Anticarcinogenic Activity of Green Tea Polyphenols

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The main physiologically active polyphenol in green tea extract is (−)-epigallocatechin gallate (EGCG). Green tea extract has an advantage over EGCG as a cancer chemopreventive agent for humans, as is apparent from the Japanese custom of injesting green tea on a daily basis. Green tea extract similarly inhibited protein kinase C activation by teleocidin, a tumor promoter, as did EGCG. In addition, EGCG and green tea extract showed inhibitory effects on the growth of lung and mammary cancer cell lines with similar potencies. An experiment using the estrogen-dependent MCF-7 cell line showed the mechanisms of action of these compounds to be inhibiting the interaction of estrogen with its receptors. Considering our previous results of a single application of EGCG to mouse skin inhibiting the specific binding of ^{3}H-12-*O*-tetradecanoylphorbol-13-acetate (^{3}H-TPA) and ^{3}H-okadaic acid, we postulated that EGCG and compounds in green tea extracts would block the interaction of tumor promoters, hormones and growth factors with their receptors: a kind of sealing effect. The sealing effect would account for reversible growth arrest, and may be induced by various kinds of compond.

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Introduction

Tea leaves contain various kinds of polyphenols which are also called tea tannins. There is evidence that the main constituent of the polyphenols, (−)-epigallocatechin gallate (EGCG), is a potential cancer chemopreventive agent for use among the general human population.[1, 2] In 1987, we first reported applications of EGCG prior to the tumor promoter, teleocidin, to inhibit tumor promotion on mouse skin initiated by 7,12-dimethylbenz(a)anthracene (DMBA).[3] Inhibitory effects of EGCG on various systems of chemical carcinogenesis have been further studied in the U.S.A. and Japan.[4–9] In addition, Taniguchi and his colleagues recently reported the peroral administration of EGCG to inhibit metastasis of mouse B16 melanoma cell lines into the lung tissue of mice.[10]

One advantage of studying the effects of EGCG lies in knowing that the Japanese customarily injest EGCG in green tea every day. It is thought to be a non-toxic agent. It is, however, expensive to isolate EGCG in a pure form and to develop it as a cancer preventive agent. If the mixture of polyphenols contained in green tea extract has a strong inhibitory activity similar to EGCG, it would be more practical to use green tea extract for cancer prevention in humans. In the present studies we therefore gave particular attention to green tea extracts containing polyphenols.

The mechanism of action by which EGCG blocks tumor promotion has not been fully elucidated. We have shown EGCG dose-dependently to inhibit the activation of protein kinase C by teleocidin *in vitro*.[3] A topical application of EGCG on mouse skin immediately blocked the specific binding of ^{3}H-12-*O*-tetradecanoylphorbol-13-acetate (^{3}H-TPA) as well as that of ^{3}H-okadaic acid to their receptors in a particulate fraction of the skin. We think the EGCG inhibited carcinogenesis by blocking the receptors within cell membranes.[11] It was named

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'a sealing effect' of EGCG.[11] If the receptors were indeed blocked, EGCG can also be expected to inhibit the growth of cancer cells. The present paper reports that, like EGCG, a green tea extract inhibited protein kinase C (PKC) activation induced by the tumor promoter, teleocidin. EGCG and green tea extract also showed inhibitory effects on the growth of the two lung cancer cell lines, PC-9 and PC-14, and the two mammary cancer cell lines, MCF-7 and BT-20. Green tea extract showed almost as strong activity as EGCG in *in vitro* biochemical and cell culture systems. In addition, a sealing effect of EGCG on the cell membrane was confirmed by an experiment using the estrogen-dependent mammary cancer cell line, MCF-7, resulting in inhibition of estrogen-receptor interaction by EGCG. Thus, EGCG physiologically retards the growth of cancer cells.

Materials and Methods

Materials

The EGCG preparation contained EGCG (85%), (−)-epicatechin (10%) and (−)-epicatechin gallate (5%), as reported previously.[3] The green tea extract, which was kindly provided by Dr. Douglas A. Balentine, U.S. Tea Association, was prepared by extracting green tea leaves (12.5g) with 500 ml boiled water, as reported previously.[5] The EGCG content of green tea extract solids is approximately 15%. Other polyphenols, such as (−)-epicatechin, (−)-epicatechin gallate, (−)-epigallocatechin, and (+)-catechin along with caffein are also present in green tea extract, as reported previously.[5]

Cells

The PC-9 and PC-14 lung cancer cell lines were provided by Dr. Nagahide Saijo of the National Cancer Center Research Institute, Tokyo. The cells were grown in RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo) supplemented with 10% fetal bovine serum (Gibco Laboratories, Tokyo), penicillin (100 U/ml) and streptomycin (100 μg/ml).[12] The MCF-7 and BT-20 mammary cancer cell lines were obtained from Dr. Ken Yamaguchi at the National Cancer Center Research Institute, Tokyo. The cells were grown in Dulbecco's modified Eagle medium (DMEM) (Nissui), supplemented with 10% fetal calf serum (Gibco), *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethane-sulfonic acid] (HEPES; 15 mM), insulin (6 ng/ml), penicillin (50 U/ml) and streptomycin (50 μg/ml). All cell lines were maintained in a humidified incubator with a 5% CO_2 atmosphere in air at 37°C.

Inhibition of Teleocidin-induced Protein Kinase C Activation by EGCG and Green Tea Extract

Protein kinase C (PKC) was partially purified from mouse brain by DEAE-cellulose column chromatography. PKC activation was induced by 2.2 μM teleocidin in the presence of phosphatidylserine and Ca^{++}, as described previously.[13] Various concentrations of EGCG and green tea extract were added to the assay mixture to determine the inhibition of PKC activation.

Inhibition of Cell Growth by EGCG and Green Tea Extract

Cells (1×10^3) were plated in 0.1 ml medium in a humidified 5% CO_2 atmosphere at 37°C. After 24 h incubation, the cells were treated with various concentration of either EGCG or green tea extract dissolved in water for 72 h. The number of cells was determined by the 3-(4,5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay.[12] The optical density (O.D.) of formazan crystals dissolved in 200 μl dimethyl sulfoxide (DMSO) was measured at 577 and 630 nm using an ELIZA Analyzer ETY-96 (Toyosokki Co. Ltd., Tokyo). The fractional absorbance was calculated as:

$$\frac{\text{Mean absorbance in five test wells} - \text{absorbance in background well}}{\text{Mean absorbance in five control wells} - \text{absorbance in background well}} \times 100$$

Inhibition of Estrogen Dependency by EGCG

An estrogen-dependent MCF-7 cell line (2×10^3 cells) was cultured in DMEM medium (Nissui), supplemented with HEPES (15 mM), bovine insulin (6 ng/ml), glutamine (2 mM), penicillin (50 V/ml), streptomycin (50 μg/ml) and 10% fetal calf serum which had been filtered through a charcoal column to minimize the estrogen content. After 24 h incubation, the cells were incubated with 10 nM estrogen alone, 10 nM estrogen plus 1, 10, 100, 200, or 500 μM EGCG, or EGCG without estrogen, at the same concentrations. The number of cells was determined by MTT assay 6 days after treatment. The fractional absorbance in the MTT assay for untreated cells at 6 days was expressed as 100%.

Results and Discussion

Inhibition of Teleocidin-induced PKC Activation by EGCG and Green Tea Extract

EGCG and green tea extract dose-dependently inhibited PKC activation, which was induced by 2.2 μM teleocidin. The inhibitions were expressed as percentages of ^{32}Pi-incorporation into histone H1, as shown in Fig. 1. The concentrations of 50% inhibition (IC_{50}) were 2.5 μg/ml (5.5 μM) for EGCG

and 10.5 μg/ml for the green tea extract. Thus, at gram equivalent weights, EGCG was only four times more effective than the green tea extract. The results indicated the green tea extract to contain inhibitory substances, in addition to EGCG. Although not much is known about how EGCG inhibits PKC activation by a tumor promoter, green tea extract is expected to act similarly to EGCG. As reported previously, a single application of EGCG to mouse skin blocked the specific binding of ^{3}H-TPA as well as that of ^{3}H-okadaic acid to their receptors in the cell membrane,[11] suggesting EGCG to inhibit the interaction of a tumor promoter with membrane components. We believe that tea polyphenols also inhibit these interactions, resulting also in the inhibition of protein kinase C activation.

Inhibition of Cell Growth by EGCG and Green Tea Extract

Effects on Lung Cancer Cell Lines. The inhibitory effects of EGCG and green tea extract on the growth of PC-9 and PC-14 cell lines are shown in Fig. 2. EGCG and green tea extract dose-dependently inhibited the growth of two cell lines. At gram equivalent weights, the IC_{50} values of EGCG were similar to those of the green tea extract for the two cell lines (Table I). Interestingly, EGCG was approximately 200 times less effective than adriamycin, the IC_{50} values being 41.5 μM for EGCG and 0.17 μM for adriamycin to PC-9 cells (Fig. 2). Similar results with stomach cancer cell lines were obtained with EGCG (S.-J. Kim *et al.*, manuscript in preparation). Recently two research groups in the United States reported green tea infusion effectively to inhibit *N*-nitrosodiethylamine- and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced tumorigenesis in the lungs of female A/J mice.[5] They reported EGCG to inhibit the tumorigenesis through the inhibition of metabolic activation of the carcinogen and through antioxidant activity, but it is not yet clear how EGCG acts on cells in lung tissue.

Effects on Mammary Cancer Cell Lines. The inhibition of the growth of MCF-7 and BT-20 cell lines by EGCG and green tea extract was determined by MTT assay, after 72 h treatment. Similarly to the results with lung cancer cell lines, EGCG and green tea extract inhibited the growth of two cell lines with the same degree of potency (Table I). It is important to note that EGCG and green tea extract, both of which mediate through similar mechanisms of action, were approximately two hundred and one thousand times less effective, respectively, than potent anti-cancer agents, based on their IC_{50} values (data not shown). We had previously tested the inhibition of spontaneous mammary tumor develop-

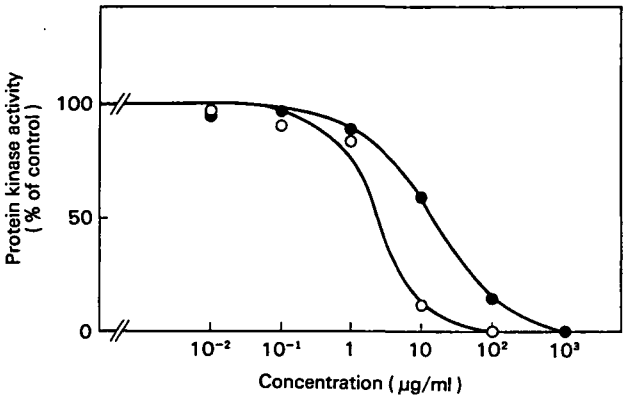

Fig. 1. Inhibition of teleocidin-induced protein kinase C activation by EGCG and green tea extract. EGCG (○) and green tea extract (●).

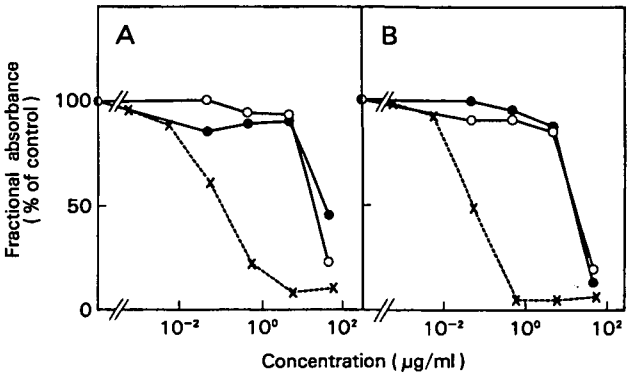

Fig. 2. Inhibition of growth of two lung cancer cell lines by EGCG and green tea extract. (A) PC-9 cell line and (B) PC-14 cell line, EGCG (○), green tea extract (●), and adriamycin (×).

Table I. Inhibition of Growth of Cancer Cell Lines by EGCG and Green Tea Extract

	EGCG IC_{50} (μg/ml)	Green Tea Extract IC_{50} (μg/ml)
Lung cancer cell line		
PC- 9	19.0	36.0
PC-14	16.0	16.0
Mammary cancer cell line		
MCF- 7	420.0	420.0
BT-20	350.0	350.0

EGCG, (−)-epigallocatechin gallate; IC_{50}, concentration of an agent reducing the fractional absorbance of the 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay to 50%.

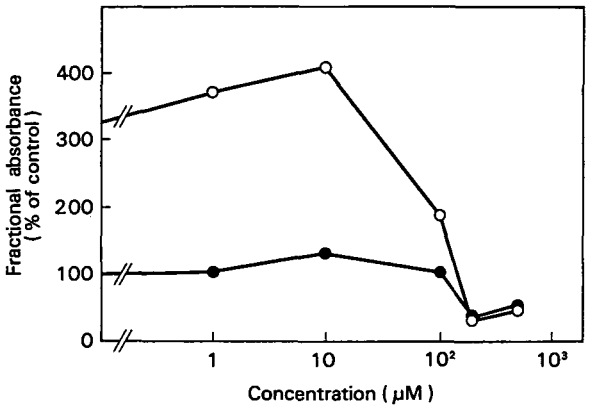

Fig. 3. Inhibition of estrogen dependency of MCF-7 cells by EGCG. Treatment with 10 nM estrogen (○) and without estrogen (●).

ment in SHN mice carrying Mouse Mammary Tumor Virus (MMTV) by the administration of 0.1% EGCG in drinking water.[14] The development of mammary tumors was not, however, inhibited. In this case, EGCG was thought to have poor bioavailability in mammary tissue.

Inhibition of Estrogen Dependency by EGCG

The MCF-7 cell line clearly showed an estrogen-dependency (Fig. 3). The numbers of cells that had been treated with 10 nM estrogen alone had increased to 326% 6 days after treatment, whereas those of cells without estrogen were expressed as 100%. Thus, treatment with 10 nM estrogen increased cell numbers by over three times in 6 days. Treatment with EGCG at concentrations of 100, 200 and 500 μM clearly inhibited the growth of estrogen-dependent cells in a medium containing 10 nM estrogen. The inhibitory effects of EGCG on cell growth were dose-dependent, that is, at 100 μM EGCG (45.9 μg/ml) the fractional absorbances in the MTT assay of the cells with estrogen were reduced from 187 to 0% and, of the cells without estrogen, from 104 to 0% at 200 μM EGCG (91.8 μg/ml). At 200 μM EGCG, most of the cells were damaged (Fig. 3). The results indicated that EGCG in the growth medium blocked the specific binding of estrogen to the estrogen receptor of the MCF-7 cells.

Preliminary results indicated the specific estrogen binding to the cells to be inhibited by treatment with EGCG at concentrations of 50 and 100 μM (data not shown). EGCG is thought to bind to the receptor molecule through the general property of polyphenolic compounds. The protein binding property results in the astringent flavor of tea beverages.[15] Although EGCG is a potent antioxidant, we do not yet know if the antioxidant activity of EGCG is related to the EGCG-protein interaction.

Previously, we reported that quercetin and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), a calmodulin antagonist, inhibited teleocidin tumor promotion on mouse skin initiated by DMBA.[16, 17] In the experiments, we also found a single application of compounds to mouse skin immediately to block the specific binding of ^{3}H-TPA to its receptors. The time-courses of the effects were similar to those of EGCG. We therefore assume that various kinds of compounds interfere with the interaction of tumor promoters, for example, estrogen and other growth factors, with their receptors, resulting in the inhibition of cell growth through sealing effects on the cell membrane. This is one of the reversible growth arrests useful in cancer chemoprevention.

Acknowledgments

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