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Suppression of Extracellular Signals and Cell Proliferation Through EGF Receptor Binding by (−)-Epigallocatechin Gallate in Human A431 Epidermoid Carcinoma Cells

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Abstract Tea polyphenols are known to inhibit a wide variety of enzymatic activities associated with cell proliferation and tumor progression. The molecular mechanisms of antiproliferation are remained to be elucidated. In this study, we investigated the effects of the major tea polyphenol (−)-epigallocatechin gallate (EGCG) on the proliferation of human epidermoid carcinoma cell line, A431. Using a [^{3}H]thymidine incorporation assay, EGCG could significantly inhibit the DNA synthesis of A431 cells. In vitro assay, EGCG strongly inhibited the protein tyrosine kinase (PTK) activities of EGF-R, PDGF-R, and FGF-R, and exhibited an IC_{50} value of 0.5–1 μg/ml. But EGCG scarcely inhibited the protein kinase activities of $pp60^{v\text{-}src}$, PKC, and PKA ($IC_{50} > 10$ μg/ml). In an in vivo assay, EGCG could reduce the autophosphorylation level of EGF-R by EGF. Phosphoamino acid analysis of the EGF-R revealed that EGCG inhibited the EGF-stimulated increase in phosphotyrosine level in A431 cells. In addition, we showed that EGCG blocked EGF binding to its receptor. The results of further studies suggested that the inhibition of proliferation and suppression of the EGF signaling by EGCG might mainly mediate dose-dependent blocking of ligand binding to its receptor, and subsequently through inhibition of EGF-R kinase activity. J. Cell. Biochem. 67:55–65, 1997.

Key words: (−)-epigallocatechin gallate; epidermal growth factor receptor; platelet-derived growth factor; fibroblast growth factor; protein tyrosine kinases; receptor tyrosine kinases; protein kinase A; protein kinase C

INTRODUCTION

Tea has been used as a daily beverage and crude medicine in China for several thousand years. The inhibitory effects of tea against tumorigenesis and tumor growth have been attributed to the biologic activities of the polyphenols in tea [1–6]. The green tea polyphenols (GTPs) comprise (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), (−)-epicatechin (EC), (+)-gallocatechin (GC), and catechin (C). Among these polyphenols, EGCG is the major component of the green tea. Many biological functions of tea polyphenols have been reported, including antioxidative activities [1–3], anticarcinogenic activities [4–6], and antiproliferative effects [7,8] on various cell lines. Several molecular mechanisms of tumor-inhibitory properties of GTPs have been studied, including inhibition of TPA-induced epidermal ornithine decarboxylase activity and cellular proliferation [9], antiinflammatory, inhibition of protein kinase C activity [10], and mouse epidermal lipooxygenase and cyclooxygenase activities [11] in different assay systems.

In recent work, we have shown that the major and most potent component of tea polyphenol EGCG inhibited the growth of S-180 and

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A431 cells [12]. The mechanism underlying these diverse effects of EGCG is not fully understood. So, we tried to explore the growth inhibiting nature and mechanism of action of EGCG on human epidermoid carcinoma A431 cells that express high levels of epidermal growth factor (EGF) receptors [13]. The EGF receptor is a 170-kDa plasma membrane glycoprotein with an extracellular ligand-binding domain, a single transmembrane region, and an intracellular domain that exhibits intrinsic tyrosine kinase activity [14]. The ligands, including epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) binding to the EGF receptor results in activation of the kinase activity and leads to autophosphorylation on at least five tyrosines located in the C-terminal tail region [15]. Activation of the EGF-R tyrosine kinase by its ligand that are thought to initiate the multiple cellular responses associated with mitogenesis and cell proliferation [16]. Overexpression of EGF receptor can produce a neoplastic phenotype in cells and really find in some human tumors including breast cancer [17], squamous cell carcinoma of the lung and oral cavity [18], bladder carcinoma, and esophageal cancer [19].

Several plant flavonoid compounds have been reported to inhibit tyrosine kinase activity of EGF-R, including quercetin [20], genistein [21], and apigenin [21,22]. In this study, we investigated the antiproliferative actions of EGCG in human epidermoid carcinoma A431 cells, and the kinase inhibitory effects of EGCG on EGF-R, PDGF-R, FGF-R, $pp60^{v\text{-}src}$, PKC, and PKA in vitro. We also studied the kinase inhibitory effects of EGCG on EGF-induced activation of EGF-R autophosphorylation in A431 cells. Furthermore, we observed that EGCG could block cellular binding of EGF by binding to its EGF-R, preventing its autophosphorylation and downstream signal transductions.

EXPERIMENTAL PROCEDURES

Materials

EGCG, EGC, and ECG were purified from Chinese tea (Longjing tea, *Camellia sinensis*) by the method of Nonaka et al. [23] with some modifications as described in our previous report [12]. EC and catechin were purchased from Aldrich. All proteinase inhibitors, phosphatase inhibitors, poly Glu_4Tyr, Triton X-100, gallic acid, and caffeine were from Sigma (St. Louis, MO). Human recombinant EGF, aa-PDGF, and bFGF were purchased from R&D Systems. [^{3}H]Thymidine, [r-^{32}p]ATP, ^{125}I-EGF (100 μCi/ml), and [^{32}P]orthophosphate were purchased from Amersham (Arlington, IL). Phosphocellulose (P-81) was from Whatman (Maidstone, England).

Cell Culture and Growth Assay

Human epidermal carcinoma A431, v-*src*-transformed NIH 3T3, and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal calf serum (FCS) (Gibco), and maintained at 37°C in a humid atmosphere of 5% CO_2 in air. For growth stimulation assay, 70% confluent cultures were serum-starved for 24 h, and then treated with EGF (40 ng/ml) for 24 h in the presence or absence of the various dose of EGCG, followed by determination of [^{3}H]thymidine (3 μl, 25 Ci/mmol) incorporation into DNA during the last 4 h of treatment [24].

Assay of Kinases Activities In Vitro and In Vivo

Isolation of membrane proteins was prepared as described previously [25]. The standard kinase assay mixture (final volume 60 μl in 50 mM Hepes buffer, pH 7.4) contained of 10 μg A431 cells membrane proteins (for EGF-R activity) or 10 μg NIH3T3 cells membrane proteins (for PDGF-R and FGF-R activities), 10 mM $MgCl_2$, 1 mM $MnCl_2$, 100 μM ATP, 10 μM [γ-^{32}p] ATP, 0.67 mg/ml $polyGlu_4Tyr$, 100 ng/ml different ligands (EGF, aa-PDGF or bFGF) and the various dose of EGCG. The mixture was incubated for 20 min at 22°C, and the reaction was terminated by adding a 20-μl stop solution (120 mM EDTA, 4 mM Na_3VO_4). Reaction mixture was transferred to phosphocellulose (p81, Whatman) papers. The papers were washed with 5% trichloroacetic acid (TCA) for several times and radioactivities were measured by scintillation counting. Tyrosine kinase activity of $pp60^{v\text{-}src}$, immunoprecipitated by the Src-specific ($p60^{src}$) monoclonal antibody (UBI) and protein A/G PLUS-agarose (Santa-Cruz Biotechnology, California) from the detergent lysate of v-*src*-transformed NIH3T3 cells was assayed in a reaction mixture (final volume of 50 μl) containing 20 mM MOPS, pH 7.0, 10 mM $MgCl_2$, 3 μg rabbit muscle enolase (Sigma) 25 μCi [γ-^{32}P] ATP, 5 μM ATP. All other procedures of immune complex kinase assay were carried out as described previously [25]. PKCs were partially purified from quiescent NIH 3T3 cells [26]. The incubation mixture (0.2 ml) contained 50 μl

(2–10 µg) of the partially purified PKC, 5 µmol Tris–HCl pH 7.4, 2 µmol $MgCl_2$, 40 µg of lysine-rich histone, 10 µg of phosphatidylserine, 0.75 µg of 1,2-diolein, 10 µg/ml leupeptin, 5 µM [γ-^{32}P] ATP (4,000 cpm/pmol), 0.35 µmol $CaCl_2$, and the EGCG [24]. The reaction mixture was incubated for 3 min at 30°C and analyzed as described above. PKA kinase activity was assayed in a reaction mixture (final volume of 40 µl) contained 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 µM [γ-^{32}P] ATP, 0.25 mg/ml bovine serum albumin (BSA), 50 µM Kemptide (peptide substrates for PKA), 10 µM cAMP, and 4 µg of the enzyme and the inhibitor. The reaction was performed at 30°C for 5 min [24].

For autophosphorylation of EGF-R or PDGF-R in vivo, cells were incubated in serum-free medium for 24 h, then treated EGF (20 ng/ml) of PDGF (10 ng/ml) and inhibitor at various conditions. Cells were lysed with 100 µl of Gold lysis buffer containing 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris–HCl pH 7.9, 100 µM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. An equal amount of protein (50 µg) was resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred onto PVDF membrane (Amersham), followed by immunoblotting with anti-phosphotyrosine first antibody (PY20, Transduction Laboratory, Lexington, KY) and then secondary antibody-conjugated horseradish peroxidase (Dako, Glostrup, Denmark). The immunocomplexes were visualized using the enhanced chemiluminescence kits (ECL, Amersham). The intensity of EGF-R or PDGF-R phosphorylation bands were quantified by densitometry (IS-1000 Digital Imaging System). Analysis of EGF-R phosphorylation, cells were labeled with 1 mCi/ml of [^{32}P]orthophosphate (Amersham, spec act 10 mCi/ml) for 4 h, using phosphate-free culture medium, and incubated with or without EGCG for 30 min and then EGF (20 ng/ml) for 10 min.

Immunoprecipitation of the EGF Receptor

Aliquots of unlabeled or ^{32}P-labeled cell extracts containing equal amounts of proteins were incubated with anti-EGF receptor mAb (Transduction Laboratory) and protein A/G PLUS-agarose (Santa-Cruz Biotechnology, California), as described [27].

Phosphoamino Acid Analysis of EGF Receptor

The polyacrylamide gel containing labeled EGF-R was first transferred to Immobilon-P membrane (Amersham), and then excised, washed, and subjected to acid hydrolysis in 6 N HCl for 1 h at 110°C. The phosphoamino acids were resolved by two-dimensional separation of electrophoresis at pH 1.9, followed by electrophoresis at pH 3.5 buffer [28].

Binding of ^{125}I-EGF to A431 Cells

A431 cells were serum-starved for 6 h and then treated various concentrations of EGCG. After 30 min, added 2 ng/ml of ^{125}I-EGF and incubated at 4°C for 1 h in 2 ml of binding medium (DMEM, 5 mM Hepes pH 7.5, 0.1% BSA). Nonspecific binding was measured with 2 µg/ml unlabeled EGF. After the incubation, the cells were washed six times with cold-DMEM and solubilized with 1 ml of 1.5 N NaOH at room temperature for 1 h. The solubilized lysates were transferred to plastic tubes for counting on a γ-spectrometer [29].

RESULTS

Inhibition of Receptor Tyrosine Kinase Activity by EGCG In Vitro

We examined the effect of different concentrations of EGCG on the activities of six different protein kinases. The results of this experiment indicated that EGCG inhibited all the kinases examined but to different extents. As shown in Table I, the inhibitory activity of EGCG is more effective in receptor-type protein tyrosine kinases (EGF-R, PDGF-R, and FGF-R, IC_{50} = 0.5–1 µg/ml) than nonreceptor-type protein tyrosine kinase ($pp60^{v\text{-}src}$, $IC_{50} > 10$ µg/ml). In receptor-type protein tyrosine kinases (PTKs), EGCG shows more selective inhibition of EGF-R (IC_{50} = 0.5 µg/ml) than other receptor-type PTKs. By contrast, EGCG scarcely inhibits serine- and threonine-specific protein kinases such as protein kinases A and C at 20 µg/ml (Table I). Thus, inhibitory activity of EGCG is highly specific for receptor-type PTKs, especially EGF-R. This observation prompted us to investigate the effects of EGCG on the DNA synthesis and phosphorylation of EGF-R in A431 cells.

Inhibition of A431 Cells DNA Synthesis by EGCG

We tested whether EGCG could inhibit EGF-induced cell proliferation in A431 cells. Table II

TABLE I. Effect of EGCG on Protein Kinase Activity In Vitro

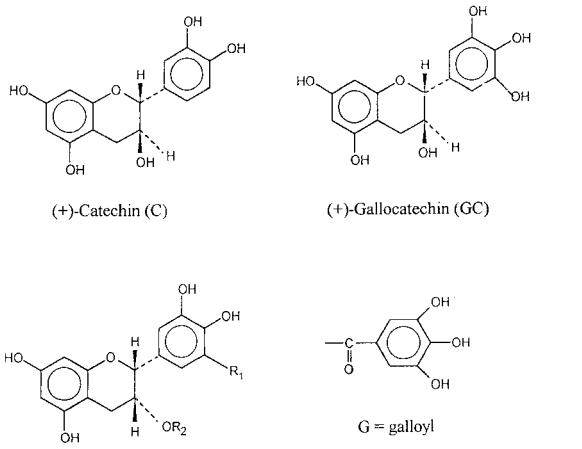
Protein kinases	IC_{50}[a] (μg/ml)
EGF receptor	0.51 ± 0.08
PDGF receptor	1.04 ± 0.20
FGF receptor	1.03 ± 0.12
$pp60^{v\text{-}src}$	>10
Protein kinase C	>20
Protein kinase A	>20

[a]Protein kinase activity was measured as described under Experimental Procedures in the absence or presence of various concentrations of EGCG. Each experiment was independently performed three times and expressed as mean ± SE.

TABLE II. Effect of EGCG on EGF-Stimulated [^{3}H]Thymidine Incorporation in A431 Cells

Treatment	[^{3}H]Thymidine incorporation (relative ratio to control)[a]
Control	1.00
EGF (40 ng/ml)	1.62 ± 0.02*
EGCG (15 μg/ml)	0.92 ± 0.11
EGF + EGCG (1 μg/ml)	1.68 ± 0.06
EGF + EGCG (5 μg/ml)	1.42 ± 0.08
EGF + EGCG (15 μg/ml)	1.06 ± 0.04**

[a]Each experiments was independently performed three times and expressed as mean ± SE.
*Compared with control, $P < 0.05$ (Students' t-test).
**Compared with *, $P < 0.05$ (Students' t-test).



	R_1	R_2
(-)-Epicatechin (EC)	H	H
(-)-Epigallocatechin (EGC)	OH	H
(-)-Epicatechin gallate (ECG)	H	G
(-)-Epigallocatechin gallate (EGCG)	OH	G

Fig. 1. Structures of green tea polyphenols (GTPs).

shows that EGCG alone has no significant effect on cell survival at 15 μg/ml but can inhibit the [^{3}H]thymidine incorporation into DNA in EGF-treated cells. This inhibition exhibit a dose-dependent manner. There are no apparent changes in A431 cell morphology within 24 h of EGCG treatment.

Effects of EGCG on the Phosphorylation of EGF Receptor In Vivo

Based on the results of the previous experiments (Tables I, II), we decided to examine the effect of EGCG on the activation of EGF-R induced by EGF in vivo. The effect of EGCG on the activation of EGF-R phosphorylation on tyrosine by using a specific anti-phosphotyrosine mAb PY-20. As shown in Figure 2, A431 cells pretreated with EGCG before EGF for 30 min showed maximal inhibition of EGF-R autophosphorylation by contrast, A431 cells were pretreated with EGF; then EGCG exhibited insufficient inhibition (lanes 3 and 4). In the next experiment, we investigated the kinetics of inhibition of EGF-R autophosphorylation by EGCG in A431 cells. Figure 3A showed that A431 cells were treated with EGCG for 30 min, then washed and changed with fresh medium, followed by treatment with EGF for 10 min. Autophosphorylation of the receptor is inhibited by about 35% and 50% at 5 μg/ml and 15 μg/ml of EGCG, respectively (lanes 5 and 6). Co-treatment of A431 cells with EGCG and EGF for 10 min demonstrated no inhibition (lanes 7 and 8). As illustrated in Figure 2, only pretreatment of A431 cells with EGCG for 30 min is sufficient to inhibit the EGF-R kinase activity (lanes 3 and 4). Therefore, we decided to pretreat A431 cells with EGCG for 30 min in the following experiments.

In the experiments shown in Figure 4, we examined the effects of different concentrations of EGCG on the EGF-R (or PDGF-R) (Fig. 4B) autophosphorylation of A431 cells (or NIH 3T3 cells) (Fig. 4B) in response to EGF (or PDGF) (Fig. 4B). Western blotting using an antiphosphotyrosine antibody shows that EGF-R (or PDGF-R) (Fig. 4A,B, lane 2) were rapidly tyrosine phosphorylation upon EGF (or PDGF) stimulation for 10 min. In other lanes, the results of dose-response tested groups indicate that 1 μg/ml of EGCG is sufficient to inhibit the

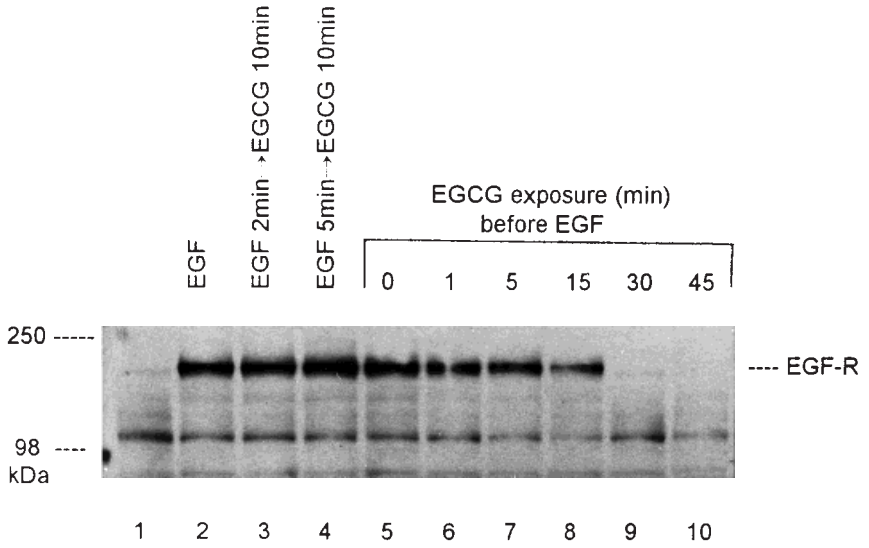


Fig. 2. Time course of the inhibition of EGF-R autophosphorylation by EGCG in A431 cells. Serum-starved cells were exposed to 5 μg/ml of EGCG and 20 ng/ml of EGF (**lanes 2, 5–10**) was treated for 10 min at varying times after EGCG treatment. The cells were exposed to EGF (20 ng/ml) for 2 or 5 min, then added EGCG (5 μg/ml) for 10 min (**lanes 3, 4**). Total cellular proteins (50 μg) were separated on SDS–PAGE (10% polyacrylamide) and blotted with anti-phosphotyrosine antibody. Immunocomplexes were detected by horseradish peroxidase second antibody and then by ECL kits. The position of the 170-kDa phosphotyrosine protein is indicated as EGF-R at the right.

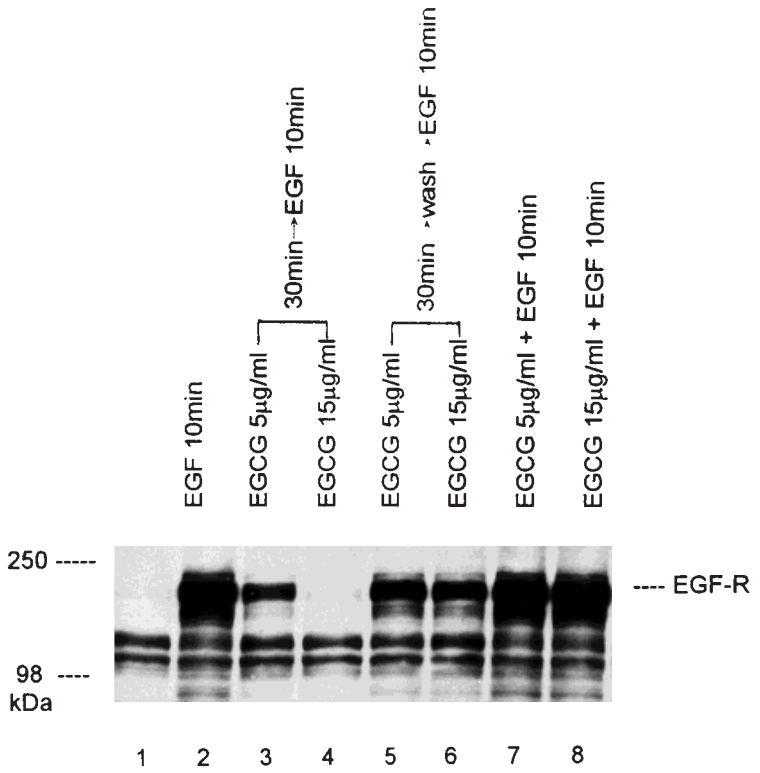


Fig. 3. Analysis of EGCG-medicated inhibition of EGF-stimulated tyrosine phosphorylation of EGF-R in A431 cells. Serum-starved cells were exposed to EGCG (lane 3, 5 μg/ml; lane 4, 15 μg/ml) for 30 min (**lanes 3, 4**), or then removed EGCG by washing of the cells twice with fresh medium (**lanes 5, 6**), and EGF (20 ng/ml) was added for 10 min. The cells were treated EGCG and EGF (20 ng/ml) as same time for 10 min (**lanes 7, 8**). Expression of tyrosine phosphorylated proteins were done as described (see Fig. 2).

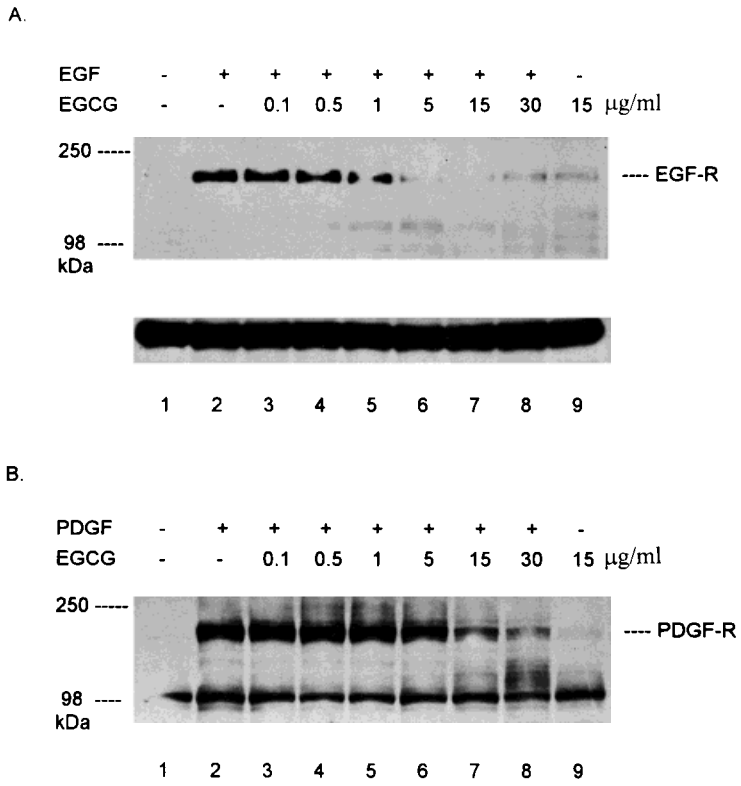


Fig. 4. Effect of EGCG on tyrosine phosphorylation in A431 cells **(A)** or NIH 3T3 **(B)** induced by EGF (A) or PDGF (B). Serum-starved cells were exposed to various concentrations of EGCG for 30 min (**lanes 3–9**) and then to 20 ng/ml of EGF (or 10 ng/ml of PDGF) (**lanes 2–9**). Expression of tyrosine phosphorylated proteins was as described (see Fig. 2). Expression of EGF-R was analyzed by immunoblotting, using first anti-EGF-R mAb, then as described (see Fig. 2).

EGF-R kinase activity by 45% (Fig. 4A, top); 5 μg/ml of EGCG is sufficient to inhibit the PDGF-R kinase activity by 53% (Fig. 4B). There is no effect of EGCG treatment (30 min) on the level of total EGF-R protein (Fig. 4A, bottom) or PDGF-R (data not shown). These results suggest that EGCG can suppress EGF-R (or PDGF-R) autophosphorylation upon EGF (or PDGF) stimulation. But the precise nature of EGCG-mediated inhibition of EGF-R kinase activity remains to be investigated. It is conceivable that EGCG may directly inhibit EGF-R kinase activity (Table I; Figs. 2, 3) or prevent EGF from binding to EGF-R by binding to EGF itself or EGF-R. To examine the latter possibility, we first incubated EGF (20 ng/ml) with different concentrations of EGCG for 10–30 min at 37°C, then treated A431 cells for 10 min. The results indicate that the autophosphorylation of EGF-R is slightly inhibited at 20 μg/ml of EGCG (data not shown). This phenomenon suggests that EGCG might not interact with EGF directly. In order to further analyze the effect of EGCG on the affinity of EGF to EGF-R, we used ^{125}I-EGF for the binding assay.

Effects of EGCG on the Binding of ^{125}I-EGF to A431 Cells

As shown in Figure 5, A431 cells were pretreated with various concentrations of EGCG for 30 min; when incubated with ^{125}I-EGF for 1 h, they showed maximal inhibition of ^{125}I-EGF binding to cells. Inhibition of ^{125}I-EGF binding to cells by EGCG occurred in a dose-dependent manner. Cells pretreated with various doses of EGCG for 30 min, washed and changed with fresh medium, and then incubated with ^{125}I-EGF for 1 h showed medial inhibition. Cotreatment of EGCG and ^{125}I-EGF for 1 h showed minimal inhibition. These phenomena suggested that EGCG might bind to cell membrane at some receptor protein molecules and block ^{125}I-EGF binding to these occupied EGF-R proteins.

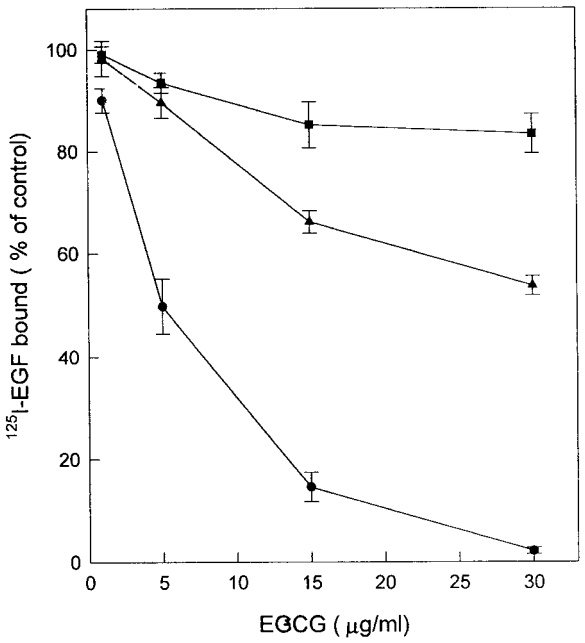


Fig. 5. Effect of EGCG on ^{125}I-EGF binding to A431 cells. A431 cells were serum-starved for 6 h and then treated with various concentrations of EGCG. After 30 min, ^{125}I-EGF (●) or washed cells were added, followed by ^{125}I-EGF (▲) for 1 h. A431 cells were treated with EGCG and ^{125}I-EGF simultaneously (■) for 1 h. Data were counted as described in Experimental Procedures and represent the mean ±SE of three samples.

Phosphoamino Acid Analysis of the EGF-R of A431 Cells Treated With EGCG

In this experiment, cells were labeled with [^{32}P]orthophosphate, then pretreated with EGCG before EGF as previously described. EGF-R was immunoprecipitated from total cell lysates and analyzed by SDS–PAGE, followed by autoradiography. The results shown in Figure 6A, there is an increase in the levels of total phosphorylation of the ^{32}P-EGF-R by EGF (20 ng/ml, lane 2), and it can be inhibited by EGCG (5 μg/ml, lane 3). Phosphoamino acid analysis of the EGF-R showed that EGCG only decreases the phosphorylation of the receptor protein on tyrosine residue (Fig. 6B), but not on serine and threonine residues.

Effects of EGCG and Related Compounds on the EGF-R Autophosphorylation and the Binding of ^{125}I-EGF to A431 Cells

To investigate structure–activity relationships, the effects of several GTPs (Fig. 1) and caffeine on EGF-R kinase activity were examined on its autophosphorylation. Among these compounds, only EGCG and ECG exhibit rather strong inhibitory activity, while the other GTPs and caffeine exhibit limited inhibitory activity (Fig. 7A). In Fig. 7B, the inhibitory capacities of these compounds on the ^{125}I-EGF binding to EGF-R were also investigated and gave the similar inhibition patterns (Fig. 7A,B).

DISCUSSION

Overexpression of EGF-R occurs frequently in human gliomas and other tumors [30]. In the present study, we have demonstrated that EGCG strongly inhibited the growth of A431 cells, which express high levels of EGF-R. The observation that EGCG-mediated inhibition of EGF binding to EGF-R is an early cellular effect of EGCG and precedes inhibition of EGF-induced phosphorylation at a later stage. However, we cannot rule out the possibility that EGCG might directly inhibit partial kinase activity of EGF-R. In the time course experiment (Figs. 2, 3), inhibition of EGF-R kinase activity was found to be insignificant when A431 cells were treated simultaneously with EGF and EGCG. But, there was a maximal inhibition effect that A431 cells were pretreated EGCG for 30 min. These phenomena might involve the mechanism of EGCG-mediated block of EGF binding to EGF-R. As we harvested cells after EGCG treatment, the cells pellet showed a brown color. This phenomenon indicated that EGCG might penetrate the cytoplasm or anchor on the membrane surface. EGCG, as with other flavanols, can form complexes with biologic macromolecules, such as lipids, carbohydrates, proteins, and nucleic acids [31]. It is possible that (1) EGCG binds to multiple sites on the fraction of cytoplasm or extracellular of EGF-R, resulting in a conformational change of EGF-R and blocking its ability to bind EGF or (2) EGCG directly binds to the EGF binding site of EGF-R, blocking the binding of EGF. Figure 4 shows that EGCG inhibits not only EGF-R, but also PDGF-R kinase activity. EGCG might not be a specific inhibitor of EGF binding. It is possible that EGCG could block most signals transduction from cell surface to nucleus. The results of Figures 2–4 indicate that EGCG inhibits the tyrosine kinase activity of the EGF-R in intact A431 cells. However, EGCG is not found to inhibit EGF-stimulated serine and threonine phosphorylation of the EGF-R. It is suggested that EGCG not only blocks EGF binding but inhibits the phosphorylation of tyrosine residues as well. These results are consistent with

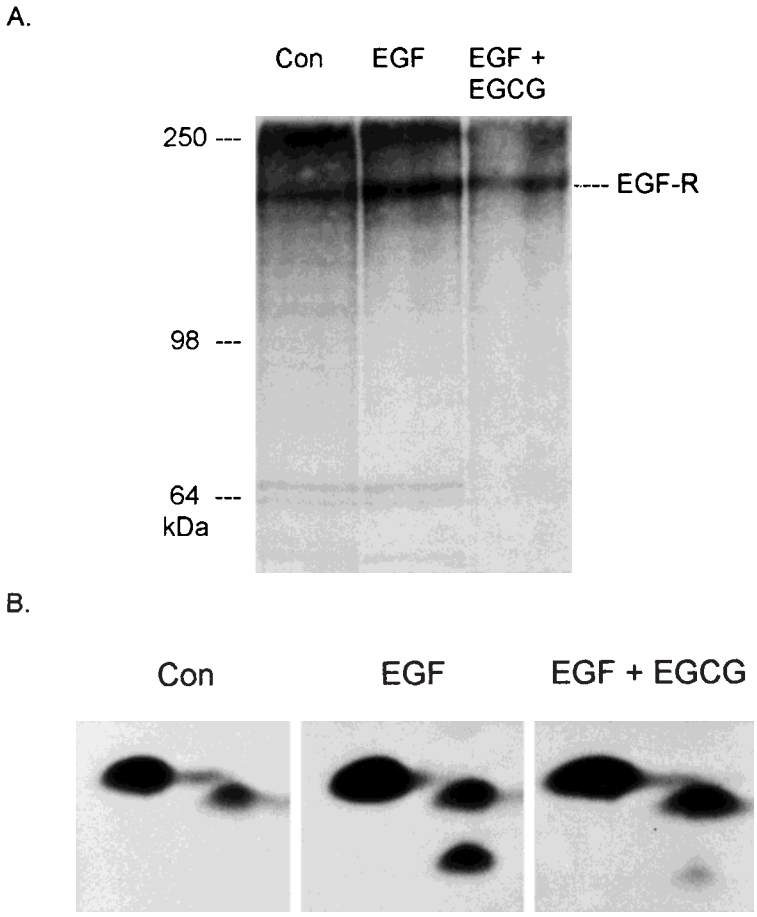


Fig. 6. Analysis of EGF-induced phosphorylation of EGF-R by EGCG. **A:** Cells were labeled with [^{32}P]orthophosphate for 4 h, and incubated with or without EGCG (5 µg/ml) for 30 min then EGF (20 ng/ml) for 10 min. ^{32}P-labeled EGF-R were immunoprecipitated with anti-EGF-R mAb and analyzed by autoradiography. **B:** Two-dimensional thin-layer electrophoresis pattern of amino acids after hydrolysis of ^{32}P-EGF-R excised from Immobilon-P membrane. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

the fact that EGCG scarcely inhibits serine and threonine kinase activity, such as PKA and PKC (Table I).

The activation of EGF receptor kinase is dependent on divalent cation [32]; tea polyphenols are usually regarded as strong metal ion chelators [31]. Therefore, it is also possible that EGCG chelates some divalent cations, leading to inhibition of the activity of EGF receptor kinase.

Both EGCG and ECG can block EGF binding and EGF-R kinase activity (Fig. 7). These two compounds share the same structure, while EGCG has one more hydroxyl group in position 3′ than does ECG (Fig. 1). The other GTPs show different structures and therefore do not inhibit EGF-R kinase activity significantly.

Up to the present, several possible biological reagents have been developed to inhibit binding of a growth factors to its receptor. Some reagents have been studied, including antibodies [33], growth factor antagonists, and suramin [34]. Antibodies to HER2/*neu* are currently undergoing clinical trials, but the development of growth factor antagonists has been unsuccessful. Suramin, initially developed as an antitrypanosome agent [35], was found to inhibit the binding of EGF (or PDGF) to EGF-R (or PDGF-R). The binding of EGF to EGF-R is block by 83% and 87% at 50 µg/ml of suramin and 15 µg/ml of EGCG, respectively (Fig. 5). This paper describes a new group of chemicals EGCG whose analogues have the ability to behave like suramin, although they have no struc-

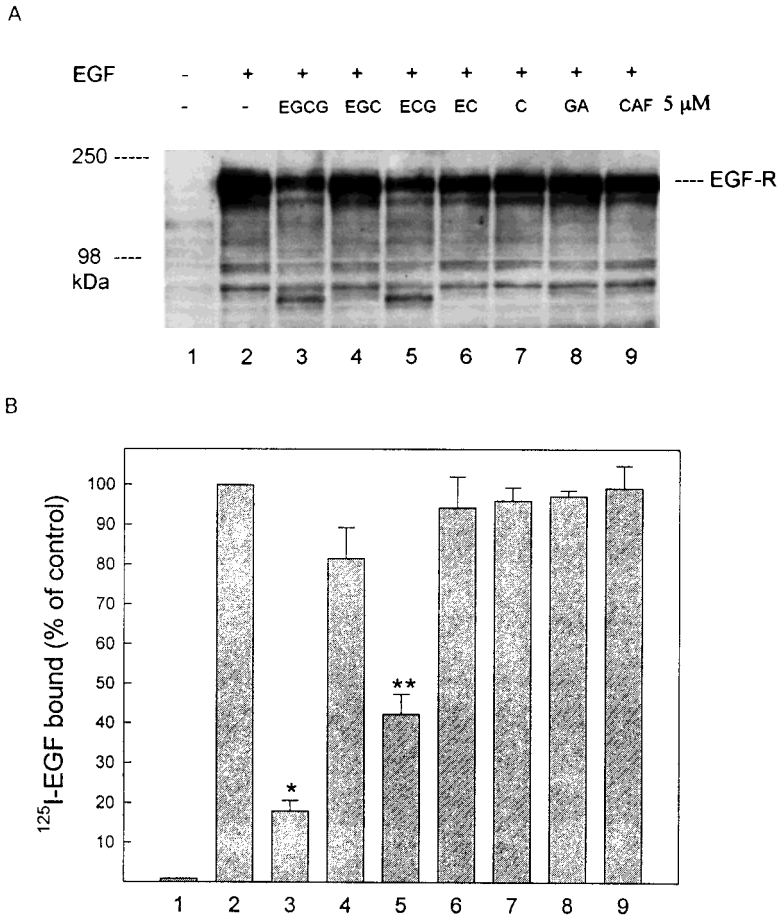


Fig. 7. Effect of EGCG and related compounds on EGF-R autophosphorylation and the binding of ^{125}I-EGF to A431 cells. **A:** Serum-starved cells were exposed to different kinds of GTPs and caffeine (5 μM) for 30 min, then to 20 ng/ml of EGF for 10 min. Purity of the EGCG, EGC, and ECG was 97%, 95%, and 93%, as determined by HPLC. Expression of tyrosine phosphorylated proteins were done as described (see Fig. 2). **B:** Serum-starved cells were treated different kinds of GTPs and caffeine (5 μM) for 30 min, ^{125}I-EGF was then added and incubated at 4°C for 1 h. The results were counted as described in Experimental Procedures. Data represent the mean ±SE from three samples. *Compared with lane 2, $P < 0.01$ (Students' *t*-test). **Compared with lane 2, $P < 0.05$ (Students' *t*-test).

ture relationships. On the other hand, the structure of EGCG is like quercetin [36] with phenolic styrene, which is a common pharmacophore for PTK inhibitors, and can be viewed as a "dehydrogenated" tyrosine mimic [38]. Based on this structure, several analogues have been developed and exhibit inhibitory activity, such as tyrophostin and its derivatives AG series. Among tyrophostins, both AG 213 and AG490 exhibit good inhibitory activity to both EGF-R and PDGF-R kinases in the range of IC_{50} = 0.7–6 μM [37]. However, EGCG inhibits EGF-R and PDGF-R kinase activity in the range of IC_{50} = 0.5–1 μg/ml in vitro. In summary, EGCG can inhibit both the binding of a growth factor to its receptor as well as autophosphorylation of the receptor tyrosine kinases (RTKs). It might be an useful compound for blocking RTKs signaling.

EGCG dose inhibits phosphorylation of not only EGF-R, but also some phosphoproteins (Fig. 6, arrow). In addition, EGCG is able to induce the tyrosine phosphorylation of some cellular proteins (Fig. 7A, lane 3, arrow). These findings suggest the possible existence of some specific targets that could be involved in the growth regulation by EGCG, as long-term or high-dose treatment of EGCG results in apoptosis.

Tea and its components have been shown to inhibit carcinogenesis by several mechanisms. The experiments reported here point to a new target for EGCG in modulating the cellular

mitogenic signals. These findings suggest that EGCG might inhibit the processes of tumor promotion through blocking cellular signal transduction.

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