Induction of Apoptosis and Cell Cycle Arrest in Cancer Cells by In Vivo Metabolites of Teas

Guoying Zhang, Yutaka Miura, and Kazumi Yagasaki

Abstract: The present study was conducted to determine in vivo possibilities of inducing apoptosis and cell cycle arrest in rat cancer cells by green, oolong, and black teas and also to further identify the mechanisms inhibiting cancer cell proliferation by the sera from tea-treated rats. The tea extracts from these three kinds of tea, the rat sera obtained after oral intubation of the tea extracts, and the tea polyphenolic compounds, (+)-epigallocatechin-3-gallate, (+)-epicatechin-3-gallate, (+)-epicatechin-3-gallate, and theaflavins, were used in the related tests. The extracts, the sera from the treated rats, and the polyphenolic compounds significantly inhibited the proliferation of a rat hepatoma cell line (AH109A) and murine B16 melanoma cells but not normal rat mesothelial (M) cells. (+)-Epicatechin exhibited synergistic effects with (+)-epigallocatechin-3-gallate, (+)-epicatechin-3-gallate, and theaflavins against AH109A cell proliferation. The fluorescence staining of the nuclei, electrophoresis detection of DNA fragmentation, and analysis of cell cycle indicated that the sera from the tea-treated rats, the tea extracts, and the polyphenolic compounds resulted in loss of viability, apoptosis, and cell cycle arrest at the G0 phase in AH109A and/or B16 cells, but not in normal M cells. Our results suggest that induction of apoptosis and cell cycle arrest may be important mechanisms of in vivo proliferation inhibition of AH109A and other cancer cells by these teas.

Introduction

Tea (Camellia sinensis) is one of the most widely consumed beverages in the world. Experimental and epidemiological studies have suggested the potentials of teas against some cancers (1–6). Our previous studies showed that green, oolong, and black tea extracts, the sera from rats treated with these teas, and related components inhibit the proliferation and invasion of a rat hepatoma cell line of AH109A (7,8). Interestingly, the three kinds of tea extract and the rat sera obtained after oral intubation of each extract have shown similar inhibitory effects against the proliferation and invasion of AH109A cells, despite great differences among the constituents in these teas (8). Recently, induction of apoptosis in some cancer cells by tea and certain tea components has been reported (9–11). Generally, the concentrations of the tea component (+)-epigallocatechin-3-gallate (EGCG) to induce apoptosis of cancer cell lines vary between ~87 and 2,000 μM (12). The level of EGCG, (+)-epigallocatechin (EGC), and (+)-epicatechin (EC) in blood was usually <60 ng/ml in rats after they drank decaffeinated green tea (13). The level of these tea components in human blood is generally <1 μg/ml (14,15). Such low concentrations in blood seem unlikely to inhibit the proliferation of cancer cells by inducing apoptosis and cell cycle arrest. To clarify the in vivo possibilities of such action in cancer cell lines by these teas and also to further determine the mechanism(s) for the inhibition of AH109A cell proliferation using the sera of tea-treated rats, we conducted a series of investigations; in the present study, we examined the effects of these tea extracts, the rat sera obtained after oral intubation of the extracts, and the characteristic components in the teas on cell viability, induction of apoptosis, and cell cycle arrest in the cancer cell lines and normal rat mesothelial (M) cells.

Materials and Methods

Preparation of Extracts From Powdered Green, Oolong, and Black Teas and the Solution of Each Tea Component

Powdered green tea (PGT), powdered oolong tea (POT), and powdered black tea (PBT) were generously provided by Yamato Tea (Nara, Japan). Two grams of each tea were extracted by 100 ml of boiling water for three minutes. After being sterilized by filtration, each extract was stored at ~20°C for later experiments. To prepare the sera from rats orally given these teas, PGT, POT, or PBT extract was evaporated to dryness, and the dried materials were dissolved in 10 ml water for the test extracts. Each concentrated extract was orally intubated to rats as described below. EGCG, ECG, EGC, and EC from green tea and the mixture of theaflavin and theaflavin gallates (TFs) from black tea were
dissolved in phosphate-buffered saline (PBS, pH 7.2). All the tea components (all >98% pure, except for TFs, which were >80% pure) were purchased from Sigma Chemical (St. Louis, MO); their chemical structures are shown in Figure 1.

Animal Experimentation and Preparation of Sera From Tea Extract-Treated Rats

Male Donryu rats (4 wk old) were purchased from NRC Haruna (Gunma, Japan). Animals were treated in accordance with guidelines established by the Animal Care and Use Committee at Tokyo Noko University. The animals were kept in animal facilities for at least one week before use, given water and a stock pellet diet (CE-2, Clea Japan, Tokyo, Japan) ad libitum, and kept in an air-conditioned room with lights on from 8 AM to 8 PM. They were deprived of their diet at 6 PM but allowed free access to water until oral administration of each tea extract, which was conducted at 10 AM on the next day. The concentrated PGT, POT, or PBT extract was orally intubated to the rats at a dose of 1 ml/100 g body wt. The blood was collected at 0, 0.5, and 2 hours thereafter. The collected blood was left to clot for two hours at room temperature and centrifuged twice at 3,000 g at 4°C for 20 minutes. The sera were sterilized by filtration and then heated at 56°C for 30 minutes. The prepared sera were added to the DM-160 medium (Kyokuto Pharmaceutical, Tokyo, Japan) at a concentration of 10% for the related experiments. Generally, the peak blood tea polyphenol levels were observed two hours after ingestion of green tea, and EGCG, EGC, and EC had half-lives of three and five hours in green tea (12). Our previous study indicated that the effective concentrations of sera from PGT-, POT-, and PBT-treated rats appeared at 0.5–5 hours after oral intubation of each tea extract. The two-hour sera from each tea-treated rat reached the peak effect (8). Therefore, 0-, 0.5-, and 2-hour sera from each tea-treated rat were prepared and used in the present study.

Primary Culture of Mesentery-Derived M Cells and Culture of AH109A and B16 Cells

For preparation of M cells, the abdominal cavity of a male Donryu rat that had been anesthetized with pentobarbital sodium (5 mg/0.1 ml/100 g body wt ip; Nembutal, Dainabot, Osaka, Japan) was opened and the mesentery was cut off with scissors. The obtained mesenteries were incubated in 0.25% trypsin (Sigma Chemical) in PBS (pH 7.2) at 37°C for 20 minutes with gentle stirring. After treatments by digestion and filtration, the M cells were seeded at a density of 1.2 × 10⁶ cells/60-mm culture dish (Nalge Nunc, Tokyo, Japan) 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, Tokyo, Japan). The cells were cultured under an atmosphere of 5% CO₂-95% humidified air at 37°C to 70% confluent state by replacing culture media every other day and were used for the experiments described below (7,8). AH109A cells were obtained from the Institute of De-
Determination of Relative Cell Viability

The AH109A cells (5 × 10^4 cells/well), B16 cells, and M cells (the initial seeded cell density was 5 × 10^4 cells/well) at ~70% confluence (cultured in 24-well culture plates) were treated with the tea extracts containing 0 (vehicle), 0.02, 0.04, 0.08, and 0.16% PGT, POT, or PBT, 0, 50, 100, 200, and 400 μM EGCG, ECG, EGC, TFs, or EC, 200 μM EC mixed with 50 μM EGCG or ECG, or 100 μM TFs. An expression of “0.16% PGT,” for example, means that PGT extract present in 1 ml of experimental medium is equivalent to extract prepared from 1.6 mg of green tea powder. This concentration is <160 μg of catechins per milliliter of medium, since PGT contains ~10% catechins. In the tests for effects of the sera from these tea-treated rats, the DM-160 medium was replaced with fresh DM-160 medium containing 0-hour rat serum or 0.5- or 2-hour sera from PGT-, POT-, or PBT-treated rats. After culture for 48 hours, the relative cell viability was determined by counting viable cells that could exclude trypan blue dye. Percentage of the relative cell viability is defined as the percentage of the number of viable test cells divided by the number of viable control cells of the same cell line at the end of each experiment.

Detection of DNA Fragmentation

The AH109A cells (2 × 10^4 cells/dish), B16 cells, and M cells (the initial seeded cell density was 2 × 10^4 cells/dish) at ~70% confluence were treated with each sample. In the tests for effects of different time treatment with the tea extracts containing 0.16% PGT, POT, or PBT on induction of apoptosis in AH109A cells, the cells were treated for 48 hours with the extracts containing 0.16% PGT, POT, or PBT, 0, 50, 100, 200, and 400 μM EGCG, ECG, EGC, TFs, or EC. In the tests for effects of the sera obtained 0.5 and 2 hours after oral intubation of PGT, POT, and PBT extracts on induction of apoptosis in AH109A cells, the cells were treated for 48 hours with 0-hour rat serum or 0.5- or 2-hour serum from each tea-treated rat instead of 10% CS. In the tests for effects of PGT, POT, and PBT extracts and the serum from each tea-treated rat on induction of apoptosis in normal rat M cells, the cells were treated for 48 hours with the extracts containing 0.16% PGT, POT, or PBT, 0, 50, 100, 200, and 2-hour serum from PGT-, POT-, or PBT-treated rats. Control groups of AH109A and M cells treated with vehicle were also used in the experiments. After these treatments, the AH109A or M cells were washed in PBS, suspended in TE buffer [10 mM tris(hydroxymethyl)aminomethane-HCl, pH 8.0, and 1 mM EDTA] containing 0.1 mg/ml ribonuclease A (Sigma Chemical). The mixture was incubated at 50°C with 1% sodium dodecyl sulfate and 0.1 mg/ml protease K for 40 minutes. The DNA was precipitated with isopropanol, 6 M NaI solution, and ethanol at room temperature. The DNA precipitate was centrifuged at 14,000 g at 4°C for 15 minutes, and the pellet was air-dried and dissolved in 20 μl of TE buffer. The DNA was electrophoresed on a 2% agarose gel stained with 0.5 μg/ml ethidium bromide in TBE [pH 8.3, 89 mM tris(hydroxymethyl)aminomethane, 89 mM boric acid, and 2 mM EDTA] and visualized by ultraviolet light. The method used to detect DNA fragmentation was based on that of Ishizawa and co-workers (17) with some modifications.

Fluorescence Staining

AH109A, B16, and M cells at ~70% confluence (the initial seeded cell density was 2 × 10^4 cells/dish) were treated for 24 hours with each extract containing 0.16% PGT, POT, or PBT, 48 hours with each extract containing 0.16% PGT, POT, or PBT, 0, 0.5-hour rat serum or 2-hour sera from PGT-, POT-, PBT-, or PBT-treated rats (10% serum in medium in the case of AH109A cells), or 200 μM EGCG, ECG, EGC, or TFs (in the case of AH109A and B16 cells). The control groups of AH109A and B16 cells treated with vehicle were used in the experiments. These treated cells were fixed with 1% glutaraldehyde in PBS for 30 minutes at room temperature, washed in PBS, and stained with 1 mM Hoechst-33258 (Funakoshi Chemical, Tokyo, Japan) for 30 minutes at room temperature (18). The morphological changes in the nuclear chromatin were observed under a fluorescent microscope (Olympus BX50, Tokyo, Japan), using a UPlan Fl 100×/1.30 oil immersion lens.

Cell Cycle Analysis

The AH109A cells (2 × 10^4 cells/dish) were serum starved for 48 hours to arrest them in the G0/G1 phase of the cell cycle. These serum-starved AH109A cells and those not subjected to serum starvation were treated with vehicle or 50 μM EGCG, 0-hour rat serum (control), or 2-hour sera from PGT-, POT-, or PBT-treated rats. After 48 hours of treatment, the cells were collected and washed twice with cold PBS. The pellet was stained with 50 μg/ml propidium iodide solution containing 0.1% Triton X-100 and 0.1% so-
dium citrate and then analyzed by flow cytometry (EPICS ELITE ESP, Coulter, Miami, FL).

**Statistical Analysis**

Values are means ± SE. Data were analyzed by a one-way analysis of variance. When the *F* value was significant (*p* < 0.05), differences among the data were inspected at *p* < 0.05 by Tukey’s *Q* test.

**Results**

Trypan blue dye exclusion assay indicated that PGT, POT, and PBT extracts dose dependently and significantly decreased the relative cell viability of AH109A (Figure 2, A1) and B16 cell lines (Figure 2, A5). The sera from PGT-, POT- or PBT-treated rats, obtained 0.5 hour (0.5-h sera) and 2 hours (2-h sera) after oral intubation of each tea extract, also reduced the relative viability of AH109A cells significantly (Figure 2, A2), but the 2-hour sera from PGT-, POT- or PBT-treated rats did not show any cytotoxicity to normal M cells (data not shown). There was similar activity for the sera from these tea-treated rats (Figure 2, A2). EGC or ECG at 50 μM could significantly decrease the relative viability of AH109A cells, but TFs and EGC had no significant effect at this concentration. EC had no significant effect at 100 μM or 400 μM (Figure 2, A3). However, the relative viability of AH109A cells was significantly and greatly reduced by 200 μM EC + 50 μM EGC or ECG or 100 μM TFs compared with that of each tea component treatment in the absence of EC. EC at 200 μM + 100 μM TFs caused the death of all cells, but it failed to show a synergistic effect with EGC (Figure 2, A4).

The characteristic cleavage of DNA into oligonucleosomal fragments has been regarded as an important biochemical marker of apoptosis (19). As evident by formation of the DNA ladder (Figure 3, A1, A2, and A3) compared with the control groups from 24-, 36-, and 48-hour samples treated with vehicle (Figure 3, A1, A2, and A3, Lanes 1–3, respectively), the treatment of AH109A cells with media containing tea extract derived from 0.16% (1,600 μg/ml) PG-T, POT-, or PBT resulted in a time-dependent induction of apoptosis. The clear DNA ladder appeared at 18, 24, 30, 36, and 48 hours (Figure 3, A1, A2, and A3, Lanes 8–12, respectively), but only the weak DNA ladder appeared in the case of the PBT extract treatment for 48 hours (Figure 3, A3, Lane 12). There is a dose-effect relationship between the concentrations of PGT, POT, and PBT extracts and induction of apoptosis in AH109A cells. The DNA ladder could be observed at the extract concentrations of 0.08% and 0.16% PGT, POT, or PBT (Figure 3B, left, middle, and right, Lanes 4 and 5, respectively), although 0.08% POT and PBT caused very weak and no detectable DNA fragmentation, respectively. At 0.02% and 0.04% PG-T, POT-, or PBT, there was no detectable DNA ladder. More importantly, the 2-hour sera from PGT-, POT-, or PBT-treated rats could directly induce the formation of the DNA ladder in AH109A cells (Figure 3C, Lanes 3, 5, and 7, respectively), although the 0.5-hour sera from PGT-, POT-, or PBT-treated rats did not produce this effect (Figure 3C, Lanes 2, 4, and 6, respectively). Furthermore, EGC, ECG (Figure 3D1), EGC, and TFs (Figure 3D2) exhibited a dose-dependent effect on induction of apoptosis in AH109A cells. Treatment with 100, 200, and 400 μM EGC or ECG or 200 and 400 μM EGC or TFs resulted in the formation of the ladder. TFs at 100 μM caused weak DNA fragmentation, but treatment with 100 μM EGC or 50 μM EGC, ECG, EGC, or TFs did not produce detectable DNA fragments. At 50–400 μM, EC did not induce formation of the DNA ladder (data not shown). Interestingly, treatment with the tea extracts derived from 0.16% PGT, POT, or PBT (Figure 3E, left, Lanes 2–4) or the 2-hour sera from PGT-, POT-, or PBT-treated rats (Figure 3E, right, Lanes 2–4) did not cause apoptosis in normal rat M cells under the same test conditions.

Hoechst-33258 stain further confirmed these findings. Under the fluorescent microscope, the apoptotic nuclei presented chromatin condensation and marginalization or nuclear beading during the apoptosis of AH109A or B16 cells induced by PGT, POT, or PBT extract, the sera from PGT-, POT-, or PBT-treated rats, or the tea polyphenolic compounds. After 24 hours of treatment with the tea extracts derived from 0.16% PGT, POT, or PBT (Figure 4, A–C), the 2-hour sera from PGT-, POT-, or PBT-treated rats (Figure 4, D–F) or 200 μM EGC, ECG, EGC, or TFs (Figure 4, G–J), AH109A cells displayed the apoptotic morphological alteration described above. B16 cells also showed apoptosis after 24 hours of treatment with the extracts derived from 0.16% PGT, POT, or PBT (Figure 4, K–M) or 200 μM EGC, EGC, ECG, or TFs (Figure 4, N–Q). There was no apoptosis for the control AH109A or B16 cells in the absence of tea (Figure 4, A1, D1, and K1). For the normal rat M cells, there was also no apoptosis after 24 hours of treatment with vehicle (Figure 3, R1) or for the tea extracts derived from 0.16% PGT, POT, or PBT (Figure 4, R–T). The normal cells showed homogeneous staining of their nuclei.

Flow cytometric analysis indicated that 50 μM EGC and the 2-hour sera from PGT-, POT-, or PBT-treated rats arrested the cell cycle at the G1 phase in the AH109A cells pretreated with serum starvation. Compared with the control groups in the absence of tea (Figure 5, A1 and A3, respectively), the 2-hour sera from PBT (Figure 5, A2), PGT-, or POT (data not shown)-treated rats and EGCG (Figure 5, A4) caused an increase in the G1 cell population and a decrease of the cell percentage at the G2 phase of the cell cycle. The time course study further confirmed that the cell cycle of the AH109A cells without serum starvation arrested at the G1 phase after 24 and 48 hours of treatment with the sera from PBT (Table 1), PGT-, or POT (data not shown)-treated rats. However, 24 and 48 hours of treatment with 50 μM EGCG did not cause such arrest in these cells without serum starvation (data not shown).
Figure 2. Effects on relative cell viability by powdered green tea (PGT), powdered oolong tea (POT), and powdered black tea (PBT) extracts, sera from rats treated with these teas, and tea polyphenolic compounds. Relative viability of AH109A and B16 cells was determined by trypan blue dye exclusion assay after AH109A cells were treated for 48 h as follows. A1, treatment of AH109A cells with media containing tea extracts derived from 0% (vehicle), 0.02%, 0.04%, 0.08%, and 0.16% PGT, POT, or PBT. A2, treatment of AH109A cells with sera from these tea-treated rats obtained at 0, 0.5, and 2 h after oral intubation of PGT, POT, or PBT extract. A3, treatment of AH109A cells with 0, 50, 100, 200, and 400 μM (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin (EGC), theaflavins (TFs), or (-)-epicatechin (EC). A4, treatment of AH109A cells with 200 μM EC only, 50 μM EGCG, ECG, or EGC, or 100 μM TFs in absence or in presence of 200 μM EC. A5, treatment of B16 cells with media containing tea extracts derived from 0% (vehicle), 0.02%, 0.04%, 0.08%, and 0.16% PGT, POT, or PBT. Percentage of relative cell viability is defined as percentage of number of viable test cells divided by number of viable control cells of the same cell line at the end of each experiment. All data were inspected at $p < 0.05$ by Tukey’s Q test. Values (means ± SE of 4 wells) are representative of ≥2 similar experiments.
Figure 3. Effects of tea extracts, sera from tea-treated rats, and related tea polyphenolic compounds on formation of DNA ladder in apoptotic AH109A cells and normal rat M cells. Cells were treated with tea extracts, sera from tea-treated rats, or related tea components at different times or doses. Cells were collected, and cellular DNA was isolated and detected by 2% agarose gel electrophoresis followed by visualization of bands as described in Materials and Methods. A: time-dependent effect of PGT, POT, and PBT extracts (A1, A2, and A3, respectively) on DNA fragmentation in AH109A cells. M, marker; Lanes 1–3, vehicle only (control AH109A cells in absence of tea for 24, 36, and 48 h, respectively); Lanes 4–12, formation of DNA ladder of AH109A cells treated with media containing tea extracts derived from 0.16% (1,600 μg/ml) PGT, POT, or PBT for 0, 3, 6, 12, 18, 24, 30, 36, and 48 h, respectively. B: dose-effect relationship between concentrations of PGT, POT, or PBT extract and induction of apoptosis in AH109A cells treated for 36 h with tea extracts. M, marker; Lane 1, vehicle only; Lanes 2–5, media containing tea extracts derived from 0.02%, 0.04%, 0.08%, and 0.16% PGT, POT, or PBT, respectively. C: formation of DNA ladder in AH109A cells treated for 48 h with sera from tea-treated rats obtained at 0.5 and 2 h after oral intubation of PGT, POT, or PBT extract. M, marker; Lane 1, 0-h rat serum (control); Lanes 2, 4, and 6, 0.5-h sera from PGT-, POT-, or PBT-treated rats, respectively; Lanes 3, 5, and 7, 2-h sera from PGT-, POT-, or PBT-treated rats, respectively. D: dose-dependent effect on DNA fragmentation in AH109A cells treated for 48 h with media containing tea extracts derived from 0.16% PGT, POT, or PBT (left Lanes 2, 3, and 4, respectively) or 2-h sera from PGT-, POT-, or PBT-treated rats (right Lanes 2, 3, and 4, respectively). M, marker; Lane 1, control. Data are from a representative experiment repeated ≥3 times with similar results.
Figure 4. Morphological changes of nuclear chromatin in treated AH109A, B16, and M cells stained by Hoechst-33258. Treated cells were fixed with 1% glutaraldehyde and then stained. Morphological changes in nuclear chromatin were observed under a fluorescent microscope as described in Materials and Methods. A1–J: AH109A cells (AH) treated for 24 h with media containing tea extracts derived from 0.16% PGT, POT, and PBT (A–C), 2-h sera from PGT-, POT-, or PBT-treated rats (D–F), or 200 μM EGCG, EGC, ECG, and TFs (G–J). K1–Q: B16 cells treated for 24 h with media containing tea extracts derived from 0.16% PGT, POT, and PBT (K–M) and 200 μM EGCG, EGC, ECG, or TFs (N–Q). A1 and D1: control AH109A cells in DM-160 medium containing 10% calf serum (CS, A1) or 10% rat serum (RS, D1). K1: control B16 cells in RPMI 1640 medium containing 10% fetal bovine serum (FBS). R1–T: M cells treated for 24 h with vehicle in DM-160 medium containing 10% CS (R1) or media containing tea extracts derived from 0.16% PGT, POT, and PBT (R–T).
EGCG has been regarded as the best inhibitor in most of the cell lines tested, with 50% inhibition (IC_{50}) values, and the most effective concentrations to induce apoptosis varying between 87 and 2,000 μM (12). Our present results indicate that 100 and 200 μM EGCG, EGC, ECG, and TFs caused apoptosis in AH109A and B16 cells, respectively (Figures 3 and 4). EGCG reduced the viability of AH109A by 80% at 100 μM (Figure 2, A3). PGT, POT, and PBT at 0.16% induced apoptosis in AH109A and B16 cells, but not in normal M cells. Clearly, different cell lines have different sensitivity to tea components, and cancer cell lines are more sensitive than normal cells to the tea treatments. Treatment with 0.04% PGT, POT, or PBT resulted in loss of 80% viability in AH109A or B16 cells (Figure 2), but not in normal M cells (data not shown). We were not able to assess the cell cycle distribution in AH109A cells treated with higher concentrations of EGCG or with 0.04% PGT, POT, or PBT, because most cells died from these treatments. To clarify the in vivo possibilities of inducing apoptosis and cell cycle arrest in cancer cells by these teas and also to further determine the mechanism(s) by which proliferation of cancer cells is inhibited, we conducted a series of related ex vivo experiments. Our results have demonstrated that the 2-hour, but not 0.5-hour, sera from PGT-, POT-, or PBT-treated rats caused abundant loss of the viability and apoptosis in AH109A cells but not in normal M cells (Figures 2–4). The cell cycle arrested at the G1 phase in the AH109A cells (pretreated with serum starvation) after 24 hours of treatment with 2-hour sera from PBT (Figure 5)-, PGT-, or POT (data not shown)-treated rats. The time course study further confirmed that the cell cycle arrested at the G1 phase 24 and 48 hours after treatment of AH109A cells (pretreated with serum starvation) after 24 hours of treatment with sera from PBT (Figure 5)-, PGT-, or POT (data not shown)-treated rats. The time course study further confirmed that the cell cycle arrested at the G1 phase 24 and 48 hours after treatment of AH109A cells (pretreated with serum starvation) after 24 hours of treatment with sera from PBT (Figure 5)-, PGT-, or POT (data not shown)-treated rats. The level of EGCG, EGC, and EC in blood was only 37, 55, and 20 ng/ml, respectively, after the rats received decaffeinated green tea at a concentration of 9 mg solids/ml (15). So low a concentration of a single tea compound in the sera from tea-treated rats may not display apoptosis and cell cycle inhibi-

### Table 1. Effects of Sera From PBT-Treated Rats on Cell Cycle Distribution in AH109A Cells

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PBT sera</td>
<td>Control</td>
</tr>
<tr>
<td>24 h</td>
<td>36.6</td>
<td>48.1</td>
<td>50.1</td>
</tr>
<tr>
<td>48 h</td>
<td>49.9</td>
<td>54.9</td>
<td>28.6</td>
</tr>
</tbody>
</table>

a: PBT, powdered black tea.

**Discussion**

EGCG has been regarded as the best inhibitor in most of the cell lines tested, with 50% inhibition (IC_{50}) values, and the most effective concentrations to induce apoptosis varying between 87 and 2,000 μM (12). Our present results indicate that 100 and 200 μM EGCG, EGC, ECG, and TFs caused apoptosis in AH109A and B16 cells, respectively (Figures 3 and 4). EGCG reduced the viability of AH109A by 80% at 100 μM (Figure 2, A3). PGT, POT, and PBT at 0.16% induced apoptosis in AH109A and B16 cells, but not in normal M cells. Clearly, different cell lines have different sensitivity to tea components, and cancer cell lines are more sensitive than normal cells to the tea treatments. Treatment with 0.04% PGT, POT, or PBT resulted in loss of 80% viability in AH109A or B16 cells (Figure 2), but not in normal M cells (data not shown). Nevertheless, there was no detectable DNA fragment in AH109A cells treated with 0.04% PGT, POT, or PBT or 50 μM EGCG, EGC, ECG, or TFs. Analysis of the cell cycle in AH109A cells showed that 24 hours of treatment with 50 μM EGCG arrested the cell cycle at the G1 phase in the serum-starved AH109A cells (Figure 5). However, 24 and 48 hours of treatment with 50 μM EGCG did not affect the cell cycle distribution in these cells without serum starvation (data not shown). We were not able to assess the cell cycle distribution of AH109A cells treated with higher concentrations of EGCG or with 0.04% PGT, POT, or PBT, because most cells died from these treatments. To clarify the in vivo possibilities of inducing apoptosis and cell cycle arrest in cancer cells by these teas and also to further determine the mechanism(s) by which proliferation of cancer cells is inhibited, we conducted a series of related ex vivo experiments. Our results have demonstrated that the 2-hour, but not 0.5-hour, sera from PGT-, POT-, or PBT-treated rats caused abundant loss of the viability and apoptosis in AH109A cells but not in normal M cells (Figures 2–4). The cell cycle arrested at the G1 phase in the AH109A cells (pretreated with serum starvation) after 24 hours of treatment with sera from PBT (Figure 5)-, PGT-, or POT (data not shown)-treated rats. The time course study further confirmed that the cell cycle arrested at the G1 phase 24 and 48 hours after treatment of AH109A cells (pretreated with serum starvation) after 24 hours of treatment with sera from PBT (Figure 5)-, PGT-, or POT (data not shown)-treated rats. The level of EGCG, EGC, and EC in blood was only 37, 55, and 20 ng/ml, respectively, after the rats received decaffeinated green tea at a concentration of 9 mg solids/ml (15). So low a concentration of a single tea compound in the sera from tea-treated rats may not display apoptosis and cell cycle inhibi-

![Figure 5. Cell cycle analysis. AH109A cells were treated with 0-h rat serum (RS control), 2-h serum from PBT-treated rat, CS without tea (CS control), or 50 μM EGCG.](image.png)

![Table 1. Effects of Sera From PBT-Treated Rats on Cell Cycle Distribution in AH109A Cells](table.png)
tory activity. The present results suggest that the combined activity of at least several tea components, the in vivo metabolites, and/or tea-induced changes in the endogenous substances may produce these effects on the cancer cells. These teas may also lead to loss of the viability, apoptosis, and cell cycle arrest of cancer cells in the human body. EGCG, ECG, EGC, EC, and/or TFs may be effective components in the sera after in vivo absorption and metabolism of these teas. Indeed, EGCG, EGC, and EC have been detected in the rat, mouse, and human plasma as mentioned previously (12–15). EC did not significantly decrease AH109A cell viability, but it does possess a synergistic effect with EGCG, EGC, and TFs (Figure 2). In addition, there may be other unidentified tea components or their in vivo metabolites in sera that partially contribute to the induction of apoptosis and cell cycle arrest in cancer cells. Actually, there are great differences among the constituents in green, oolong, and black teas, but these teas and the sera from tea-treated rats have shown similar effects on AH109A cells in the present and previous studies (8). The rat sera obtained 0.5, 1, 2, 3, 4, and 5 hours after oral intubation of PGT, POT, and PBT significantly suppressed proliferation and invasion of AH109A cells. The effective concentrations of these teas in blood reached a peak two hours after oral intubation of each tea (8). The time course of absorption of tea in rats is similar to that in humans (12–15), although the absorption of tea components, including catechins, is more efficient in humans than in rats (15). These findings suggest the bioavailability and the therapeutic and preventive importance of the three kinds of tea extracts against cancers.

Acknowledgments and Notes

Address correspondence to Dr. Kazumi Yagasaki, Dept. of Applied Biological Science, Tokyo Noko University, Saiwaicho 3-5-8, Fuchu, Tokyo 183-8509, Japan.

Submitted 3 December 1999; accepted in final form 28 August 2000.

References
