Composition of Polyphenols in Fresh Tea Leaves and Associations of Their Oxygen-Radical-Absorbing Capacity with Antiproliferative Actions in Fibroblast Cells

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The polyphenols in various parts of the tea plant used in manufacture of tea products (young leaves, old leaves, and stem) were analyzed by HPLC. The young leaves (apical bud and the two youngest leaves) were found to be richer (2.7-fold) in polyphenols than old leaves (from the tenth to the fifth leaf). Also, the tea polyphenols were found to be higher (1.4-fold) in summer than in spring. Ten different types of commercial tea (manufactured tea), including unfermented, semifermented, and fermented tea, were analyzed for their polyphenol compounds, and it was found that both yields of solids in tea water extracts (TWEs) and the amount of (−)-epigallocatechin 3-gallate (EGCG) in these products varied with different tea leaves and processing methods. Longjing tea (unfermented green tea) contained the highest concentration of EGCG and polyphenols, whereas Assam black tea (most fermented) contained the least. Longjing TWEs showed strong inhibitory effect on DNA synthesis in A-431 tumor cells. We also found that green tea polyphenols (GTPs) and EGCG strongly inhibited tumor cell DNA synthesis and peroxyl-radical generation. The evidence suggests that the strong antioxidative and antiproliferative activities of Longjing TWEs and GTPs are mainly due to the higher contents of tea polyphenols. EGCG (the major component of GTPs) showed strong inhibitory action on the growth of immortalized cells (NIH3T3) and tumor cells (S-180II) but almost no effect on the growth of normal cells (C3H10T1/2).

Keywords: *Polyphenols; EGCG; antiproliferative effect; antioxidative effect; tea water extracts*

INTRODUCTION

Tea plants are widely cultivated in Southeast Asia. Tea is one of the most popular beverages in the world because of its attractive flavor, aroma, and taste. Over 300 different kinds of tea are now produced, but there are only three general forms of tea: the unfermented green tea, the partially fermented pauchong tea or oolong tea, and the fermented black tea. Green tea is manufactured by steaming or drying fresh tea leaves to prevent oxidation of the green tea polyphenols (Takeo, 1992). The manufacture of black tea is characterized by a high degree of enzymatically catalyzed aerobic oxidation of the leaf polyphenols followed by a series of chemical condensations (Hampton, 1992). Pauchong tea or oolong tea is partially oxidized (Takeo, 1992; Graham, 1992). The composition of tea varies with species, season, the age of the leaf (plucking position), climate, and horticultural practices. Green tea contains polyphenols, which include flavanols, flavandiols, flavonoids (Hertog et al., 1993), and phenolic acids; these compounds may account for up to 30% of the dry weight. The polyphenols are the most significant group of tea components, especially certain catechins. The major tea catechins are (−)-epigallocatechin 3-gallate, (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin 3-gallate (ECG), (−)-epicatechin (EC), (+)-gallocatechin, and (+)-catechin (C). In the manufacture of black tea, the monomeric flavan-3-ols undergo polyphenol oxidase-dependent oxidative polymerization leading to the formation of bisflavanols, theaflavins, thearubigins, and other oligomers in a process commonly known as "fermentation". Theaflavins (about 1−2% of the total dry weight of black tea), including theaflavin, theaflavin 3-*O*-gallate, theaflavin 3′-*O*-gallate, and theaflavin 3,3′-*O*-digallate, possess benzotropolone rings with dihydroxy or trihydroxy substitution systems. About 10−20% of the dry weight of black tea is due to thearubigens, which are more extensively oxidized and polymerized, have a wide range of molecular weights, and are less well characterized. Pauchong tea or oolong tea contains monomeric catechins, theaflavins, and thearubigins.

Many biological functions of tea polyphenols have been studied (Yang and Wang, 1993), including antioxidative activity (Ho et al., 1992; Yoshino et al., 1994; Katiyar et al., 1994; Yen et al., 1995), antimutagenic effects (Wang et al., 1989; Jain et al., 1989; Shiraki et al., 1994), and anticarcinogenic effects (Oguni et al., 1988; Huang et al., 1992; Mukhtar et al., 1992; Wang et al., 1992; Katiyar et al., 1993) in several systems. But, there have been relatively few reports on the action of tea polyphenols on the growth of transformed cells (Nakane and Ono, 1990; Lea et al., 1993) and on the relationship between antioxidative activity and the content of EGCG from various tea extracts (Ho et al., 1992).

In the present study, we have investigated the composition of polyphenols in various parts of the fresh tea (young leaves, old leaves, and stem) in spring or summer and in commercial tea by HPLC analysis. We also studied the antiproliferative actions of TWEs, GTPs, and EGCG in human epidermoid carcinoma A-431 cells, and the antioxidant activity was measured by ORAC (oxygen-radical absorbance capacity) assay. Furthermore, we also investigated the growth inhibitory and the cytotoxic effects of the pure EGCG on fibroblast cell lines.

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MATERIALS AND METHODS

Chemicals. Gallic acid (GA) was purchased from the Sigma Chemical Co. (St. Louis, MO). Methanol and caffeine were purchased from E. Merck Co. (Darmstadt, Germany). Catechin, EC, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Aldrich Chemical Co. EGCG standard and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Porphyridium cruentum β-phycoerythrin (β-PE) was obtained from Boehringer Mannhein (Germany). EGCG, ECG, and EGC were purified from our laboratory as described below.

Isolation of Polyphenolic Catechins from Green Tea Leaves (Mukhtar et al., 1992). One hundred grams of Chinese green tea, Longjing tea (produced by Wangs' Tea Enterprise Co., Ltd., Taipei, Taiwan), was suspended in 1 L of distilled water at 75 °C for 30 min; then the supernatant was collected. This step was repeated three times. The supernatant were filtered to eliminate chlorophylls and undissolved particles. The total aqueous layers were concentrated to 0.5 L under reduced pressure using a rotary vacuum evaporator. The concentrated solution was extracted with an equal volume of chloroform three times to eliminate caffeine and pigments. The remaining aqueous phase was then extracted with an equal volume of ethyl acetate three times to extract tea polyphenols. The ethyl acetate was combined and evaporated *in vacuum*. The residue was dissolved in a small volume of distilled water and freeze-dried. This gold-brown solid matter was called green tea polyphenols (GTPs). Three grams of GTPs was dissolved in acetone and then applied to a Sephadex LH-20 column (Nonaka et al., 1983) and eluted with ethanol. Eight fractions were obtained, and the yields of fractions 6−8 were 213.2, 190.5, and 983.2 mg, respectively. Bondapak C18 chromatography of fraction 6 was used; water−methanol with an increasing amount of methanol (20−80%) furnished EGC (108.7 mg). Fraction 7 was similarly chromatographed over a Bondapak C18 column followed by repeated Sephadex LH-20 chromatography using 50% aqueous methanol to give ECG (85.6 mg). Fraction 8 was dissolved in cold water, and the solution was passed through a column of Bondapak C18, preswollen in a mixture of water−methanol (7:3, v/v). Elution with the same solvent followed by crystallization from H_2O afforded EGCG (501.7 mg). The homogeneity of all the purified polyphenolic catechins was compared by physical (mp) and NMR spectral data with those of authentic samples (Nonaka et al., 1983).

Preparation of Fresh Tea Extracts. In spring (April 1995), we plucked fresh young tea leaves of 10 different varieties at the Taiwan Tea Experiment Station (TTES). In summer (August 1994), we plucked fresh tea from three positions: young leaves (containing the apical bud and the two youngest leaves), old leaves (from the tenth to the fifth leaf), and stem (the 6 cm segment counted downward from the bottom of the two youngest leaves) of different varieties. In order to compare the composition of fresh tea in spring and summer, we plucked fresh tea in the same group of each tea at TTES. The fresh teas were stored in a sealed plastic bag at 4 °C before use and extracted as soon as possible. The polyphenol oxidase of fresh tea was inactivated by a microwave oven (Model TMO 7580) at 110 V/60 Hz for 1.5 min; then the water content of the fresh tea was partially removed during this process to form a dry sample. The amount of moisture in the dry sample was estimated to be 10.7 ± 1.2% after drying at 105 °C for 2 h. The same weight of dry sample was added to 50% aqueous methanol at 75 °C for 30 min. The fresh tea extracts were analyzed by HPLC.

Preparation of Commercial Tea Water Extracts (Wang et al., 1992). The different varieties of tea were kindly obtained from the TTES. The moisture content of each commercial tea was 5.1 ± 0.6%. Each of the dry tea leaves (12.5 g) was added to 500 mL of boiling water and steeped for 15 min. The infusion was cooled to room temperature and then filtered. The tea leaves were extracted a second time with 500 mL of boiling water and filtered, and the two filtrates were combined to obtain a 1.25% tea water extract and then analyzed by HPLC. This 1.25% tea water extract was presumably similar in composition to the tea beverages consumed by humans. The tea water extract was also concentrated under vacuum and freeze-dried. The light-brown solid matter was called TWEs (tea water extracts) powder and stored in dark brown bottles at −70 °C until used.

HPLC Analysis of Tea Extracts. The composition of tea extract was determined by HPLC analysis using a Waters 600E system controller. The Waters 484 turnable absorbency detector was used to detect tea extract at 280 nm, and all peaks were plotted and integrated by a Waters 745 data module. The HPLC method used a Cosmosil C18-MS packed column (5 μm, 4.6 mm i.d. × 250 mm). The tea extracts were filtered through a 0.45 μm filter disk, and 20 μL was injected onto the column. The mobile phase was methanol/water/formic acid (20:79.7:0.3) and ran by an isocratical method at a flow rate of 1.0 mL/min. Identification of the individual epicatechin derivatives was based on the comparison of the retention times of unknown peaks to those of reference standards.

Oxygen-Radical Absorbance Capacity (ORAC) Assay (Cao et al., 1993). The final reaction mixture for the assay contained 8.35×10^{-9} M β-PE and 3×10^{-3} M AAPH in 7.5×10^{-2} M phosphate buffer, pH 7.0. A final volume of 2 mL was used in 10 mm wide cuvettes. Into each sample tube, 20 μL of various concentrations of TWEs, GTPs, EGCG, EGC, ECG, EC, C, GA, and caffeine was added. Each sample was dissolved in acetone and then added to the reaction mixture. For the blank, 20 μL of acetone was used. AAPH was used as the peroxyl-radical generator to start the reaction. Once AAPH was added, the reaction mixture was incubated at 37 °C. Fluorescence was measured by time-scanning function at the emission of 565 nm and excitation of 540 nm using a Model F-4500 fluorescence spectrophotometer (Hitachi) until zero fluorescence occurred. For a standard, 20 μL of a 100 μM (1 μM in final concentration) Trolox stock solution was assayed similarly during each run. The ORAC value refers to the net protection area under the quenching curve of β-PE in the presence of an antioxidant. One ORAC unit has been assigned by the net protection area (S) provided by 1 μM Trolox. The ORAC value (U/mL) = $50k(S_{\text{Sample}} - S_{\text{Blank}})/(S_{\text{Trolox}} - S_{\text{Blank}})$, where k is the dilution factor and S refers to the area under the quenching curve of β-PE; this area is integrated by a computer connected directly to the output of the fluorescence spectrophotometer.

Cell Culture. A-431 cell line was established from human epidermoid carcinoma cells. This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 2 mM glutamine. The cells were harvested by incubating with 0.1% trypsin and 1 mM EDTA at 37 °C for 8 min. The NIH3T3 mouse embryo fibroblast cells were maintained on DMEM containing 10% calf serum, 100 U/mL penicillin, and 50 μg/mL streptomycin. The C3H10T1/2 mouse embryo fibroblast cells were maintained in Eagle's basal medium (BME) supplemented with 10% FCS, 2 mM glutamine, and Earle's basal salts. The S-180II murine sarcoma cells were maintained in Eagle's minimal essential medium (EMEM) with nonessential amino acids, Earle's basal salts, and 10% FCS. All cultures were incubated at 37 °C in an atmosphere of 5% CO_2:95% air.

[^{3}H]Thymidine Incorporation Assay. A-431 cells (2×10^5) were plated with 10 mL of medium in a 10 cm dish. When 70% confluence was achieved, starvation occurred for 24 h. Afterward, 0.5 mL of 10% FCS and various concentrations of TWEs, GTPs, and EGCG were added, and then the mixture was incubated for 12 h. The [^{3}H]thymidine (1 μL, 25 Ci/mmol) was added during the last 4 h of incubation. The cells were washed two times with cold phosphate-buffered saline (PBS) and incubated with 5% cold trichloroacetic acid to remove excess thymidine. The cells were lysed with 0.5 mL of 1 N NaOH and placed in scintillation vials containing liquid scintillation cocktail. The radioactivity (cpm) was counted by a Beckman L-600 scintillator.

Cell Growth Inhibition Assay. Cells were plated at a density of 1.0×10^5 viable cells/100 mm of culture dish. After 24 h, the optimal concentrations of EGCG were applied to the dishes, and the cultures were incubated at 37 °C and harvested

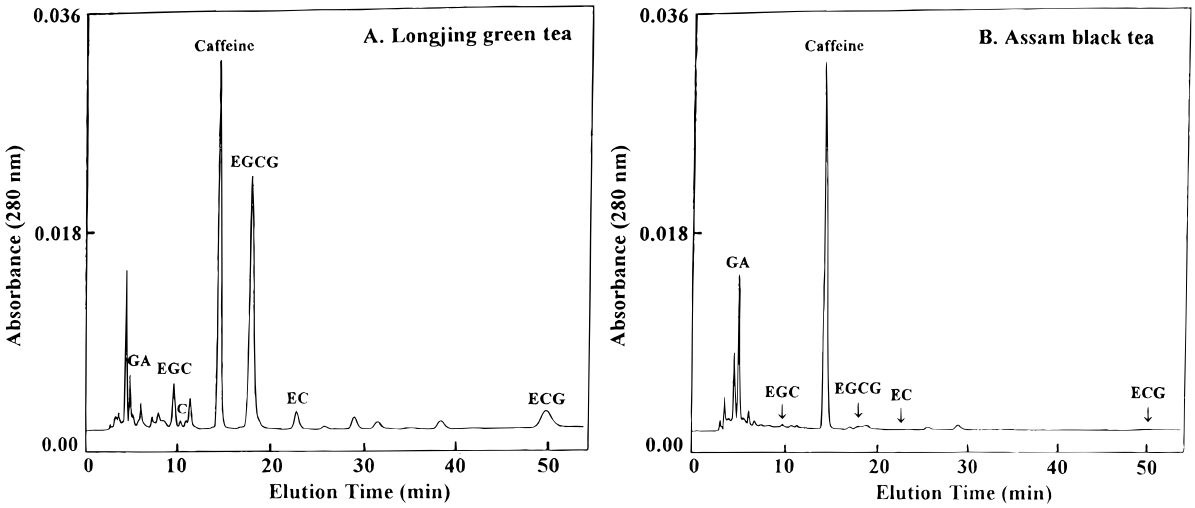

Figure 1. Reverse-phase HPLC of TWEs on a Cosmosil 5 μm C18-MS column: (A) 4 μg of Longjing green tea (200 μg/mL in 50% aqueous methanol, v/v) and (B) 3 μg of Assam black tea (150 μg/mL in 50% aqueous methanol, v/v). The mobile phase was methanol/water/formic acid (20:79.7:0.3) and ran by an isocratical method at a flow rate of 1.0 mL/min.

Table 1. Composition[a] of Various Fresh Young Tea Leaves Plucked in Summer (August 1994) and Spring (April 1995)

		catechins						
varieties	total catechins	EGCG	EGC	ECG	EC	C	GA	caffeine
				Summer				
Gau-Lu	5.83	2.89	1.10	1.22	0.39	0.23	0.09	1.41
Chin-Shin-Dah-Pan	4.13	2.22	0.96	0.63	0.21	0.11	0.15	3.09
Tiee-Guan-In	4.33	2.08	0.80	1.14	0.23	0.08	0.11	1.98
Wun-Yi	6.01	2.83	1.29	1.31	0.44	0.14	0.22	4.35
Gan-Tzy	3.95	1.97	0.97	0.76	0.19	0.06	0.11	1.59
Suragasho	6.16	2.72	1.40	1.37	0.45	0.22	0.08	3.18
Shiang-Eel	6.90	3.54	0.95	1.91	0.35	0.15	0.22	3.36
TTE No. 8	5.33	3.17	0.97	0.87	0.21	0.11	0.19	2.82
TTE No. 12	3.62	1.60	1.01	0.76	0.19	0.06	0.06	1.50
TTE No. 13	6.25	2.99	1.54	0.98	0.60	0.14	0.08	2.50
mean ± SE	5.25 ± 0.36	2.60 ± 0.19	1.10 ± 0.07	1.10 ± 0.12	0.33 ± 0.04	0.13 ± 0.02	0.13 ± 0.02	2.58 ± 0.30
				Spring				
Gau-Lu	3.72	1.78	0.72	0.89	0.21	0.12	0.03	0.68
Chin-Shin-Dah-Pan	2.12	0.95	0.65	0.31	0.15	0.06	0.06	1.26
Tiee-Guan-In	2.81	1.46	0.46	0.64	0.19	0.06	0.06	1.80
Wun-Yi	6.12	2.68	1.48	1.37	0.48	0.11	0.09	2.60
Gan-Tzy	3.85	2.09	0.77	0.65	0.24	0.10	0.10	1.54
Suragasho	3.78	1.64	0.67	1.00	0.36	0.11	0.04	1.94
Shiang-Eel	5.15	2.60	0.72	1.46	0.23	0.14	0.05	1.68
TTE No. 8	3.28	1.85	0.71	0.55	0.11	0.06	0.03	1.94
TTE No. 12	3.44	1.97	0.53	0.67	0.20	0.07	0.08	1.34
TTE No. 13	3.38	1.56	0.90	0.63	0.24	0.05	0.01	1.66
mean ± SE	3.77[b] ± 0.36	1.86[b] ± 0.16	0.76[b] ± 0.09	0.82 ± 0.12	0.24 ± 0.03	0.09 ± 0.01	0.06[b] ± 0.003	1.64[c] ± 0.16

[a] The values are averages from duplicate samples and are given in % of sample weight. [b] Compared with summer, $p < 0.05$ (Student's *t*-test). [c] Compared with summer, $p < 0.01$ (Student's *t*-test).

at 24, 48, or 72 h after addition of EGCG. Numbers of viable cells were determined by counting trypan blue dye-excluding cells in a hemacytometer.

In Vitro Cytotoxicity. Cells (3×10^4) were distributed into each well of a 24-well tissue culture plate (Coster). The cells were incubated at 37 °C for 24 h. EGCG was added to each well so as to give a final concentration of 1, 5, 10, 20, 30, 40, and 50 μg/mL. The cells were incubated for a further 24 h. At the end of the incubation period, the media were decanted and the cells washed twice with cold PBS. The cells were then fixed in 2.5% (v/v) glutaldehyde for 0.5 h and washed with distilled water. Viable cells were stained with 300 μL of 1% aqueous crystal violet solution for 0.5 h and then washed exhaustively with distilled water. Acetic acid (1 mL; 33% (v/v)) was added to solubilize the cells for 5 min and the absorbance read at 580 nm (Matthews et al., 1987). The percentage of cytotoxicity was calculated according to the following formula: $[(b - c) \times 100]/(a - c)$, where *a*, *b*, and *c* are the absorbance values of cells in medium without EGCG, cells in medium with EGCG, and medium alone, as background, respectively.

RESULTS

HPLC Analysis of Fresh Tea (Young Leaves, Old Leaves, and Stem). The HPLC profiles of Longjing TWEs are shown in Figure 1. The retention times of GA, EGC, C, caffeine, EGCG, EC, and ECG were 4.7, 9.6, 10.4, 14.6, 18.2, 23.1, and 50.9 min, respectively. Table 1 shows the composition of fresh tea polyphenols derived from different plucking seasons (in spring and summer). We found that Wun-Yi and Shiang-Eel strains contain the highest concentration of polyphenols in spring and summer, respectively, and the amount of polyphenols was generally higher in summer than in spring. The mean contents of total catechins and caffeine were 1.4- and 1.57-fold higher in summer than

Table 2. Comparison of the Composition[a] of Various Fresh Teas Derived from Different Plucking Positions (Young Leaves, Old Leaves, and Stem) in Summer (August 1994)

varieties		total catechins	catechins					GA	caffeine
			EGCG	EGC	ECG	EC	C		
Wun-Yi	young leaves	6.01	2.83	1.29	1.31	0.44	0.14	0.22	4.35
	old leaves	2.60	1.02	0.84	0.39	0.28	0.07	0.04	1.32
	stem	1.04	0.32	0.38	0.11	0.20	0.03	0.02	0.39
TTE No. 8	young leaves	5.33	3.17	0.97	0.87	0.21	0.11	0.19	2.82
	old leaves	2.49	1.16	0.69	0.42	0.18	0.04	0.06	2.55
	stem	0.74	0.35	0.23	0.07	0.06	0.02	0.03	0.42
TTE No. 13	young leaves	6.25	2.99	1.54	0.98	0.60	0.14	0.08	2.50
	old leaves	1.36	0.41	0.36	0.23	0.31	0.05	0.02	0.73
	stem	0.78	0.26	0.20	0.11	0.20	0.01	0.04	0.25
mean ± SE	young leaves	5.86 ± 0.28	3.00 ± 0.10	1.27 ± 0.17	1.05 ± 0.13	0.42 ± 0.11	0.13 ± 0.01	0.16 ± 0.04	3.22 ± 0.57
	old leaves	2.15[b] ± 0.40	0.86[b] ± 0.23	0.63 ± 0.14	0.35[b] ± 0.06	0.26 ± 0.04	0.05[c] ± 0.008	0.04 ± 0.01	1.53 ± 0.54
	stem	0.85[c] ± 0.09	0.31[c] ± 0.03	0.27[b] ± 0.06	0.10[b] ± 0.01	0.15 ± 0.05	0.02[b] ± 0.006	0.03 ± 0.006	0.35[b] ± 0.05

[a] The values are averages from duplicate samples and are given in % sample weight. [b] Compared with young leaves, $p < 0.05$ (Student's *t*-test). [c] Compared with young leaves, $p < 0.01$ (Student's *t*-test).

in spring, respectively. As shown in Table 2, we compared fresh tea polyphenols from three different plucking positions: young leaves, old leaves, and stem. The content of polyphenols, especially EGCG, in each fresh tea is in the following order: young leaves > old leaves > stem. The ratios of total catechins in fresh young tea leaves, old leaves, and stem are 5.6:2.9:1, and the ratios of caffeine in the three positions are 7.9:4.6:1.

HPLC Analysis of Commercial Tea Water Extracts. The yield and composition of 1.25% various commercial TWEs are shown in Table 3. Comparison of the content of each catechin was in the order EGCG > EGC > ECG > EC > C. EGCG is the major constituent, especially in Longjing tea. Assam black tea contained the least amount of EGCG, but the content of gallic acid was higher than in other tea leaves. The yield and composition of polyphenols in various TWEs varied with tea varieties, the origin of the leaf, and manufacturing conditions.

Antioxidative Effect of EGCG, GTPs, and TWEs. The scavenging effects of EGCG, GTPs, and TWEs on the peroxyl radical were in the following order: Longjing tea (GTPs) > EGCG > Longjing tea (TWEs) > Assam green tea (TWEs) > Assam black tea (TWEs), and their ORAC values were 1.41, 1.32, 0.95, 0.86, and 0.77 at a dose of 0.5 μg/mL, respectively (Figure 2). Especially in EGCG and GTPs, both ORAC values were higher than 1.0, even at very low concentration (0.25 μg/mL).

Antioxidative Effect of Various Polyphenols and Caffeine. The peroxyl-radical-absorbing capacity of various polyphenols and caffeine was assayed and showed the following order: EGCG > EGC > ECG > GA > EC > C > caffeine. The ORAC values were 1.40, 1.19, 1.06, 0.98, 0.48, 0.20, and 0.05 at a dose of 1 μM, respectively (Figure 3). Especially in EGCG, EGC, and ECG, their ORAC values were higher than 1.0 at 1 μM.

Inhibition of A-431 Cell DNA Synthesis by EGCG, GTPs, and TWEs. In order to compare the antiproliferative effect in A-431 cells, we selected the TWEs of Longjing tea, Assam green tea, and Assam black tea because they contained different concentrations of EGCG. The compositions of three TWEs and GTPs are shown in Tables 3 and 4. Compared to control, the incorporation of [^{3}H]thymidine into DNA of A-431 cells was inhibited by 50.7%, 27.4%, and 7.4% after treatment with 200 μg/mL Longjing tea, Assam green tea, and Assam black tea extracts, respectively (Figure 4). In the same cell line, GTPs at 200 μg/mL caused 56.3% inhibition of incorporation of [^{3}H]thymidine into DNA, and EGCG at 200 μg/mL caused a 85.3% strong inhibition of DNA synthesis. The inhibition of DNA synthesis was dose-dependent on polyphenols, especially in EGCG.

Growth Inhibitory Effect of EGCG on Mouse Normal and Tumor Cells. Tea component and GTPs have been shown to inhibit the growth of some cancer cell lines (Lea et al., 1993). Since little is known about the effects of GTPs on normal cells, we applied the most potent compounds of GTPs—EGCG—to mouse normal, immortal, and tumor cells. Mouse embryonic fibroblast C3H10T1/2 cell line was well characterized by Reznikoff et al. In this study, it is used as a normal cell line because it is genetically stable, is nontumorigenic, and exhibits low rates of spontaneous transformation. NIH3T3 is a nontumorigenic but immortal cell line derived from NIH Swiss mouse embryo cultures. S-180II is a tumorigenic cell line established by Foley et al. in 1960 from a S-180 sarcoma maintained in adult CFW mice.

A dose of 10 μg/mL EGCG did not inhibit the growth of either C3H10T1/2 or NIH3T3 cells by 24−72 h posttreatment, and no cytotoxicity was observed (Figure 5A,B). However, 30 μg/mL EGCG severely inhibited the growth of both NIH3T3 and S-180II cells and caused dead cells and cytotoxicity (Figure 5B,C).

Cytotoxic Effect of EGCG on Mouse Normal and Tumor Cells. The cytotoxic effects of EGCG on normal and tumor cells (assay by crystal violet vital staining) are shown in Figure 6. EGCG led to a significant dose-dependent inhibition of the cell viability. The concentration of EGCG required to produce a 50% cytotoxic effect of NIH3T3 and S-180II cells was 27.4 and 16.3 μg/mL, respectively. But, for the normal mouse fibroblast cells, C3H10T1/2, LD_{50} was higher than 50 μg/mL. The EGCG-induced cytotoxicity was also assessed by the colony formation of C3H10T1/2, NIH3T3, or S-180II cells and had the same results (data not shown).

DISCUSSION

The composition of tea varies with species, season, age, climate, and horticultural practices. In Table 1, we found that the amount of total catechins was higher in summer than in spring and higher in young leaves than in old leaves. Presumably, the growth rate and metabolic activities of both tea trees and young leaves were higher in summer, so they were rich in total catechins. Tables 2 and 3 show that the content of total catechins significantly varied with leaf age, tea species, and processing methods.

Many previous studies have focused on the anticarcinogenic and antimutagenic effects of tea. The mech-

Table 3. Yield and Composition of Various Commercial Tea Water Extracts[a]

varieties	teas	areas	yield[d]	total catechins[d]	catechins[b]					GA[b]	caffeine[b]
					EGCG	EGC	ECG	EC	C		
Chin-Shin-Dah-Pan	green tea	Head Station	12.2	4.0	2.0 ± 0.1	1.3 ± 0.1	0.5 ± 0.0	0.15 ± 0.06	0.07 ± 0.02	0.01 ± 0.00	1.6 ± 0.1
TTE No. 12	green tea	Taitung Substation	26.5	10.1	4.9 ± 0.3	3.2 ± 0.2	1.4 ± 0.1	0.42 ± 0.02	0.17 ± 0.00	0.04 ± 0.01	4.0 ± 0.1
TTE No. 12	paochung tea, unroasted	Taitung Substation	23.1	7.9	3.8 ± 0.1	2.6 ± 0.1	1.0 ± 0.1	0.33 ± 0.06	0.13 ± 0.01	0.03 ± 0.00	3.1 ± 0.2
TTE No. 12	paochung tea, roasted at 80 °C for 4 h	Taitung Substation	27.8	10.3	5.1 ± 0.2	3.2 ± 0.1	1.4 ± 0.2	0.40 ± 0.06	0.17 ± 0.00	0.06 ± 0.00	3.9 ± 0.2
TTE No. 12	paochung tea, roasted at 100 °C for 4 h	Taitung Substation	24.1	9.0	4.4 ± 0.4	2.9 ± 0.2	1.2 ± 0.0	0.36 ± 0.06	0.17 ± 0.03	0.03 ± 0.01	3.5 ± 0.0
Assam	green tea	Yu-Chih Substation	24.2	12.2	6.0 ± 0.4	3.9 ± 0.3	1.6 ± 0.1	0.53 ± 0.03	0.14 ± 0.00	0.05 ± 0.00	5.0 ± 0.2
Assam	black tea	Yu-Chih Substation	23.4	3.2	1.3 ± 0.1	1.4 ± 0.1	0.4 ± 0.1	0.07 ± 0.00	ND[c]	0.76 ± 0.03	5.4 ± 0.2
Wun-Yi	green tea	Head Station	18.3	6.7	3.1 ± 0.1	2.1 ± 0.2	0.9 ± 0.1	0.46 ± 0.00	0.18 ± 0.01	0.01 ± 0.00	2.4 ± 0.1
Wun-Yi	paochung tea	Head Station	18.4	8.0	3.8 ± 0.4	2.5 ± 0.1	1.0 ± 0.1	0.47 ± 0.02	0.19 ± 0.02	0.02 ± 0.00	3.0 ± 0.2
Gan-Tzy	green tea (Longjing tea)	San-Shya, Taipei	32.4	22.2	12.2 ± 1.8	5.8 ± 0.4	2.8 ± 0.2	1.11 ± 0.02	0.30 ± 0.01	0.04 ± 0.00	5.7 ± 0.1

[a] Each of the commercial tea leaves (12.5 g) was extracted twice with 500 mL of boiling water and filtered, and the two filtrates were combined to obtain 12.5% TWEs. [b] Each value represents mean ± SE of four individual determinations and is given in % of dry tea leaves (w/w). [c] ND, not detected. [d] Percent of dry tea leaves (w/w).

Figure 2. ORAC values of EGCG, GTPs, and TWEs. Each point represents the mean value of duplicate experiments.

Figure 3. ORAC values of EGCG, EGC, ECG, EC, C, GA, and caffeine. The concentration of each sample was 1 μM. The purity of the EGCG, EGC, and ECG is 97%, 95%, and 93% as determined by HPLC. Each point represents the mean value of duplicate experiments.

anisms contributed to the anticarcinogenic and antimutagenic effects of tea may involve the antioxidative activity (Ho et al., 1992; Yoshino et al., 1994; Katiyar et al., 1994; Yen et al., 1995), induction of phase II enzymes (Khan et al., 1992), blocking the biosynthesis of ultimate carcinogen (Nakamura and Kawabata, 1981), inhibition in the covalent binding of carcinogen to DNA (Shi et al., 1994), and inhibition of DNA synthesis and cell proliferation (Lea et al., 1993).

Reactive oxygen species might be important causative agents of a number of human diseases, cancers, atherosclerosis, and aging. Tea polyphenols such as EGCG, EGC, ECG could react with peroxy radical and thus terminate lipid peroxidation chain reactions (Katiyar et al., 1994). We found that EGCG has a more potent scavenging peroxyl-radical action than other tea polyphenols. The result indicated that the hydroxy group at the 3′ position and the galloyl group on EGCG were responsible for its strongly antioxidative activity. As illustrated in Figure 2, the antioxidative activity was in the order of Longjing tea (GTPs) > Longjing tea

Table 4. Composition of Various TWEs (300 μg/mL) and GTPs (300 μg/mL) Preparations[a]

component	TWEs, μg/mL (%)[b]			
	Assam black tea	Assam green tea	Longjing tea	Longjing tea (GTPs), μg/mL (%)
total catechins	32.04 (10.7)	76.83 (25.5)	188.70 (62.9)	239.40 (79.8)
catechins				
EGCG	9.81 (3.3)	38.93 (13.0)	97.80 (32.6)	133.41 (44.5)
EGC	16.71 (5.6)	23.91 (7.9)	53.46 (17.8)	51.30 (17.1)
ECG	4.83 (1.6)	9.74 (3.2)	24.87 (8.3)	33.15 (11.1)
EC	0.69 (0.2)	3.32 (1.1)	9.87 (3.3)	16.56 (5.5)
C	ND[c]	0.93 (0.3)	2.70 (0.9)	4.98 (1.7)
gallic acid	9.60 (3.2)	0.48 (0.2)	0.27 (0.1)	ND
caffeine	68.85 (23.0)	62.04 (20.7)	53.37 (17.8)	ND

[a] TWEs and GTPs samples were prepared as described in Materials and Methods. TWEs, tea water extracts; GTPs, green tea polyphenols. [b] Percent of total TWEs or GTPs solids (w/w). [c] Not detected.

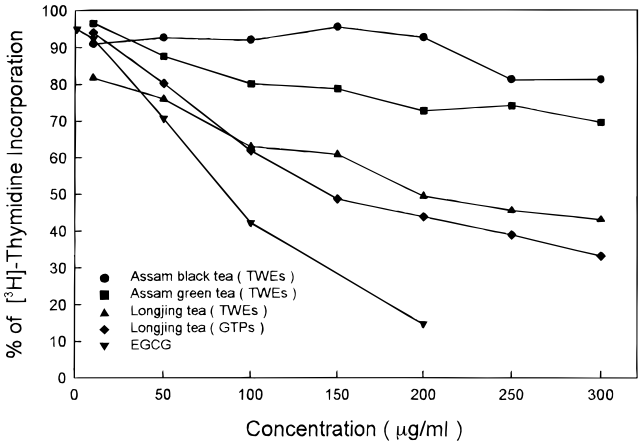

Figure 4. Effects of EGCG, GTPs, and TWEs on DNA synthesis in A-431 cells. The compositions of TWEs and GTPs are described in Table 4, and the preparations are described in Materials and Methods. Each point represents the mean value of duplicate experiments. The DNA synthesis is assayed by [^{3}H]thymidine incorporation.

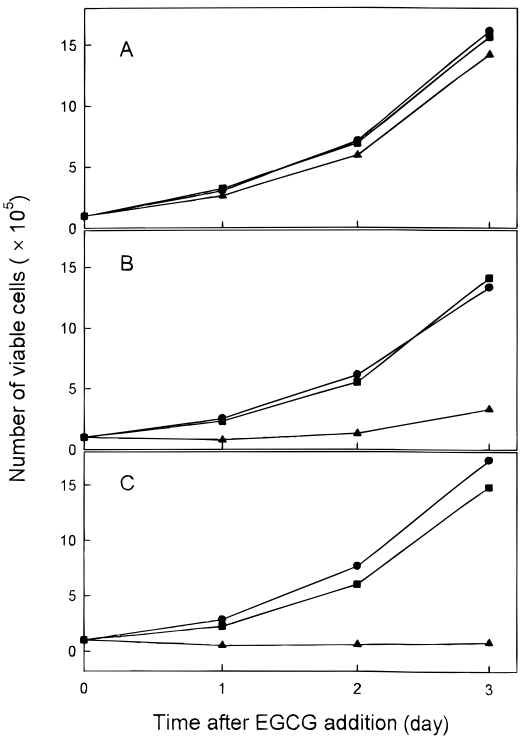

Figure 5. Inhibitory effects of EGCG on the growth of C3H10T1/2 (A), NIH3T3 (B), and S-180 II (C) cells. Cells were treated without (●) and with 10 μg/mL (■) or 30 μg/mL (▲) EGCG and counted daily as described in Materials and Methods. The data represent the mean value from four samples.

(TWEs) > Assam green tea (TWEs) > Assam black tea (TWEs), and these results correlated intimately with their EGCG contents. In Figure 3, we found that caffeine has a poor oxygen-radical-absorbing capacity, but it has been reported that caffeine effectively scavenges hydroxyl radicals (•OH), as investigated by electron spin resonance (ESR) spin trapping (Shi et al., 1991). The difference may be due to different test systems for different oxygen-radical species.

In the present studies, we also demonstrated that EGCG, GTPs, and TWEs inhibited DNA synthesis in A-431 cells. The content of caffeine in TWEs of Longjing tea, Assam green tea, and Assam black tea was similar, but the amount of total catechins was in the order of Longjing tea > Assam green tea > Assam black tea; the antiproliferative activity was also in the order of Longjing tea > Assam green tea > Assam black tea. These results indicated that the antiproliferative effect of tea was mainly due to the higher content of total catechins. The contents of EGCG in decaffeinated GTPs and Longjing TWEs were 133.41 and 97.80 μg/mL, and the antiproliferative effects were 66.8% and 56.97%, respectively. It was evident that the antiproliferative activity of TWEs or GTPs was correlative with the concentration of EGCG. Similar observations were made by Lea et al. In 1993, they found that tea extracts and EGCG inhibitied DNA synthesis and proliferation in HTC rat hepatoma cells and DS19 mouse erythroleukemia cells. However, little is known about the effects of EGCG on normal cells. In Figures 5 and 6, we found that tumor cells were more sensitive to EGCG treatment than normal cells, so EGCG had selective cytotoxicity on tumor cells. These results indicated that EGCG may selectively inhibited some types of cancers, but the mechanism is not clear. In 1990, Nakane and Ono found that EGCG and ECG can inhibit the activities of cellular DNA and RNA polymerases. Therefore, it is possible that EGCG inhibits the activities of DNA/RNA polymerases or oncoproteins or other enzymes that are associated with cell proliferation and then suppresses the growth of some tumor cells.

In this study, we did not analyze the content of the oxidized catechins in TWEs and test the antioxidative and antiproliferative effects of the oxidized catechins. Oxidized products of catechins, such as theaflavins (TFs) and thearubigin (TR), are known to be contained in the infusions of oolong tea and black tea, although the content of the infusion of oolong tea is lower than that of black tea. The antioxidative activity of TFs and TR was examined using the *tert*-butyl hydroperoxide-induced lipid peroxidation of rat liver homogenates (Yoshino et al., 1994). The antioxidative activity of TFs and TR was higher than that of glutathione, L-(+)-ascorbic acid, *dl*-

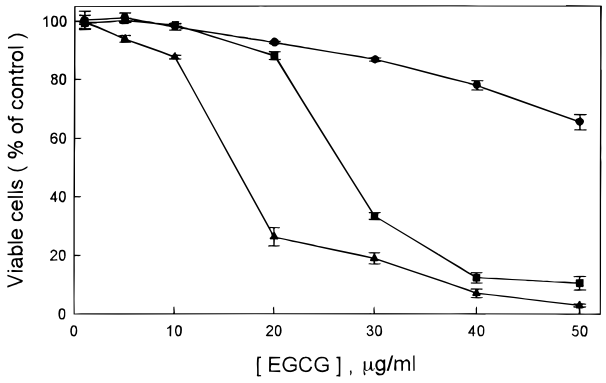

Figure 6. Cytotoxic effect of EGCG on C3H10T1/2 (●), NIH3T3 (■), and S-180 II (▲) cells in vitro. Cells were treated various concentrations of EGCG for 24 h and counted as described in Materials and Methods. The data represent the mean ± SE of the results obtained from six cultures.

α-tocopherol, etc., but was lower than the activity of ECG, EGC, and EGCG. In our studies, the oxygen-radical-absorbing capacity of Assam green tea and Assam black tea was similar, although that of Assam green tea was slightly higher than that of black tea. The yields of these lyophilized infusions from Assam green and black teas were about 24.2% and 23.4%, respectively, but the content of total catechins was higher in Assam green tea (12.2%) than in black tea (3.2%). Thus, the antioxidative activity of black tea would be due not only to catechins but also to TFs and TR.

Taken together, our results provided that antioxidative and antiproliferative activities have a good correlation with EGCG content. Longjing GTPs and TWEs have more antioxidative and antiproliferative activities than other teas. It is suggested that tea polyphenols indeed play an important part in the function of antioxidation and antiproliferation. Further work is required to determine the molecular mechanism involved in the antioxidative and antiproliferative effects of EGCG, GTPs, and TWEs.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihyrdochloride; C, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin 3-gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin 3-gallate; GA, gallic acid; GTPs, green tea polyphenols; HPLC, high-pressure liquid chromatography; ORAC, oxygen-radical absorbance capacity; β-PE, β-phycoerythrin; TTES, Taiwan Tea Experiment Station; TWEs, tea water extracts.

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LITERATURE CITED

Cao, G.; Alessio, H. M.; Cutler, R. G. Oxygen-radical absorbance capacity assay for antioxidants. *Free Radicals Biol. Med*. **1993**, *14*, 303−311.

Foley, G. E.; Drolet, B. P.; McCarthy, R. E.; Goulet, K. A.; Dokos, J. M.; Filler, D. A. Isolation and serial propagation of malignant and normal cells in semi-defined media origins of CCRF cell lines. *Cancer Res*. **1960**, *20*, 930−939.

Graham, H. N. Green tea composition, consumption, and polyphenol chemistry. *Prev. Med*. **1992**, *21*, 334−350.

Hampton, M. G. Production of black tea. In *Tea: Cultivation to Consumption*; Willson, K. C., Clifford, M. N., Eds.; Chapman & Hall: London, U.K., 1992; pp 459−511.

Hertog, M. G. L.; Hollman, P. C. H.; Putte, B. van de. *J. Agric. Food Chem*. **1993**, *41*, 1242−1246.

Ho, C.-T.; Chen, Q.; Shi, H.; Zhang, K.-Q.; Rosen, R. T. Antioxidative effect of polyphenol extract prepared from various Chinese teas. *Prev. Med*. **1992**, *21*, 520−525.

Huang, M.-T.; Ho, C.-T.; Wang, Z.-Y.; Ferraro, T.; Finnegan-Olive, T.; Lou, Y.-R.; Mitchell, J. M.; Laskin, J. D.; Newmark, H.; Yang, C. S.; Conney, A. H. Inhibitory effect of topical application of a green tea polyphenol fraction on tumor initiation and promotion in mouse skin. *Carcinogenesis* **1992**, *13*, 947−954.

Jain, A. K.; Shimoi, K.; Nakamura, Y.; Kada, T.; Hara, Y.; Tomita, I. Crude tea extracts decrease the mutagenic activity of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine in vitro and in intragastric tract of rats. *Mutat. Res*. **1989**, *210*, 1−8.

Katiyar, S. K.; Agarwal, R.; Zaim, M. T.; Mukhtar, H. Protection against *N*-nitrosodiethylamine and benzo[*a*]pyrene-induced forestomach and lung tumorigenesis in A/J mice by green tea. *Carcinogenesis* **1993**, *14*, 849−855.

Katiyar, S. K.; Agarwal, R.; Mukhtar, H. Inhibition of spontaneous and photo-enhance lipid peroxidation in mouse epidermal microsomes by epicatechin derivatives from green tea. *Cancer Lett*. **1994**, *79*, 61−66.

Khan, S. G.; Katiyar, S. K.; Agarwal, R.; Mukhtar, H. Enhancement of antioxidant and phase II enzymes by oral feeding of green tea polyphenols in drinking water to SKH-1 hairless mice: possible role in cancer chemoprevention. *Cancer Res*. **1992**, *52*, 4050−4052.

Lea, M. A.; Xiao, Q.; Sadhukhan, A. K.; Cottle, S.; Wang, Z.-Y.; Yang, C. S. Inhibitory effects of tea extracts and (−)-epigallocatechin gallate on DNA synthesis and proliferation of hepatoma and erythroleukemia cells. *Cancer Lett*. **1993**, *68*, 231−236.

Matthews, N.; Neale, M. L. Cytotoxicity assays for tumor necrosis factor and lymphotoxin. In *Lymphokines and Interferons. A Practical Approach*; Clemens, M. J., Morris, A. G., Gearing, A. J. H., Eds.; IRL Press: Oxford, **1987**; pp 221−225.

Mukhtar, H. M.; Wang, Z.-Y.; Katiyar, S. K.; Agarwal, R. Tea components: antimutagenic and anticarcinogenic effects. *Prev. Med*. **1992**, *21*, 351−360.

Nakamura, M.; Kawabata, T. Effect of Japanese green tea on nitrosamine formation in vitro. *J. Food Sci*. **1981**, *46*, 306−307.

Nakane, H.; Ono, K. Differential inhibitory effects of some catechin derivatives on the activities of human immunodeficiency virus reverse transcriptase and cellular deoxyribonucleic and ribonucleic acid polymerases. *Biochemistry* **1990**, *29*, 2841−2845.

Nonaka, G.-I.; Kawahara, O.; Nishioka, I. Tannins and related compounds. XV. A new class of dimeric flavan-3-ol gallates, theasinensins A and B, and proanthocyanidin gallates from green tea leaf. *Chem. Pharm. Bull*. **1983**, *31*, 3906−3914.

Oguni, I.; Nasu, K.; Yamamoto, S.; Nomura, T. On the antitumor activity of fresh green tea leaf. *Agric. Biol. Chem*. **1988**, *52*, 1879−1880.

Reznikoff, C. A.; Bertram, J. S.; Brankow, D. W.; Heidelberger, C. Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division. *Cancer Res*. **1973**, *33*, 3239−3249.

Shi, S. T.; Wang, Z.-Y.; Smith, T. J.; Hong, J.-Y.; Chen, W.-F.; Ho, C.-T.; Yang, C. S. Effects of green tea and black tea on 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone bioactivation, DNA methylation, and lung tumorigenesis in A/J mice. *Cancer Res*. **1994**, *54*, 4641−4647.

Shi, X.; Dalal, N. S.; Jain, A. C. Antioxidant behavior of caffeine: efficient scavenging of hydroxyl radicals. *Food Chem. Toxicol*. **1991**, *29*, 1−6.

Shiraki, M.; Hara, Y.; Osawa, T.; Kumon, H.; Nakauama, T.; Kawakishi, S. Antioxidative and antimutagenic effects of theaflavins from black tea. *Mutat. Res*. **1994**, *323*, 29−34.

Takeo, T. Green and semi-fermented teas. In *Tea: Cultivation to Consumption*; Willson, K. C.; Clifford, M. N., Eds.; Chapman & Hall: London, U.K., 1992; pp 413−457.

Wang, Z.-Y.; Cheng, S. J.; Zhou, Z. C.; Athar, M.; Khan, W. A.; Bickers, D. R.; Mukhtar, H. Antimutagenic activity of green tea polyphenols. *Mutat. Res.* **1989**, *223*, 273−285.

Wang, Z.-Y.; Huang, M.-T.; Ferraro, T.; Wong, C.-Q.; Lou, Y.-R.; Reuhl, K.; Iatropoulos, M.; Yang, C. S.; Conney, A. H. Inhibitory effect of green tea in the drinking water on tumorigenesis by ultraviolet light and 12-O-tetradecanoylphorbol-13-acetate in the skin of SKH-1 mice. *Cancer Res.* **1992**, *52*, 1162−1170.

Yang, C. S.; Wang, Z. Y. Tea and cancer. *J. Natl. Cancer Inst.* **1993**, *85*, 1038−1049.

Yen, G.-C.; Chen, H.-Y. Antioxidant activity of various tea extracts in relation to their antimutagenicity. *J. Agric. Food Chem.* **1995**, *43*, 27−32.

Yoshino, K.; Hara, Y.; Sano, M.; Tomita, I. Antioxidative effects of black tea theaflavins and thearubigin on lipid peroxidation of rat liver homogenates induced by *tert*butyl hydroperoxide. *Biol. Pharm. Bull.* **1994**, *17*, 146−149.

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