Molecular and cellular effects of green tea on oral cells of smokers: A pilot study

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Studies in cell culture and laboratory animals have shown that green tea and its major component, epigallocatechin-3-gallate, inhibit cell growth and reduce tumor incidence. However, results of epidemiological studies have generated inconsistent, sometimes conflicting data regarding protection by green tea against human cancers. To clarify the findings of these laboratory studies in application to humans, we conducted a pilot intervention study with three heavy smokers (>10 cigarettes/day) and three nonsmokers (never smokers) in order to evaluate the molecular and cellular effects of drinking green tea using human oral cells as an investigative tool. Green tea total extract (400 – 500 mg/cup, 5 cups /day) was administered in drinking water to the subjects for four weeks. Two oral cytology samples were taken weekly for measurements of tobacco carcinogen-induced DNA damage, including bulky adducts and oxidized bases, cell growth, DNA content, and apoptosis. The study showed that during the course of green tea administration smoking-induced DNA damage was decreased, cell growth was inhibited, and the percentage of cells in S phase was reduced, cells accumulated in G_1 phase (cyclin D_1 positive), DNA content became more diploid and less aneuploid, and p53, Caspase-3, and TUNEL, markers of apoptosis, were increased. The study, although preliminary, indicates that drinking green tea reduced the number of damaged cells in smokers by inducing cell growth arrest and apoptosis, a mechanism similar to that observed in cultured cells and animals. These results warrant a large-scale intervention trial to further verify the role of green tea in the prevention of oral cancer in smokers.

Keywords: Apoptosis / Cell proliferation / DNA content / DNA damage / Green tea / Oral cells / Oral cytology / Smokers

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1 Introduction

Since the early 1990s, the number of research articles about tea and its role in human health has grown exponentially [1, 2]. The role of tea, both green and black, in the prevention of cancer is supported by evidence from a large number of studies conducted in cell culture and animal bioassays. Over the past decade, there have been more than 80 published studies demonstrating protective effects of tea against tumorigenesis induced by different carcinogens in various organs in animal models. These organs include skin, lung, liver, mammary gland, and colon [3]. These studies have shown that tea can inhibit tumorigenesis at various stages, including initiation, promotion, and progression. The beneficial effects of tea are attributed to a handful of active compounds present in tea, primarily a family called "polyphenolics". The polyphenolic compounds, notably epigallocatechin-3-gallate (EGCG), are potent antioxidants. *In vitro* and *in vivo* studies have provided evidence that supports the role of polyphenolic compounds in the prevention of tumorigenesis.

While laboratory studies have provided evidence for the protective role of tea against cancer, results from epidemiological studies have been inconsistent [4]. This discrepancy remains a major challenge to research on tea and its effects on human cancer. To address this important issue, it will be pivotal to conduct studies on the cellular and molecular mechanisms evoked by tea, not only in cells/tissues and animals, but more importantly in humans. The mechanisms demonstrated to operate in cell culture and/or animal models need to be verified in humans by carrying out intervention trials.

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Abbreviations:, **B[*a*]P**, benzo[*a*]pyrene; **EGCG**, epigallocatechin-3-gallate; **8-OH-dG**, 8-hydroxy-2′-deoxyguanosine

Unlike studies in cultured cells and animals, only limited data are available on effects of drinking tea on oral cells in humans. In a double-blind intervention trial conducted earlier, 64 patients of both sexes, mostly smokers with oral leukoplakia, (a preneoplastic lesion,), took daily capsules containing a total of 0.38 g of green tea extract, green tea polyphenols, and black tea polyphenols (theaflavins and thearubigins), roughly equivalent to the consumption of 800 mL of regular tea per day, for six months [5]. During the study period, tea mixed in glycerin was also topically applied to the lesions. The placebo group received the same amount of capsules containing only glycerin with added starch and coloring agents. Results of this trial demonstrated that oral lesions were significantly reduced in 38% of patients treated with tea mix as compared with 10% of patients in the placebo group. This study also reported that the incidence of micronucleated exfoliated oral mucosa cells was significantly lowered in the tea treated group (5.4/1000 cells) as compared with that of the control placebo group (11.3/1000 cells). The cell proliferative index (PCNA) in the oral mucosa cell nuclei also appeared to be lower than in the control placebo group. Another study demonstrated metabolism of green tea catechins through examination of the serum and urine [6]. Therefore, the oral mucosa model offers a unique opportunity to examine the mechanism of cancer prevention by tea in humans. In this pilot study, we used oral keratinocytes of smokers and nonsmokers as an experimental system to glean data on the mechanisms of green tea efficacy against malignancies by measuring effects on the DNA damage induced by tobacco carcinogen, and measuring DNA content, cell growth, and apoptosis. The results, however preliminary, do provide initial evidence for prevention before an oral premalignant or malignant lesion is observed. This study demonstrates the reversal of molecular/cellular effects of tobacco smoke exposure following drinking of green tea.

2 Materials and methods

2.1 Treatment protocol

Three nonsmokers and three smokers (>10 cigarettes/day) from age 22 to 55 were recruited. Green tea was administered (35 bags of green tea powder were provided for each subject per week after the first visit for baseline sample collection, and each bag contained 400–500 mg of green tea powder (provided by Dr. Agarwal of Unilever, Bestfoods). The composition of the green tea powder is given in Table 1. The 0.15% tea solution was prepared by dissolving the green tea powder in 300 mL of hot water for consumption. The schedule for treatment and visits are shown in Fig. 1. Subjects were asked to use two bags in the morning, two in the afternoon, and one after dinner. Tea bags were dunked into hot water two to three times for 30–40 s. The water was at a temperature of 100°C. The counting of returned empty tea bags after each visit was used to control for compliance. At each visit, cells were harvested from the right and left lateral surfaces of the tongue using a nylon bristle cytobrush of medium stiffness. Two samples were pooled for each week and stored at −80°C. Subjects did not indicate any discomfort during tea treatment. The cell surface layer from the lateral border of the tongue was discarded because of a high apoptotic rate (30 ± 11.8% by mebstain, see below). After rinsing, the cells were carefully removed and placed into phosphate-buffered saline (1 ×), washed at least twice (800 rpm at 40°C for 8 min) and fixed with 1.0% paraformaldehyde. After final precipitation of cells and the removal of the supernatant containing 1 × PBS, an equal volume of 1.0% paraformaldehyde was added to the cell pellet. Generally, this was in the range of 200 mL. Cell viability using trypan blue dye exclusion before fixation indicated the presence of nucleated cells (Papanicolaou stain) [7]. Viability and number of cells harvested from an average smoker or nonsmoker reported in Section 3.

Table 1. Average composition of tea extracts

Tea components	Green tea extract
Epicatechin	3.80%
Epigallocatechin	8.70%
Epicatechin gallate	4.30%
Epigallocatechin gallate	15.10%
Caffeine	5.40%

2.2 DNA adducts

The detailed immunohistochemical assays for detecting DNA damage in oral cells were described previously [7]. Briefly, an aliquot of cells was stained using a monoclonal antibody (1:50) against the DNA adducts 8-hydroxy-2′-deoxyguanosine (8-OH-dG) (clone: MC-1001; commercially available from Trevigan, Gaithersburg, MD, USA) or using a polyclonal antibody against benzo[*a*]pyrene-7,8-dihydroxy-9,10-oxide-deoxyguanosine (B[*a*]P-dG; from Trevigan) for 1 h at 23°C. The antibody bound to the adduct present in oral keratinocytes is detected with a secondary fluorescent. A rabbit anti-mouse secondary antibody (1:50) with a fluoresceinated isothiocyanate conjugate (FITC) was used to detect the primary antibody. This antibody was purchased from Zymed (San Francisco, CA, USA). The amount of adduct was assessed for sensitivity (*e.g.*, area *versus* pixel density) as previously documented. Following fixation the cells are placed on lysine-coated glass slides and allowed to air-dry. The primary antibody is added for 1 h and washed at least twice with 1 × PBS and a secondary detection antibody is added and washed at least twice with 1 × PBS. Cells are analyzed using a laser scan-

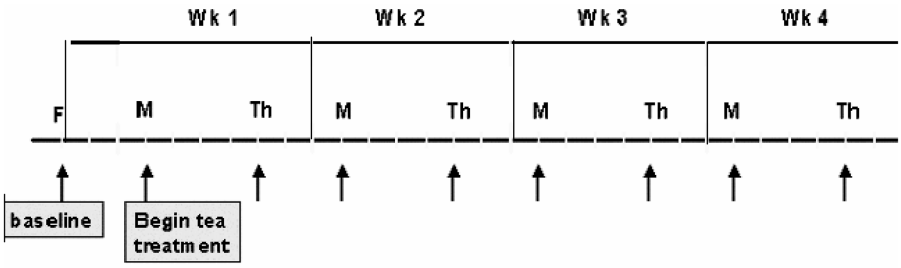

Figure 1. Protocol used to study effects of green tea on oral cells in smokers and nonsmokers. Subjects were asked to drink green tea prepared by dissolving 400 – 500 mg total green tea extract (provided by Unilever Bestfoods) in water five times daily for four weeks. Oral cell samples were collected twice a week as indicated by arrows by a procedure described in Section 2. Two samples obtained in each week were pooled for analyses for 4 weeks. Oral cytology harvest began with a baseline assessment Friday. This initial harvest of oral cells was proceeded by a Monday (M), and Thursday (Th) harvest.

ning cytometer purchased from CompuCyte (Cambridge, MA, USA). The fluorescence was detected at 480 nm using an argon laser. The laser scans the total slide area which is gated in the instrument. The settings for threshold, background, pixel density, and countour to detect the cell structures were fixed at the start of the study and were unchanged during the course of the study. These settings optimize the level of detection for the flourescence in the cells. The software used to detect each fluoresceinated antibody conjugate in scatterplot (FITC *versus* cell area, pixel intensity *versus* cell area, FITC1 *versus* FITC2), or histogram distributions (percentage of cells in population *versus* FITC) is proprietary for this instrument. A report is generated for each FITC or fluoresceinated labeled antibody that indicated the percentage of cells expressing this labeled protein, number of cells, and sizes of the cells. These results were based upon the region, counts, percentage, mean, coefficient of variation, median, and standard deviation. Calibration of instrument sensitivity for detection of benzo[*a*]pyrene (B[*a*]P) adduct was based upon an assessment of detection of B[*a*]P adducts using standard methods but enhanced using FITC-labeled detection using an automated laser scanning approach. Recognition by the primary antibody for B[*a*]P-N^2-dG adduct in a cell control was intiated. Oral keratinocytes were exposed to ^{3}H-B[*a*]P 1.0 mM of B[*a*]P for 6 h. Antibody detection and radiolabeled HPLC detection demonstrated B[*a*]P adducts detected by the antibody. In an identically treated cell population the level was assessed using state of the art, counting of fluoresceinated cells using microscopic visualization and addition of a nonbiased, blinded determination of adduct detection by fluorescence using laser scanning cytometry [7]. For example, antibodies used in this study have recognized 10^6 nucleotides for the 8-OH-dG and 10^{10} nucleotides for B[*a*]P-dG DNA adduct. As a control, oral cells exposed to B[*a*]P or benzo[*a*]pyrene diol epoxide verified the dose and time of exposure required to develop detectable levels of B[*a*]P-dG and 8-OH-dG adducts.

To validate the relationship between clinical lesion formation and the presence of DNA adduct formation, biopsy tissue sections were obtained from smokers that had presented oral carcinoma. Histopathology analysis disclosed sites of normal, mild, moderate, severe dysplasia, and invasive oral carcinoma. Laser microdissection using an Arturus PixCell II Laser Capture Microdissection System (Arturus Bioscience, Mountain View, CA, USA), a 7.5 mm spot size and a near-infrared diode laser at 40 × magnification of 10 μm thick sections stained with hematoxylin were a source of oral cytology. From one tissue section at least five sites of each histopathology provided oral cytology. A single-cell suspension of cells was obtained from each tissue section following incubation in 0.25% trypsin-EDTA at 37°C for 1 h and gentle pipetting using a Pasteur pipette to break apart the tissue and free single cells [7] from clinical biopsy samples and exhibiting containing moderate – severe – moderately severe dysplasia. Oral carcinoma sites were analyzed using an identical laser scanning and flow cytometry methods. Cells were placed on lysine-coated glass slides using cytospin centrifugation. These cells were analyzed for changes in DNA content and cell proliferation (cell cycle) [7]. Harvested cells from the tongue were routinely placed onto lysine-coated glass slides using cytospin centrifugation, and analyzed using laser scanning cytometry. A complete scan over the entire gated surface contained on average 10 000 cells. Contouring of the cells data was used to achieved an optimum threshold, pixel density, and background for each sample before scanning for final data collection was begun. Histogram analyses included a comparison of area to pixel density assessed for the FITC-conjugated antibodies against 8-OH-dG or B[*a*]P-N^2-dG.

2.3 Cell cycle determination

Cell cycle software is a modified version of WinCycle developed by Flow Systems (San Diego, CA, USA) [7]. The normalized DNA content data obtained from the laser scanning cytometer (Compucyte, Boston) was analyzed for cell cycle determinations (Epics XL; Beckman Coulter; Palo Alto, CA, USA). This analysis includes G_1, S, and G_2 + mitosis and reports % of cells in each stage for at least

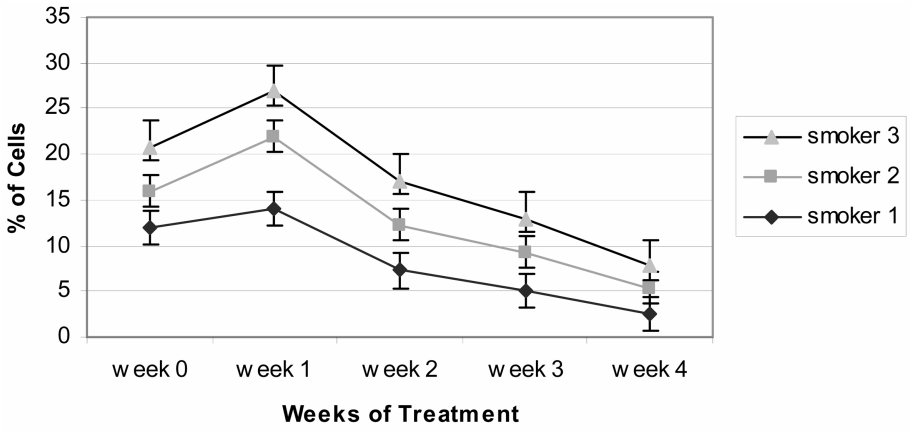

Figure 2. Percent of oral cells expressing B[*a*]P-dG adduct determined by using a polyclonal antibody for smokers before and during drinking of green tea from week 0 to week 4. A mean and standard deviation was calculated from the Monday and Thursday samples from each week for each patient. Initially a significant difference was found between the three individuals ($p > 0.05$) but at week 4 no significant difference was noted between the patients. The final level was also not significantly different from the nonsmoker level, 4.7 ± 1.3%.

10 000 cells. Fragmented nuclear DNA, number and sizes of DNA nuclei were evaluated and quantified for apoptosis using a DNA fluorescence histogram. The cells for these discriminators are diploid and the G_2/G_1 ratio is fixed to reduce variability (below 1.9). The "Background, Aggregate, and Debris" (B.A.D.) is eliminated from these calculations and is observed to be consistently, $\leq 0.01\%$ of the quantified population (Beckman Coulter) [7].

2.4 DNA content

The same aliquot of cells obtained for DNA adduct determination was analyzed for DNA content using the laser scanning cytometer following staining with the fluorescent dye, propidium iodide. The procedure used as follows: 10–20 000 cells were gated for each analysis. The cytometric data included nuclear content (*e.g.*, amount of DNA) staining with propidium iodide (PI) (1 μg/μL) with (0.1%) Triton X-100, 1 h at 23°C. This method stains isolated nuclei. The data are presented as a scatter plot with dual staining for DNA content (Sigma Chemical, St. Louis, MO, USA) (PI, stains red) and fluoresceinated isothiocyanate-conjugated anti mouse antibody (1 : 200) (Sigma) (FITC) (*e.g.*, p53) [7].

2.5 Apoptosis quantitation

The primary immunohistochemical staining of cells was analyzed for tumor suppressor activity with p53 expression (clone: Pab240; NeoMarkers, Fremont, CA, USA), and cyclin D_1 (clone : CD1.1; AbCam, Cambridge, MA, USA). Cells were screened for apoptosis, followed by apopain/caspase 3: A fluorescent equivalent, Apo 2.7 (Immunotech, Beckman Coulter) and TUNEL, nuclesomal fragmentation as characterized by terminal deoxynucleotidyl transferase (TdT) mediated dUTP fluorescein activity, mebstain, to assess the level of histone DNA fragmentation as previously described (Trevigan) [7].

3 Results

A typical sample collected at a single visit on week 0 (baseline) was 9.2 ± 5.2 × 10^6 cell/mL for nonsmokers and 2.6 ± 2.0 × 10^6 cells/mL for smokers. Viability of smoker's oral keratinocytes was 79.3 ± 4.4% at the baseline, whereas that of nonsmokers was significantly higher reaching 91.7 ± 2.7%. Among smokers, cell viability improved slightly to 89.7 ± 3.6% by week 4 with drinking green tea. Number of nucleated cells obtained from smokers also rose to 6.0 ± 4.0 × 10^6 cells/mL from the baseline value by week 4.

3.1 DNA adducts

The distribution of B[*a*]P-dG DNA adduct in oral cells collected from smokers over the four weeks of drinking tea is shown in Fig. 2. The initial percentage of cells expressing this adduct varied among the three smokers ranging from 13.1 ± 3.5 to 20.2 ± 4.1%. On week 4, oral cells with B[*a*]P-dG adducts decreased to about 3.2 ± 1.8 to 6.6 ± 5.0% among smokers. In contrast, cells harvested from nonsmokers showed 4.7 ± 1.3% with B[*a*]P-dG DNA adducts which did not vary more than 0.5% during the four weeks of green tea administration (data not plotted). Oxidative DNA lesion formation, 8-OH-dG, also showed a trend of gradual decline. Smokers showed 50.1 ± 4.1% to 80.0 ± 18.2% of oral cells with this lesion at the beginning on week 0 (Fig. 3). At the end of week 4, the percentages were reduced to between 20.1 ± 4.8% and 39.9 ± 11.3%. Nonsmokers showed a lower level of oxidative damage; 8-OH-dG lesions were expressed in only 4.7 ± 0.9% of the oral cells (data not plotted). After tea treatment for four weeks, 3.8 ± 2.2% of the oral cells showed 8-OH-dG expression among non-

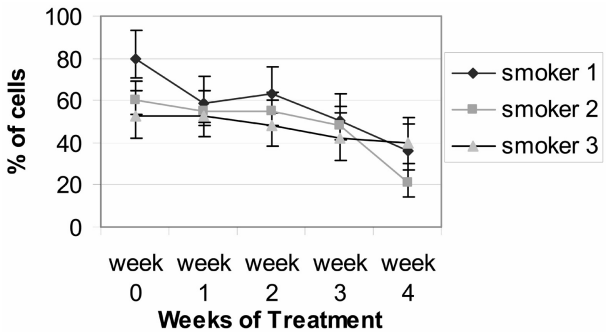

Figure 3. Percent of oral cells expressing 8-OH-dG adduct determined by a polyclonal antibody for smokers before and during drinking of green tea from week 0 to week 4. A mean and standard variation was calculated from the Monday and Thursday samples from each week for each patient. Initially and at the end of 4 weeks of treatment no significant difference was recorded between the patients but a significant difference was noted between nonsmokers 8-OH-dG levels and smokers ($p < 0.003$).

smokers. A trend of reduction in tobacco carcinogen-induced DNA damage among smokers, which included bulky and oxidative adducts, were observed as a result of green tea drinking.

3.2 Cell cycle

Oral keratinocytes harvested from tongue surfaces from smokers showed an increased number of cells in S phase relative to that of nonsmokers. Before the green tea administration (week 0), 61.7 ± 10.0% of the cells obtained from smokers were in S phase, whereas 20.9 ± 6.5% of the cells analyzed from nonsmokers were in S phase. After 4 weeks of drinking green tea, 40.2 ± 3.2% of the cells obtained from the tongue of nonsmokers were in S phase. Similarly, oral keratinocytes harvested from the tongue surfaces from smokers showed a significant decrease in S phase to 32.5 ± 3.1% after 4 weeks of treatment (Table 2). These opposing trends resulted in a similar percentage of cells in S phase from both groups of individuals after 4 weeks of drinking green tea. The rate of decrease of cells in S phase was fairly consistent among all three smokers during the treatment period (Table 2).

Studying the number of cells in G_1 from the same samples of cells described above, an initial and significantly ($p \leq 0.03$) higher number of cells in G_1 were found among nonsmokers, 67.4 ± 5.3%. In comparison, 28.0 ± 15.0% of the cells among smokers on week 0 were in G_1. After 4 weeks of drinking green tea the numbers of cells in G_1 decreased to 38.9 ± 2.5% for nonsmokers and increased to 43.9 ± 13.7% for smokers. To substantiate these changes in cell accumulation, we examined cyclin D_1 expression in oral cells. The percentage of cells that expressed cyclin D in smokers increased from 5.5 ± 3.0% on week 0 to 20.2 ± 2.4% on week 4 (Fig. 4). Nonsmokers showed a relatively steady trend with an average of 20.7 ± 1.8 for cyclin D_1 expression week 1 and 14.4 ± 3.9% by week 4. In contrast, the number of cells in G_2/M increased among nonsmokers from time 0 (11.4 ± 1.9) to 18.6 ± 2.8% by week 4. A significant level of increase was recorded ($p \leq 0.02$). Drinking green tea resulted in a decrease in oral cell proliferation from the tongue surface of smokers. Likewise, nonsmokers showed a significant increase in cell growth but a nonsignificant decline in cells accumulating in G_1 of the cell cycle.

3.3 DNA content

Oral keratinocytes from smokers showed a greater number of aneuploid cells, 21.9 ± 6.2%, than nonsmokers, 10.1 ± 6.0% ($p \leq 0.07$) (Table 3). Oral cell populations from nonsmokers contained more diploid cells, 89.8 ± 4.6%, than smokers, 76.0 ± 5.8% (time 0). In nonsmokers, these values changed only slightly from week 0 to week 4 (±1.5%) with drinking green tea, but the percentage of oral cells from smokers that were diploid by week 4 increased (83.0 ± 2.8%) ($p \leq 0.15$, not significant, comparison between time 0 and 4). A simultaneous reduction in the number of aneuploid cells was also recorded (14.2 ± 2.8%) (Table 3) ($p \leq 0.15$, not significant, comparison between time 0 and 4).

To substantiate these observations, a scatter-plot analysis comparing p53 expression and DNA content was performed on identical cell population. The data from the scatter-plot

Table 2. Cell cycle changes following administration of green tea

	Week 0			Week 1			Week 2			Week 3			Week 4		
	G	S	G_2/M	G	S	G_2/M	G	S	G_2/M	G	S	G_2/M	G	S	G_2/M
Smoker 1	43.8	50.1	6.1	39.2	52.7	8.1	45.3	48.0	6.7	34.9	41.1	22.0	63.0	31.5	5.5
Smoker 2	13.8	67.8	8.5	31.1	61.8	7.1	37.3	59.2	3.6	50.5	46.8	2.6	59.4	36.1	4.5
Smoker 3	26.6	67.3	6.1	32.4	65.3	2.3	37.3	59.2	3.6	46.5	46.7	6.8	65.1	30.1	4.7
Nonsmoker	71.2	15.2	12.6	49.9	30.2	18.4	44.6	34.2	9.0	40.9	40.1	18.0	36.0	38.1	20.7
Nonsmoker	69.8	18.0	12.5	55.0	30.1	14.3	57.1	29.0	14.4	49.0	37.5	14.1	40.4	38.5	19.9
Nonsmoker	61.3	28.8	9.1	50.1	34.0	13.8	49.2	34.9	13.8	42.1	40.3	16.9	40.5	44.0	15.4

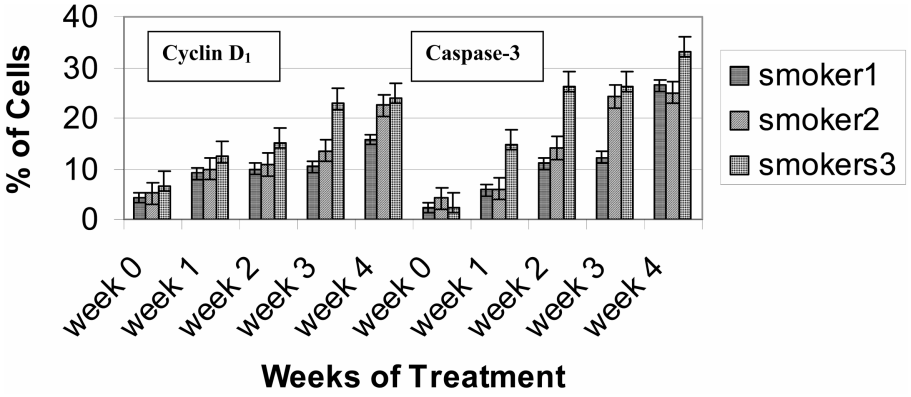

Figure 4. Cyclin D_1 and caspase-3 expressions in oral cells of smokers before and during drinking green tea. Bothe cyclin D and caspase-3 levels show a gradual increase in oral cells of smokers after drinking green tea.

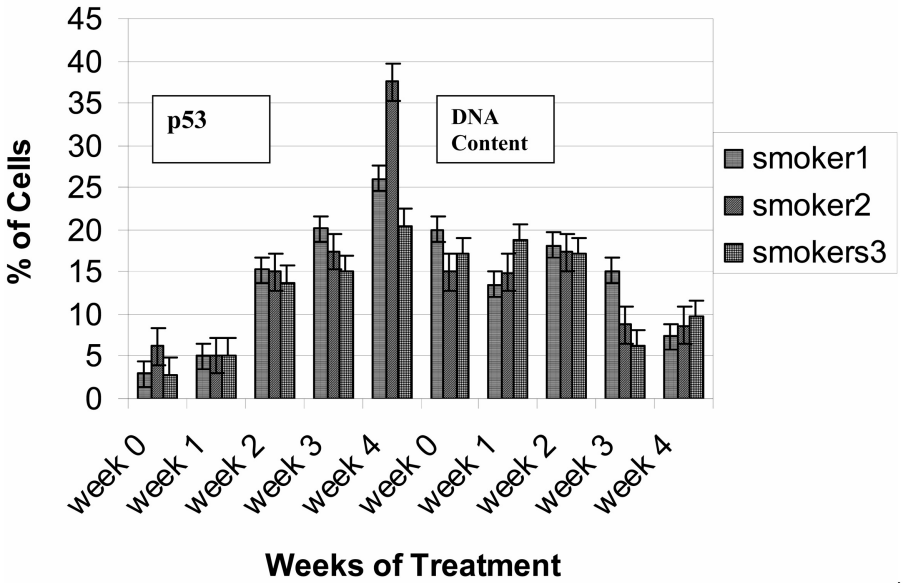

Figure 5. p53 expression and DNA content (aneuploid) in oral cells of smokers before and during drinking green tea. p53 expression levels increases as green tea drinking progresses from week 0 to 4. DNA content (aneuploid) levels of oral cells also exhibits an increase during the green tea drinking period.

Table 3. Percentage of diploid/aneuploid cells in smokers administered green tea

	Week 0	Week 1	Week 2	Week 3	Week 4
Smoker 1	79.5/17.5	68.3/27.7	86.4/11.6	84.2/12.8	85.5/11.5
Smoker 2	69.3/29.1	66.9/28.1	76.8/20.2	71.5/26.5	79.9/17.1
Smoker 3	79.4/19.3	76.9/21.5	84.3/23.2	77.5/20.5	83.8/14.2
Nonsmoker	93.0/7.0	93.0/7.0	89.5/10.5	94.0/5.0	93.0/6.0
Nonsmoker	92.0/8.0	91.4/8.6	89.0/10.0	91.0/8.0	92.6/7.4
Nonsmoker	84.5/5.5	90.0/9.9	91.0/8.0	88.5/11.5	93.0/6.0

was converted to graphic form to facilitate analysis. Among smokers, a reduction in aneuploid DNA content coincided with an increase in p53 expression (Fig. 5). For example, smokers in week 0 showed 3.4 ± 2.3% of cells expressing p53 while 17.7 ± 2.5% of these cells also contained a high DNA content (aneuploid) in week 0. After 4 weeks of green tea drinking, 28.2 ± 8.4% of the cells expressed p53 and 9.3 ± 3.5% of these cells had a high DNA content (aneuploid). In general, green tea consumption reduced the number of aneuploid cells and increased the number of cells that expressed p53 among smokers.

3.4 Apoptosis

p53 expression is an indicator of apoptosis, but to confirm apoptosis induction required the additional analysis of nucelosomal fragmentation (TUNEL) and analysis of caspase-3 expression level. A significantly increased mebstain (TUNEL) expression in cells from smokers from week 0 (2.0 ± 1.2%) to week 4 (20.3 ± 2.0%) ($p \leq 0.004$) (Table 4) and caspase-3 expression from 1.9 ± 0.9%, week 0 to 28.9 ± 4.3% by week 4 ($p \leq 0.003$), confirmed enhanced apoptosis induction.

Cells derived from the tongue of nonsmokers exhibited week 0, 9.7 ± 2.4% positive for mebstain (TUNEL) and after drinking green tea for 4 weeks 14.5 ± 1.7% exhibited apoptosis induction in a significant manner ($p \leq 0.05$) (Fig. 4). These results demonstrate that drinking green tea by smokers or nonsmokers increases the levels of apoptosis.

Taken together, this pilot study, although including only a small number of subjects, demonstrates that drinking green tea causes a decrease in tobacco carcinogen-induced bulky

Table 4. Percentage of mebstain (TUNEL) cells in smokers administered green tea

	Week 0	Week 1	Week 2	Week 3	Week 4
Smoker 1	3.3 ± 0.1	4.0 ± 2.2	5.3 ± 1.1	10.0 ± 2.2	18.2 ± 2.3
Smoker 2	2.0 ± 0.2	4.8 ± 2.5	5.8 ± 0.7	12.0 ± 2.5	20.7 ± 7.6
Smoker 3	0.8 ± 0.6	5.4 ± 1.8	6.6 ± 2.1	6.6 ± 3.0	22.2 ± 1.3
Nonsmoker	9.0 ± 5.5	9.8 ± 7.7	10.4 ± 5.9	13.0 ± 3.5	16.5 ± 6.2
Nonsmoker	7.7 ± 4.0	8.2 ± 3.9	15.0 ± 6.2	10.1 ± 4.9	13.0 ± 4.9
Nonsmoker	12.5 ± 6.0	12.9 ± 8.2	13.0 ± 8.8	13.3 ± 7.1	14.1 ± 9.2

DNA adduct and oxidative lesion, cell proliferation, and DNA content with a concomitant induction of apoptosis characterized by increased cyclin D_1, p53 expression, caspase-3 mitochondrial activation, and histone nucleosomal fragmentation.

4 Discussion

Evidence obtained from studies in cultured cells, including oral premalignant and malignant cells, and laboratory animals supports the induction of apoptosis as a likely mechanism whereby tea, polyphenolic compounds to inhibit tumorigenesis [3, 9–13]. Cells exposed tea polyphenols showed increased caspase-3 activity, and a specific p53-dependent apoptosis marker, thus suggesting a mitochondrion activation pathway [13, 14]. TUNEL, a terminal apoptosis marker, has also been identified with treatment of oral carcinoma cells with polyphenols [15]. Oral cytology harvest combined with flow cytometric analysis provided continuous information of oral cells harvested from the tongue surfaces of smokers and nonsmokers. Both groups of individuals showed an induction of apoptosis, but the smokers showed a reduction from a high level of cell proliferation while nonsmokers demonstrated an increase in cell growth. This apparent divergent effect resulted in similar levels of cell growth and apoptosis by the end of the study period. These data suggests drinking green tea has a low level cleansing activity by increasing normal diploid cell growth and removing damaged cells through apoptosis.

A series of apoptosis indicators is also found in the p57/KIP2 pathway that links apoptosis and cell cycle regulation. Induction of p57 is required for cell survival when exposed to green tea polyphenols and p57 is a general apoptotic signal for increased stress-activated protein kinase (SAPK) and c-Jun NH2-terminal kinase (JNK) [14–18]. Several cyclin proliferation markers are suppressed following exposure of oral premalignant or malignant cells to tea polyphenols, such as EGCG [11, 12, 14, 16]. These markers include tritiated thymidine uptake, proliferating cell nuclear antigen (PCNA), and bromodeoxyuridine (BrdU). Accumulation of cells in G_1, a marker for increased p53, following EGCG treatment of premalignant dysplastic oral cells was also noted [9, 10]. Accumulation of cells in G_1 is linked to enhanced cyclin D_1, phosphorylated retinoblastoma, activation of the p53/$p21^{CIPI}$ (cyclin-dependent protein kinase inhibitor), and signal activation under the control of AP-1, transcription factor c-jun/c-fos dimer complex [18, 19].

Cigarette smoke contains polycyclic aromatic hydrocarbons that increase expression of growth factor receptors in oral cancer patients. In one study, polyphenols were shown to downregulate one of the sites of insulin-like growth factor binding protein-5 (IGFBP-5) in normal oral keratinocytes (NHOKs) and oral carcinoma cells (SCC-25) during malignant transformation [20, 21]. Increased differentiation and enhanced apoptosis was linked to this process [10].

Unresolved in previous laboratory studies, the question arises whether drinking green or black teas is associated with benefit or risk in human cancer. While some epidemiological studies have concluded that tea drinking is associated with a reduction in the risk of head and neck cancers such as esophageal, and squamous carcinoma of the mouth [22, 23], several other studies have shown that tea drinking has little influence on risk for oral cancers [23–27]. The epidemiological investigations of tea and its possible influence on cancer could be difficult to interpret due to many confounding factors, such as diversity in types of tea used, preparation methods, including temperature of tea infusion, and frequency of tea drinking, just to name a few. The inconsistent data and conclusions, thus far, on tea and its relationship to cancer risk in population studies point to an urgent need to obtain data from intervention trials.

A limited number of intervention studies with green tea in smokers have been conducted to date. For example, a phase I trial was recently conducted with the oral administration of green tea extract in patients with solid tumors [26]. The pilot study described here is the first to examine the mechanisms of green tea in its role of reducing risk of oral cancer in healthy smokers. This study confirms that the molecular and cellular responses, that are attributable to the tumor preventive activity of this beverage that are demonstrated in cells and in animal bioassays with green tea or ECGC can also be demonstrated in human tissues. The results of this study reinforce the notion that green tea is likely to play a role as a protective dietary agent against human oral cancer.

In this study we also identified several potential molecular and cellular markers linked to oral cancer risk by smoking. This pilot trial used a novel modification of a noninvasive oral cytology sampling from the tongue of smokers and nonsmokers. Flow cytometry and laser scanning cytometer analysis of DNA adduct formation, cell proliferation assay, quantitation of DNA content, and quantitation of induction of apoptosis were all performed in parallel on aliquots of cells from patient samples. Data from this study demonstrated green tea drinking to increase cell growth of oral cells harvested from nonsmokers, but to reduce growth of

cells from smokers. Both groups of cells were observed to contain more apoptotic cells after treatment with tea. The distinctly opposite responses between nonsmoker and smoker oral keratinocytes upon administration of tea are expected to depend upon the presence of DNA damage. Relatively high levels of DNA damage in the oral cells from smokers were expected from exposure to tobacco smoke. DNA damage from bulky covalent and oxidation DNA adducts is expected to lead to cell growth, increased DNA content and apoptosis abnormalities not observed in the oral cells from nonsmokers. Higher numbers of aneuploid cells are expected to develop from a higher rate of DNA synthesis (high DNA content) [28]. Aneuploidy, a significant marker for risk for oral cancer, arises from a defect in segregation of chromosomes during mitosis, which becomes burdened by a greater number of cells exiting from the S phase of the cell cycle [29].

One prominent mechanism for inhibiting tumorigenesis involves damaged cells undergoing cell cycle check-point control as exemplified by induction of cyclin D_1 and p53 expressions. A capacity to maintain the relatively high percentage of diploid cells while increasing the elimination of aneuploid cells by inducing apoptosis will also result in a reduction in risk for premalignant lesion formation. One pathway for triggering apoptosis is through mitochondrial activation and the p53-dependent caspase-3 pathway. This was examined in this study and the data obtained suggests green tea induces apoptosis in oral cells of smokers through this mechanism and ultimately leads to histone nucleosomal fragmentation. Evidence from studies in Syrian hamsters treated with B[*a*]P to produce oral cancers in the floor of the mouth and lateral border of the tongue also confirms the requirement for these events during oral carcinogenesis, as well as the necessity to reverse these events in order to inhibit oral carcinogenesis, after administration of a preventive agent [8].

Finally, this pilot study demonstrated the feasibility of a noninvasive approach to obtain oral keratinocytes from smokers and nonsmokers to assess molecular and cellular characteristics of oral cells in response to tobacco smoke and green tea. Oral cells may have the potential to serve as surrogates of other target cells, such as lung, for the study of effects of dietary compounds on tobacco carcinogenesis. While this study has shown that short-term daily exposure to green tea can reverse molecular/cellular processes associated with increased risk of smoking, a large intervention study is required in order to further understand of the detailed mechanisms and firmly establish the preventive role of green tea in human cancers.

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