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Effects of green, oolong and black teas and related components on the proliferation and invasion of hepatoma cells in culture

Guoying Zhang, Yutaka Miura & Kazumi Yagasaki*
Department of Applied Biological Science, Tokyo Noko University, Saiwaicho 3-5-8, Fuchu, Tokyo 183-8509, Japan
*(*Author for all correspondence)*

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Abstract

The effects of teas and related components on the proliferation and invasion of cancer cells were examined by employing both *in vitro* proliferation and invasion assay systems. Powdered green, oolong and black tea extracts dose-dependently inhibited proliferation and invasion of a rat ascites hepatoma cell line of AH109A but did not affect the proliferation of the normal mesentery-derived mesothelial cells (M-cells) isolated from rats; higher concentrations of powdered oolong and black teas could restrain the proliferation of another tumor cell line of L929. The AH109A cells were found to penetrate underneath the monolayer of M-cells in the presence of 10% calf serum. When each rat serum obtained at 0.5, 1, 2, 3 and 5 h after oral intubation of each tea extract was added to the culture media instead of calf serum at a concentration of 10%, both the invasion and proliferation of AH109A were significantly suppressed. These *ex vivo* results suggest the potential bioavailability of effective tea components in rats. Furthermore, (–)-epigallocatechin gallate, (–)-epicatechin gallate and (–)-epigallocatechin from green tea as well as the mixture of theaflavin and theaflavin gallates from black tea were shown to be the most effective components against the invasion and proliferation of AH109A. These results show that the inhibitory effects of the teas and related components against AH109A cells are due to the cell-specific and higher sensitivity of the cell line to tea components.

Introduction

The endless proliferation and metastasis of tumor cells are regarded as two biological characteristics of cancers. The agent that could effectively suppress the proliferation and/or metastasis of cancer cells possesses the potential to have the therapeutical effect on the patients with cancer. Metastasis is a series of processes by which malignant cells leave their primary site and spread to distant locations throughout the body. During the complicated process of metastasis, the invasion is the most important and characteristic step. In theory, an agent inhibiting the invasion of cancer cells could suppress the metastasis of cancer. There have been not a few reports about the inhibitory effects of teas and tea components against the proliferation of cancer cells such as breast, colon, lung, prostate, liver, forestomach cancer cells (Hirose et al., 1993; Nishida et al., 1994; Okabe et al., 1997; Valcic et al., 1996; Wang et al., 1992a; Wang et al., 1992b), and so forth. However, there are few reports about the inhibitory effects of teas and the related components against the invasion and matastasis of cancer (Sazuka et al., 1995; Taniguchi et al., 1992). Tea (*Camellia sinensis*) is one of the most popular beverages consumed worldwide. Tea could be divided into three major kinds: black, oolong and green teas, depending on the process of tea manufacture. The black tea, oolong tea and green tea are also called as fermented tea, half-fermented tea and unfermented tea, respectively, because they are separately made by the so called fermentation, partial fermentation and non-fermentation during the processes of tea manufacture. For the three kinds of teas, there are great differences among the tea con-

stituents even if they are manufactured by the same material of fresh tea leaves.

Therefore, it is necessary to conduct a deep study on the effects of green, oolong and black teas on cancer and the mechanisms of action of the teas. From therapeutical standpoint, we here studied the effects of powdered green tea (PGT), powdered oolong tea (POT) and powdered black tea (PBT) extracts and the related components on the proliferation and invasion of a rat ascites hepatoma cell line of AH109A, using the successful *in vitro* and *ex vivo* culture models (Miura et al., 1997). In the present study, we have demonstrated that the PGT, POT and PBT extracts could effectively inhibit both the *in vitro* and *ex vivo* proliferation and invasion of AH109A cells, and also screened out the related effective components against both the proliferation and invasion of AH109A cells.

Materials and methods

Preparation of extracts from powdered green tea (PGT), powdered oolong tea (POT) and powdered black tea (PBT) and the solution of each tea components

PGT, POT and PBT were generously provided by Yamato Tea Co., Ltd, Nara, Japan. Two grams of PGT, POT and PBT were respectively extracted by 100 ml of boiling water for 3 min. After being sterilized by filtration, each tea extract was stored at –20 °C for later experiments. For the *ex vivo* experiments, the obtained solution of each tea extract was evaporated to dryness. The dried materials were redissolved in 10 ml water (that is, 10 fold concentration). (–)-Epigallocatechin gallate (EGCG), (–)-epicatechin gallate (ECG), (–)-epigallocatechin (EGC), (–)-epicatechin (EC) and theophylline (TPL) from green tea as well as the mixture of theaflavin and theaflavin gallates (TFs) from black tea were respectively dissolved in DM-160 medium (Kyokuto Pharmaceutical Co., Tokyo, Japan), while TPL was dissolved in 0.5% dimethyl sulfoxide (Wako Pure Chemical Industries Co. Osaka, Japan) and then mixed with DM-160 medium. All the tea components were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The chemical structures of these tea components are shown in Figure 1.

Primary culture of mesentery-derived mesothelial cells (M-cells) and culture of L929 mouse fibroblasts

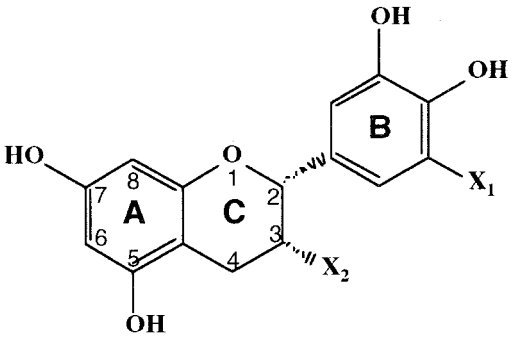
The abdominal cavity of a male Donryu rat (5 weeks old, obtained from NRC Haruna, Gunma, Japan) was opened and the mesentery was cut off with scissors. The obtained mesenteries were incubated in 0.25% trypsin (Sigma) in phosphate-buffered saline (PBS) at 37 °C for 20 min with gentle stirring. After treatments by digestion and filtration, the M-cells were seeded at a density of 1.2×10^5 cells/60 mmϕ culture dish with 2 mm grids (Corning, Cambridge, MA, U.S.A.) in 3 ml of 10% calf serum (CS) in DM-160 medium supplemented with streptomycin (100 μg/ml, Meiji-seika Kaisha, Tokyo, Japan) and penicillin (100 U/ml, Ban-yu Pharmaceutical Co., Tokyo, Japan). The cells were cultured under an atmosphere of 5% CO_2–95% humidified air at 37 °C to the confluent state by replacing culture media every other day and were used for the invasion assays described below (Miura et al., 1997). L929 cells were obtained from Riken Cell Bank, Tsukuba, Japan. L929 cells were seeded at 5×10^4 cells/well of a 24-well-plate (Falcon) in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) for the experiments (Komatsu et al., 1996). RPMI 1640 and FBS were obtained from Nissui Pharmaceutical Co., Tokyo and JRH Biosciences, Lenexa, KS, U.S.A., respectively.

Culture of AH109A hepatoma cells

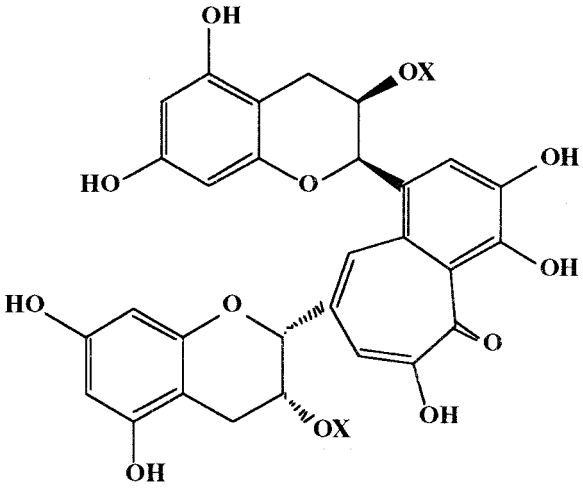
AH109A cells were provided by SRL (Tokyo, Japan), and were maintained in the peritoneal cavities of male Donryu rats. AH109A cells prepared from accumulated ascites could be cultured *in vitro* in 10% CS/DM-160 for up to 2 months. AH109A cultured for at least 1 week after preparing them from rat ascites were used for the assays.

In vitro proliferation assay

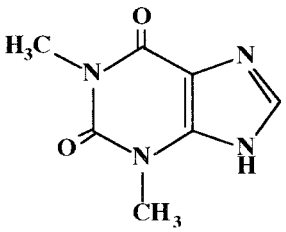
Effects of PGT, POT and PBT extracts on AH109A proliferation were examined by WST-1 method (Ishiyama et al., 1993; Ishiyama et al., 1996). For WST-1 assay, 5×10^3 AH109A cells were cultured for 44 h in each well of a 96-well-plate containing 10% CS/DM-160 in the absence or presence of PGT, POT and PBT extracts. WST-1 solution was then added and the cells were cultured for another 4 h. Viable cells can make WST-1 produce highly colored formazan dyes and the absorbance at 415 and 595 nm was measured with a microplate reader (BIO-RAD, Model



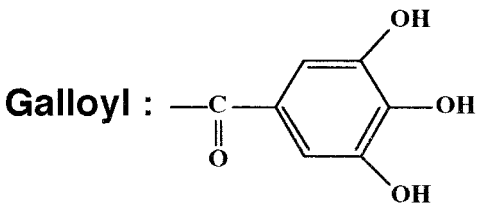
Catechins



Theaflavins (TFs)



Theophylline (TPL)



	X	X_1	X_2
(-)-Epicatechin (EC)		H	OH
(-)-Epicatechin-3-gallate (ECG)		H	O-galloyl.
(-)-Epigallocatechin (EGC)		OH	OH
(-)-Epigallocatechin-3-gallate (EGCG)		OH	O-galloyl.
Theaflavins (TFs)	galloyl./H		

Figure 1. Structures of catechins, TFs and TPL.

450, California, U.S.A.). The relative proliferation rate of AH109A was calculated between the control group treated with no tea and test groups treated with each tea extract, tea extract-loaded serum or tea component. Effects of PGT, POT and PBT extracts on proliferation of L929 cells and M-cells were examined by the MTT method (Miura et al., 1997). For MTT method, 5×10^4 L929 cells or M-cells were seeded on each well of a 24-well-plate for 24 h and then the nonadherent cells were washed with PBS. These adherent cells were respectively cultured for 44 h with 10% FBS/RPMI-1640 (L929 cells) and 10% CS/DM-160 (M-cells) in the absence or presence of the different concentrations of PGT, POT and PBT extracts. MTT solution was then added and the cells were cultured for another 4 h. The relative prolifertion rate of L929 cells or M-cells was evaluated after measuring the absorbance at 570 and 630 nm with U-1100 spectrophotometer (Hitachi, Tokyo, Japan).

In vitro invasion assay

When M-cells became confluent after 6–8 days culture, 2.4×10^5 AH109A cells were seeded on the M-cell monolayer with the 10% CS/DM-160 medium in the absence or presence of different concentrations of each tea extract or tea component and cultured for 48 h. The invasive activity of AH109A was measured by counting the invading AH109A cells and colonies under a phase contrast microscope. Usually at least 10 areas were counted and the invasive activity of the cells was indicated by the number of invading cells and colonies/cm^2 (Miura et al., 1997).

Ex vivo proliferation and invasion assay

Each concentrated PGT, POT and PBT extract was respectively intubated to male Donryu rats (5 weeks old) that had been fasted overnight at a dose of 1.0 ml/100 g body weight and blood was collected at 0, 0.5, 1, 2, 3 and 5 h after oral intubation. The sera were prepared, sterilized by filtration, and added to culture media at a concentration of 10% instead of calf serum for *in vitro* proliferation and invasion assays of AH109A. The proliferative and invasive activities of AH109A in the presence of these sera were respectively measured as described above.

Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA). When F value was significant ($P<0.05$), differences among the data were inspected at $P<0.05$ by Tukey's Q test.

Results

Effects of PGT, POT and PBT extracts on proliferation and invasion of AH109A cells

Figures 2A and B show the inhibitory effects of PGT, POT and PBT against the proliferation and invasion of AH109A cells in the *in vitro* experiment. The 0.02, 0.04, 0.08 and 0.16% of each powdered tea extract dose-dependently and significantly inhibited both the proliferation and invasion of AH109A cells.

Effects of PGT, POT and PBT extracts on proliferation of L929 cells and M-cells

Figures 3A and B show the effects of PGT, POT and PBT extracts on proliferation of L929 cells and M-cells. The 0.08 and 0.16% POT and PBT extracts dose-dependently inhibited the proliferation of L929 tumor cells, while PGT extract did not significantly inhibit the proliferation of L929 cells. The 0.02, 0.04, 0.08 and 0.16% of PGT, POT and PBT extracts did not suppress the proliferation of the normal rat M-cells except for 0.16% POT extract which restrained the proliferation of the M-cells.

Effects of PGT, POT and PBT extract-loaded rat sera on proliferation and invasion of AH109A cells

Figures 4A and B show the effects of PGT, POT and PBT extract-loaded rat sera against the proliferation and invasion of AH109A cells in the *ex vivo* experiment. PGT, POT and PBT extract-loaded rat sera obtained at 0.5, 1, 2, 3 and 5 h after oral intubation significantly suppressed the proliferation and invasion of AH109A cells except for the POT extract-loaded rat sera obtained at 0.5, 3 and 5 h after oral intubation, which did not significantly inhibit AH109A cell proliferation. Each tea extract-loaded rat serum obtained at 2 h after oral intubation had the strongest inhibitiory effects against both the proliferation and invasion of AH109A cells.

Effect of tea components on proliferation and invasion of AH109A cells

Figures 5A and B show the effects of tea components against the proliferation and invasion of AH109A cells

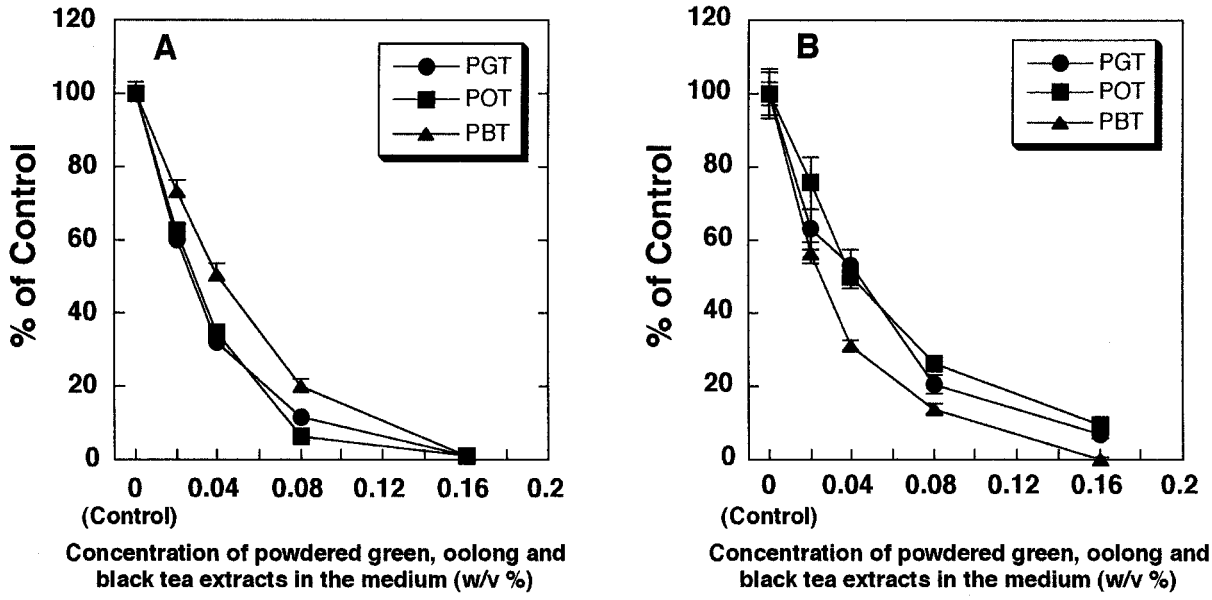


Figure 2. In vitro effects of powdered green tea (PGT), powdered oolong tea (POT) and powdered black tea (PBT) extracts on the proliferation and invasion of AH109A cells. Each extract from PGT, POT and PBT was added at the concentrations indicated in figure and their effects on the proliferation (A) and invasion (B) were respectively examined by WST-1 assay and invasion assay *in vitro* as described in the Section 'Materials and methods'. Each point and vertical bar represents the mean and SEM for 8 wells (A) or 10 areas (B). All data were inspected at $P<0.05$ by Tukey's Q test. This figure is the representative of at least 3 similar experiments.

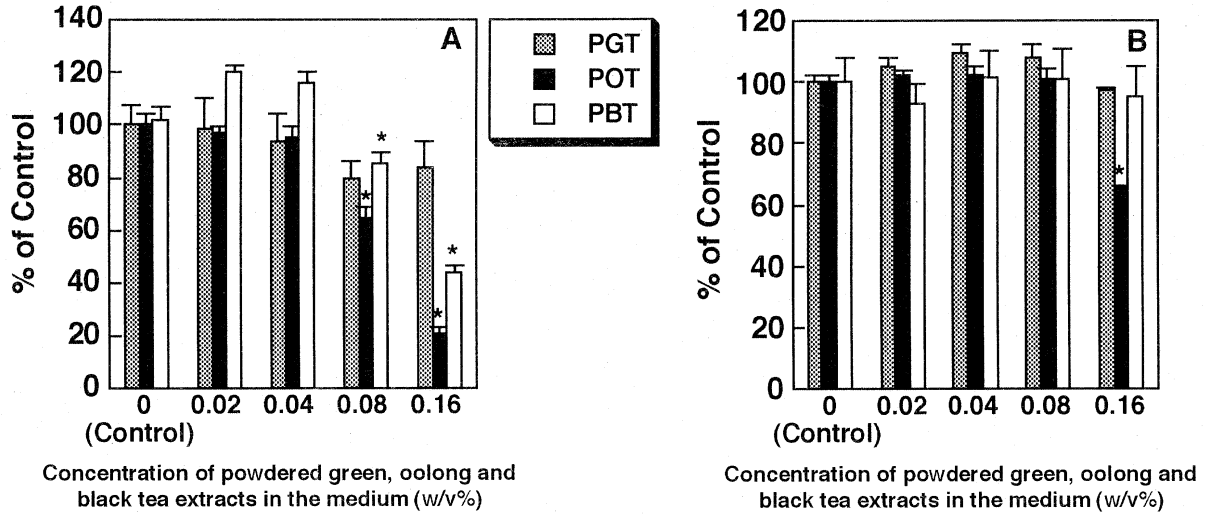


Figure 3. In vitro effects of powdered green tea (PGT), powdered oolong tea (POT) and powdered black tea (PBT) extracts on the proliferation of L929 tumor cells and M-cells. Each extract from PGT, POT and PBT was added at the concentrations indicated in figure and their effects on the *in vitro* proliferation of L929 cells (A) and M-cells (B) were examined by MTT assay described in the Section 'Materials and methods'. Each column and vertical bar represents the mean and SEM for 4 wells. All data were inspected at $P<0.05$ by Tukey's Q test. This figure is the representative of at least 3 similar experiments. Asterisk represents significant difference between the test group and control group.

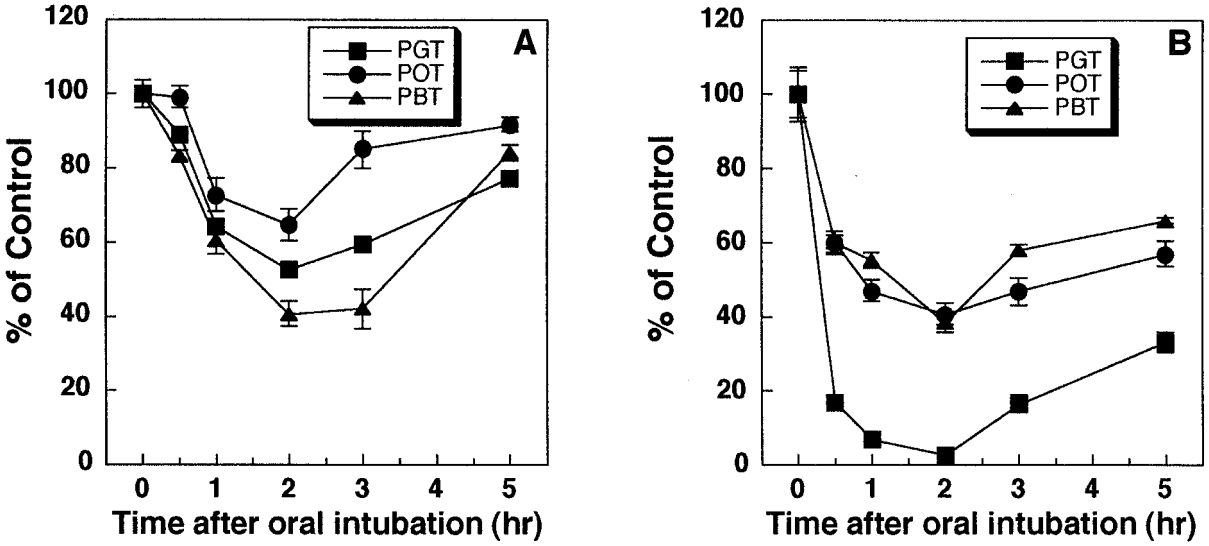


Figure 4. Ex vivo effects of powdered green tea (PGT), powdered oolong tea (POT) and powdered black tea (PBT) extracts on the proliferation and invasion of AH109A cells. Each tea extract-loaded rat serum was obtained at the indicated time after oral intubation. The effects on the *ex vivo* proliferation of AH109A by WST-1 assay (A), and on the *ex vivo* invasion of AH109A underneath the cultured M-cell monolayer (B) were examined as described in the Section 'Materials and methods'. Each point and vertical bar represents the mean and SEM for 8 wells (A) or 10 areas (B). All data were inspected at $P<0.05$ by Tukey's Q test. This figure is the representative of at least 3 similar experiments.

in the *in vitro* experiment. EGCG, ECG, EGC and TFs dose-dependently inhibited both the proliferation and invasion of AH109A cells. EC had weak inhibitory effect against the proliferation but was unable to suppress the invasion of AH109A cells. TPL did not restrain either the proliferation or invasion. The strongest inhibitory effects against both proliferation and invasion of AH109A cells were shown by EGCG. EGCG at 50 μM could effectively suppress about 60% of proliferation and 70% of invasion of AH109A cells.

Discussion

In the present study, we found that not only PGT but also POT and PBT extracts could effectively inhibit the *in vitro* proliferation and invasion of AH109A cells; the 0.02% of each extract had significantly inhibitory effects; the 0.04% of each extract showed strong inhibition; the 0.16% of each extract could almost completely suppress both the proliferation and invasion of AH109A cells (Figure 2). In order to make sure whether or not the inhibitory effects of PGT, POT and PBT extracts are selective to AH109A cells, we also tested the effects of these kinds of tea extracts on another tumor cell line of L929 and the normal rat M-cells used in the invasion assay system. It could be seen from Figure 3 that the L929 cell line is not so high sensitive to the three kinds of tea extracts and generally these extracts did not inhibit the proliferation of the normal rat M-cells. The results show that the inhibitory effects of PGT, POT and PBT against the proliferation and invasion of AH109A cells are due to the cell-specific and higher sensitivity of the cell line to these teas. As shown in Figure 4, each extract-loaded rat serum obtained after oral intubation significantly inhibited the proliferation and invasion of AH109A cells. Figure 4 also suggest that the effective concentrations of the related tea components or metabolites in blood could last at least 5 h for each tea extract against both the proliferation and invasion of AH109A cells except for POT extract which could last about 2 h for inhibitng AH109A proliferation, and could reach the peak at 2 h after oral intubation of each tea extract. Among all the tested tea components, EGCG, ECG, EGC and TFs have been screened out to be the most effective components against both the proliferation and invasion of AH109A cells (Figure 5). These effective tea components may be mainly responsible for the inhibitory effects against both the proliferation and invasion of AH109A cells by each tea extract-loaded rat serum in present study. A report has shown that EGCG, EGC and EC could be detected in rat plasma after rats drank decaffeinated green tea liquor (Lee et al., 1995). EGCG has been demonstrated to have the strongest inhibitory effects against both the

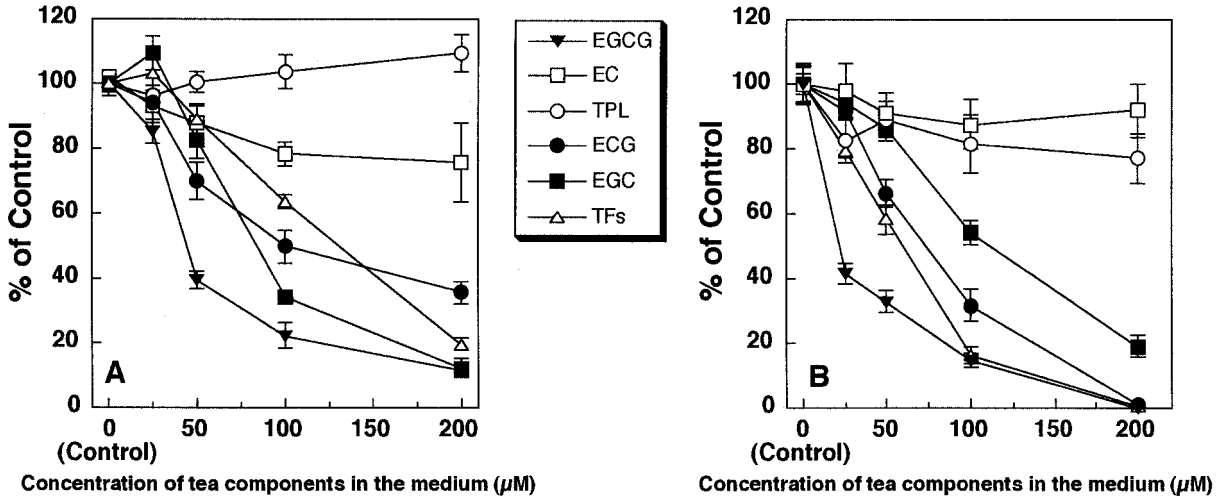


Figure 5. In vitro effects of EGCG, ECG, EGC, EC and TPL from green tea as well as the TFs from black tea on the proliferation and invasion of AH109A cells. Each tea component was added at the concentrations indicated in figure. The effects on the *in vitro* proliferation of AH109A by WST-1 assay (A), and on the *in vitro* invasion of AH109A underneath the cultured M-cell monolayer (B) were examined as described in the Section 'Materials and methods'. Each point and vertical bar represents the mean and SEM for 8 wells (A) or 10 areas (B). All data were inspected at $P<0.05$ by Tukey's Q test. This figure is the representative of at least 3 similar experiments.

proliferation and invasion of AH109A cells (Figure 5). Generally, the amount of EGCG in the black tea is about 30 to 50% of that in green tea. The amount of EGCG in the green tea accounts for about 5 to 15% of the total dry matter of green tea. The amount of TFs, the characteristic group of compounds in black tea, accounts for about 1 to 2% of the total dry matter of black tea. There is trace amount of TFs and about 3.5 to 12% of EGCG (total dry matter) in oolong tea. However, PBT, POT and PGT extracts have shown the similar inhibition of proliferation and invasion of AH109A cells *in vitro* experiment (Figure 2). These suggest that not only TFs but also other components in the PBT extract may contribute to the inhibitory effects against AH109A cells. Other components in POT extract also may have anticancer activity. Comparing with the result in the proliferation assay, the IC_{50} of TFs had greatly decreased in the invasion assay (Figure 5). This may suggest that TFs play a more important role in anti-invasion than anti-proliferation.

Then, what is the mechanisims of action of the teas against the proliferation and invasion of AH109A cells? Some relevant reports may give us some hints. A research result based on the effect of tea polyphenols on E1A-3Y1 cell-specific toxicity indicated that the inhibitory effect of polyphenolic lipoxygenase inhibitor (catechins) on E1A-3Y1 cells is due to their antioxidant activity (Mitsui et al., 1995). There are other reports that superoxide radicals potentiate the invasive activity of AH130 and that superoxide dismutase and catalase could inhibit AH130 invasion *in vitro* (Mukai et al., 1987, Shinkai et al., 1986). The involvement of arachidonic acid metabolites in tumor cell invasion and metastasis was also reported (Reich and Martin, 1996: Liu et al., 1996). From these results, it may be considered that the mechanisims of action of the catechins and theaflavins are at least referred to their antioxidative activity. Their antioxidative activity have been demonstrated by a lot of experiments (Okuda et al., 1983; Namiki and Osawa, 1986; Salah et al., 1995; Nanjo et al., 1996). It is reported that the free radical (diphenylpicrylhydrazyl radical) sacvenging activity of tea catechins are respectively in the order of EGCG>ECG>EC (Fourneau et al., 1996) and EGCG>EGC>ECG>EC (Nanjo et al., 1996), and the inhibitory activity of tea polyphenols on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase induction is in the order of EGCG>EGC=ECG>EC (Agarwal et al., 1992). In our present study, the anti-proliferative and anti-invasive activity of the catechins and theaflavins are respectively in the order of EGCG>EGC>ECG>TFs>EC and EGCG>TFs>ECG>EGC>EC (Figure 5). These results may suggset the relationship between the structure and the effect of the catechins and their oxidized derivatives TFs. The ortho-dihydroxyl group in the B ring of the catechins is necessary for their biological ef-

fects; the ortho-trihydroxyl group in the B ring and/or the galloyl group at the 3-position (Figure 1) increase the effects of catechins.

In conclusion, we have demonstrated that PGT, POT and PBT extracts could dose-dependently inhibit the *in vitro* and *ex vivo* proliferation and invasion of AH109A cells; EGCG, ECG, EGC and TFs are the most effective components against AH109A cells among all components screened. Our results show that the inhibitory effects of the teas and related components against AH109A cells are due to the cell-specific and higher sensitivity of the cell line to tea components. From these results, inhibitory effects of teas and their components on the *in vivo* growth and metastasis of AH109A are anticipated. Further studies are needed to clarify the *in vivo* effects and the precise mechanisms for anti-proliferative and anti-invasive activity of tea extracts and their components, and are now in progress in our laboratory.

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