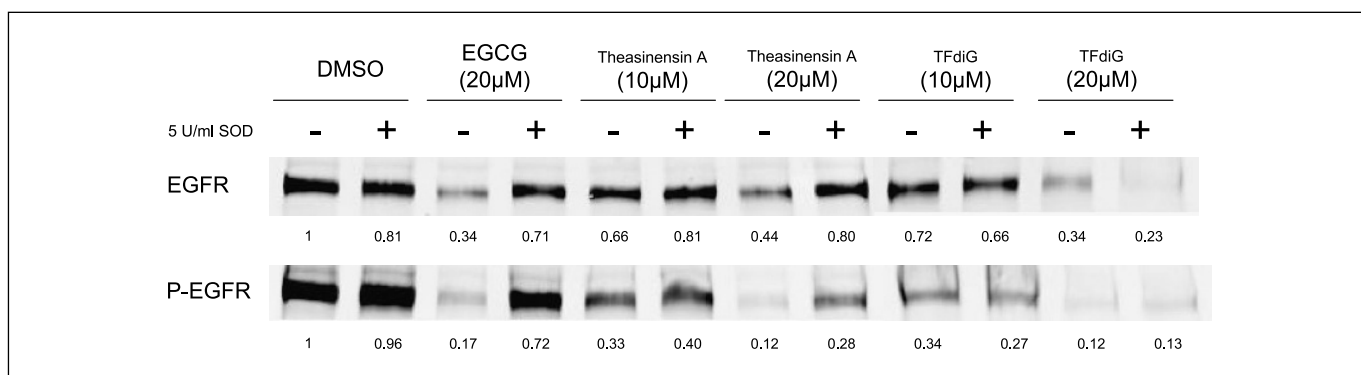


Figure 4. Phosphorylation of EGFR at Tyr¹⁰⁶⁸ under treatment of theasinensin A or TFdiG. KYSE 150 cells were seeded into 6-well plates and cultured in RPMI 1640 and HAM's F12 mixed medium (1:1, v/v, 5% FBS, 1% streptomycin/penicillin). The cells were serum starved for 24 hours and preincubated with theasinensin A (10 or 20 μmol/L) or TFdiG (10 or 20 μmol/L) in the absence or presence of SOD (5 units/mL) for 8 hours in serum-free medium. Fifteen minutes before harvesting, EGF (50 ng/mL) was added to activate EGFR. Proteins were extracted and analyzed as above.

Effect of theasinensin A or theaflavin digallate on phosphorylation of epidermal growth factor receptor. Among the oxidative products, EGCG dimers may be suspected as a possible reactive intermediate that inactivates EGFR. To test this possibility, we treated the cells with 10 or 20 μmol/L theasinensin A for 8 hours (Fig. 4). The preincubation of theasinensin A inhibited EGFR phosphorylation, and the effect of 20 μmol/L theasinensin A was comparable with that of 20 μmol/L EGCG. The presence of SOD prevented the inactivation of EGFR but only partially prevented the inhibitory effect on the phosphorylation of EGFR. Under our experimental conditions, the incubation with 20 μmol/L EGCG produced theasinensin A at an estimated maximal concentration of 4 μmol/L. Therefore, the formation of theasinensin A could account for only part of the inhibitory activity on EGFR

phosphorylation caused by EGCG auto-oxidation. TFdiG, a characteristic polyphenol in black tea, was also included in this study. At 20 μmol/L, TFdiG effectively decreased the protein level and phosphorylation of EGFR. In contrast to the situation with EGCG, the effect was not prevented by SOD (Fig. 4).

Effects of (-)-epigallocatechin-3-gallate in the presence of superoxide dismutase on cancer cell growth. A colony formation assay was done to study the effects of EGCG on KYSE 150 cell growth. The cells were treated with different concentrations of EGCG (5, 10, 20, and 50 μmol/L) in the absence or presence of SOD. EGCG caused a growth inhibitory effect on KYSE 150 cells at a concentration of 5 μmol/L and the effect increased with increasing concentrations of EGCG (Fig. 5). At 20 μmol/L of EGCG, the colony formation was inhibited by 50%. The presence of

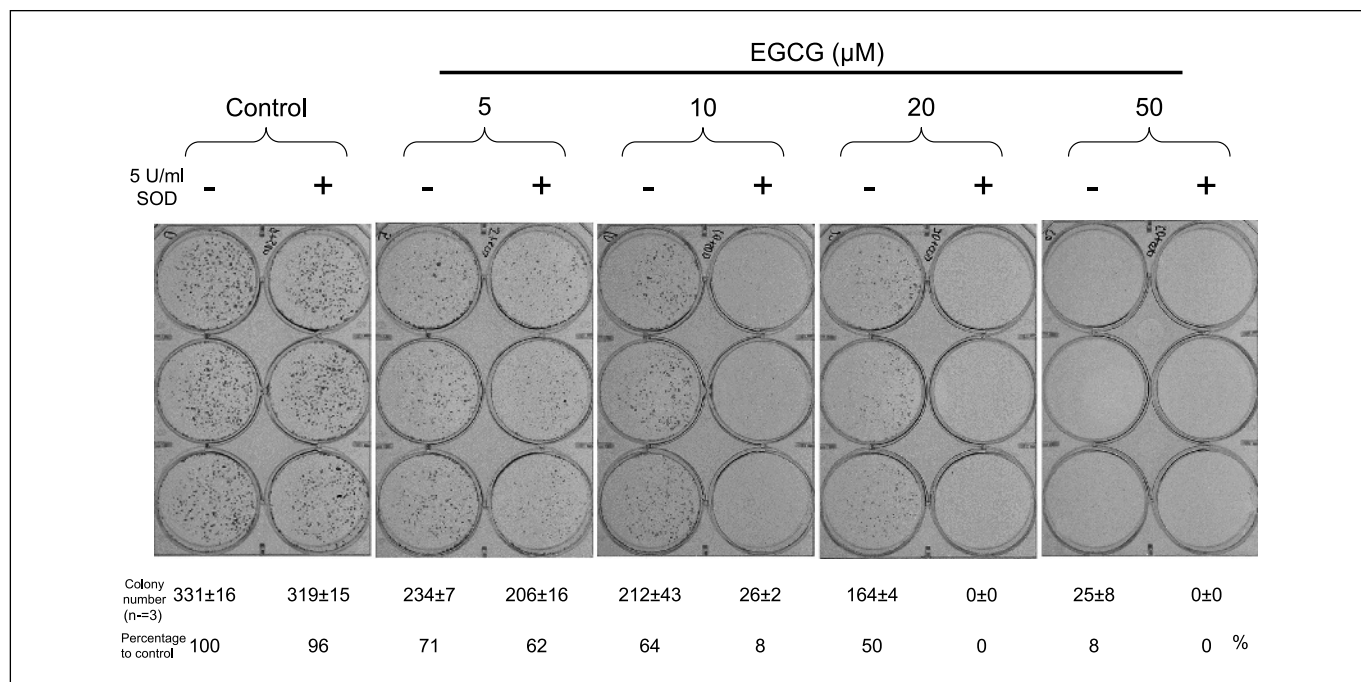


Figure 5. Effect of EGCG on KYSE 150 cell growth. KYSE 150 cells were seeded into 6-well plates and treated with EGCG (0, 5, 10, 20, and 50 μmol/L) for 24 hours in the absence or presence of SOD (5 units/mL). Fresh medium was then used to replace the EGCG-containing medium, and the culture continued for another 7 days before the colonies were stained with methylene blue. The colony numbers (mean ± SD, $n = 3$) and percentage relative to control were shown below each band.

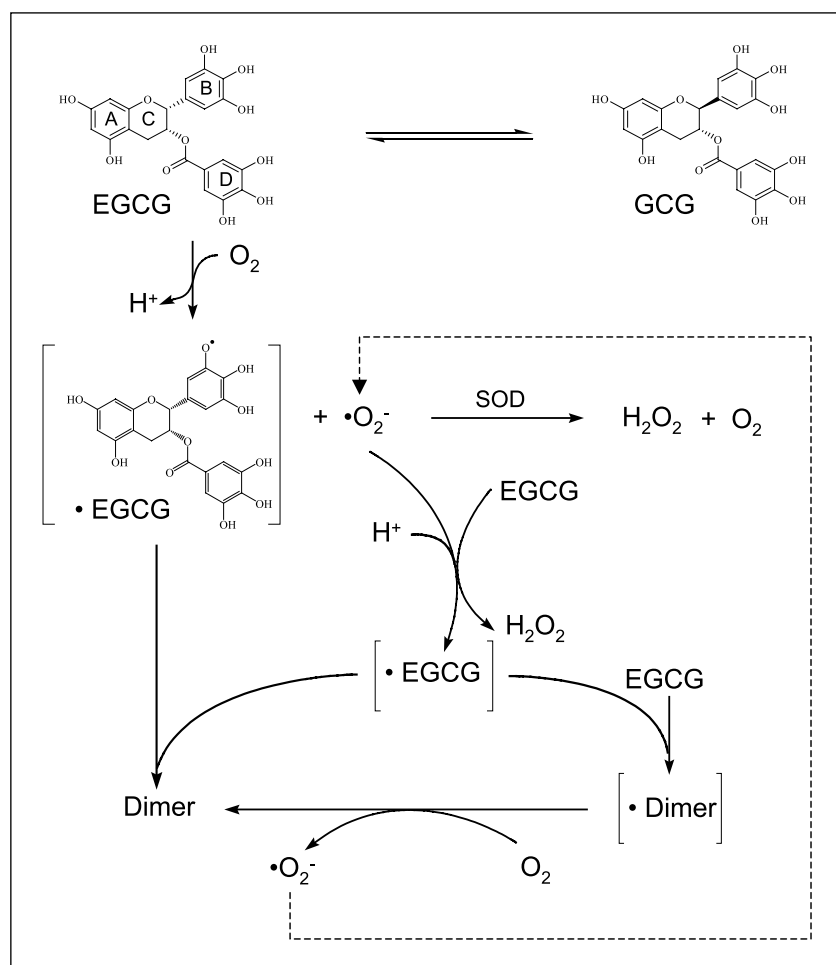


Figure 6. Proposed mechanism of EGCG auto-oxidation.

SOD dramatically augmented the inhibitory effect of EGCG on colony formation, showing 38% inhibition at 5 $\mu\text{mol/L}$, 92% inhibition at 10 $\mu\text{mol/L}$, and 100% inhibition at higher concentrations of EGCG. These results showed that when EGCG was stabilized by SOD, the inhibitory effect on cell growth was significantly increased.

One observation that may be related to the auto-oxidation was the appearance of a brownish-red color upon treatment of cells with 20 $\mu\text{mol/L}$ EGCG for 24 hours, if the cells were previously serum starved for 24 hours. The cells also became sticky in the trypsinization step, and single cell suspension could not be made. However, this phenomenon was not observed when the cells were incubated in the presence of SOD or if the cells were not serum starved. The serum starvation dependent effect of EGCG on cell adhesion needs to be further studied.

Discussion

Based on the above observations and the results of previous studies (24–26), we propose a mechanism for the auto-oxidation of EGCG and its stabilization by SOD (Fig. 6). EGCG is auto-oxidized under cell culture conditions, and the half-life of EGCG is rather short. The reaction, probably catalyzed by metal ions such as Cu^{2+} in the culture medium, produces superoxide radicals and EGCG radicals ($\cdot EGCG$). The unpaired electron should be delocalized around the B ring (26). The superoxide radical can further react

with another EGCG molecule to produce H_2O_2 and $\cdot EGCG$. Two $\cdot EGCG$ molecules may collide to form a dimer. It is more likely, however, for the $\cdot EGCG$ to attack the B ring of another EGCG molecule, which is more abundant, to form a dimer radical ($\cdot dimer$). The dimer radical can react with molecular oxygen to form the EGCG dimer and regenerate the superoxide radical. An alternative mechanism is that the $\cdot EGCG$ is oxidized by molecular oxygen to form $\cdot O_2^-$ and EGCG quinone, and the quinone would react with another molecule of EGCG to form the dimer. In either case, the reaction is propagated by the reaction of superoxide with EGCG, generating EGCG dimers and H_2O_2 . Dimers can be further transformed to other compounds, presumably polymers, in a similar manner of oxidation. The addition of SOD facilitates the conversion of $\cdot O_2^-$ to H_2O_2 and inhibits the propagation of the chain reactions. Therefore, the auto-oxidation of EGCG is inhibited. More work is needed to substantiate these mechanisms.

The epimerization of EGCG to GCG is known to occur at 120°C and pH 5 to 6 (33). The conversion of EGCG to GCG was also observed under an anaerobic condition in a phosphate buffer system in the absence of SOD.² Under our experimental conditions in cell culture, GCG formation was observed once EGCG was stabilized by the presence of SOD. In the absence of SOD, GCG is

² Unpublished data.

probably formed and oxidized; thus, GCG was not detected. GCG has a similar inhibitory effect on KYSE 150 cell growth, with an IC_{50} of 15 $\mu\text{mol/L}$ (data not shown).

The observations that the inhibitory effects of EGCG on phosphorylation and protein levels of EGFR and HER-2/*neu* (and PDGFR β) require a preincubation period suggest a mechanistic link between this inhibition and EGCG auto-oxidation. We hypothesize that superoxide, $\cdot\text{EGCG}$, and other radicals, and perhaps theasinensin A, generated from EGCG auto-oxidation in the cell culture medium can attack and inactivate these receptors, and the inactivated receptors lose their ability to be autophosphorylated upon the addition of ligands (i.e., EGF or PDGF-BB). The inactivated receptors may be recognized by the cellular degeneration machinery, such as the proteasomes, to clear off from the membrane. As shown in Fig. 3B, theasinensin A was formed and then degraded. Based on these facts, we believe that theasinensin A, being a polyphenolic compound, can undergo the auto-oxidation process similar to EGCG. The radical species generated during this process can inactivate EGFR and HER-2/*neu* as well. EGCG has been suggested to affect the autophosphorylation of EGFR by direct binding to the tyrosine kinase active sites or by altering the conformation of the proteins required for autophosphorylation to take place. These mechanisms of action should not be abolished by SOD and are likely not important in our experimental system.

This mechanism of the inhibitory action of EGCG may be generalized to other membrane receptor proteins as well. These proteins have one domain facing the extracellular space and one domain to relay the extracellular signals into the intracellular space. The former domain may be vulnerable and could be targets for radicals generated through EGCG auto-oxidation occurring in the surrounding medium. These proteins, usually the most upstream components in signal transduction relays, are essential for numerous biological functions of the cell; injuries to the proteins will cause dramatic changes in cell behavior (e.g., decreased cell proliferation rate and induction of apoptosis *in vitro*). Whether EGCG can be oxidized intracellularly remains an intriguing question. The effects of EGCG oxidation on DNA and intracellular proteins need to be investigated carefully.

Significant amount of H_2O_2 was generated from EGCG in many cell culture systems (24, 25, 34, 35). For example, 50 $\mu\text{mol/L}$ EGCG generated up to 25 $\mu\text{mol/L}$ H_2O_2 in HT-29 cell culture medium (24), and 25 $\mu\text{mol/L}$ EGCG generated 4 $\mu\text{mol/L}$ H_2O_2 in 21BES cell culture (25). The fact that the addition of catalase, which eliminates H_2O_2 , can almost completely or partially prevent EGCG-induced

apoptosis suggests that the H_2O_2 generated from EGCG auto-oxidation may be responsible or partially responsible for the induction of apoptosis (25, 36). In the present study with KYSE 150 cell culture system, the presence of SOD or catalase did not change the apoptotic cell population significantly (around 1-2%; data not shown). In the present cell culture system for KYSE 150 cells, a peak level of 1.2 $\mu\text{mol/L}$ H_2O_2 was observed (Fig. 3B). It seems that the contribution of H_2O_2 to apoptosis depends on the level of H_2O_2 produced in the system.

In the present study, the inhibition of cell growth is not driven by auto-oxidation and not related to the degradation of EGFR. In contrast, the addition of SOD, which stabilizes EGCG by blocking auto-oxidation, resulted in extensive inhibition of cell growth of KYSE 150 cells (Fig. 5). This suggests that the stabilized EGCG is responsible for the growth inhibition. On the other hand, the presence of SOD did not affect the IC_{50} value of EGCG on growth inhibition (data not shown). The mechanisms for the growth inhibition and the discrepancy between the effects in the two cell lines need to be investigated.

A critical question is whether the auto-oxidation initiated inactivation of EGFR, observed in the cell culture systems, occurs in animals. This is an important topic for future investigation. Under normal conditions, the oxygen partial pressure in the internal organs is much lower than that under cell culture conditions (<40 versus 160 mm Hg), and cells are endowed with antioxidative enzymes such as superoxide dismutase and glutathione peroxidase. We have no evidence for the auto-oxidation of EGCG in the mouse; the EGCG are rather stable and no EGCG dimers have been detected in the blood. If the auto-oxidation of EGCG does not occur in most tissues, then the inactivation of EGFR or HER-2/*neu* by EGCG, as observed in our cell line systems, may not occur in these tissues. It remains to be determined whether EGCG auto-oxidation occurs in special tissues, at sites of inflammation, or in tumor cells. Before this issue is resolved, in future studies with EGCG and many other compounds that can undergo auto-oxidation, the addition of SOD in the cell culture system may be a convenient approach to avoid possible artifacts caused by auto-oxidation.

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