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SHORT COMMUNICATION

Suppression of extracellular signals and cell proliferation by the black tea polyphenol, theaflavin-3,3′-digallate

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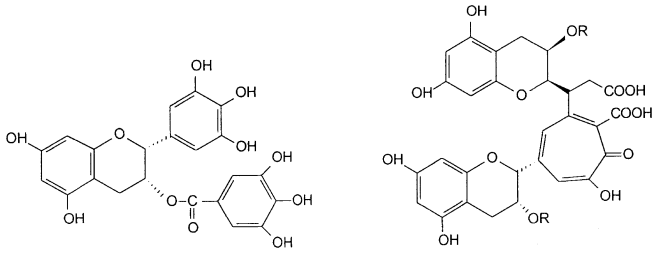
Previous studies in our laboratory have shown that the major green tea polyphenol, (–)-epigallocatechin-3-gallate (EGCG), suppressed autophosphorylation of epidermal growth factor (EGF) receptor induced by EGF in human A431 epidermoid carcinoma cells. In this study, we examined the inhibitory effects of black tea polyphenols, including theaflavin (TF-1), a mixture (TF-2) of theaflavin-3-gallate (TF-2a) and theaflavin-3′-gallate (TF-2b), theaflavin-3,3′-digallate (TF-3) and the thearubigin fraction on the autophosphorylation of the EGF and PDGF receptors in A431 cells and mouse NIH3T3 fibroblast cells, respectively. First, we examined the effects of these polyphenols on the proliferation of A431 and NIH3T3 cells. Both EGCG and TF-3 strongly inhibited the proliferation of A431 and NIH3T3 cells more than the other theaflavins did. In cultured cells with pre-treatment of tea polyphenol, TF-3 was stronger than EGCG on the reduction of EGF receptor and PDGF receptor autophosphorylation induced by EGF and PDGF, respectively. Other theaflavins slightly reduced the autophosphorylation of the EGF and PDGF receptors; furthermore, TF-3 could reduce autophosphorylation of the EGF receptor (or PDGF receptor) even with co-treatment with EGF (or PDGF) and TF-3, but EGCG was inactive under these conditions. In addition, TF-3 was stronger than EGCG in blocking EGF binding to its receptor. These results suggest that not only the green tea polyphenol, EGCG, but also the black tea polyphenol, TF-3, have an antiproliferative activity on tumor cells, and the molecular mechanisms of antiproliferation may block the growth factor binding to its receptor and thus suppress mitogenic signal transduction.

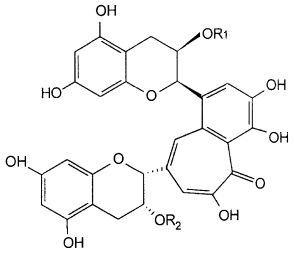
Green tea is thought to exert a possible inhibitory effect against tumorigenesis and tumor growth because of the biological activities of its polyphenols. (–)-Epigallocatechin-3-gallate (EGCG) is the major polyphenol component of green tea and a potential component for anticarcinogenesis. Green tea polyphenols have been demonstrated to have several inhibitory properties on the growth of tumor cell lines (4,5). The molecular mechanism of antitumor growth might operate through blocking the signal transduction pathway (6). Recently, we and others have indicated that the molecular mechanisms of antipromotion activity of EGCG involves blocking epidermal growth factor (EGF) binding to its receptor (7) and inhibition of activator protein 1 (AP-1) activity induced by EGF or 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) (8). We have recently reported that EGCG inhibited the activities of several key G_1 regulatory proteins such, as Cdk2 and Cdk4, and could induce the protein expression of p21 and p27 of Cdk inhibitors in human breast carcinoma cells (9). Khafif *et al*. (10) reported that EGCG could block cell cycle progression in G_1 phase in human oral epithelial cells. Ahmad *et al*. (11) reported that EGCG induced apoptosis and cell cycle arrest in human carcinoma cells. A study from our laboratory used lipopolysaccharide-activated peritoneal macrophages to demonstrate that EGCG could reduce NO radical production through preventing the binding of nuclear factor-κB to the inducible nitric oxide synthase promoter (12). These results suggest that green tea polyphenols, especially EGCG, can act as antitumor promotors, antiproliferators, be anti-inflammatory, and may be useful for cancer chemoprevention.

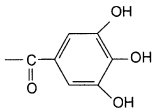
Tea (*Camellia sinensis*) is the most popular beverage worldwide. The major tea beverage is black tea, especially in Western nations. For the manufacture of black tea, the 'fermentation' process causes green tea polyphenols to oxidize and form oligomeric flavanols, including theaflavins, thearubigin and other oligomers. Theaflavins are a mixture of theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3′-gallate (TF-2b) and theaflavin-3,3′-digallate (TF-3). Thearubigins are the most abundant phenolic fraction of black tea and their structures are not well characterized. Black tea is assumed to be much less beneficial compared with green tea. However, reports have demonstrated that black tea could be as effective as green tea in cancer chemoprevention. Recently, Lu *et al*. (13) reported that black tea significantly inhibited proliferation and enhanced apoptosis of skin tumors in mice. Halder and Bhaduri (14) reported that theaflavins and thearubigins have antioxidative properties on human red blood cells. Among black tea components, theaflavins are generally considered to be the more effective components for the inhibition of carcinogenesis, but which one of these theaflavins is the most effective is unclear. In this study, we compared TF-1, TF-2 (a mixture of TF-2a and TF-2b), TF-3 and thearubigin (TR) with EGCG, to assess the inhibitory effects of signal transduction induced by EGF or PDGF. Previous evidence has indicated that activation of the EGF receptor by its ligand is associated with mitogenesis and cell proliferation (15): overexpression of the EGF receptor can produce a neoplastic phenotype in some human tumors (16,17). It was thought that this study might enable understanding of the molecular mechanisms of antitumor proliferation of black and green tea.

EGCG (purity >95%) was purified from Chinese tea

Abbreviations: DMEM, Dulbecco's modified essential medium; EGCG, (–)-epigallocatechin-3-gallate; EGF, epidermal growth factor; FBS, fetal bovine serum; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; TF-1, theaflavin; TF-2, mixture of theaflavin-3-gallate and theaflavin-3′-gallate; TF-2a, theaflavin-3-gallate; TF-2b, theaflavin-3′-gallate; TF-3, theaflavin-3,3′-digallate; TR, thearubigin.







Theaflavins	R_1	R_2
Theaflavin (Theaflavin-1; TF-1)	H	H
Theaflavin-3-gallate (Theaflavin-2a; TF-2a)	G	H
Theaflavin-3′-gallate (Theaflavin-2b; TF-2b)	H	G
Theaflavin-3,3′-digallate (Theaflavin-3; TF-3)	G	G

Fig. 1. Structures of individual theaflavin, thearubigin and (–)-epigallocatechin-3-gallate molecules.

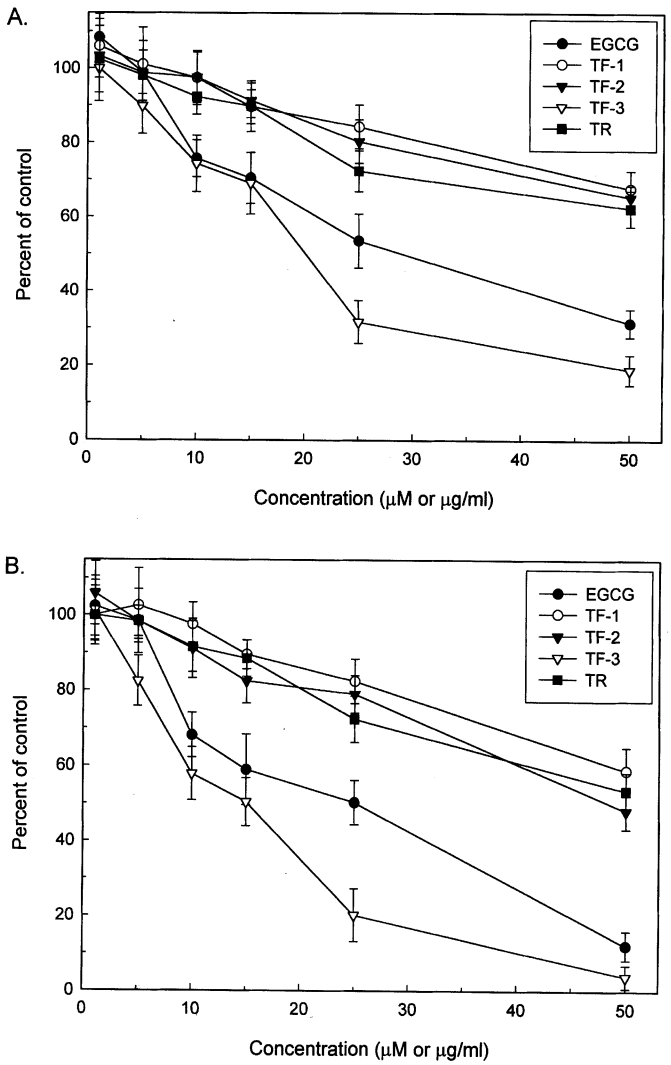


Fig. 2. Effects of EGCG, individual theaflavin and thearubigin on the growth of A431 (**A**) and NIH3T3 (**B**) cells. Cells were cultured in 12-well plates with Dulbecco's modified medium (DMEM), which contained 5% fetal bovine serum (FBS). Cells were changed to serum-free DMEM during drug treatment, treated with various concentrations of drugs for 30 min, and then added to FBS to reach a concentration of 5% FBS, and continuously cultured for 2 days. The numbers of viable cells were determined by counting the trypan blue-excluding cells in a hemocytometer. Data represent means ± SE of three experiments. The concentration of thearubigin was expressed in μg/ml.

(Longjing tea, *Camellia sinensis*) as described in our previous report (4). TF-1, TF-2, TF-3 and TR were purified from black tea as described previously (18). The structures of these compounds are shown in Figure 1. First we examined the antiproliferative effects of these compounds on mouse NIH3T3 fibroblasts and human A431 epidermoid carcinoma cells. TF-3 and EGCG were more effective at inhibiting the growth of both cells than were TF-1 and TF-2 (Figure 2). TF-3 was more able to inhibit cell growth than EGCG. The IC_{50} of TF-3 was as low as 15 and 18 μM for the growth of NIH3T3 and A431 cells, respectively. The IC_{50} values for EGCG were 26 and 28 μM for the growth of NIH3T3 and A431 cells, respectively. There was no difference in the inhibition of cell growth between TF-1 and TF-2. Based on results from a previous report (7), the inhibition of cell growth by EGCG might mediate block ligand binding to its receptor and lead to the inhibition of membrane receptor kinase activity. We examined the effects of EGCG, theaflavins and thearubigin on the activation of the EGF receptor (or PDGF receptor) induced by EGF (or PDGF) in cultured cells. As shown in Figure 3A, A431 cells were pre-treated with tea polyphenols before EGF and gained a better inhibition compared with co-treating these tea polyphenols and EGF on EGF receptor autophosphorylation (Figure 3A, top versus middle panels). When A431 cells were pre-treated with these tea polyphenols for 30 min, the inhibition capacity of EGF receptor autophosphorylation was in the following order: TF-3 > EGCG > TF-1 or TF-2 (Figure 3A, top panel). Only TF-3 exhibited an effective inhibition of autophosphorylation of EGF receptor when A431 cells were co-treated with these tea polyphenols and EGF (Figure 3A, middle panel). Thearubigin scarcely inhibited the proliferation of both cells, and suppressed the autophosphorylation of the EGF receptor at a concentration of 10 μg/ml. To confirm that the inhibited autophosphorylation accurately reflects suppressed intrinsic kinase activity, we performed *in vitro* an immune complex kinase assay using enolase as an exogenous substrate. The results showed that the inhibition of EGF receptor intrinsic kinase activity by TF-3 and EGCG was similar to the inhibition of EGF receptor autophosphorylation (Figure 3A). These results indicate that TF-3 was more able than EGCG to inhibit EGF receptor autophosphorylation. We examined the effects of different concentrations of TF-3 on EGF receptor autophosphorylation of A431 cells in response to EGF (Figure 3A, lower panel). The results indicate that 5 μM of TF-3 was sufficient to inhibit EGF receptor kinase activity by ~75% and

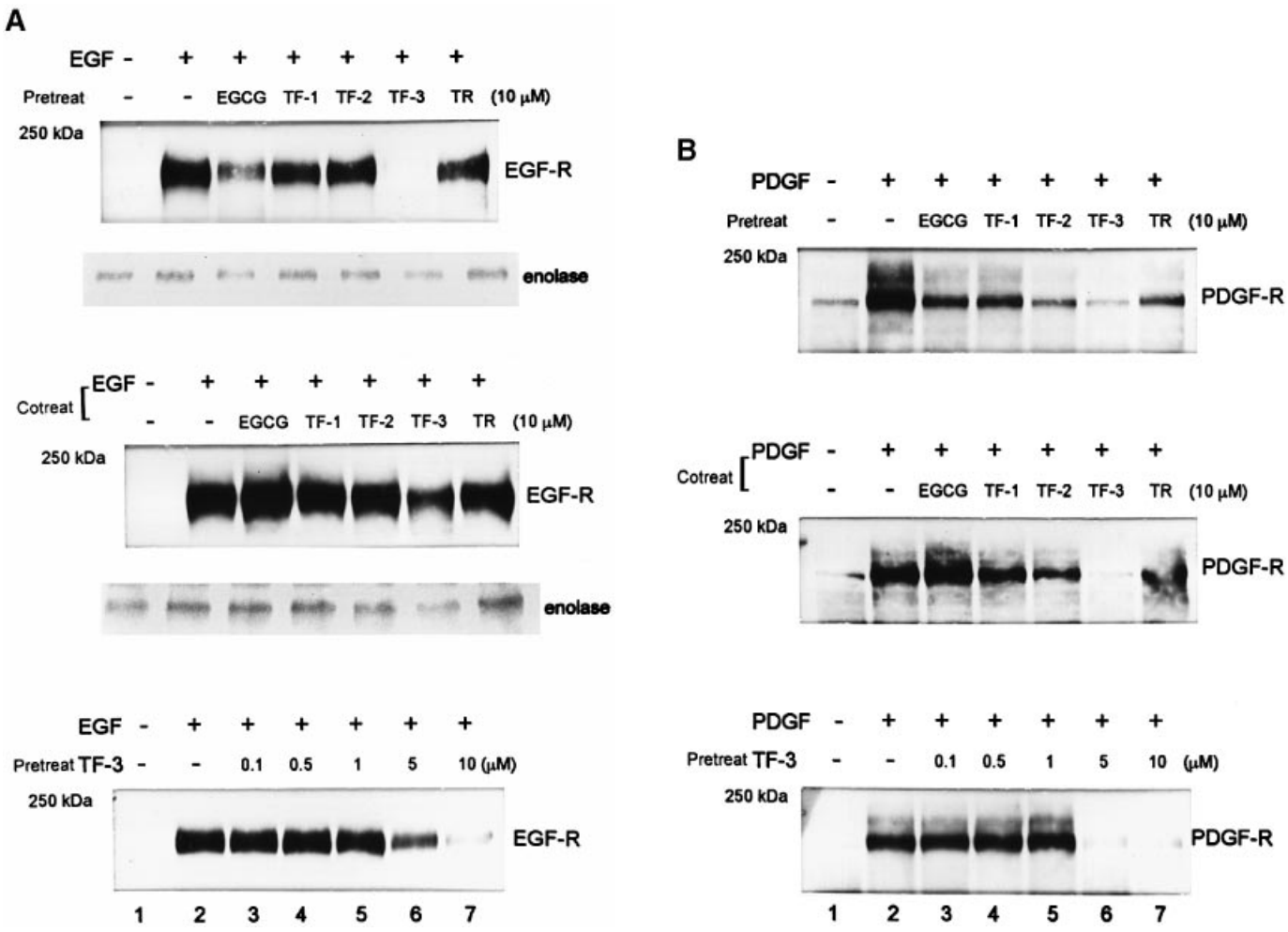


Fig. 3. Effects of EGCG, individual theaflavin and thearubigin on the kinase activities of the EGF or PDGF receptors induced by EGF or PDGF, respectively, in A431 or NIH3T3 cells. (**A**) A431 cells were cultured in 10 cm dishes with DMEM containing 10% FBS. Cells were changed to serum-free DMEM for 24 h before drug treatment. Serum-starved cells were treated with different kinds of drug (top panels) or various concentrations of TF-3 (lower panels) for 30 min, and were then treated with 20 ng/ml of EGF for 10 min. Alternately, cells were co-treated with different types of drug (middle panels) and 20 ng/ml of EGF at same time for 10 min (7). Total cellular proteins (50 μg) were separated on SDS–PAGE (9% polyacrylamide) and transferred to a PVDF membrane (Amersham, Arlington, IL). The membrane was blotted with antiphosphotyrosine antibody (PY20; Transduction Laboratory, Lexington, KY) and then horseradish peroxidase antibody (Transduction Laboratory). Immunocomplexes were detected by ECL kits (Amersham). Under the same conditions, the EGF receptor proteins were immunoprecipitated and incubated in 40 μl kinase buffer with 10 μCi of [γ-^{32}P]ATP (Amersham) and 5 μg of enolase (Sigma, St Louis, MO) as an exogenous substrate (19). The reaction was stopped by adding 8 μl of 6× SDS polyacrylamide gel sample loading buffer. After separation on a 8% SDS–polyacrylamide gel and drying, the phosphorylated proteins were visualized by autoradiography. (**B**) Serum-starved NIH3T3 cells were treated with the drugs as in (A), but PDGF was used (10 ng/ml) instead of EGF. Expression of tyrosine phosphorylated proteins was performed as described above (A). The position of the 170 or 180 kDa phosphotyrosine protein is indicated as EGF-R (EGF receptor) and PDGF-R (PDGF receptor), respectively, on the right. Thearubigin concentration was expressed in μg/ml.

10 μM of TF-3 completely inhibited kinase activity. There was no effect on protein expression in the EGF receptor after treatment with these tea polyphenols (data not shown). (We used NIH3T3 cells instead of A431 cells and stimulated them with PDGF (Figure 3B), and treated them with drugs as in Figure 3A.) The inhibition of PDGF receptor autophosphorylation by EGCG and TF-3 was similar to that of the EGF receptor, but TF-2 and thearubigin (Figure 3B, top and middle panels) also slightly inhibited the autophosphorylation of the PDGF receptor. These results indicate that inhibition of tumor growth by black tea might occur through the suppression of extracellular signals. TF-3 was the strongest inhibitor and the major effector in black tea. We investigated the inhibition mechanism of TF-3 on EGF receptor kinase activity by a [^{125} I]EGF binding assay. As shown in Figure 4A, EGCG significantly inhibited [^{125}I]EGF binding to the cells when A431 cells were pre-treated with EGCG for 30 min (white bars). On the other hand, co-treatment of EGCG and [^{125}I]EGF showed less inhibition (black bars). However, TF-3 significantly inhibited [^{125}I]EGF binding to cells both pre- and co-treated (white and black bars) in a concentration-dependent manner (Figure 4B). In contrast, TF-1, TF-2 and thearubigin (at 10 μg/ml) slightly inhibited [^{125}I]EGF binding to cells in both treatments. These results suggest that the inhibition of EGF receptor kinase activity by EGCG and TF-3 might block EGF binding to its receptor. However, it might be possible that EGCG or TF-3 directly inhibited partial kinase activity of the EGF receptor. In our previous report (7) and this study, we found that EGCG, epicatechin-3-gallate (ECG) and TF-3 blocked EGF binding to its receptor and inhibited EGF receptor kinase activity among green and black tea polyphenols. These compounds share a similar structure and have the galloyl group

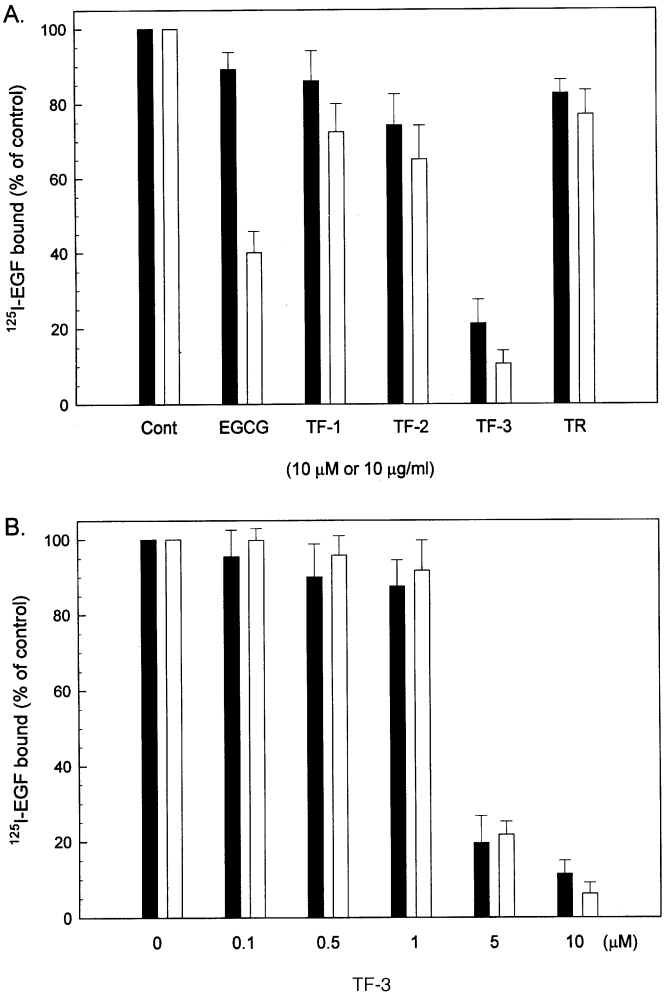


Fig. 4. Effects of EGCG, individual theaflavin and thearubigin on [^{125}I]EGF binding to A431 cells. A431 cells were cultured in 24-well plates with DMEM, containing 10% FBS. Cells were changed to serum-starved media for 6 h before drug treatment. Serum-starved cells that were pre-treated with drugs for 30 min (**A** and **B**, white bars) and then were added with [^{125}I]EGF, and incubated with gentle mechanical agitation at 4°C for 1 h. Alternately, cells were treated with drugs and [^{125}I]EGF simultaneously (A and B, black bars) and incubated with gentle mechanical agitation at 4°C for 1 h. After incubation, the cells were washed three times with PBS, solubilized with 1 ml of 1.5 M NaOH, then counted with a γ-spectrometer (7). Data represent means ± SE of three experiments. Thearubigin concentration was expressed as μg/ml.

in position 3 or 3′. EGCG has one more hydroxyl group than does ECG and the structure of TF-3 is similar to the dimer form of EGCG.

Black tea is one of the most popular beverages worldwide. Several reports have indicated that black tea can inhibit tumor cell proliferation in animal models (5,13), but the active components in black tea and the specific theaflavins responsible for this activity are not known. The present studies clearly demonstrate that TF-3 may be the major active component that contributes to the antiproliferative activity in black tea. Moreover, TF-3 appears to be a better inhibitor of tyrosine receptor kinase than green tea polyphenol EGCG. However, it must be pointed out that TF-3 is only a minor component of black tea. In summary, we have provided evidence for a mechanism of antitumor proliferation by TF-3 and EGCG. The inhibition of EGF receptor (or PDGF receptor) kinase activity by TF-3 and EGCG may be mediated through blocking of EGF (or PDGF) by binding to its receptor.

Acknowledgements

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