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In vitro Cytotoxicity of Epigallocatechin Gallate and Tea Extracts to Cancerous and Normal Cells from the Human Oral Cavity

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Abstract: This study compared the *in vitro* responses of malignant and normal cells from the human oral cavity to tea extracts and to its main polyphenolic component, (−)-epigallocatechin gallate (EGCG). The antiproliferative effects of tea polyphenolic extracts and EGCG were more pronounced towards immortalized, tumourigenic (CAL27, HSC-2, and HSG_1) and non-tumourigenic (S-G) cells than towards normal (GN56 and HGF-1) fibroblasts and green tea was more toxic than black tea. As the addition of tea extract or EGCG to cell culture medium led to the formation of hydrogen peroxide (H_2O_2), the research then focused on EGCG as an inducer of oxidative stress, using CAL27, the cancerous cells most sensitive to EGCG, HSG_1, the cancerous cells least sensitive to EGCG, and GN56 cells. The toxicity of EGCG was decreased in the presence of catalase, an enzyme that degrades H_2O_2, or of deferoxamine, a chelator of Fe^{3+}. Conversely, pretreatment of the cells with the glutathione depleters, 1-chloro-2,4-dinitrobenzene and 1,3-bis(2-chloroethyl)-*N*-nitrosourea, potentiated the toxicity of EGCG. A 4-hr exposure to EGCG lessened the intracellular level of reduced glutathione in the CAL27 and HSG_1 cells, but not in the GN56 fibroblasts. Whereas EGCG itself did not induce lipid peroxidation, Fe^{2+}-induced lipid peroxidation was potentiated by EGCG. A 72-hr exposure to cytotoxic concentrations of EGCG induced significant cytoplasmic vacuolization in all cell types. The results presented herein are consistent with EGCG acting as a prooxidant, with the cancerous cells more sensitive to oxidative stress than the normal cells.

Although the causative agents of human disease are varied and manifold, much evidence has accumulated that changes in dietary habits and lifestyles may reduce health risks. Research has indicated that many foods have non-nutritive constituents, termed nutraceuticals, which may provide protection against human illnesses, including cancer and heart disease. One such class of nutraceuticals is the dietary polyphenolics, which are ubiquitous in edible vegetables, fruits, and nuts, as well as in beverages, such as tea and wine. The estimated daily intake of dietary polyphenolics is about 1 g (Scalbert & Williamson 2000).

Epidemiologic findings and research with laboratory animal model systems have shown that the polyphenolic compounds in tea reduce the risk of cancer (Mukhtar & Ahmad 2000; Fujiki & Suganuma 2002). Green tea, consumed primarily in China and Japan, has more cancer preventive effects than black tea, consumed in Western countries. Although green and black teas are derived from the same plant, *Camellia sinensis*, they undergo different manufacturing processes. In production of green tea, freshly harvested leaves are rapidly stemmed or pan-fried to inactivate enzymes, thereby producing a dry, stable product rich in epicatechins, with (−)-epigallocatechin gallate (EGCG) (fig. 1) the major polyphenolic of green tea and the constituent thought to exhibit cancer preventive effects. In the production of black tea, the leaves are withered and then rolled and crushed to initiate fermentation reactions, in which some epicatechins are converted to theaflavins (Mukhtar & Ahmad 2000).

Interest in the chemopreventive properties of green tea, in particular EGCG, has directed much *in vitro* research using cells derived from malignant tissues. Studies with human cancerous cell lines have shown EGCG to inhibit cell proliferation (Uesato *et al.* 2001; Takada *et al.* 2002); to alter progression through the cell cycle (Ahmad *et al.* 1997; Salucci *et al.* 2002); to induce apoptosis (Ahmad *et al.* 1997; Chen *et al.* 2003; Hsu *et al.* 2003a); and to promote telomere shortening (Naasani *et al.* 2003).

Those relatively few studies comparing the *in vitro* responses of malignant and normal cells to tea polyphenols noted that immortalized cells were more sensitive, in terms of growth inhibition and apoptosis induction, than normal cells (Ahmad *et al.* 2000; Chen *et al.* 1998 & 2003). The initial portion of this study delineated the comparative responses of six cell lines, including both immortalized and normal cells, to EGCG and green and black tea polyphenolic extracts. All cell lines were derived from tissues of the human oral cavity, as this is the site initially exposed upon intake of tea. Thereafter, the study was directed to the effect of EGCG on oxidative stress. For comparisons, these latter studies used CAL27 cells, the cancerous cell line most sensi-

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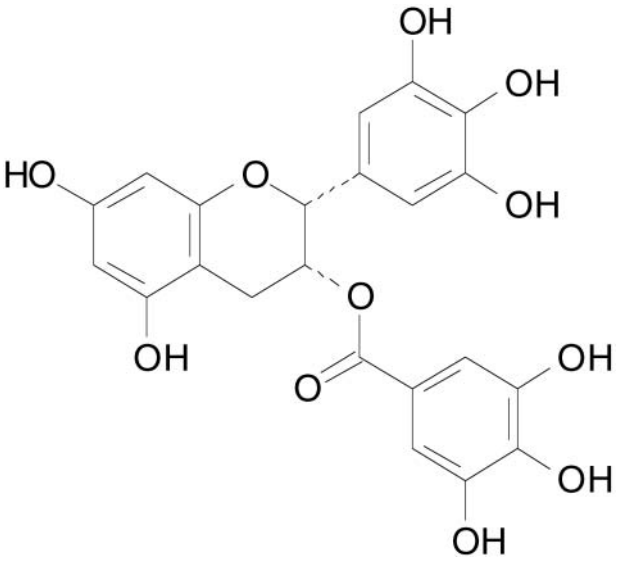

Fig. 1. Molecular structure of (-)-epigallocatechin gallate.

tive to EGCG, HSG_1 cells, the cancerous cell line least sensitive to EGCG, and GN56 fibroblasts, a normal cell line.

Materials and Methods

Cell cultures. Normal human gingival (GN56) fibroblasts and human tongue squamous carcinoma (CAL27) cells were obtained from D.A. Tipton, University of Tennessee, College of Dentistry, Memphis, TN, U.S.A.; human salivary gland carcinoma (HSG_1) cells and human squamous carcinoma (HSC-2) cells derived from the floor of the oral cavity were obtained from H. Sakagami, Department of Dental Pharmacology, Meikai University School of Dentistry, Saitama, Japan; human gingival epithelial (S-G) cells were obtained from F.H. Kasten, East Tennessee State University, Quillen College of Medicine, Johnson City, TN, U.S.A. (Kasten *et al.* 1989 & 1990; Rawls *et al.* 1990); and normal human gingival (HGF-1) fibroblasts were obtained from the American Type Culture Collection (Manassa, VA, U.S.A.). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 50 U/ml penicillin G, 50 µg/ml streptomycin sulfate, and 1.25 µg/ml amphotericin B (Fungizone), termed the growth medium, and maintained in a humidified atmosphere with 5.5% CO_2 at 37°. Cultures were dissociated with 0.05% trypsin-0.02% EDTA.

Chemicals. (−)-Epigallocatechin gallate (lot# A0402001), black tea polyphenolic extract (lot# A0202905), and green tea polyphenolic extract (lot# A0202907) (table 1) (Unilever Bestfoods North America, Englewood Cliffs, NJ, U.S.A.), 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma Chemical Co., St. Louis, MO, U.S.A.), and 1,3-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) (Bristol-Myers Squibb, Princeton, NJ, U.S.A.) were solubilized in ethanol. Stock solutions were made at high enough concentrations so that when diluted prior to use, the residual solubilizing ethanol concentration (0.5% and less) was not cytotoxic. Hydrogen peroxide (H_2O_2), deferoxamine mesylate, and catalase (from bovine liver) (Sigma Chemical Co., St Louis, MO, U.S.A.) were prepared directly in exposure medium, consisting of DMEM, 10% Serum Plus (JRH Biosciences, Lenexa, KS, U.S.A.), 2% FBS, and antibiotics. Serum Plus is a supplement containing low levels of foetal serum proteins and enhanced with specific growth promoting factors.

Neutral red cytotoxicity assay. For exposures to the test agents, individual wells of a 96-well microtiter tissue culture plate were inoculated with 0.2 ml of the growth medium containing $3.2–3.4\times10^4$ cells for a 24-hr exposure or $1–1.2\times10^4$ cells for a 72-hr exposure. After the 1 day of incubation, the growth medium was replaced with exposure medium, with or without varied concentrations of the test agents. Six to eight replicate wells were used per treatment. After 24 or 72 hr of exposure, viability was assessed with the neutral red assay, which is based on the uptake and accumulation of the supravital dye, neutral red (Borenfreund *et al.* 1990).

The protocol for the neutral red assay was as follows. A foil-wrapped 4 mg/ml aqueous stock suspension of neutral red was stored at room temperature. The stock was diluted to a working concentration of 0.04 mg/ml neutral red in exposure medium and incubated overnight at 37°. Prior to use, this solution was centrifuged to remove fine dye crystals. After a 24 or 72-hr exposure with the test agents, the medium was removed, 0.2 ml of neutral red-containing medium was added per well, and incubation was continued for 1 hr at 37°. Cells were then rapidly washed and fixed with 0.2 ml of 0.5% formalin-1% $CaCl_2$ (vol/vol) and the neutral red incorporated into viable cells was released into the supernatant with 0.2 ml of 1% acetic acid-50% ethanol. Absorbency was recorded at 540 nm with a microtiter plate spectrophotometer.

H_2O_2 assay. Measurement of H_2O_2 was carried out by the ferrous ion oxidation xylenol orange (FOX) method using the PeroXOquant Quantitative Peroxide Assay Kit, lipid-soluble formulation (Pierce Biotechnology, Rockford, IL, U.S.A.). The protocol was followed as suggested by the manufacturer, but with some exceptions. Briefly, a 90 µl sample of exposure medium (without phenol red) amended with EGCG, green tea polyphenolic extract, or black tea polyphenolic extract was mixed with 10 µl of methanol and incubated at room temperature for 30 min. The FOX reagent (0.9 ml) was added, followed by vortexing and 30 min. incubation at room temperature. Solutions were centrifuged at 12,000×g for 10 min. and absorbance of the supernatant was read at 580 nm against a blank consisting of 90 µl exposure medium (without phenol red), 10 µl methanol, and 0.9 ml FOX reagent.

Lipid peroxidation. Lipid peroxidation was estimated by measuring the production of malondialdehyde in culture supernatants as described previously (Babich *et al.* 2002). Briefly, confluent monolayers of cells in 6-well plates were washed twice with phosphate buffered saline (PBS) and treated for 4 hr with 2 ml PBS (control), 2 mM Fe^{2+} (as $FeCl_2\cdot4H_20$) in PBS, 500 µM EGCG in PBS, or a combination of 2 mM Fe^{2+} and 500 µM EGCG in PBS. Thereafter, 1 ml of 24% trichloroacetic acid was added, the cells were scraped, and the resulting suspensions were centrifuged to remove precipitated proteins. Then, 1 ml aliquots of the supernatant, to which was added 0.5 ml of 1% thiobarbituric acid in 0.05 M NaOH, were heated at 95–100° for 20 min. After cooling, the samples were cen-

Table 1.

Composition of polyphenols in tea extracts.

	Percent of total flavonoids	
Tea component	Black tea	Green tea
(−)-Epicatechin	13.50	9.08
(−)-Epigallocatechin	8.74	16.10
(−)-Epicatechin gallate	25.98	16.70
(−)-Epigallocatechin gallate	35.59	58.12
Theaflavin	5.52	–
Theaflavin-3-gallate	5.54	–
Theaflavin-3′-gallate	3.29	–
Theaflavin-3,3′-digallate	2.64	–

Black tea extract – lot #A0202905.
Green tea extract – lot #A0202907.
Source: Unilver Bestfoods North America, Englewood Cliffs, NJ, U.S.A.

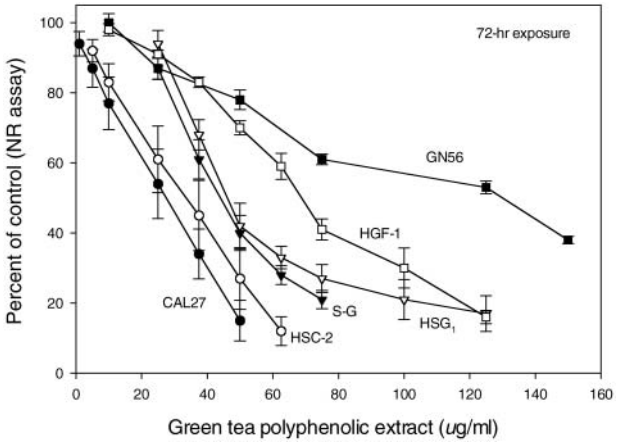

Fig. 2. Comparative 72-hr cytotoxicity of green tea extract towards proliferation of immortalized tumourigenic CAL27, HSC-2, and HSG_1 cells, immortalized, non-tumourigenic S-G cells, and normal GN56 and HGF-1 fibroblasts. Cytotoxicity was assessed with the neutral red (NR) assay. The data are presented as the mean percentages of the control±S.E.M. $P<0.05$ as compared to the control for concentrations of ≥25 μg/ml for CAL27 and HSC-2 cells, of ≥37.5 μg/ml for HSG_1 and S-G cells, and of ≥50 μg/ml for GN56 and HGF-1 cells.

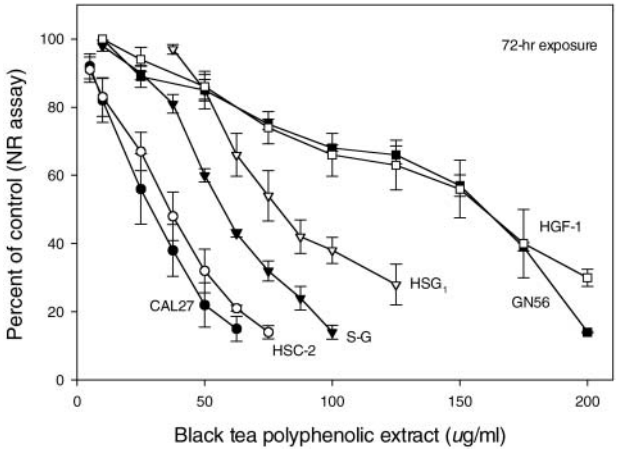

Fig. 3. Comparative 72-hr cytotoxicity of black tea extract towards proliferation of immortalized tumourigenic CAL27, HSC-2, and HSG_1 cells, immortalized, non-tumourigenic S-G cells, and normal GN56 and HGF-1 fibroblasts. Cytotoxicity was assessed with the neutral red (NR) assay. The data are presented as the mean percentages of the control±S.E.M. $P<0.05$ as compared to the control for concentrations of ≥25 μg/ml for CAL27 and HSC-2 cells, of 37.5 μg/ml ≥ for S-G cells, of ≥62.5 μg/ml for HSG1 cells, of ≥50 μg/ml for GN56 cells, and of ≥100 μg/ml for HGF-1 cells.

trifuged at 1,300×g for 10 min. Upon heating, the malondialdehye formed in the peroxidizing lipid system reacted with thiobarbituric acid to produce an orange-pink chromogen, which was measured at 532 nm (Gutteridge & Halliwell 1990).

Glutathione assay. Measurements of cellular reduced glutathione (GSH) were performed according to the procedures of Anderson (1981), with minor modifications (Babich *et al.* 2002). Cells, maintained in growth medium and grown to about 70% confluence in 60 mm diameter dishes, were washed with PBS and treated for 4 hr with PBS or with PBS amended with 500 μM EGCG. Thereafter, the cells were washed with PBS, lysed with 0.2% Triton X-100 and made to contain 2.5% sulfosalicyclic acid in a total volume of 0.12 ml. After harvesting by scraping and centrifugation, acid-soluble extracts were assayed. GSH was determined in 0.1 ml aliquots of the acid-soluble extract by determining the oxidation of GSH by 5% 5,5′-dithiobis(2-nitrobenzoic acid), prepared in phosphate buffer/EDTA (pH 7.5), to glutathione disulfide (GSSG), with stoichiometric formation of 5-thio-2-nitrobenzoic acid, a yellow chromogen measured spectrophotometrically at 412 nm. With each assay, a standard curve was generated with known amounts of GSH.

Microscopy. Cells were seeded to alcohol-cleansed, glass coverslips in 35 mm diameter tissue culture dishes containing growth medium. After the monolayers achieved 60–70% confluence, the cells were treated for 72 hr with exposure medium amended with the test agents. Thereafter, the cells were washed with PBS, fixed in methanol, stained with Giemsa solution, and examined microscopically.

Statistical analyses. All experiments were performed at least three times. Cytotoxicity graphic data were presented as the mean percentages of control±standard errors of the mean (S.E.M.). Linear regression analysis was used to compute the concentration of test agent needed to reduce absorbency of neutral red by 50%, termed the midpoint cytotoxicity, or neutral red_{50}, value. Experimental data were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant differences. The P-value of the effect had to be ≤0.05% to be considered significant.

Results

Concentration-response cytotoxicity curves for a 72-hr exposure of the human-derived cells to green tea polyphenolic extract are presented in fig. 2. Initial toxicity occurred at 25 μg/ml for CAL27 and HSC-2 carcinoma cells, at 37.5 μg/ml for HSG_1 carcinoma cells and immortalized, non-tumourigenic epithelial S-G cells, and at 50 μg/ml for normal

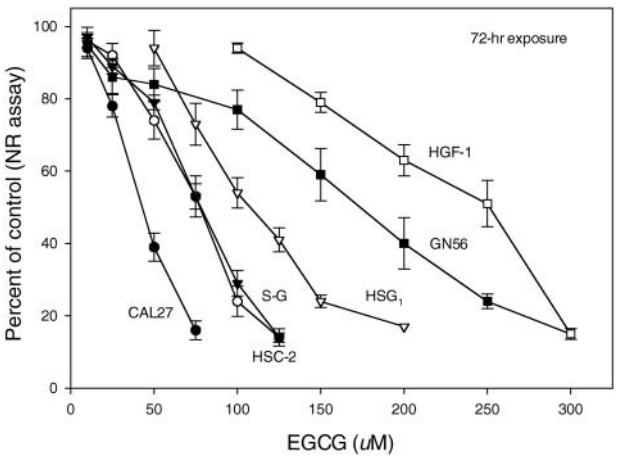

Fig. 4. Comparative 72-hr cytotoxicity of (−)-epigallocatechin gallate towards proliferation of immortalized tumorigenic CAL27, HSC-2, and HSG_1 cells, immortalized, nontumorigenic S-G cells, and normal GN56 and HGF-1 fibroblasts. Cytotoxicity was assessed with the neutral red (NR) assay. The data are presented as the mean percentages of the control±S.E.M. $P<0.05$ as compared to the control for concentrations of ≥25 μM for CAL27 cells, of ≥50 μM for HSC-2 and S-G cells, of ≥100 μM for HSG_1 cells, of ≥150 μM for GN56, and of ≥200 μM for HGF-1 cells.

Table 2.

Midpoint cytotoxicity values neutral red (NR_{50}) for a 72-hr exposure of test agents towards human cells in culture.

		NR_{50} (ug/ml)		NR_{50} (uM)
Cell line	Description	Green tea extract	Black tea extract	EGCG
CAL27	carcinoma; tongue	28±3.9	30±4.2	45±0.8
HSC-2	carcinoma; roof of oral cavity	35±5.7	42±7.3	78±5.6
S-G	immortal; gingival epithelial	48±2.0	59±1.3	80±4.9
HSG_1	carcinoma; salivary gland	55±4.9	87±6.6	109±5.7
HGF-1	normal; gingival fibroblast	71±2.6	120±14.4	246±17.9
GN56	normal; gingival fibroblast	110±9.1	163±13.8	171±15.6

Data are expressed as the mean percentage of control±S.E.M.

GN56 and HGF-1 fibroblasts. For the black tea polyphenolic extract, initial toxicity occurred at 25 μg/ml for CAL27 and HSC-2 cells, at 37.5 μg/ml for S-G cells, at 62.5 μg/ml for HSG_1 cells, at 50 μg/ml for GN56 cells, and at 100 μg/ml for the HGF-1 cells (fig. 3). Calculated midpoint cytotoxicity values (neutral red_{50}) are presented in table 2.

Similar studies were performed with EGCG, the most abundant polyphenolic in the tea extracts (table 1). For a 72-hr exposure to EGCG, initial toxicity was noted at 25 μM for CAL27 cells, at 50 μM for HSC-2 and S-G cells, at 100 μM for HSG_1 cells, at 150 μM for GN56 cells, and at 200 μM for HGF-1 cells (fig. 4). Calculated midpoint cytotoxicity values (neutral red_{50}) are presented in table 2.

Attention was directed to the production of H_2O_2 in exposure medium amended with EGCG, green tea polyphenolic extract, and black tea polyphenolic extract. The amount of H_2O_2 produced by EGCG increased in a concentration dependent manner and more H_2O_2 was produced by green, rather than by black, tea polyphenolic extract (table 3).

Table 3.

Generation of H_2O_2 in cell culture medium amended with test agents.

Test agent	Concentration	H_2O_2 in medium(uM)
EGCG	50 uM	20±4.2
	100 uM	45±3.8
	500 uM	100±21.9
green tea extract	100 ug/ml	85±12.5
black tea extract	100 ug/ml	63±12.2

Test agents were added to exposure medium (without phenol red) and incubated for 0.5 hr at room temperature.

Table 4.

Midpoint cytotoxicity values neutral red (NR_{50}) for a 24-hr exposure of test agents towards human cells in culture.

	NR_{50} (mM)	
Cell line	EGCG	H_2O_2
CAL27	0.18±0.02	1.1±0.10
HSG_1	0.42±0.03	1.0±0.05
GN56	0.44±0.05	1.0±0.09

Data are expressed as the mean percentage of control±S.E.M.

To better focus the research towards the prooxidant effects of EGCG, the subsequent studies were limited to CAL27, the cancer cell line most sensitive to a 72-hr exposure to EGCG, to HSG_1, the cancer cell line least sensitive to a 72-hr exposure to EGCG, and to GN56 fibroblasts, a normal cell line. For these latter studies, 24-hr exposures to EGCG were performed. CAL27 cells were more sensitive to EGCG than were the HSG_1 and GN56 cells, which exhibited similar responses. The CAL27, HSG_1, and GN56 cells were more sensitive to EGCG than to H_2O_2, but demonstrated comparable responses to H_2O_2 (table 4).

As the formation of H_2O_2 in medium amended with EGCG indicated that, under the conditions of these experiments, EGCG acted as a prooxidant, attention was directed to evaluating EGCG as a potential causative agent of oxidative stress. The involvement of H_2O_2 in EGCG-mediated cytotoxicity was shown by the decrease the EGCG cytotoxicity upon addition of exogenous catalase (100 Units/ml) (fig. 5). In tissue culture medium EGCG presumably reduces Fe^{3+} to Fe^{2+}, which interacts with H_2O_2 to yield the hydroxyl radical (i.e., the Fenton reaction, $Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+OH^{\cdot}+OH^{-}$). To confirm the involvement of Fe^{3+} in EGCG-mediated cytotoxicity, cells were coexposed to EGCG in the absence and presence of deferoxamine (2

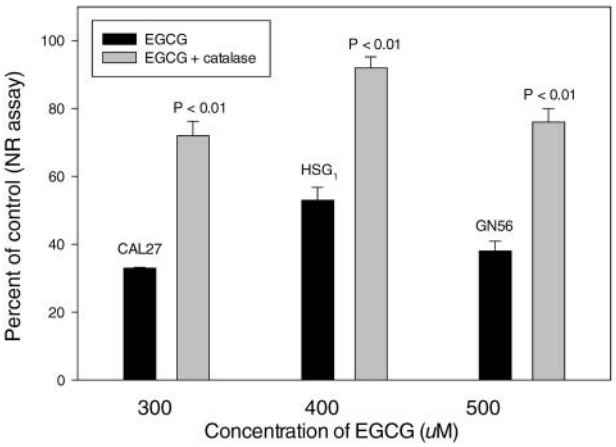

Fig. 5. Toxicity of (−)-epigallocatechin gallate in the absence and presence of 100 units/ml catalase. Cytotoxicity was assessed with the neutral red (NR) assay. The data are presented as the mean percentages of the control±S.E.M.

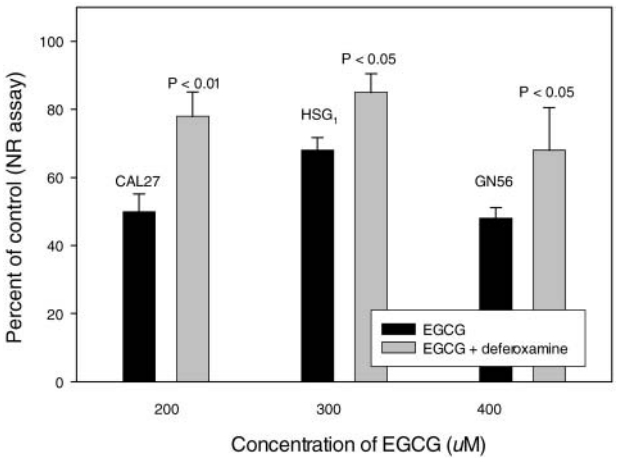

Fig. 6. Toxicity of (−)-epigallocatechin gallate in the absence and presence of 2 mM deferoxamine. Cytotoxicity was assessed with the neutral red (NR) assay. The data are presented as the mean percentages of the control±S.E.M.

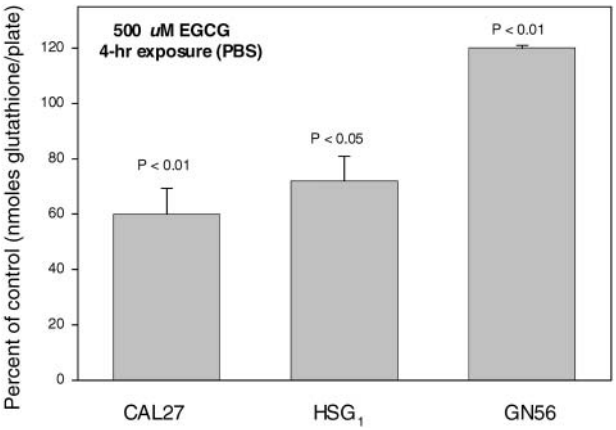

Fig. 8. The level of intracellular glutathione following a 4 hr exposure to (−)epigallocatechin gallate. In unexposed cells the control levels were 17.7±1.06 nmoles glutathione/10^6 CAL27 cells, 14.2±2.58 nmoles glutathione/10^6 HSG_1 cells, and 27.3±3.72 nmoles glutathione/10^6 GN56 cells. The data are presented as the mean percentages of the control±S.E.M.

mM), a chelator of Fe^{3+}. As seen in fig. 6, deferoxamine decreased the cytotoxicity of EGCG.

Glutathione (GSH), a thiol-containing tripeptide, is part of the cellular defense against reactive oxygen species. The three cell types, pretreated with the non-toxic concentrations of GSH depleters, CDNB (0.025 mM in PBS for $^3/_4$ hr) or BCNU (0.5 mM in PBS for $^1/_2$ hr), were hypersensitive to a subsequent exposure to EGCG (fig. 7). For CAL27 cells exposed to 500 μM EGCG, the intracellular level of GSH was lowered by 15% after 1.5 hr and by 30% after 3 hr (data not presented); by 4 hr of exposure to EGCG, the intracellular level of GSH was decreased by 40% (fig. 8). A 3 hr exposure to 500 μM EGCG did not lower the intracellular GSH content in HSG_1 cells (data not presented), however, after 4 hr of exposure to EGCG the intracellular GSH level was lowered by 30%. After 4 hr of exposure to 500 μM EGCG the GN56 cells exhibited an increase in intracellular GSH content (fig. 8).

Although a 4-hr exposure to EGCG in PBS did not induce lipid peroxidation in CAL27, HSG_1, and GN56 cells, it potentiated Fe^{2+}-induced lipid peroxidation, albeit to the same extent, in the three cell lines (fig. 9).

Morphological changes of CAL27, HSG_1 and GN56 cells exposed to EGCG for 72 hr were observed. Unexposed CAL27 cells maintained a polygonal shape and the HSG_1 maintained a variety of shapes, from polygonal to spindle-like. Upon confluence, both the CAL27 and HSG_1 cells exhibited the typical cobblestone pattern of monolayer growth. The unexposed GN56 fibroblasts were spindle-shaped. Following a 72 hr exposure to cytotoxic concentrations of EGCG, cells showed marked changes, such as

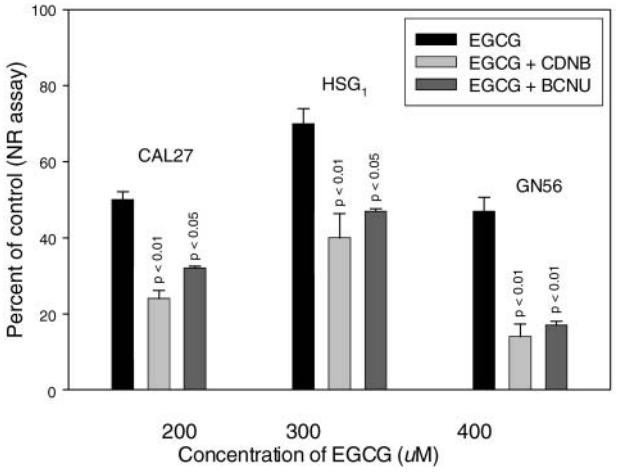

Fig 7. Enhanced toxicity of (−)-epigallocatechin gallate towards cells pretreated with the glutathione depleters, 1-chloro-2,4-dinitrobenzene (0.025 mM CDNB for $^3/_4$ hr) and 1,3-bis(2-chloroethyl)-*N*-nitrosourea (0.5 mM BCNU for $^1/_2$ hr). Cytotoxicity was assessed with the neutral red (NR) assay. The data are presented as the mean percentages of the control±S.E.M.

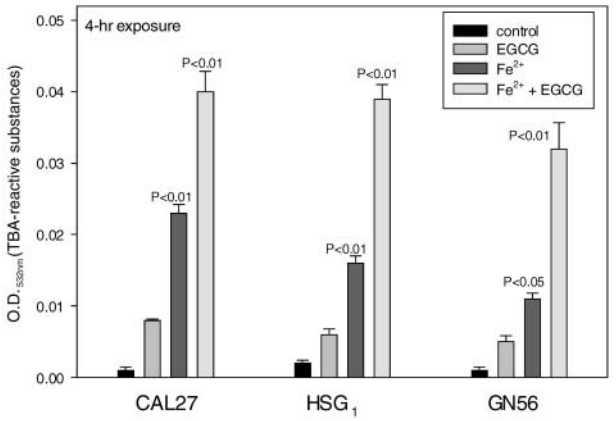

Fig. 9. Fe^{2+}-induced lipid peroxidation, as mediated by (−)-epigallocatechin gallate, following a 4 hr incubation. The data are presented as the arithmetic means±S.E.M.

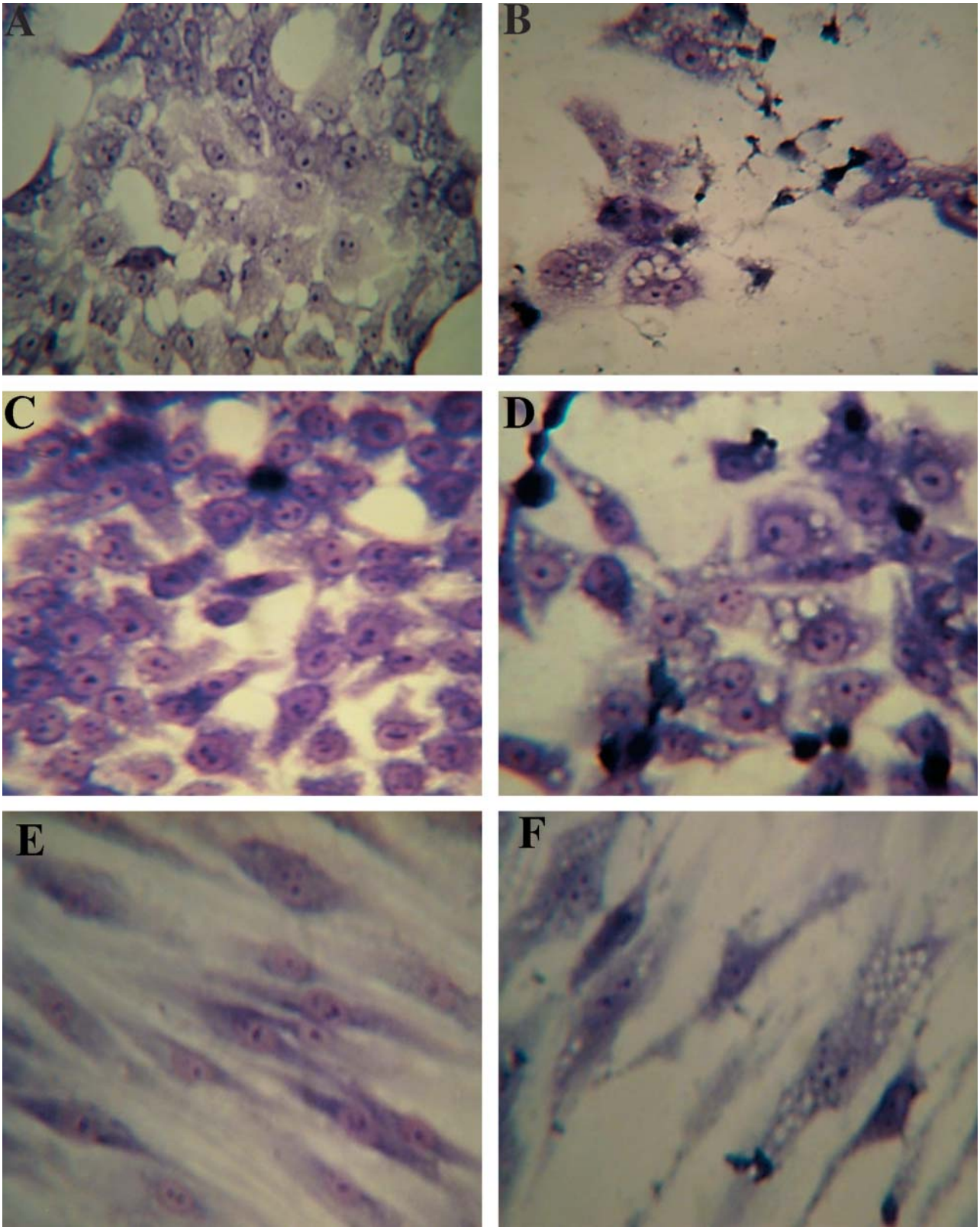

Fig. 10. Brightfield microscopy. (A) CAL27 carcinoma cells grown in control, unamended exposure medium. (B) CAL27 carcinoma cells exposed to 250 μM EGCG. (C) HSG_1 carcinoma cells grown in control, unamended exposure medium. (D) HSG_1 carcinoma cells exposed to 300 μM EGCG. (E) GN56 fibroblasts grown in control, unamended exposure medium. (F) GN56 fibroblasts exposed to 400 μM EGCG. Giemsa stain; original magnification ×320.

cellular flattening, irregularity, and the development of cytoplasmic vacuoles, indicative of oncosis, and other cells showed hypercondensed nuclei indicative of apoptosis (fig. 10).

Discussion

Both the immortalized and normal cells were more sensitive to the antiproliferative effects of green, than of black, tea polyphenolic extract, apparently reflecting its higher concentration of EGCG. The immortalized cells, both those derived from malignant tissues (i.e., CAL27, HSC-2, and HSG_1 cells) and the non-tumourigenic S-G cells derived from normal tissue, were more sensitive to the tea polyphenolic extracts and to EGCG than were the non-immortalized, finite-growth cells (i.e., GN56 and HGF-1 fibroblasts). These findings are in accord with Chen *et al.* (1998 & 2003), Ahmad *et al.* (2000), and Yamamoto *et al.* (2004) who observed that immortalized cells were more sensitive than normal cells to the growth inhibitory effects of EGCG.

The concentrations of EGCG used in the 24- and 72-hr cytotoxicity studies herein were in accord with those reported by others (Chen *et al.* 2003; Tanaka 2000; Salucci *et al.* 2002). Wide differences in sensitivity among cancerous cell lines to EGCG as seen by Uesato *et al.* (2001), were also noted herein.

The detection of H_2O_2 in exposure medium amended with EGCG and with green and black tea polyphenolic extracts was reported by others (Sakagami *et al.* 2001; Hong *et al.* 2002; Long *et al.* 2002; Roques *et al.* 2002; Chai *et al.* 2003). The greater amount of H_2O_2 generated by green, than by black, tea polyphenolic extract probably reflected its higher content of EGCG. Presumably, the formation of H_2O_2 involves interactions between EGCG and inorganic salts (Roques *et al.* 2002) in the culture medium.

As noted in this study and by Sakagami *et al.* (2001) and Yamamoto *et al.* (2004), EGCG cytotoxicity was only partially abrogated by catalase, suggesting that other factors, in addition to the generation of extracellular H_2O_2, were involved in its cytotoxicity. EGCG is unstable in cell culture medium and, in addition to H_2O_2, yielded oxidative products (e.g., theasinesin) (Hong *et al.* 2002), the EGCG radical, and hydroxyl radicals (Hagerman *et al.* 2003). Naasani *et al.* (2003) noted that the oxidative degradation products of EGCG in culture medium were more toxic than the parent EGCG molecule. Thus, the growth inhibitory effects of EGCG may be due to a combination of EGCG *per se*, the EGCG radical, metabolic products of EGCG, H_2O_2, and hydroxyl radicals.

Although studies promoting the health benefits of green tea consumption focus on its antioxidant activity (e.g., Webb 2000), EGCG-induced generation of H_2O_2 in culture medium indicated its prooxidant potential. Nakagawa *et al.* (2002) showed that EGCG reduced Fe^{3+} to Fe^{2+}, which, via a Fenton reaction, generated hydroxyl radicals, thereby inducing oxidative stress. Pretreatment of osteoclast-like cells with the deferoxamine, a Fe^{3+} chelator, phenanthroline, a Fe^{2+} chelator, and exogenously added catalase suppressed EGCG-induced cell death. They postulated that upon entry into the cells, EGCG reduced Fe^{3+} to Fe^{2+}, thereby igniting the Fenton reaction and leading to the generation of hydroxyl radicals. Yang *et al.* (2000) detected intracellular H_2O_2 in cells treated with EGCG. Others noted that EGCG-induced apoptosis of human cancerous cells was blocked by coexposures with the antioxidants, GSH and *N*-acetyl-L-cysteine (Chen *et al.* 2003), and with catalase (Chen *et al.* 2003; Yang *et al.* 2000).

In our studies, H_2O_2 was detected in culture medium amended with EGCG and tea polyphenolic extracts. Most probably, H_2O_2 was a contributing factor in the cytotoxicity of EGCG to the CAL27, HSG_1, and GN56 cells. The cytotoxicity of EGCG was lessened, but not abolished, by catalase and deferoxamine, a hydrophilic molecule that enters by endocytosis and is selectively transported to the lysosomal apparatus (Ollinger & Brunk 1995), in which there is a pool of Fe^{2+} (Sakaida *et al.* 1990).

GSH reduces H_2O_2 directly to water with the formation of oxidized glutathione and is one of the cell's prime defenses against oxidative stress. Cells pretreated with the nontoxic concentrations of the GSH depleters, BCNU and CDNB, were hypersensitive to a subsequent exposure to EGCG. BCNU, an irreversible inhibitor of glutathione reductase, inhibits the recycling of oxidized glutathione to GSH and CDNB depletes the intracellular storage of GSH by forming a thioether conjugate with glutathione-*S*-transferase. Decreases in intracellular GSH occurred in the CAL27 and HSG_1 cells exposed to 500 μM EGCG for 4 hr. However, for the GN56 cells, a 4 hr exposure to 500 μM EGCG stimulated the production of intracellular GSH. Apparently, EGCG evoked a minor stress to the GN56 fibroblasts, stimulating their fortification against oxidative stress. Arrick *et al.* (1982) showed that after brief exposure to GSH depleters, cells rapidly resynthesized GSH, often to levels overshooting normal. Interestingly, the sequence of EGCG-induced lessening of intracellular GSH in the three cell types paralleled their sensitivities to the cytotoxicity of EGCG. Furukawa *et al.* (2003) noted that human leukaemia HL-60 cells pretreated with the GSH depleter, L-buthionine-[*S,R*]-sulfoximine (BSO), exhibited increased DNA damage upon subsequent exposure to EGCG.

EGCG potentiated Fe^{2+}-dependent lipid peroxidation to an equivalent extent among the 3 cell types. Similarly, protocatechuic acid, a polyphenolic in olive oil, potentiated Fe^{2+}-induced lipid peroxidation of S-G and HSG_1 cells (Babich *et al.* 2002).

EGCG (Hong *et al.* 2002; Sugisawa & Umegaki 2002) and H_2O_2 (Zhang & Brunk 1993) readily penetrate the plasma membrane by passive diffusion. Upon a 72-hr exposure to EGCG, the CAL27, HSG_1, and GN56 cells developed cytoplasmic vacuoles that occasionally increased to balloon-like dimensions. Similar aberrations were reported in cells exposed to agents generating reactive oxygen species (Babich *et al.*1996; Kawaguchi *et al.* 1992). The involvement of cytoplasmic vacuoles in oxidative stress has received

some attention (Zhang & Brunk 1993), particularly, as the Fenton reaction may occur within lysosomes (Sakaida *et al.* 1990).

As shown herein and by others, EGCG can act as a prooxidant. EGCG generates extracellular H_2O_2 in cell culture medium (Chai *et al.* 2003; Long *et al.* 2002) and, upon cell entry, generates reactive oxygen species (ROS), including H_2O_2 and, by the Fenton reaction, the hydroxyl radical (Nakagawa *et al.* 2002; Yamamoto *et al.* 2003 & 2004; Yang *et al.* 2000); its cytotoxicity is reduced by extracellular catalase, GSH, *N*-acetyl-L-cysteine and by the Fe chelators, deferoxamine and phenanthroline (Chen *et al.* 2003; Nakagawa *et al.* 2002; Yang *et al.* 2000), but potentiated in cells treated with the GSH depleters, BSO (Furukawa *et al.* 2003), CDNB and BCNU; it decreases the level of intracellular GSH; and it enhances Fe^{2+}-mediated lipid peroxidation. The greater sensitivities to EGCG of the cancer cells versus the normal cells appeared, in part, to be related to their greater sensitivities to oxidative stress damage. These findings are in accord with studies by Yamamoto *et al.* (2004), who demonstrated the formation of reactive oxygen species in oral carcinoma cells, but not in normal keratinocytes, incubated with EGCG.

Findings of the prooxidant nature of EGCG appear to conflict with the generally recognized concept that tea polyphenolics, including EGCG, act as antioxidants (Webb 2000). The apparent contradiction in the biological activity of EGCG may reflect differences in experimental design, specifically the concentration of EGCG, the cell line, and the cell culture medium. Sakagami *et al.* (2001) suggested that the antioxidant activity of EGCG might be due to the radical scavenging activity of its flavin unit, whereas its prooxidant activity may be due to the flavonoid unit of catechin. However, the mechanism whereby EGCG changes from antioxidant activity to prooxidant activity, and vice versa, was uncertain. Johnson & Loo (2000) suggested that EGCG at low levels scavenges free radicals, whereas, at high concentrations, it induces cellular damage. Hastak *et al.* (2003) and Weinreb *et al.* (2003) noted that low concentrations of EGCG induced an anti-apoptotic pattern of gene expression thereby modulating cell survival, whereas high concentrations of EGCG induced a pro-apoptotic pattern of gene expression thereby modulating cell death.

The involvement of H_2O_2 in the *in vitro* cytotoxicity of EGCG to carcinoma cells has raised questions regarding whether EGCG is an effective chemopreventive agent *in vivo.* Halliwell (2003) suggested that the *in vitro* induction of apoptosis in carcinoma cells by exposure to elevated concentrations of EGCG is, in fact, artifactual, in that high levels of H_2O_2 cannot be achieved *in vivo* and that the maximum plasma concentration (C_{max}) of EGCG is only about 10 μM. It is difficult to correlate *in vitro* with *in vivo* data, as the C_{max} data in humans and animal models represents plasma EGCG in its conjugated forms and as the cellular concentrations of EGCG in laboratory animals or humans as compared to cultured cells are not known (Yang *et al.* 1998). Furthermore, EGCG-induced apoptosis to cancer cells *in vitro* may involve mechanisms in addition to the generation of extracellular H_2O_2 in the culture medium. EGCG penetrates cells to induce oxidative stress intracellularly, either by the generation of H_2O_2 (Yang *et al.* 1998 & 2000), of hydroxyl radical by a Fenton reaction (Nakagawa *et al.* 2002), of reactive oxygen species (ROS) (Yamamoto *et al.* 2003 & 2004), and/or of a semiquinone radical (Oikawa *et al.* 2003). Yamamoto *et al.* (2003) noted that EGCG induced intracellular oxidative stress in carcinoma cells *in vitro* but reduced ROS levels in normal cells, indicating the activation of different biochemical pathways. They suggested that the *in vivo* administration of green tea/EGCG may enhance the effectiveness of chemo- and radiation therapy to promote cancer cell death, while protecting normal cells. However, as noted, the intracellular induction of oxidative stress by green tea/EGCG in malignant cells *in vivo* still needs to be demonstrated.

Using DNA fragments from human cancer-related genes, Furukawa *et al.* (2004) showed that EGCG, in the presence of metal ions, generated levels of H_2O_2 and hydroxyl radical that caused oxidative DNA damage. Interestingly, they hypothesized that the DNA-damaging capacity of EGCG may explain those few epidemiological studies (Lu *et al.* 1999) and laboratory animal studies (Hirose *et al.* 2001) indicating a positive correlation between tea consumption and an increased risk of cancer. There are no data that consumption of green tea, even at elevated levels of intake, induces oxidative stress *in vivo* and, in contrast, a reduced risk of cancer of the prostate gland was correlated with a high consumption of green tea consumption (Jian *et al.* 2004).

In the studies presented herein, the cell types were isolated from the human oral cavity and concentrations of EGCG up to 500 μM were employed, with cytotoxicity occurring at much lower levels of EGCG in carcinoma than in normal cells. The concentration range of EGCG in normal green tea beverage may extend to 300 μM, as a single cup of green tea beverage may contain 150–200 mg EGCG (Chen *et al.* 2003). EGCG is rapidly absorbed through the oral mucosa in humans and secreted back into the oral cavity by saliva. After drinking green tea, the salivary levels of EGCG, as well as other green tea catechins, were much higher than those observed in the blood and holding a tea solution in the mouth for a few minutes without swallowing produced even more elevated levels of salivary catechins (Yang *et al.* 1999). The levels of salivary catechins during the first couple of hours after drinking the green tea (Yang *et al.* 1999) were high enough to inhibit the *in vitro* growth of preneoplastic cells from the human oral cavity (Khafif *et al.* 1998). After 6 months of treatment with a mixed green tea treatment, patients with oral precancerous lesions exhibited a significant inhibitory effect on oral mucosa cell proliferation as compared to controls given a placebo (Li *et al.* 1999). Fujiki *et al.* (2002) suggested that as a chemopreventive measure, 10 cups of green tea should be consumed per day, in addition to taking green tea supplements. Yamamoto *et al.* (2003) observed that EGCG, even at levels higher than C_{max}, did not induce oxidative stress in normal

human cells derived from the oral cavity, whereas with carcinoma cells from the human oral cavity EGCG induced oxidative stress and signaled the tumour cells to undergo apoptosis. Using the hamster buccal pouch as the model system, Li *et al.* (2002) showed that green tea inhibited the induction of oral carcinogenesis by 7,12-dimethylbenz[a]-anthracene at the post-initiation stage and that such inhibition may be related to the suppression of cell proliferation and the induction of apoptosis. Hsu *et al.* (2002 & 2003b) and Yamamoto *et al.* (2004) postulated that normal cells in potentially frequent contact with plant-derived polyphenols, such as cells found in the oral mucosa, have developed a tolerance to mitigate cytotoxicity, whereas normal cells from internal organs and tumour cells, in general, are sensitive.

Data from the literature suggest that EGCG has the dual functions of prooxidant/carcinogenic and antioxidant/anticarcinogenic potentials. Further research should be directed on the safety and risk assessment of catechins, especially as EGCG is widely available as part of multivitamin pills and tablets.

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