Antitumor Promoting Activity of (−)-Epigallocatechin Gallate, the Main Constituent of "Tannin" in Green Tea

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(−)-Epigallocatechin gallate (EGCG) is the main polyphenolic constituent of extracts of green tea. EGCG slightly inhibited specific binding of [^{3}H]12-*O*-tetradecanoylphorbol-13-acetate ([^{3}H]TPA) to a particulate fraction of mouse skin, and caused a prompt decrease in the phorbol ester receptor number in mouse skin. It also inhibited the activation of protein kinase C by teleocidin. In week 25 of a two-stage carcinogenesis experiment in mouse skin, tumors were found in 53% of the mice treated with 7,12-dimethylbenz(a)anthracene (DMBA) plus teleocidin, but in only 13% of those treated with DMBA plus teleocidin and EGCG. The average number of tumors per mouse in these groups was 2.1 and 0.1, respectively. No tumors were found in other groups treated with DMBA alone or teleocidin and EGCG alone. Thus EGCG inhibited tumor promotion by teleocidin.

INTRODUCTION

Various kinds of compounds have been found to inhibit tumor promotion in mouse skin. These include protease inhibitors, vitamin A derivatives, anti-inflammatory steroids, phosphodiesterase inhibitors, lipoxygenase inhibitors, phospholipase A_2 inhibitors, inhibitors of polyamine synthesis and of histidine decarboxylase, and calmodulin antagonists (Troll *et al.*, 1970; Hozumi *et al.*, 1972; Verma *et al.*, 1979; Schwarz *et al.*, 1977; Fischer *et al.*, 1982; Nakadate *et al.*, 1982; Takigawa *et al.*, 1982; Umezawa *et al.*, 1983; Belman and Troll, 1974; Nishino *et al.*, 1984a; Slaga *et al.*, 1982). Tumor promotion can be inhibited by use of various known inhibitors of specific biochemical reactions induced by tumor promoters, but we considered that the inhibitory effects of natural compounds present in daily foods should also be investigated.

Recently, quercetin was found to inhibit the tumor promoting activity of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or teleocidin in two-stage carcinogenesis experiments in mouse skin initiated with 7,12-dimethylbenz(a)anthracene (DMBA) (Kato *et al.*, 1983; Nishino *et al.*, 1984a; Fujiki *et al.*, 1986). In addition, we demonstrated that other flavonoids inhibit the *in vitro* and *in vivo* effects induced by tumor promoters (Horiuchi *et al.*, 1986). Since quercetin and other flavonoids are present in foods, such as vegetables and fruits, we realized that antitumor promoters are present in daily foods.

Every day Japanese people drink green tea, which contains (−)-epigallocatechin gallate, EGCG (Fig. 1), as its main polyphenolic constituent. Therefore, in this work, we studied the influence of EGCG on tumor promotion such as its effects on the specific binding of [^{3}H]TPA to a mouse particulate fraction *in vitro*, its effect on phorbol ester receptors *in vivo* and its effect on activation of protein kinase C by teleocidin *in vitro*. EGCG inhibited tumor promotion by teleocidin in a two-stage chemical carcinogenesis experiment on mouse skin.

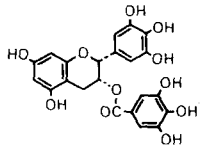

Figure 1. Structure of (−)-epigallocatechin gallate, EGCG.

MATERIALS AND METHODS

Materials. The preparation of EGCG used contained EGCG (85%), (−)-epicatechin (10%) and (−)-epicatechin gallate (5%), as determined by high performance liquid chromatography (HPLC) on a YMC-A312 (ODS) column (6 × 150 mm), with water-acetonitrile cetic acid (85 : 10 : 5) as a solvent, monitoring the UV absorption at 254 nm. This EGCG was isolated from Japanese green tea leaves as follows: the tea leaves were extracted with a boiling mixture of methanol–water (1 : 1), the solvent was evaporated, and the residue was dissolved in water and applied to a column of DIAION HP–20 [Mitsubishi, Kasei, Japan]. Material was eluted with stepwise increasing concentrations of methanol, and fractions containing EGCG were detected by HPLC. These fractions were pooled, concentrated, and extracted with ethyl acetate. The ethyl acetate extract was chromatographed on a column of Toyo-pearl HW-40 (coarse) [Toyo Soda, Japan], by elution with a water-

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to-ethanol gradient. 7,12-Dimethylbenz(a)anthracene (DMBA) was purchased from Sigma Chemicals (St. Louis, MO, USA). Teleocidin was isolated from *Streptomyces mediocidicus* (Fujiki and Sugimura, 1983). TPA was obtained from Consolidated Midland Corp. (Brewster, NY, USA) and [^{3}H]TPA was purchased from New England Nuclear (MA, USA). [γ-^{32}P]ATP was obtained from Amersham (Amersham, UK).

Inhibition of specific [^{3}H]TPA binding. A particulate fraction containing phorbol ester receptors was prepared from mouse skin as described previously (Ashendel and Boutwell, 1981; Suganuma *et al.*, 1984). Specific [^{3}H]TPA binding to the particulate fraction was measured by the cold acetone filter method described previously (Ashendel and Boutwell, 1981; Suganuma *et al.*, 1984).

Assay of protein kinase C activity. Protein kinase C was partially purified from mouse brain by DEAE-cellulose chromatography. The activity of protein kinase C activated with 2.2 μM teleocidin was determined as described previously (Fujiki *et al.*, 1984).

Two-stage chemical carcinogenesis experiment. Female CD-1 mice of 7 weeks old were obtained from Japanese Charles River Co. Ltd. (Kanagawa, Japan). The skin of their back was initiated by a single application of 50 μg of DMBA dissolved in 0.1 ml acetone. From one week later, teleocidin with or without EGCG was applied to the initiated area twice a week until week 25. In practice, a solution of 5 mg of EGCG in 0.1 ml acetone was applied topically before each treatment with 2.5 μg of teleocidin. The percentage of tumor-bearing mice, and the average number of tumors per mouse were determined, as described previously (Fujiki *et al.*, 1982).

RESULTS AND DISCUSSION

EGCG is a polyphenolic compound (Fig. 1). It is not structurally related to phorbol esters, but we first studied whether it binds to phorbol ester receptors in the cell membrane. The specific binding of [^{3}H]TPA to a mouse particulate fraction was 37 000 cpm per mg protein in the presence of 4 nM [^{3}H]TPA. Figure 2 shows that EGCG caused slight dose-dependent inhibition of the specific binding of [^{3}H]TPA: 500 nM of EGCG caused only 28% inhibition of the binding, whereas 4 nM unlabelled TPA caused 50% inhibition.

For determination of the direct interaction of EGCG with phorbol ester receptors in mouse skin, a solution of 5 mg of EGCG in 0.2 ml of acetone, or 0.2 ml of vehicle only, was applied to the skin of the back of mice, and the particulate fraction of the skin was isolated at various times. Thereafter, the specific [^{3}H]TPA binding per 100 μg of particulate fraction was measured. As Fig. 3 shows, the specific binding of [^{3}H]TPA, expressed as a percentage of that in untreated skin, decreased immediately after application of EGCG, and remained at the reduced level for at least 120 min. This phenomenon, named modulation of phorbol ester receptors in mouse skin, has also been observed after application of a

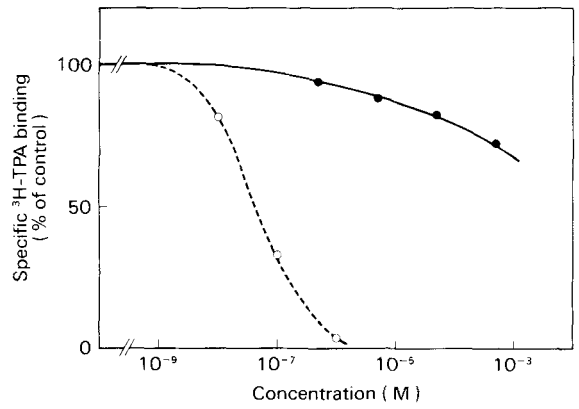

Figure 2. Effects on the specific binding of 4 nM [^{3}H]TPA of EGCG (●——●) and unlabelled TPA (○--○).

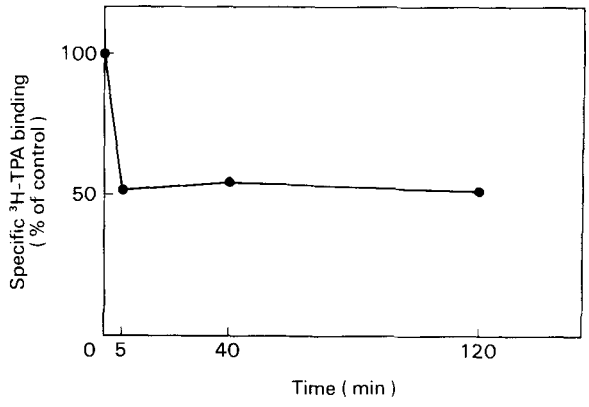

Figure 3. Time-course of the effect of EGCG application on specific [^{3}H]TPA binding to a particulate fraction.

calmodulin antagonist, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and quercetin (Horiuchi *et al.*, 1986; Nishino *et al.*, 1984). As we reported previously, this modulation of phorbol ester receptors is associated with the mechanism of the antitumor promoting activities of W-7 and quercetin.

Since phorbol ester receptors are known to be Ca^{++}-activated, phospholipid-dependent protein kinase (protein kinase C) (Nishizuka, 1984), we studied whether EGCG inhibited the phosphorylation of H1 histone by protein kinase C *in vitro*. The results in Fig. 4, expressed as percentages of ^{32}P-incorporation into H1 histone by protein kinase C with 2.2 μM of teleocidin, show that EGCG inhibited the activation of protein kinase C dose-dependently, and that 1.4 μM EGCG caused 50% inhibition. Thus it was 10 times more effective than quercetin.

From the above evidence, EGCG was thought to have antitumor promoting activity in mouse skin. Therefore, we tested it in a two-stage carcinogenesis experiment with DMBA plus teleocidin. In week 25, the percentage of tumor-bearing mice in the group treated with DMBA plus teleocidin was 53% whereas that in the group treated with DMBA plus teleocidin and EGCG was 13%, and the average number of tumors per mouse was 2.1 and 0.1, respectively (Fig. 5). No tumors developed in groups treated with DMBA alone, or teleocidin plus EGCG up to week 25. Thus EGCG possesses antitumor promoting activity against teleocidin in mouse skin.

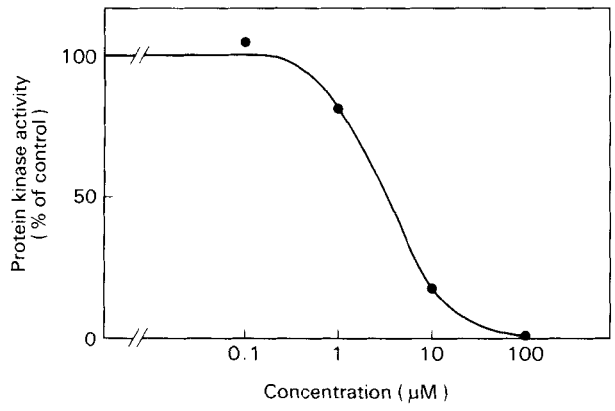

Figure 4. Inhibition by EGCG of the activation of protein kinase C by 2.2 μM teleocidin.

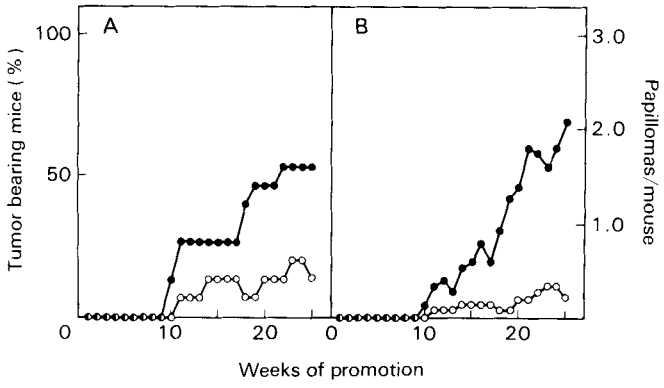

Figure 5. Inhibition by EGCG of tumor promotion by teleocidin. DMBA plus teleocidin (●——●), and DMBA plus teleocidin and EGCG (○--○).

Okuda *et al.* (1983) reported that EGCG inhibits lipid peroxidation in rat liver mitochondria stimulated by adenosine 5′-diphosphate and ascorbic acid. Inhibition of lipid peroxidation is not well correlated with anti-tumor promoting activity in mouse skin, but some anti-tumor promoters, such as flavonoids and tannins, are known to act as radical scavengers (Fujita *et al.*, 1987). One cup of green tea infusion often contains 100–200 mg of polyphenolic constituents which are thought to be tannins judging from their relative astringency, and relative affinity to methylene blue (Okuda *et al.*, 1985) and their behavior on HPLC. EGCG is one of the main constituents of green tea, and also of partly fermented tea, oolong tea, in which some of the EGCG is converted to condensates. Black tea contains various further condensates of EGCG due to its more advanced fermentation.

It is noteworthy that EGCG and extracts of tea inhibit the mutagenicity of Trp-P-1 and MNNG (Okuda *et al.*, 1984). Moreover, Okuda *et al.*, (1984) found by the preincubation method using *Salmonella typhimurium* TA 98 and TA 100 that tannins interfere with the activity of direct-acting mutagens, such as *N*-OH-Trp-P-2 and benzo(a)pyrene diol epoxide. Therefore, EGCG in green tea inhibits both mutation and tumor promotion. Vital statistics from the Ministry of Health and Welfare (1984) show that the mortality rates from total cancer and stomach cancer were significantly lower in Shizuoka prefecture, an area producing Japanese tea plants, than in other Japanese prefectures. Therefore, EGCG in Japanese green tea may play a role in preventing cancer formation, especially cancers of the gastro-intestinal tract, such as esophageal, stomach and colon cancers in humans. We calculated that in Japan a tea-lover may take about 1 g of EGCG per day in green tea. Further experiments are necessary on whether EGCG actually inhibits tumor promotion in the gastro-intestinal tract, and whether it is effective in chemoprevention.

Acknowledgements

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