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Mechanisms of Growth Inhibition of Human Lung Cancer Cell Line, PC-9, by Tea Polyphenols

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(−)-Epigallocatechin gallate (EGCG), the main constituent of green tea, and green tea extract show growth inhibition of various cancer cell lines, such as lung, mammary, and stomach. We studied how tea polyphenols induce growth inhibition of cancer cells. Since green tea extract contains various tea polyphenols, such as EGCG, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC), the inhibitory potential of each tea polyphenol on the growth of a human lung cancer cell line, PC-9 cells, was first examined. EGC and ECG inhibited the growth of PC-9 cells as potently as did EGCG, but EC did not show significant growth inhibition. The mechanism of growth inhibition by EGCG was studied in relation to cell cycle regulation. Flow cytometric analysis revealed that treatment with 50 μM and 100 μM EGCG increased the percentages of cells in the G_2-M phase from 13.8% to 15.6% and 24.1%, respectively. The DNA histogram after treatment with 100 μM EGCG was similar to that after treatment with genistein, suggesting that EGCG induces G_2-M arrest in PC-9 cells. Moreover, we found by microautoradiography that [^{3}H]EGCG was incorporated into the cytosol, as well as the nuclei. These results provide new insights into the mechanisms of action of EGCG and green tea extract as cancer-preventive agents in humans.

Key words: EGCG — [^{3}H]EGCG — G_2-M arrest — Tea polyphenol — Cancer prevention

(−)-Epigallocatechin gallate (EGCG), the main constituent of green tea polyphenols, and green tea extract are acknowledged cancer-preventive agents in humans in Japan.[1–4] Their anticarcinogenic activities have been established in various organs, such as rat glandular stomach,[5] mouse duodenum,[6] rat colon,[7] hamster pancreas,[3] mouse liver,[8] mouse lung,[9] mouse skin,[1–3, 10] and metastasis in the lung of mice.[11] Regarding the mechanisms of action of tea polyphenols, we previously showed that topical application of EGCG to mouse skin inhibited the specific binding of both [^{3}H]12-*O*-tetradecanoylphorbol-13-acetate and [^{3}H]okadaic acid to a particulate fraction of mouse skin, suggesting that EGCG blocks the interaction of a tumor promoter with its receptor.[12] This was named the "sealing effect of EGCG."[12]

EGCG and green tea extract inhibited the growth of various cancer cell lines, such as PC-9, PC-14, MCF-7 and KATO III cells.[13] Flavonoids, such as quercetin and genistein, were reported to inhibit cell growth by inhibiting cell cycle progression at the G_1-S or G_2-M phase, respectively.[14–16] Since green tea extract contains various tea polyphenols, such as EGCG, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG) and (−)-epicatechin (EC), we firstly examined the potential of each tea polyphenol to inhibit the growth of PC-9 cells, and secondly, how EGCG influences the cell cycle regulation of PC-9 cells.

In addition to the "sealing effect" of EGCG, we previously reported that EGCG specifically inhibited estrogen-dependent growth of MCF-7 cells, mediated through inhibition of the receptor-binding of estrogen.[13] Since all the results suggest that EGCG inhibits the interaction of tumor promoters, hormones, cytokines, and growth factors with their receptors in the membrane as well as in the cytosol of cells, we think EGCG is multifunctional. If this is so, it is not yet clear whether the "sealing effect" of EGCG is the primary event for growth inhibition of various cancer cell lines. In order to analyze the effects of EGCG in more detail, we studied whether EGCG is incorporated into the cells, using [^{3}H]EGCG in cultured cells.

MATERIALS AND METHODS

Materials EGCG was purified from Japanese green tea leaves; its purity was 99.7%.[1] Other tea polyphenols, EGC, EC and ECG were purchased from Funakoshi Co., Ltd., Tokyo. [^{3}H]EGCG (48.1 GBq/mmol) was labeled with tritium gas.

Cells The human lung cancer cell line PC-9 was 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 (Biocell Laboratories, Victoria, Australia) and kanamycin.[17]

Growth inhibition of PC-9 cells PC-9 cells (2×10^5/ml) in 24-well plates were incubated with each of the tea

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polyphenols at various concentrations for 3 days. The number of viable cells was counted by use of the trypan blue dye exclusion test; the number of non-treated cells was taken as 100%. The results were obtained from three different experiments.

Cell cycle analysis by flow cytometry PC-9 cells (2.5×10^5/ml) were incubated with 50 and 100 μM EGCG, or 50 μM genistein as a control for 24 h. Then, they were washed with phosphate-buffered saline (PBS) and fixed with ice-cold 70% ethanol solution for 30 min at 4°C. After filtration through 50 μm nylon mesh, the cells were fixed and stained with 50 μg/ml propidium iodide in the presence of 0.1% RNase. DNA contents of about 6,000 stained cells in each group were measured with an EPIC Elite flow cytometer (Coulter Co., Ltd., Hialeah, FL).

Mitotic index PC-9 cells were incubated with 100 μM EGCG or 50 μM genistein for 24 h, and the number of mitotic cells in Giemsa-stained cells was counted microscopically.

[^{3}H]EGCG incorporation into PC-9 cells PC-9 cells (1×10^6/ml) were incubated with 100 μM [^{3}H]EGCG (4×10^7 dpm) for one or 24 h, and control cells were untreated. The cells were washed 3 times with cold PBS, fixed on slide glasses, and then coated with Hypercoat emulsion (Amersham, Buckinghamshire). After 13 days in a dark room at 4°C, the slides were treated with D-19 developer (Eastman Kodak Co., Rochester, NY) and then stained with Giemsa solution (Wako Chemical Co., Tokyo). Silver grains were examined under a microscope.

RESULTS AND DISCUSSION

Growth inhibition of PC-9 cells by various tea polyphenols Fig. 1 shows the structures of EGCG, EGC, EC, and ECG. EGCG, EGC and ECG inhibited the growth of PC-9 cells dose-dependently (Fig. 2), whereas EC, which does not contain a galloyl moiety, did not significantly inhibit the cell growth. The concentrations giving 50% growth inhibition (IC_{50} values) were 140 μM for EGCG, 275 μM for EGC and 78 μM for ECG. These results supported our previous finding that green tea extract, a mixture of tea polyphenols, inhibits the growth of PC-9 cells as potently as does EGCG alone. Treatment with 100 μM EGCG for 3 days did not reduce the cell number from the beginning of the experiment, and the cell number increased after removal of EGCG from medium. The results suggest that the effects of tea polyphenols on PC-9 cells are cytostatic rather than cytotoxic.

Effects of EGCG on cell cycle regulation The flow cytometric analysis revealed that the percentages of non-treated PC-9 cells in G_1, S and G_2-M phases were 35.8%, 50.4%, and 13.8%, respectively (Fig. 3 and Table I). Treatment with 50 μM and 100 μM EGCG increased the percentages of G_2-M phase cells from 13.8% to 15.6% and 24.1%, again showing a dose-dependence. Treatment with 50 μM genistein as a positive control increased the number of cells in the G_2-M phase from 13.8% to 24.4%. It is important to note that the DNA histogram

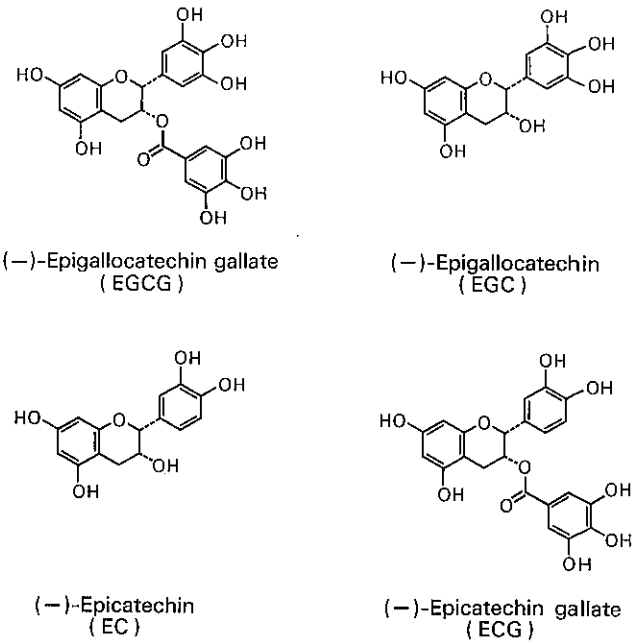

Fig. 1. Structures of tea polyphenols.

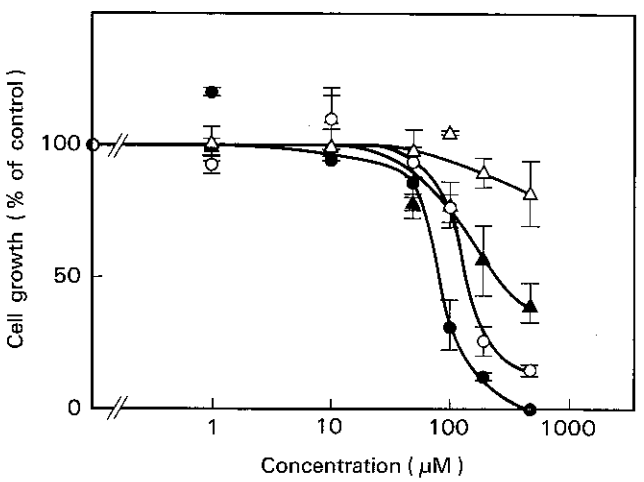

Fig. 2. Growth inhibition of PC-9 cells by tea polyphenols. PC-9 cells (2×10^5/ml) were treated with various concentrations of ECG (●), EGCG (○), EGC (▲) or EC (△) for 3 days. The number of viable cells was counted by means of the dye exclusion method.

Table I. Analysis of Cell Cycle Progression and Mitotic Index

Culture	(μM)	Frequency (%)			Mitotic index ratio of mitotic cells (%)
		G_1	S	G_2–M	
Control		35.8	50.4	13.8	11.16
EGCG	50	34.2	50.2	15.6	n. d.
EGCG	100	22.9	53.0	24.1	7.25
Genistein	50	28.9	46.7	24.4	0.81

after treatment with 100 μM EGCG was similar to that after treatment with genistein, which induces G_2-M arrest mediated through inhibition of protein tyrosine kinase.[15, 18] Whether G_2-M arrest is induced only by inhibitors of protein tyrosine kinase, and whether EGCG has inhibitory activity against protein tyrosine kinase have not yet been studied. The ratio of mitotic cells was examined by incubation with 100 μM EGCG or 50 μM genistein for 24 h. The mitotic index of non-treated PC-

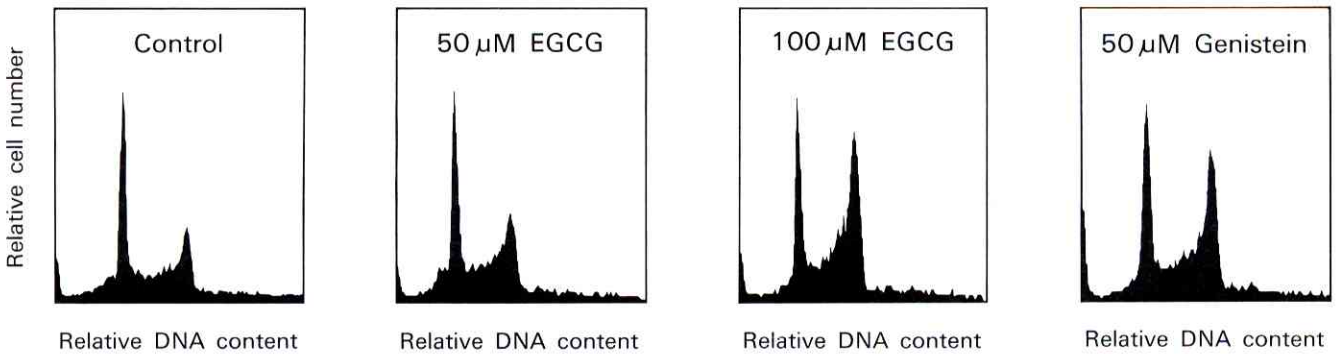

Fig. 3. Effects of EGCG on cell cycle regulation. PC-9 cells (1×10^6) were incubated with EGCG or genistein for 24 h. After propidium iodide staining, the DNA contents of about 6000 cells in each group were measured with an EPIC Elite flow cytometer.

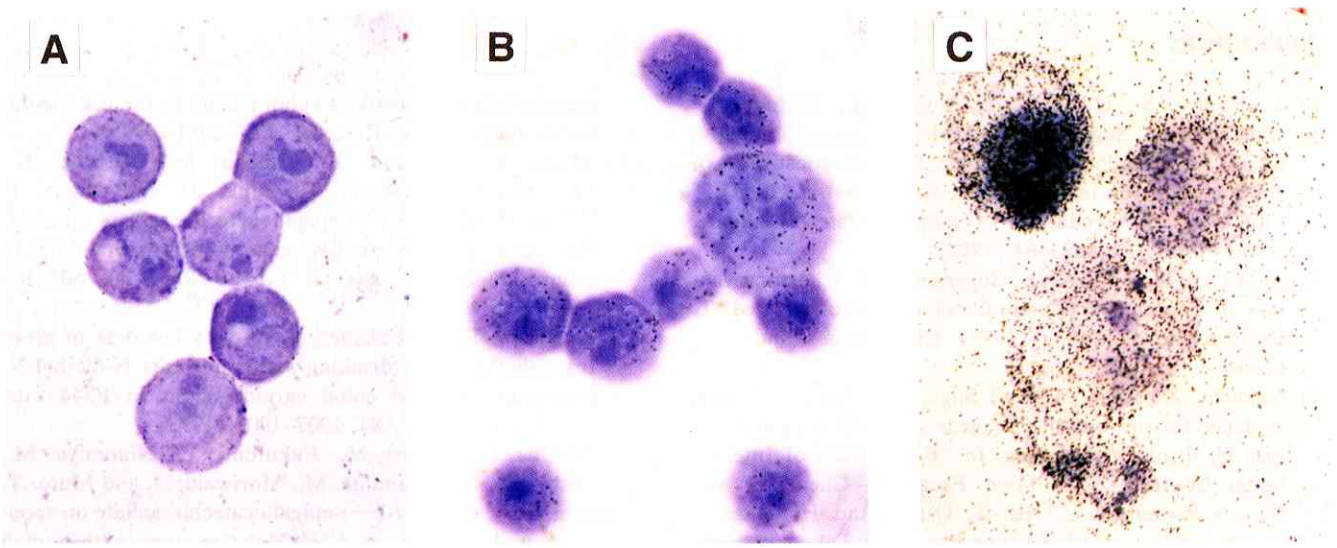

Fig. 4. Microautoradiography of PC-9 cells treated with [^{3}H]EGCG. PC-9 cells were incubated with [^{3}H]EGCG for 1 h and 24 h. Silver grains indicating radioactivity appeared in the membrane, cytosol and nuclei. A, Control; B, after incubation for 1 h; C, after incubation for 24 h.

9 cells was 11.16%, whereas those of EGCG-treated cells and genistein-treated cells were 7.25% and 0.81%, respectively. EGCG weakly decreased the mitotic index compared with genistein, suggesting that EGCG induces G_2-M arrest through a different mechanism from that of genistein. In addition, EGCG induced G_2-M arrest in a human stomach cancer cell line, KATO III, at concentrations of 60 to 200 μM, which were growth-inhibitory (data not shown). Thus, G_2-M arrest of cancer cell lines by EGCG was confirmed. However, EGC, EC and ECG did not show significant G_2-M arrest, indicating that each tea polyphenol induces a different pattern of G_1, S and G_2-M phase cells (data not shown). This point should be further investigated.

Incorporation of [^{3}H]EGCG into PC-9 cells Incorporation of [^{3}H]EGCG into PC-9 cells was determined up to 24 h. The radioactivity of the cells was 64,000 dpm after incubation for 1 h, and increased to 255,000 dpm after 24 h at 37°C, suggesting that the incorporation of radioactivity into PC-9 cells is time-dependent. The results imply that the cells incorporate EGCG at a rate of 1,300 pmol EGCG per 1×10^6 cells, upon incubation with 100 μM EGCG. We present the results with [^{3}H]EGCG, because EGCG is the main constituent of tea polyphenols. Since we have not yet labeled the other tea polyphenols, we have no direct evidence of their incorporation into the cells. However, incorporation of [^{3}H]EGCG was inhibited by excess amounts of unlabeled EGCG as well as ECG (data not shown), suggesting that other tea polyphenols are also incorporated into the cells. It is important to investigate further the incorporation of EC, because EC is an inactive compound.

Next, we studied the localization of [^{3}H]EGCG in the cells by microautoradiography. Fig. 4 shows silver grains in the treated cells 1 h and 24 h after incubation, indicating that the radioactivity appears in the cytosol as well as the nuclei. Numerous silver grains were found in membrane, cytosol and nuclei after incubation for 24 h. The number of silver grains in cells also increased time-dependently. We did not attempt to differentiate [^{3}H]-EGCG from its metabolites in cells. However, the results clearly indicate that radioactivity derived from [^{3}H]-EGCG was present in cytosol and nuclei, as well as the membrane. Since this is the first observation of this phenomenon, we believe the results provide an important clue for understanding the mechanisms of growth inhibition of cancer cells by EGCG.

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