Effect of black and green tea polyphenols on c-jun phosphorylation and \( \text{H}_2\text{O}_2 \) production in transformed and non-transformed human bronchial cell lines: possible mechanisms of cell growth inhibition and apoptosis induction

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The biological activities of theaflavin (TF), theaflavin gallate (TFG) and theaflavin digallate (TFdiG) from black tea and \((-\)-epigallocatechin 3-gallate (EGCG) and \((-\)-epigallocatechin (EGC) from green tea were investigated using SV40-immortalized (33BES) and Ha-ras gene transformed (21BES) human bronchial epithelial cell lines. Growth inhibition and cell viability were measured by trypan blue dye exclusion assay following 24 h treatment with the tea polyphenols. TFdiG, EGC and EGCG displayed comparable inhibitory effects on the growth of 21BES cells, with estimated IC\(_{50}\) values of 22–24 \( \mu \text{M} \). TFG exhibited a lower inhibitory activity (IC\(_{50}\) 37 \( \mu \text{M} \)) and TF was even less effective (IC\(_{50}\) 47 \( \mu \text{M} \)) in this cell line. A similar effect was also observed in 33BES cells. These results suggest that the gallate structure of theaflavins is important for growth inhibition. Exposure of 21BES cells to 25 \( \mu \text{M} \) TFdiG, EGC and EGCG for 24 h led to induction of cell apoptosis/death as determined by the Annexin V apoptosis assay. With TFdiG treatment cell death occurred early, and quickly peaked at 8–12 h. Morphological observations showed that TFdiG-treated cells appeared irregular in shape, with cytoplasmic granules, suggesting a cytotoxic effect. On the other hand, EGC and EGCG showed a lag phase before a rapid increase in apoptosis between 16 and 24 h, without any marked morphological changes, which was similar to that induced by \( \text{H}_2\text{O}_2 \). TFdiG, EGC and EGCG induced similar amounts of \( \text{H}_2\text{O}_2 \) formation in 21BES cells. Exogenously added catalase significantly prevented EGC- and EGCG-induced cell apoptosis, but did not prevent TFdiG-induced cell death, suggesting that \( \text{H}_2\text{O}_2 \) is involved in the apoptosis induced by EGCG and EGC, but not in TFdiG-induced cell death. EGCG and TFdiG were shown to decrease c-jun protein phosphorylation in 21BES cells. Such inhibition is expected to result in lowered AP-1 activity, which may contribute to the growth inhibitory activity of tea polyphenols.

Introduction

Tea and tea polyphenol preparations have been shown to inhibit tumorigenesis at different organ sites in animal models, including skin, lung, forestomach, stomach, duodenum and small intestine, colon, pancreas, liver and mammary gland (1–5). Our previous studies have shown that tea inhibits 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK)-induced mouse pulmonary cell proliferation, tumorigenesis and progression of adenoma to adenocarcinoma (3, 6–8). In this model activation of the Ki-ras oncogene due to mutation is a key mechanism of NNK-induced lung tumorigenesis (9–11). However, the mechanism of inhibition of lung cell proliferation and tumorigenesis by tea is not known.

Studies on cell lines by us and others have demonstrated the growth inhibitory activities of different tea and tea polyphenol preparations (12–14). Tea polyphenol-induced cell cycle arrest and cell apoptosis are the proposed mechanisms for inhibition of cancer cell growth (12–14). We have found that \((-\)-epigallocatechin 3-gallate (EGCG) can trigger \( \text{H}_2\text{O}_2 \) production and \( \text{H}_2\text{O}_2 \) production may induce apoptosis and contribute to growth inhibition in the human lung cancer cell line H661 (12). EGCG and theaflavins (a mixture of theaflavin and its gallate derivatives) have been shown to inhibit 12-O-tetradecanoylphorbol 13-acetate (TPA)- and epidermal growth factor-induced mouse JB6 cell transformation; such effects are associated with inhibition of AP-1 activity (15). A recent study showed that EGCG inhibited the growth of SV40-immortalized human fibroblasts, but not normal fibroblasts (16). However, the biological activities of different theaflavin components in black tea are not known. Whether or not tea polyphenols have differential effects on cancer and normal cells requires further studies.

Most of the studies on inhibition of carcinogenesis and cancer cell growth have been conducted with green tea and green tea components. Although black tea is the most common type of tea consumed in America and Europe, few studies have investigated the effects of black tea components on cancer formation and growth. The inhibitory activity against tumorigenesis of black tea is comparable with that of green tea in some animal models (3), but the effective components in black tea are not clearly understood. Theaflavins are characteristic polyphenol compounds of black tea and account for 2–6% of the water-extractable activity, which may contribute to the growth inhibitory activities of different tea polyphenol preparations (12–14). Tea polyphenol-induced cell cycle arrest and cell apoptosis are the proposed mechanisms for inhibition of cancer cell growth (12–14). We have found that \((-\)-epigallocatechin 3-gallate (EGCG) can trigger \( \text{H}_2\text{O}_2 \) production and \( \text{H}_2\text{O}_2 \) production may induce apoptosis and contribute to growth inhibition in the human lung cancer cell line H661 (12). EGCG and theaflavins (a mixture of theaflavin and its gallate derivatives) have been shown to inhibit 12-O-tetradecanoylphorbol 13-acetate (TPA)- and epidermal growth factor-induced mouse JB6 cell transformation; such effects are associated with inhibition of AP-1 activity (15). A recent study showed that EGCG inhibited the growth of SV40-immortalized human fibroblasts, but not normal fibroblasts (16). However, the biological activities of different theaflavin components in black tea are not known. Whether or not tea polyphenols have differential effects on cancer and normal cells requires further studies.

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In the present study SV40-immortalized non-transformed (33BES) and Ha-ras gene transformed (21BES) human bronchial epithelial cell lines were used to investigate growth inhibition and apoptosis induction...
inhibition and apoptosis induction by purified black tea polyphenols as well as by the green tea catechins EGCG and (-)-epigallocatechin (EGC). The time course of apoptosis induction by these compounds and involvement of H₂O₂ formation were studied. c-Jun protein expression and its phosphorylation were analyzed by Western blot. Inhibition of the phosphorylation of c-jun and induction of H₂O₂ formation may be key mechanisms for growth inhibition and apoptosis induction by tea polyphenols.

Materials and methods

**Chemicals and cells**

The purified green tea polyphenols, EGCG, EGC and epicatechin (EC), were provided by Thomas J. Lipton Co. (Englewood Cliffs, NJ). The black tea polyphenols, TF, TFG and TFdiG, were separated from a preparation of theaflavins (a mixture of all four compounds provided by Thomas J. Lipton Co.) by chromatography on a LH-20 column to a purity of >98%. The structures of these tea polyphenols are shown in Figure 1. EGCG, EGC and EC were dissolved in sterilized 0.85% NaCl solution. TF, TFG and TFdiG were dissolved in ethanol; the final concentration of ethanol in the culture medium was <0.1%.

SV40-immortalized non-transformed (33BES) and Ha-ras gene transformed (21BES) human bronchial epithelial cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). These cell lines were maintained in LHC-9 medium (Biofluids, Rockville, MD).

**Trypan blue dye exclusion growth assay**

The 21BES and 33BES cells were plated into 60 mm culture dishes (1×10⁶ cells/dish) overnight to allow for cell attachment. Fresh medium (50 µl/dish) containing different concentrations of tea polyphenol compounds was added and incubated for 24 h. The cells (both in suspension and attached) were harvested by trypsinization and centrifuged. The cell pellet was resuspended in 2 ml of phosphate buffer. The cells were stained with trypan blue. The number of dead and viable cells was counted using a hemacytometer. Growth inhibition by polyphenols is expressed as a percentage of the control (cells treated with the vehicle). Cell viability is the percentage of viable cells among the total (dead and viable) number of cells.

**ApoloAlert Annexin V apoptosis assay**

The 21BES cells were seeded into 60 mm culture dishes (1×10⁵ cells/dish) and were maintained in the medium overnight. For time-dependent induction of apoptosis, EC, EGC, EGCG, TFGdiG or H₂O₂ (each at 25 µM) was added to the medium for 4, 8, 12, 16, 20, 24, 28, 32 or 36 h. The role of H₂O₂ was examined by adding EGCG, EGC or TFGdiG (each at 25 µM) to the medium with/without catalase for 24 h. The cells were collected by trypsinization, centrifuged and washed in phosphate-buffered saline (PBS). The cells were then resuspended in 200 µl of 1× binding buffer. The apoptotic cells were analyzed using an Annexin V apoptosis assay kit (Clontech, Palo Alto, CA). In brief, 10 µl of Annexin V–FITC (1 µg/ml) and 10 µl of propidium iodide (2.5 µg/ml) were added to the cell suspension for 5–15 min in the dark. The cells were then analyzed under a fluorescence microscope using a dual filter set for FITC and rhodamine. The cells which showed green staining only (early stage) or both green and red staining (middle or late stage) were counted as apoptotic cells. The apoptosis index was calculated as percent apoptotic cells in the total number of cells counted using a hemacytometer.

**Dichlorofluorescein flow cytometry assay for H₂O₂**

21BES cells were seeded into 100 mm dishes (5×10⁵ cells/dish) overnight. The cells were treated with 25 or 50 µM EGCG, EGC or TFGdiG for 24 h. The cells were collected using a cell scraper, after being washed with cold PBS and lysed with 40 mM Tris buffer, pH 8.0, containing 2% 2-mercaptoethanol, 1% SDS, 5% glycerol, 10 mM EDTA, 50 µg/ml aprotinin (Sigma, St Louis, MO), 50 µg/ml leupeptin (Sigma), 10 µg/ml pepstatin A (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma). The protein concentration of the cell lysate was determined with a BCA protein assay kit (Pierce). The proteins were separated by 10% SDS–PAGE. The separated proteins were transferred to nitrocellulose or PVDF membrane. The membrane was probed with primary anti-c-jun (Santa Cruz) and anti-phosphorylated c-jun antibody followed by an enhanced chemiluminescence system (Amersham) and exposed to Hyperfilm ECL (Amersham). The intensity of each band was measured with an Image-Pro Plus system.

**Results**

**Growth inhibitory effects of theaflavins and catechins in transformed and non-transformed human bronchial cell lines**

The inhibitory activities of TF, TFG and TFdiG were dose dependent (Figure 2A). TFdiG displayed the strongest inhibitory activity (estimated IC₅₀ 22 µM) in transformed 21BES cells (Figure 2A). TFdiG was less effective (estimated IC₅₀ 37 µM) in transformed and non-transformed human bronchial cell lines (25 µM EGCG, EGC or TFdiG for 24 h). The cells were collected using a cell scraper, after being washed with cold PBS and lysed with 40 mM Tris buffer, pH 8.0, containing 2% 2-mercaptoethanol, 1% SDS, 5% glycerol, 10 mM EDTA, 50 µg/ml aprotinin (Sigma, St Louis, MO), 50 µg/ml leupeptin (Sigma), 10 µg/ml pepstatin A (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Sigma). The protein concentration of the cell lysate was determined with a BCA protein assay kit (Pierce). The proteins were separated by 10% SDS–PAGE. The separated proteins were transferred to nitrocellulose or PVDF membrane. The membrane was probed with primary anti-c-jun (Santa Cruz) and anti-phosphorylated c-jun (New England Biolabs) antibodies. The bound antibody was detected with peroxidase-conjugated anti-mouse or anti-rabbit Ig antibody followed by an enhanced chemiluminescence system (Amersham) and exposed to Hyperfilm ECL (Amersham). The intensity of each band was measured with an Image-Pro Plus system.

**Trypan blue dye exclusion growth assay**

The viability of 21BES and 33BES cells was not significantly affected by TFdiG up to a concentration of 30 µM or by TF and TFG up to 50 µM (Figure 3). Similar results on 33BES cells were observed (data not shown). Similar effects were also observed for both 21BES and 33BES cells for EGCG and EGCG in that up to a concentration of 30 µM cell viability was not significantly affected.
Inhibition of human cell growth by tea catechins

Fig. 2. Inhibitory effects of theaflavins and catechins on the growth of human bronchial epithelial cells. The cells were treated with different purified theaflavins and catechins for 24 h. Then the harvested cells were stained with trypan blue. The number of dead and viable cells was counted using a hemocytometer. (A) Transformed human bronchial epithelial cell line 21BES; (B) immortalized human bronchial epithelial cell line 33BES.

Fig. 3. Effects of different theaflavins on cell viability. 21BES cells were treated with different concentrations of theaflavins for 24 h. Cell viability was determined by trypan blue staining and expressed as a percentage of viable cells in the total number of cells counted.

Inhibition of human cell growth by tea catechins

understand these processes, we conducted time course studies on the induction of cell apoptosis/death by different tea polyphenols and H$_2$O$_2$. Based on the results of growth inhibition, 25 µM TFdiG, EGCG and EGC were selected for this study, because at this concentration the tea polyphenols inhibited cell growth but did not affect cell viability. 21BES cells treated with 25 µM TFdiG resulted in cell apoptosis/death (Annexin V–FITC-stained cells visualized as green fluorescent membrane staining), which occurred as early as 4 h and quickly peaked at 8 h (18% of cells undergoing apoptosis); the cell death index remained steady after 8 h (Figure 4). Morphological observations after TFdiG treatment revealed that some cells appeared irregular in shape with cytoplasmic granules, but these cells were resistant to staining by trypan blue. The majority of EGCG-, EGC- and H$_2$O$_2$-treated cells maintained membrane integrity without the appearance of morphological changes. A lag phase in the induction of apoptosis in 21BES cells by EGCG, EGC and H$_2$O$_2$ was observed (Figure 4). The number of apoptotic cells increased gradually for the first 16 h and then increased rapidly from 16 to 24 h. After 24 h the percentage of cells undergoing apoptosis gradually decreased, which can be explained by the growth of unaffected cells. EGC and H$_2$O$_2$ (11% of cells undergoing apoptosis) appeared to be slightly more potent than EGCG in induction of apoptosis (7.5% of cells undergoing apoptosis). EC did not significantly induce apoptosis in 21BES cells at 25 µM (Figure 4).

To test the hypothesis that tea polyphenol-induced H$_2$O$_2$ production may mediate cell apoptosis, the effect of catalase was investigated. When catalase (50 U/ml) was added to the culture medium 5 min before 25 µM TFdiG, EGCG or EGC, the percentage of 21BES cells undergoing apoptosis decreased from 8.9 to 5.3% and from 9.8 to 5.9%, respectively. In contrast, catalase did not prevent TFdiG-induced cell death (Figure 5), suggesting the involvement of a different mechanism.

**Tea polyphenol-induced H$_2$O$_2$ production**

A fluorescent dye, DCFH-DA, was used to measure the amount of intracellular H$_2$O$_2$ present following tea polyphenol treatment. Treatment of 21BES cells with 25 µM EGCG, EGC or TFdiG significantly induced H$_2$O$_2$ production. Similar amounts of H$_2$O$_2$ were produced by these three tea polyphenols
Fig. 5. Effects of catalase on tea polyphenol-induced apoptosis in 21BES cells. The cells were seeded overnight and catalase (50 U/ml) was added to the cell culture medium 5 min before adding different polyphenols. After culturing for 24 h, the cells were harvested and stained with an ApoAlert Annexin V apoptosis kit. Apoptosis was determined under a fluorescence microscope. A statistically significant difference between cells with catalase and cells without catalase was observed in the cells treated with EGCG (P < 0.05), EGCG (P < 0.05) or H$_2$O$_2$ (P < 0.01). However, there was no statistical difference between the cells treated with TFdiG or EC with/without catalase (P > 0.05).

Fig. 6. Production of H$_2$O$_2$ in 21BES cells treated with different tea polyphenols. The cells were incubated with DCFH-DA (10 µM) for 15 min and then catalase (50 U/ml) was added. Five minutes later, the purified polyphenols (25 µM) and H$_2$O$_2$ (10 µM, used as a positive control) were added. After incubation for 30 min at 37°C, H$_2$O$_2$-induced fluorescence was analyzed by flow cytometry. The H$_2$O$_2$-dependent fluorescence values in the cells treated with EGCG, EGCG and TFdiG were significantly higher than control cells (P < 0.05) and catalase significantly suppressed H$_2$O$_2$ production in the cells treated with EGCG, EGCG or TFdiG (P < 0.05).

Fig. 7. Effects of EGCG and TFdiG on c-jun protein expression and phosphorylation in 21BES cells. The cell treatments and Western blotting were as described in Materials and methods. (A) Levels of c-jun non-phosphorylated protein in cells; (B) phosphorylated protein in 21BES cells treated with 0, 10, 30 and 50 µM EGCG. (C) Levels of c-jun non-phosphorylated protein in cells treated with 0, 10 and 20 µM TFdiG; (D) phosphorylated protein in 21BES cells treated with 0, 10 and 20 µM TFdiG.

Discussion

Theaflavins, a group of characteristic polyphenol compounds in black tea, are responsible for the characteristic reddish color and stringency of black tea (17). The present results demonstrate that TFdiG, EGCG, and EGC possess strong growth inhibitory activity against human bronchial epithelial 33BES and 21BES cell lines. The transformed cells showed similar susceptibility to these tea polyphenols to the immortalized cells. TFG exhibited a lower inhibitory activity and TF was even less effective. Recent results from studies with H-ras transformed JB6 cells (30.7b Ras12) indicated that theaflavin 3-gallate and theaflavin 3'-gallate had similar inhibitory activities against cell growth. Our present results suggest that the gallate structure of theaflavins is important in cell growth inhibitory activity. TFdiG displayed a comparable inhibitory effect on the growth of the 21BES cell line as EGCG and EGC. In general, the galloyl structure on the B ring of catechins are important in metal chelation, antioxidant activity and binding to cellular molecules (18–20). The mechanism of biological action of these compounds needs to be further investigated.

In order to detect an early stage of apoptosis or cell injury and examine the time course of apoptosis, the Annexin V apoptosis assay was utilized. In the progression of apoptosis the externalization of phosphatidylinerse from the interior face of the cell membrane to the exterior surface is an early event (21). The probe Annexin V–FITC can specifically bind to the externalized phosphatidylinerse, which can be analyzed by fluorescence microscopy. EGCG, EGC and TFdiG displayed different time courses for induction of cell death in 21BES cells. After treatment with EGCG or EGC there was a lag phase before apoptosis began to increase rapidly from
16 to 24 h, which is the time course for H2O2-induced apoptosis. However, TFdiG-induced cell apoptosis/death occurred rapidly, starting at 4 h and quickly peaking at 8–12 h, suggesting the involvement of a different mechanism in induction of cell death. Morphological observations indicated that TFdiG treatment resulted in an irregular cell shape and cytoplasmic granules, which imply a different mechanism of cell death.

Our previous observation in human lung cancer cell line H661 indicated that EGCG-induced production of H2O2 may mediate apoptosis and partially contribute to the growth inhibitory activity (12). In the present study production of H2O2 and induction of apoptosis were further analyzed for different polyphenol compounds. TFdiG, EGCG and EGC, at 25 μM, induced similar amounts of H2O2 formation. Tea polyphenols are generally recognized as antioxidants, but they do have pro-oxidative activities, due to chemical reactions of the polyphenolic groups (18–20). The fact that catalase significantly inhibited formation of H2O2 and induction of apoptosis caused by EGCG and EGC but did not inhibit TFdiG-induced cell death suggests that TFdiG causes cell death by a mechanism different from the apoptosis induced by EGCG and EGC, in which H2O2 is a likely mediator. The triggering of the cell redox system intracellularly to generate reactive oxygen species is involved in the induction of cell apoptosis (22). Recent studies indicate that intracellular reactive oxygen species are involved in p53-dependent apoptosis (23,24). The possibility that EGCG and EGC induce H2O2 production and trigger the expression of redox-related genes needs to be investigated.

AP-1 activity plays a key role in cell proliferation and transformation (25,26). An early study in the mouse epidermal cell line JB-6 indicates that superoxide promotes JB-6 cell transformation and that H2O2 can inhibit this effect (27). Further studies demonstrated that transformation of JB-6 cells is associated with AP-1 activity and EGCG and theaflavins have been shown to inhibit AP-1 activity and TPA-induced cell transformation (15). Our previous data have shown that exogenously added catalase, but not superoxide dismutase, can block EGCG-induced apoptosis (12). In the present study both EGCG and TFdiG were shown to inhibit c-jun protein phosphorylation. This effect implies that the inhibition of c-jun phosphorylation by EGCG and TFdiG, which results in lowered AP-1 activity, may be a key mechanism of cell growth inhibition. It remains to be investigated whether such an action can be demonstrated in vivo.

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References

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