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Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols

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In order to study the biological activities of tea preparations and purified tea polyphenols, their growth inhibitory effects were investigated using four human cancer cell lines. Growth inhibition was measured by [^{3}H]thymidine incorporation after 48 h of treatment. The green tea catechins (–)-epigallocatechin-3-gallate (EGCG) and (–)-epigallocatechin (EGC) displayed strong growth inhibitory effects against lung tumor cell lines H661 and H1299, with estimated IC_{50} values of 22 μM, but were less effective against lung cancer cell line H441 and colon cancer cell line HT-29 with IC_{50} values 2- to 3-fold higher. (–)-Epicatechin-3-gallate, had lower activities, and (–)-epicatechin was even less effective. Preparations of green tea polyphenols and theaflavins had higher activities than extracts of green tea and decaffeinated green tea. The results suggest that the growth inhibitory activity of tea extracts is caused by the activities of different tea polyphenols. Exposure of H661 cells to 30 μM EGCG, EGC or theaflavins for 24 h led to the induction of apoptosis as determined by an annexin V apoptosis assay, showing apoptosis indices of 23, 26 and 8%, respectively; with 100 μM of these compounds, the apoptosis indices were 82, 76 and 78%, respectively. Incubation of H661 cells with EGCG also induced a dose-dependent formation of H_2O_2. Addition of H_2O_2 to H661 cells caused apoptosis in a manner similar to that caused by EGCG. The EGCG-induced apoptosis in H661 cells was completely inhibited by exogenously added catalase (50 units/ml). These results suggest that tea polyphenol-induced production of H_2O_2 may mediate apoptosis and that this may contribute to the growth inhibitory activities of tea polyphenols *in vitro*.

Introduction

Tea (*Camellia sinensis*) is one of the most common beverages consumed worldwide, and the possible beneficial health effects have received a great deal of attention. The inhibitory action of tea against experimental carcinogenesis has been demonstrated in many animal models, including those involving cancers of the lung, skin, esophagus, liver and stomach (1–4). In our previous studies on lung tumorigenesis induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) (5–7) as well as skin tumorigenesis induced by chemicals and UV light (8–10) in mice, tea preparations have been shown to inhibit carcinogenesis at the initiation, promotion and progression stages. Tea is one of the few agents known to inhibit carcinogenesis at the post-initiation stages.

In general, ~10% of the dry weight of green tea is flavan-3-ols, commonly known as catechins, which include (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG) and (–)-epicatechin (EC) (11). In the manufacturing of black tea, the tea catechins are oxidized and polymerized to form the characteristic black tea polyphenols, theaflavins and thearubigins (11). These polyphenol compounds are known to have antioxidative activities due to their radical scavenging and metal chelating functions, as well as anti-mutagenic activities (12,13). Such activities have been suggested to be the mechanisms for the inhibitory activity against carcinogenesis (1,2,4). EGCG has been considered to be a major constituent of tea and has received a great deal of attention in previous studies (14–16). Some authors even single out EGCG as the active anti-cancer component (17). However, other tea constituents, such as EGC and theaflavins, also have anti-proliferative or anti-carcinogenic activities (18–20). The active components and mechanisms involved in the anti-carcinogenic action of tea need to be studied further.

Black tea polyphenols, green tea extract and EGCG have been shown to inhibit the growth of rat hepatoma, mouse erythroleukemia and several human cancer cell lines (18,20). Induction of apoptosis in human lymphoid leukemia cells has also been demonstrated (21). However, the mechanisms of the growth inhibition and apoptosis are not known. When added to tumor cells in culture, tea and tea polyphenol compounds display inhibitory effects on the activities of many enzymes, including teleocidin-induced protein kinase C, 12-*O*-tetradecanoylphorbol-13-acetate-induced epidermal ornithine decarboxylase, reverse transcriptase, DNA topoisomerase II and urokinase (14,17,22–24). However, the effective doses of tea polyphenols of the above experiments varied greatly, from 1 μM to 200 mM. Among these possible biological activities, it is difficult to identify the most pertinent mechanisms.

In order to study the mechanism of the anti-cancer activities of tea preparations and the active components involved, we compared the growth inhibitory activities of different tea preparations and isolated tea polyphenols in four human cancer cell lines using the [^{3}H]thymidine incorporation assay. In addition, the induction of apoptosis was investigated using the ApoAlert TM Annexin V and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) methods. The involvement of H_2O_2 in tea polyphenol-induced growth inhibition and apoptosis was also studied.

Materials and methods

Tea and tea polyphenol preparations

Green tea solids were dehydrated water extracts of regular or decaffeinated green tea leaves. The regular green tea solids contained EGCG, EGC, ECG,

***Abbreviations:** EGCG, (–)-epigallocatechin-3-gallate; EGC, (–)-epigallocatechin; ECG, (–)-epicatechin-3-gallate; EC, (–)-epicatechin; GTPP, preparation of green tea polyphenols; BrdU, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PBS, phosphate buffered saline.

EC and caffeine at concentrations of 14.2, 11.7, 3.3, 3.4 and 5.4%, respectively. Decaffeinated green tea solids contained EGCG, EGC, ECG, EC and caffeine at concentrations of 7.3, 6.8, 2.2, 2.5 and 0.3%, respectively. The preparation of green tea polyphenols (GTPP) contained EGCG, EGC, ECG, EC and caffeine at concentrations of 44.7, 12.5, 12.8, 8.2 and 1.7%, respectively. The preparation of theaflavins contained theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate and theaflavin-3,3′-digallate at concentrations of 21.4, 29.9, 15.2 and 27.5%, respectively. These tea preparations as well as purified tea polyphenols EGCG, EGC, ECG and EC were generously provided by Thomas J.Lipton Company (Englewood Cliffs, NJ). Tea polyphenols EGCG, EGC, ECG and EC were dissolved in 0.85% NaCl solution before being added to the culture medium. Theaflavins were dissolved in ethanol, and the final concentration of ethanol in culture medium was <0.1%.

Cell growth and the [3H]thymidine incorporation assay

Human lung adenocarcinoma cell lines (NCI-H661, NCI-H441 and NCI-H1299) and a human colon cancer cell line (HT-29) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The lung cancer cell lines were maintained in RPMI 1640 medium, and the HT-29 cells were maintained in McCoy's 5A medium. Both media were supplemented with 10% fetal bovine serum (Lot No. A149016, Germini Bio-Products, Inc., Calabasas, CA), 100 U/ml penicillin and 100 μg/ml streptomycin.

The cells were plated into 24-well culture plates (2×10^4 cells/well) overnight to allow for cell attachment. Then, fresh medium (50 μl/well) containing different concentrations of tea polyphenol compounds was added, and the culture was continued for 48 h. [3H]Thymidine (from DuPont NEN, Boston, MA) was added to the cells, at a final concentration of 1 μCi/ml, 3 h prior to the termination of the experiment. The cells were harvested by trypsinization and lysed with cold distilled water. The cell lysates were put onto the filter (Glass microfibre filters, Whatman International Ltd, Maidstone, England) and were washed three times with 5% trichloracetic acid, and one time with acetone on a filter. The cell pellet on the filter was dissolved in 10 ml of Biofluor (Fisher Scientific, Pittsburgh, PA) and the radioactivity was determined by liquid scintillation counting. The cell growth was expressed as the percentage of the control.

Apoptosis assays

H661 cells were seeded into 60-mm culture dishes (1×10^5 cells/dish) and were maintained overnight in a medium containing 10% fetal bovine serum. The culture medium was then replaced with a serum-free medium and continued to culture for 24 h. Subsequently, EGC, EGCG or theaflavins were added to the serum-free medium for 24 h. The cells were collected by trypsinization and washed with phosphate buffered saline (PBS). The cells (1×10^5 and 1×10^6 cells) were resuspended in 200 μl of binding buffer. Annexin V staining was accomplished following the product instruction (Clontech, Palo Alto, CA). In brief, 10 μl Annexin V-FITC (1 mg/ml) and 10 μl propidium iodide (2.5 mg/ml) were added to the samples for 5–15 min in the dark. The cells were analyzed under a fluorescence microscope using a dual filter set for FITC + propidium iodide or analyzed by flow cytometry. The cells that showed green staining (early stage) or green with red staining (middle or late stage) were counted as apoptotic cells. The percent of apoptotic cells is referred to as the apoptosis index. The total number of cells was counted using a hemacytometer. Apoptosis indices were also determined by flow cytometry.

The TUNEL assay was performed following the protocol provided by the manufacturer (PharMingen, San Diego, CA). In brief, H661 cells were seeded into 100 mm-dishes (2.5×10^5 cells/chamber) overnight and treated with EGCG as described above. After harvesting the cells with trypsin treatment, the cells were fixed with 4% paraformaldehyde for 15 min on ice, and washed with PBS. The cells were then incubated in DNA labeling solution [containing terminal deoxynucleotidyl transferase enzyme, bromodeoxyuridine (BrdU), and TdT reaction buffer] for 60 min at 37°C. After removing the DNA labeling solution by rinsing cells with PBS, the cells were incubated with the FITC-conjugated anti-BrdU antibody for 30 min at room temperature in the dark, and then with the propidium/RNase A solution for 30 min. The cells were analyzed by flow cytometry within 3 h of staining.

Flow cytometry assay for H_2O_2

Flow cytometric determination of cellular peroxide levels was conducted using established methods (25). In brief, H661 cells were seeded into 100 mm-dishes (5×10^5cells/dish) overnight. The cells were then pretreated with 30 or 100 μM EGCG for 24 h, collected by trypsinization, and 200 μl of the cell suspension (1×10^6 cells/ml) was added into 800 μl PBS. The cells were incubated with 10 μM of 2′,7′-dichlorofluorescein diacetate (Molecular Probes, Eugene, OR) for 15 min, then EGCG or H_2O_2 was added and incubation was continued for 20 min at 37°C. The production of intracellular H_2O_2 was measured using flow cytometry.

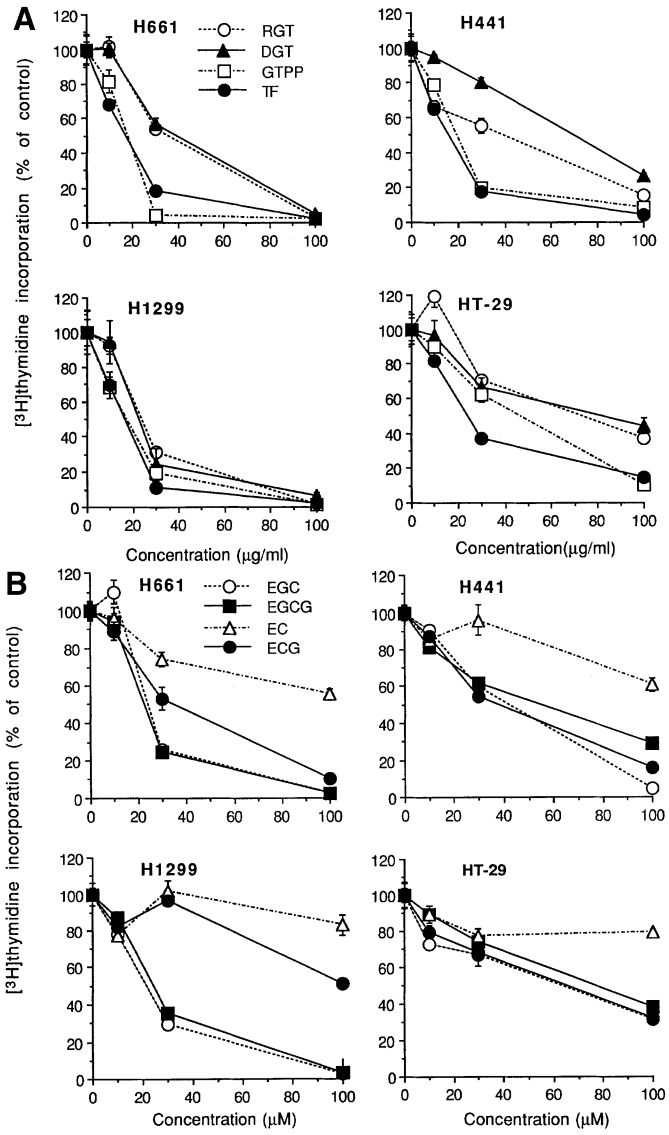

Fig. 1. Inhibitory effects of tea and tea polyphenol preparations on human cancer cell growth as determined by the [3H]thymidine incorporation assay. Lung cancer cell lines H661, H441 and H1299 and colon cancer cell line HT-29 were treated as described in Materials and methods with tea or tea polyphenol preparations (**A**) or different purified tea catechins (**B**). RGT, regular green tea solids; DGT, decaffeinated green tea solids; GTPP, preparation of green tea polyphenols; TF, theaflavins.

Results

Inhibition of human cancer cell growth by tea and tea polyphenols

Inhibition of cell growth was observed following treatments of different green tea and tea polyphenol preparations (Figure 1). The inhibitory activity was dose-dependent. Regular green tea and decaffeinated green tea appeared to display similar inhibitory activities in H1299 cells (estimated IC_{50}, 25 μg/ml), H661 cells (IC_{50}, 40 μg/ml) and HT-29 cells (IC_{50}, 75 μg/ml); but the decaffeinated green tea was less effective than regular tea in H441 cells (IC_{50} of 70 versus 40 μg/ml). With H661, H1299 and H441 cell lines, GTPP and theaflavins displayed approximately the same extent of cell growth inhibition, showing IC_{50} values of ~20 μg/ml. On a weight basis, this inhibitory activity was higher than that of the green tea preparations. The HT-29 cells appeared to be less susceptible

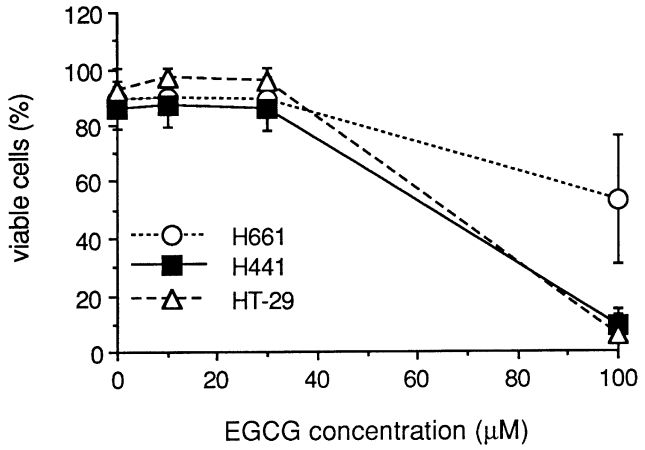

Fig. 2. Effects of EGCG on cell viability. H661, H441 and HT-29 cells were treated with different concentrations of EGCG for 48 h. Cell viability was determined by trypan blue staining and expressed as a percentage of viable cells in the total number of cells counted.

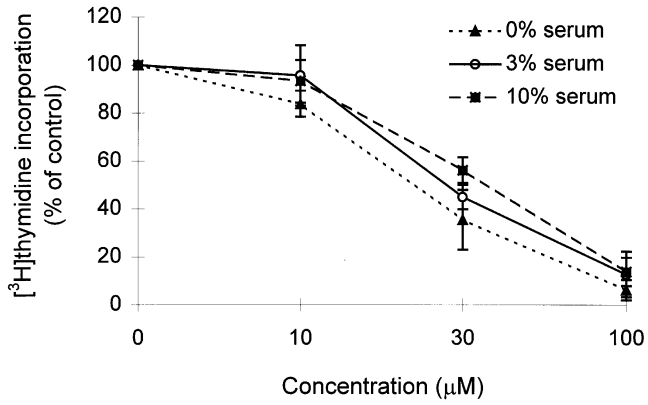

Fig. 3. Inhibition of H661 cell growth by EGCG in the presence of different concentrations of serum. H661 cells were treated with 0, 10, 30 and 100 μM of EGCG in the medium supplemented with 0, 3 or 10% fetal bovine serum for 4 h. Afterwards, the medium was replaced with fresh culture medium containing 10% serum and the incubation was continued for 48 h. The cell growth was determined by the [^{3}H]thymidine incorporation assay.

to the inhibition by GTPP (with IC_{50} of 47 μg/ml) than with other cell lines. Among the four major constituents of green tea polyphenols, EGCG and EGC had similar activities in tumor cell growth inhibition, with estimated IC_{50} values of 22 μM (equivalent to 10.1 μg EGCG and 6.7 μg EGC per ml) for cell lines H661 and H1299, and with IC_{50} values of 40 to 65 μM for H441 and HT-29 cells. ECG was less active than EGC with H661 and H1299, but had similar activities to EGC with H441 and HT-29. EC was the least active among the catechins studied, displaying only moderate inhibition at 100 μM.

To determine whether the observed growth inhibition was caused by cytostatic or cytotoxic effects, cells were exposed to tea or tea polyphenols for 48 h, and cell viability was measured using trypan blue stain. The viability of H661, H441 and HT-29 cells was not significantly affected by EGCG up to a concentration of 30 μM, but the viability was much lower with 100 μM EGCG (Figure 2). With these cells, similar results were also obtained for EGC and theaflavins (data not shown).

Polyphenolic compounds such as EGCG are known to bind strongly to proteins (11), and the serum content in the medium may alter the effective concentrations of these polyphenols. In order to determine the relationship between serum concentration and the growth inhibitory activity of tea polyphenols, H661 cells were incubated with EGCG in culture medium containing 0, 3 and 10% fetal bovine serum for 4 h, and then the cells were cultured in fresh medium containing 10% serum for 48 h. Cell growth was measured by [^{3}H]thymidine incorporation. With 30 μM EGCG, the growth inhibition rate appeared to be more pronounced in the serum-free medium than in the medium supplemented with 10% serum, but this effect was not statistically significant (Figure 3).

Induction of apoptosis by EGCG, EGC and theaflavins

Induction of apoptosis was observed in cell line H661 treated with EGC, EGCG and theaflavins. In the first experiment, this was determined by the ApoAlert™ Annexin V Apoptosis Kit, which measures the transfer of phosphatidylserine from the inner to the outer membrane of cells and can detect both early and late apoptotic cells with fluorescence microscopy (Figure 4). After treatment with 30 μM of EGCG, EGC or TF for 24 h, the apoptosis indices were 22, 26 or 8%, respectively;

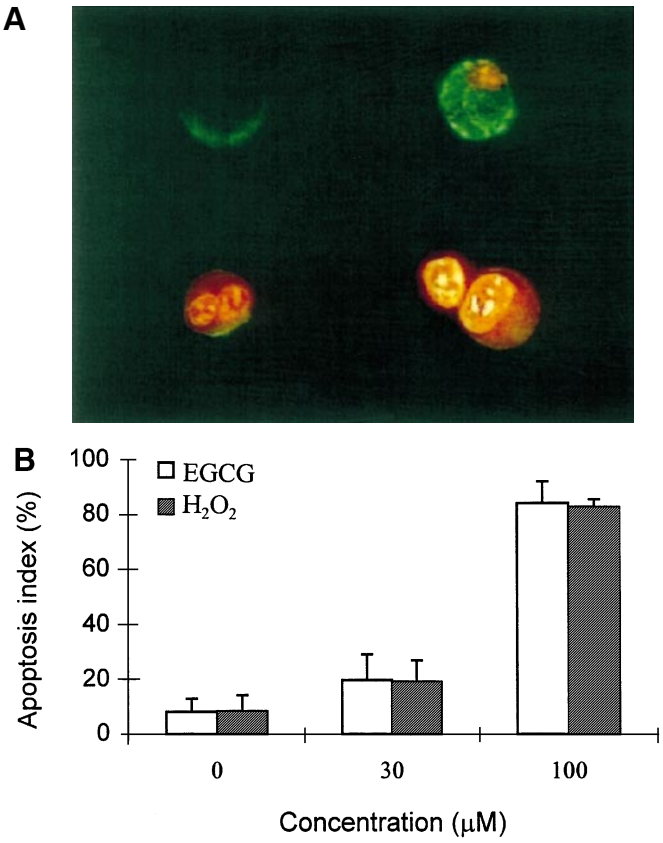

Fig. 4. Induction of apoptosis by EGCG and H_2O_2 in H661 cells as determined by Annexin V apoptosis assay. The cells were treated with 30 and 100 μM of EGCG for 24 h. The cells were then stained with Annexin V-FITC (1 μg/ml) and 10 μl propidium iodide (2.5 μg/ml) for 5–15 min in the dark. The apoptotic cells were detected under a fluorescence microscope using a dual filter set for FITC + propidium iodide. (**A**) Photomicrograph showing EGCG-induced apoptotic cells at early stage (green), middle or late stage (green and red), and dead cells (orange-red). (**B**) Induction of apoptosis by 30 and 100 μM of EGCG and H_2O_2.

with 100 μM of EGCG, EGC or theaflavins, the apoptosis indices were 81, 76 or 77%, respectively. In this experiment, induction of apoptosis by exogenously added H_2O_2 was also observed. The induction of apoptosis by EGCG was also determined by flow cytometry using the same kit; and similar

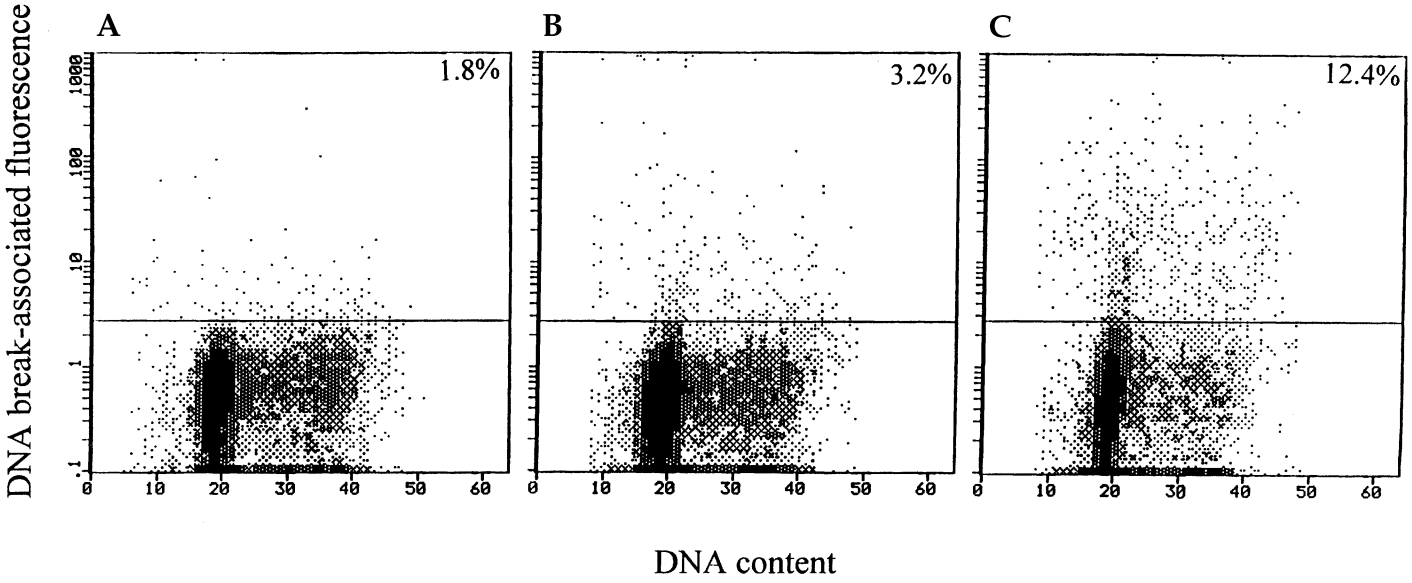

Fig. 5. EGCG-induced apoptosis of H661 cells as determined by the TUNEL assay using flow cytometry. The cells were treated with 10 and 30 μM EGCG for 24 h and then incubated with BrdU in the presence of TdT enzyme. The percentage of apoptotic cells (apoptosis index) was determined by flow cytometry. The apoptosis indices were 1.8, 3.2 and 12.4% for control (**A**), 10 μM EGCG (**B**) and 30 μM EGCG (**C**), respectively.

results were observed (data not shown). To further confirm the induction of programmed cell death by EGCG, the TUNEL assay, which detects DNA breakage that occurs during late stage of apoptosis, was performed. By the TUNEL assay, the apoptosis index was 1.8% in the control cells, and it increased to 3.2% and 12.4% in the cells treated with 10 and 30 μM EGCG, respectively (Figure 5).

In order to investigate the mechanism involved in the induction of apoptosis by EGCG, the effects of catalase and superoxide dismutase on EGCG-induced apoptosis were studied. The addition of catalase (50 U/ml) to the incubations completely prevented apoptosis induced by 30 or 100 μM EGCG (Figure 6A). The inhibitory effect of EGCG on cell growth, however, was only partially blocked by catalase (Figure 6B). No effect on the EGCG-induced cell growth inhibition and apoptosis was observed by incubation of the cells with superoxide dismutase (data not shown). Upon incubation of H661 cells with EGCG, H_2O_2 formation was observed (Figure 7). The presence of exogenously added catalase inhibited H_2O_2 formation.

Discussion

The present results demonstrate that EGCG and EGC possess growth inhibitory activities against human lung tumor cell lines H661 and H1299. ECG has lower inhibitory activity and EC was much less effective. Cell lines H441 and HT-29 appear to be less susceptible to inhibition by these polyphenols. The reasons for the observed differences in inhibition potency in terms of chemical structure and cell lines are not known. The observation that decaffeinated green tea extract had comparable inhibitory activity as regular green tea extract suggests that the decaffeination process did not remove components with significant inhibitory activities. Based on the estimated IC_{50} values for H661 and H1299 cells and the amounts of EGCG, ECG and EGC in GTPP and regular green tea solids, it was calculated that the inhibitory activities of EGCG, EGC and ECG could account for the inhibitory effects displayed by the GTPP and green tea preparations. The results also indicate

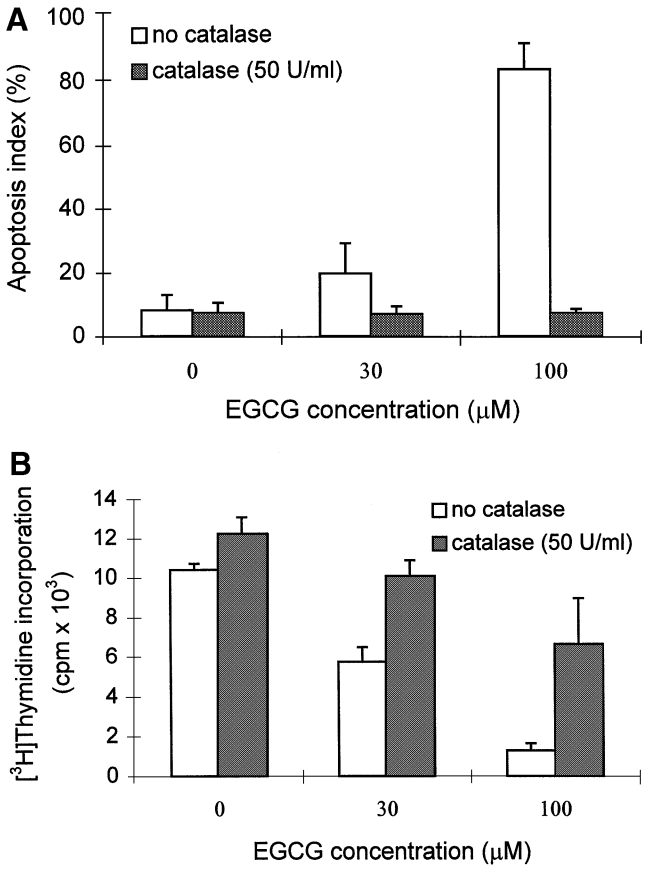

Fig. 6. Effects of catalase on EGCG-induced apoptosis and cell growth inhibition in H661 cells. (**A**) The cells were seeded overnight and catalase (50 units/ml) was added to the cell culture medium 5 min before adding EGCG. After culturing for 24 h, the cells were harvested and stained with ApoAlertTM Annexin V apoptosis kit. Apotosis index was determined under a fluorescence microscope. (**B**) In a similar experiment, the cell growth was determined by the [^{3}H]thymidine incorporation assay.

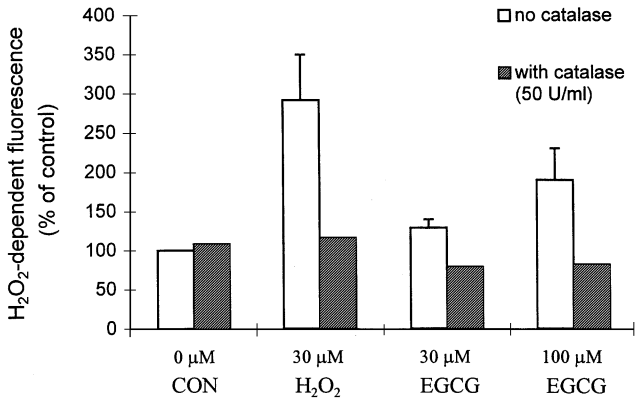

Fig. 7. EGCG-induced formation of H_2O_2 in H661 cells. The cells were incubated with 2′,7′-dichlorofluorescein diacetate (10 μM) for 15 min, and then catalase (50 U/ml) was added 5 min before the addition of H_2O_2 or EGCG. After incubation for 20 min at 37°C, H_2O_2-induced fluorescence was analyzed by flow cytometry.

that theaflavins from black tea exhibit growth inhibitory activities in cancer cells, although the activities are lower than those observed with EGC and EGCG.

At concentrations of 30 μM or lower, EGCG, EGC and theaflavins did not significantly affect the viability of the cells, whereas at 100 μM, a cytotoxic effect was observed as judged by the low viability, especially with H441 and HT-29 cells. This result is consistent with the observation that 100 μM and 30 μM of tea polyphenols induce apoptosis, showing apoptosis indices of ~80% and 8–26%, respectively. It appears that apoptosis is a major mechanism for cell killing in the presence of 100 μM of polyphenols, but it only contributes to a fraction of the growth inhibitory effect when the polyphenol concentrations were 30 μM or less. This conclusion is in agreement with the result that the addition of catalase, which completely prevented apoptosis in the presence of either 30 or 100 μM EGCG, prevented most of the growth inhibition by 100 μM EGCG, but only had a modest effect on the growth inhibition by 30 μM EGCG. The apoptosis-independent growth inhibition mechanism requires more investigation.

The effect of catalase on tea polyphenol-induced apoptosis is rather intriguing. Tea polyphenols are generally recognized to be antioxidants (26–29), but they do possess pro-oxidative activities. Indeed, H_2O_2 was produced in H661 cells during incubations with EGCG (Figure 7). H_2O_2-induced apoptosis and its inhibition by catalase have been observed by many investigators (30–32). The fact that exogenously added catalase can prevent apoptosis is probably because the intracellular and extracellular H_2O_2 are rapidly equilibrated. On the other hand, superoxide radicals are probably rapidly converted to H_2O_2 in the cells, and the exogenously added superoxide dismutase, which is not permeable to the cell membrane, would not affect H_2O_2-induced apoptosis as was observed herein.

Our previous studies indicate that under conditions in which inhibition of tumorigenesis by tea could be demonstrated in mice, the average EGCG and EGC concentrations were in the range of 0.2 to 0.3 μM (4). After ingestion of two or three cups of tea by human subjects, the average peak plasma values of EGCG and EGC were also in this range, and the highest individual values observed for EGCG and EGC were 0.65 μM (33). These values are much lower than the IC_{50} values for the growth inhibition observed herein. The plasma EGCG and EGC were mostly in the conjugated forms as glucuronides and sulfates. The cellular concentrations of EGCG and EGC in animals and in cultured cells are not known. It is possible that the presently observed growth inhibitory action against tumor cells in culture involves different mechanisms from the anti-proliferative effect of tea and tea polyphenols against pre-neoplastic cells observed in intact animals (7). The possible pro-oxidation activity of tea catechins needs to be investigated further. Although inhibition of human lung cancer cells can be demonstrated *in vitro*, it remains to be determined whether EGCG and ECG can effectively inhibit the growth of lung cancer *in vivo*. The possible application of these compounds for inhibition of lung cancer growth will also depend on the effective concentrations that can be delivered to the cancer cells.

Acknowledgements

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