

Green Tea Polyphenol Causes Differential Oxidative Environments in Tumor versus Normal Epithelial Cells

TETSUYA YAMAMOTO, STEPHEN HSU, JILL LEWIS, JOHN WATAHA, DOUGLAS DICKINSON, BALDEV SINGH, WENDY B. BOLLAG, PETRA LOCKWOOD, EISAKU UETA, TOKIO OSAKI, and GEORGE SCHUSTER

Department of Oral Biology and Maxillofacial Pathology, School of Dentistry, Medical College of Georgia, Augusta, Georgia (S.H., J.L., J.W., D.D., B.S., P.L., G.S.); Kochi Medical School, Kochi, Japan (T.Y., E.U., T.O.); and Institute of Molecular Medicine and Genetics, Medical College of Georgia, Augusta, Georgia (W.B.B.)

Received May 20, 2003; accepted July 1, 2003

ABSTRACT

Green tea polyphenols (GTPPs) are considered beneficial to human health, especially as chemopreventive agents. Recently, cytotoxic reactive oxygen species (ROS) were identified in tumor and certain normal cell cultures incubated with high concentrations of the most abundant GTPP, (–)-epigallocatechin-3-gallate (EGCG). If EGCG also provokes the production of ROS in normal epithelial cells, it may preclude the topical use of EGCG at higher doses. The current study examined the oxidative status of normal epithelial, normal salivary gland, and oral carcinoma cells treated with EGCG, using ROS measurement and catalase and superoxide dismutase activity assays. The results demonstrated that high concentrations of EGCG induced oxidative stress only in tumor cells. In contrast, EGCG reduced ROS in normal cells to back-

ground levels. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and 5-bromodeoxyuridine incorporation data were also compared between the two oral carcinoma cell lines treated by EGCG, which suggest that a difference in the levels of endogenous catalase activity may play an important role in reducing oxidative stress provoked by EGCG in tumor cells. It is concluded that pathways activated by GTPPs or EGCG in normal epithelial versus tumor cells create different oxidative environments, favoring either normal cell survival or tumor cell destruction. This finding may lead to applications of naturally occurring polyphenols to enhance the effectiveness of chemo/radiation therapy to promote cancer cell death while protecting normal cells.

Understanding the potential chemopreventive mechanisms of naturally occurring compounds is key to the future applications of such agents for human health. Green tea polyphenols (GTPPs) found in the tea plant (*Camellia sinensis*), either as a mixture or as the most abundant GTPP, (–)-epigallocatechin-3-gallate (EGCG), induce apoptosis in many types of tumor cells and have been proposed as chemopreventive or therapeutic agents (Stoner and Mukhtar, 1995; Lambert and Yang, 2003). Green tea constituents have been characterized as antioxidants that scavenge free radicals to protect normal cells (Ruch et al., 1989; Huang et al., 1992; Lee et al., 1995; Wei et al., 1999; Bors et al., 2000; Katiyar et al., 2001a,b; Higdon and Frei, 2003). However, recent reports have linked GTPPs to reactive oxygen species (ROS) production, especially hydrogen peroxide (H_2O_2), and subsequent

apoptosis in both transformed and nontransformed human bronchial cells (Yang et al., 2000). ROS are normal by-products of aerobic metabolism. Most intracellular ROS are generated via mitochondrial electron transport, although other normal biological processes contribute. To maintain a proper redox balance, many defense systems have evolved. A major cellular defense against ROS is provided by superoxide dismutase (SOD) and catalase, which together convert superoxide radicals first to H_2O_2 , and then to water and molecular oxygen. Other enzymes such as glutathione peroxidase and thioredoxin reductase use the thiol-reducing power of glutathione and thioredoxin, respectively, to reduce oxidized lipid and protein targets of ROS. H_2O_2 has been detected when a colon adenocarcinoma HT29 cell line was incubated with EGCG (Hong et al., 2002). It has been suggested that in a human B lymphoblastoid cell line, concentrations of EGCG higher than physiological levels (10 μM) induced the production of ROS, especially H_2O_2 , which inflict damage (Sugisawa and Umegaki, 2002). In an immortalized normal breast epithelial cell line (MCF10A), EGCG induced growth arrest prior to the cell cycle restriction point, with elevated p21,

This study was supported in part by funding from the Dental Research Foundation, the Department of Oral Biology and Maxillofacial Pathology, School of Dentistry, and the Medical College of Georgia Research Institute to S.H.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

DOI: 10.1124/jpet.103.054676.

ABBREVIATIONS: GTPP, green tea polyphenol; EGCG, (–)-epigallocatechin-3-gallate; ROS, reactive oxygen species; SOD, superoxide dismutase; NHEK, normal human primary epidermal keratinocyte; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DFDA, dihydrofluorescein diacetate; HPS, Hallam's physiological saline; SDH, succinate dehydrogenase; ANOVA, analysis of variance.

hypophosphorylation of Rb, and decreased cyclin D1, suggesting that higher concentrations (50–200 μM) of EGCG found in green tea may be toxic to normal mammary epithelial cells (Liberto and Cobrinik, 2000). We have reported the apoptotic effect of EGCG on human primary mammary epithelial cells, in which 50 μM EGCG induced apoptosis 24 to 96 h after treatment (Hsu et al., 2002b). Although the apoptosis-inducing factor(s) in these normal cells is(are) unknown, a trend was evident: normal cells originating from the epidermis, oral cavity, and digestive tract are tolerant of high doses of the polyphenols, whereas cells from elsewhere show sensitivity to high concentrations of GTPPs.

We have also described differential responses of normal epidermal keratinocytes versus certain tumor cells to GTPPs and proposed that GTPPs activate multiple pathways in different cell types (Hsu et al., 2001, 2002a,b, 2003a,b). This may apply to the oxidative status imposed by GTPPs or EGCG in various cell types. Primates closely related to humans rely predominantly on fresh leafy plants for their energy needs. If humans maintained a diet similar to that of their ancestors, an adult human would consume approximately 10 kg of fresh leafy plant food daily to meet daily energy requirements (Milton, 1999). Many leafy plants, either fruits or vegetables, have high levels of the polyphenols/tannins (Bravo, 1998; Nepka et al., 1999). Primates, including humans, may have evolved a tolerance to exposure to tannin-rich plants. We hypothesize that cells in frequent contact with plant-derived polyphenols, such as cells found in the epidermis, oral mucosa, and digestive tract, have developed mechanism(s) to mitigate the toxicity and benefit from these compounds. However, GTPPs, when applied in high doses, are cytotoxic to other human cells that lack this tolerance and to cancer cells that have lost these protective mechanisms. We tested EGCG concentrations up to 50 times higher than the maximum plasma concentration (C_{max}) on human oral carcinoma cells, normal epidermal keratinocytes, and immortalized normal salivary gland cells. The results demonstrate that EGCG at high concentrations failed to produce ROS and in fact lowered ROS to background levels in these normal cells. In contrast, the oral carcinoma cells, which respond to GTPPs by undergoing apoptosis, elevated ROS levels upon treatment in a dose-dependent manner. The ROS levels were significantly higher in the cell line that possesses low catalase activity, and their persistence was extended. These observations suggest that EGCG is able to create differential oxidative environments in normal epithelial versus tumor cells by exploiting compromised redox homeostasis in the tumor cells.

Materials and Methods

Cell Lines. Pooled normal human primary epidermal keratinocytes (NHEKs) were obtained from Cambrex Corporation (East Rutherford, NJ) and maintained in keratinocyte growth medium-2 (Cambrex Corporation). The OSC-2 and OSC-4 cell lines, which were isolated from cervical metastatic lymph nodes of patients with oral squamous cell carcinoma (Osaki et al., 1994), were cultured in Dulbecco's modified Eagle's medium/Ham's F12 50/50 mix medium (Cellgro; Mediatech, Herndon, VA) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 5 $\mu\text{g}/\text{ml}$ hydrocortisone. OSC-2 and OSC-4 cells have one missense mutation (exon 8, codon 280: AGA $\rightarrow$ ACA) and one silent mutation (exon 5, codon 174: AGG $\rightarrow$ AGA) in the p53 gene, respectively

(Yoneda et al., 1999). Immortalized normal salivary gland cells (NS-SV-AC), selected after transfection of origin-defective SV40 mutant DNA, were provided by Dr. M. Azuma and maintained in KGM-2 medium (Azuma et al., 1993).

Reagents. EGCG, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), catalase, and diamide were purchased from Sigma-Aldrich (St. Louis, MO). Dihydrofluorescein diacetate (DFDA) and SOD were obtained from Molecular Probes (Eugene, OR) and ICN Biomedicals Inc. (Costa Mesa, CA), respectively.

Measurement of Intracellular ROS Levels. The ROS assay measures the accumulation of intracellular ROS levels. The nonfluorescent dye DFDA passively diffuses into cells, where the acetates are cleaved by intracellular esterases. The metabolites are trapped within the cells and oxidized by ROS, mainly H_2O_2 , to the fluorescent form, 2',7'-dichlorofluorescein, which can be measured by fluorescent plate reader to reflect levels of intracellular ROS (mainly H_2O_2). Thus, values of the fluorescence in the cell cultures are constantly rising in this assay. Cells (1.5×10^4 cells/well) were incubated with Hallam's physiological saline (HPS) containing DFDA (10 μM) in a 96-well microplate for 30 min at 37°C. After the incubation, cells were washed three times with HPS and then incubated with HPS containing EGCG (15–200 μM) or diamide (5 mM) for the indicated time periods. The intracellular ROS levels were measured by using a fluorescence plate reader (BIO-TEK FL600, Bio-Tek Instruments, Winooski, VT), at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

DNA Synthesis Assay. DNA synthesis was analyzed by a BrdU Cell Proliferation Assay Kit (Oncogene Research Products, San Diego, CA). Briefly, cells (1×10^4 cells/well) were seeded in a 96-well microplate and treated with the indicated doses of EGCG for 24 h at 37°C. After the treatment, cells were labeled with BrdU for 2 h at 37°C and reacted with anti-BrdU antibody. Unbound antibody in each well was removed by rinsing, and horseradish peroxidase-conjugated goat anti-mouse antibody was added to each well. The color reaction was visualized according to the protocol provided by the manufacturer. The color reaction product was quantified using a Thermo MAX microplate reader (Molecular Devices Corp., Sunnyvale, CA) at dual wavelengths of 450 to 540 nm.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. This method directly detects the activity of mitochondrial succinate dehydrogenase (SDH). Changes in SDH activity is a measurement of cell viability when stress is introduced in cell culture through chemical or physical means. Cells (1.5×10^4 cells/well) were seeded in a 96-well microplate and treated with the indicated doses of EGCG for 24 h. After the treatment, the cells in each well were washed with 200 μl of phosphate-buffered saline, incubated with 100 μl of 2% MTT in a solution of 0.05 M Tris, 0.5 mM MgCl_2 , 2.5 mM CoCl_2 , and 0.25 M disodium succinate as substrate (Sigma-Aldrich) at 37°C for 30 min. Cells were fixed in situ by the addition of 100 μl of 4% formalin in 0.2 M Tris (pH 7.7), and after a 5-min incubation at room temperature, liquid was removed and the wells were allowed to dry. Each well was rinsed with 200 μl of water and cells were solubilized by the addition of 100 μl of 6.35% 0.1 N NaOH in dimethyl sulfoxide. The colored formazan product was measured by a Thermo MAX microplate reader (Molecular Devices Corp.) at a wavelength of 562 nm. Experiments were repeated three times with triplicate samples for each experiment.

Assays for SOD and Catalase Activities. Cells (1×10^6 cells/well) were incubated with or without EGCG (50 μM) in FilterCap 50-ml flasks (Nalge Nunc International, Naperville, IL) for 30 min at 37°C. After the incubation, cells were harvested and disrupted in 100 μl of 10 mM Tris-HCl (pH 7.4) containing 0.1% (v/v) Triton X-100, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, and 100 mM phenylmethylsulfonyl fluoride by three cycles of freezing/thawing. After centrifugation at 17,000g for 20 min at 4°C, the supernatants were used for SOD and catalase assays using the SOD Assay Kit-WST (Dojindo Molecular Technologies, Gaithersburg, MD) and the Amplex Red Catalase Assay Kit (Molecular Probes), respectively. The activities of

SOD and catalase were calibrated using a standard curve prepared with purified human SOD and catalase. The activities of SOD and catalase were expressed as units (U)/ 10^6 cells.

Statistical Analysis. All data are reported as mean $\pm$ S.D. A one-way ANOVA and unpaired Student's *t* tests were used to analyze statistical significance. Differences were considered statistically significant at $p < 0.05$.

Results

ROS Assay. Figure 1A shows that ROS levels similar to those induced by diamide were generated in OSC-2 cells immediately after the addition of 50 or 200 μ M EGCG into the cell culture and matched diamide's levels up to 15 min. After this period, diamide-induced ROS levels increased at a faster rate than did EGCG-induced levels. At 60 min, an EGCG dose response was detectable, with 200 μ M EGCG inducing higher levels of ROS than 50 μ M treatments. The EGCG-induced ROS levels remained significantly higher than the control levels beyond the 120-min time point, but lower than the ROS levels produced by diamide. In OSC-4 cells, an EGCG dose response was apparent 10 min after EGCG was applied (Fig. 1B). As found in OSC-2 cells, EGCG-generated ROS levels rose at a rate similar to that of diamide-induced ROS throughout the first 15 min postexposure. Beyond 15 min, the diamide-induced ROS levels increased at a faster rate than did the EGCG-induced levels. The rate of ROS production in OSC-4 cells incubated with EGCG peaked at 60 min and then decreased to less than either diamide-treated or untreated controls. Thus, at the 120-min time point, 50 μ M EGCG-treated cells had ROS levels identical to those of the control cells, whereas ROS in 200 μ M EGCG-treated cells remained higher than in the control cells. For NHEKs, diamide induced ROS in the cells after 1 min of incubation when compared with the endogenous ROS levels (Fig. 1C). In contrast to OSC-2 or OSC-4 cells, the ROS levels in NHEKs were significantly reduced immediately after the addition of EGCG, and the ROS were maintained at basal levels throughout the testing period of 120 min. In addition, there was no apparent EGCG dose effect in these normal cells. In NS-SV-AC cells, EGCG at various concentrations was also able to inhibit ROS production at background levels when measured at the 60-min time point (Fig. 2).

Catalase Activity Assay. Significant changes in catalase activity were not observed in any cell type when these cells were treated with 50 μ M EGCG for 30 min. However, significant differences in the levels of endogenous catalase activity were found among the three cell types: NHEKs had the highest endogenous catalase activity (per 10^6 cells), OSC-4 cells showed moderate levels of catalase activity, and OSC-2 cells exhibited the lowest levels of catalase activity (Fig. 3).

SOD Activity Assay. All three cell types possess significant amounts of SOD activities (Fig. 4). Incubation with 50 μ M EGCG for 30 min did not alter SOD activity in any of the cell types.

MTT and BrdU Assays. OSC-4 cells did not show significant changes in the mitochondrial SDH activity (as measured by MTT assays, Fig. 5A) and DNA synthesis (measured by the BrdU assay, Fig. 5B) following incubation with 50 μ M EGCG for 24 h. However, when EGCG concentration increased to 200 μ M, OSC-4 cells demonstrated significantly reduced SDH activity and DNA synthesis. In comparison to previously reported SDH activity and DNA synthesis in

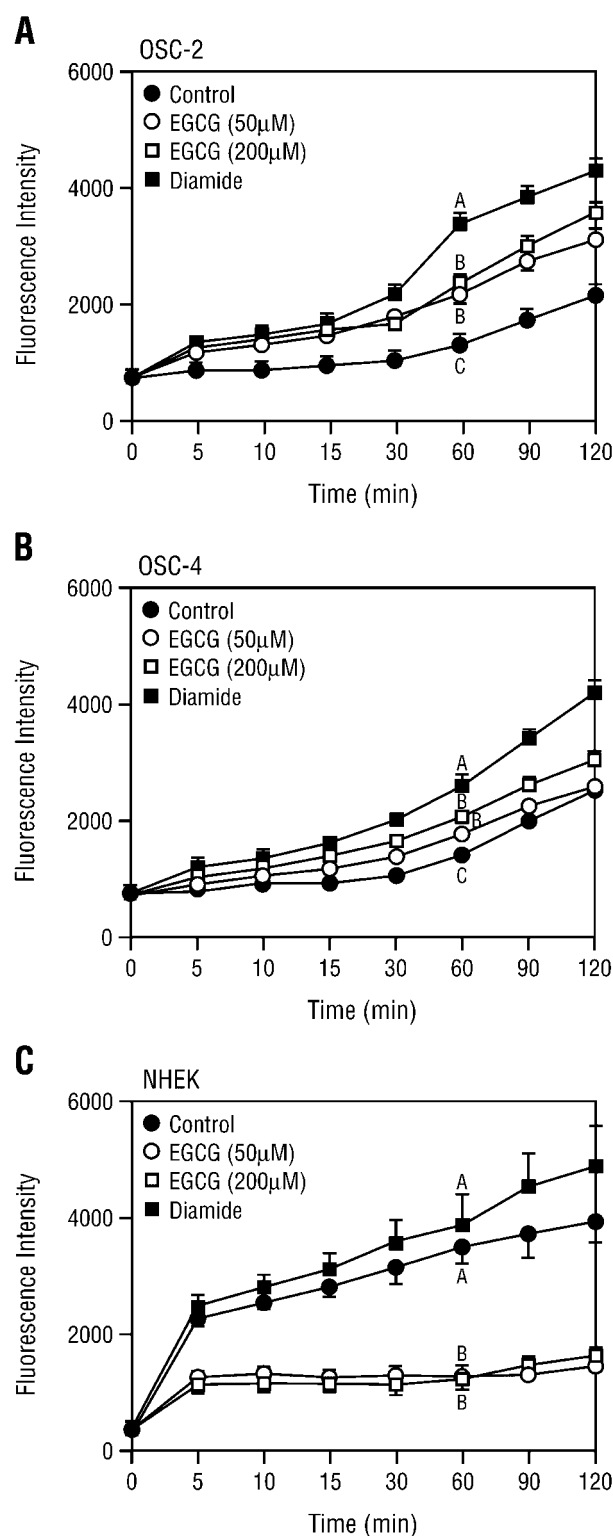


Fig. 1. Differential responses in intracellular ROS production in oral squamous cell carcinoma cells and normal epidermal keratinocytes. A, OSC-2 cells were treated with 50 or 200 μ M EGCG, or 5 mM diamide, and the intracellular ROS levels were determined at the time points indicated, with untreated cells as control. B, OSC-4 cells underwent identical treatment and ROS levels were recorded as in A. C, NHEKs were treated identically to OSC-2 and OSC-4 cells followed by ROS determination. Other concentrations of EGCG, 15, 30, or 100 μ M, produced results identical to those of NHEKs in C (data not shown). Error bars indicate one standard deviation of the mean ($n = 3$). Letters at 60 min denote statistical groupings (ANOVA, Tukey, $\alpha = 0.05$).

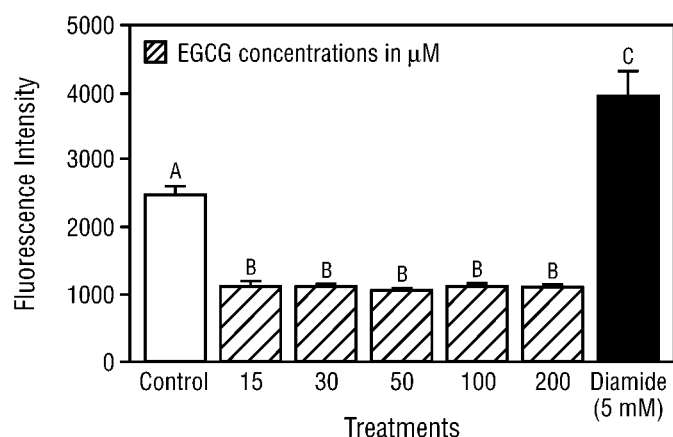


Fig. 2. Intracellular ROS level determination in NS-SV-AC cells treated with various concentrations of EGCG for 60 min. Experiments were repeated three times with similar patterns. Error bars represent standard deviations ($n = 3$). Letters denote statistical groupings (ANOVA, Tukey, $\alpha = 0.05$).

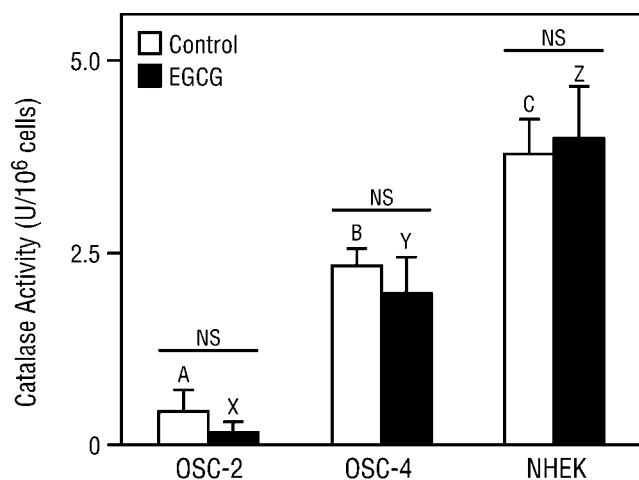


Fig. 3. Intracellular catalase activities in cells treated with EGCG compared with untreated cells. Data are presented as catalase activities versus cell numbers. Each experiment determined the activities of catalase in all three cell types in a single plate after incubation with 50 μ M EGCG for 30 min. Experiments were repeated three times. Error bars represent standard deviations ($n = 3$). Letters in each series denote statistical groupings (ANOVA, Tukey, $\alpha = 0.05$). NS denotes no statistical difference between controls and EGCG-treated cells (t test, two-sided, $\alpha = 0.05$).

EGCG-treated OSC-2 cells (Hsu et al., 2003b), where 50 μ M EGCG reduced both SDH activity and DNA synthesis, OSC-4 cells appeared less sensitive to EGCG.

Discussion

Previous reports have suggested that EGCG at high concentrations produces ROS, especially H_2O_2 , in cell cultures (Yang et al., 2000; Sakagami et al., 2001; Chai et al., 2003). The current findings confirmed this observation from two oral carcinoma cell lines, which demonstrated the formation of intracellular ROS when incubated with EGCG in a dose-dependent manner (Fig. 1, A and B). According to previous reports, EGCG-induced ROS formation can also occur in certain normal cells. However, the current study demonstrated that high concentrations of EGCG (up to 200 μ M) failed to induce ROS formation in normal epidermal keratinocytes cultured in growth media. In contrast, intracellular ROS

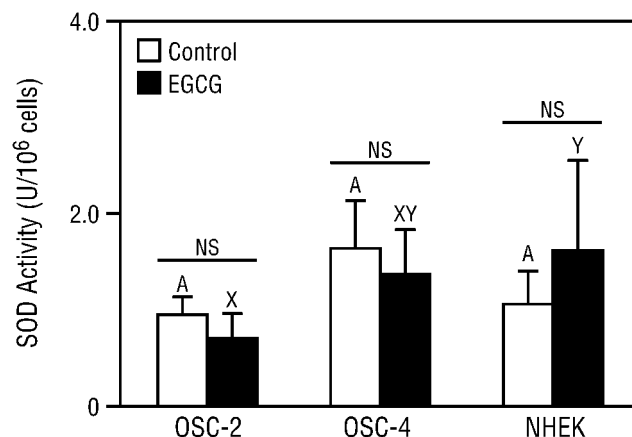


Fig. 4. Total SOD activities determined in cell lysates from three cell types treated with EGCG in comparison to untreated controls. Experiments were repeated three times with similar results. Each experiment tested the SOD activities versus cell numbers in three cell types in a single plate after incubation with 50 μ M EGCG for 30 min. Error bars represent standard deviations ($n = 3$). Letters in each series denote statistical groupings (ANOVA, Tukey, $\alpha = 0.05$). NS denotes no statistical difference between controls and EGCG-treated cells (t test, two-sided, $\alpha = 0.05$).

levels in these EGCG-treated normal cells persistently decreased to, and were maintained at, insignificant levels. Conversely, ROS levels in the untreated cultures continued to climb, at rates near those of diamide-treated cell cultures (Fig. 1C). These results demonstrated that EGCG may act as a ROS inducer or a strong ROS scavenger, depending upon specific cell type, suggesting that the mechanisms of EGCG-induced ROS formation could be more complicated than predicted. Whereas it appears that the concentrations of EGCG used might play a role in the rate of production of ROS in tumor cells, normal epithelial cells were able to tolerate very high concentrations of EGCG (approximately 50 times higher than the C_{max} in plasma) and to reduce ROS to background levels 5 min after EGCG was added in the culture, regardless of concentration (15–200 μ M). We previously proposed that GTPPs or EGCG activate multiple pathways, depending upon cell types (Hsu et al., 2001). The differential effects of GTPPs or EGCG in normal epithelial versus tumor cells signal the tumor cells to undergo apoptosis but direct the normal epithelial cells toward a survival pathway associated with cell differentiation (Hsu et al., 2003a,b). Results from the current study identified the differential impact of EGCG on oxidative status in normal versus tumor cells and support our hypothesis that GTPPs are cytotoxic to human cells that have not developed a tolerance for tannins/polyphenols, such as tumor cells and cells from internal organs, whereas cells in potentially frequent contact with plant-derived compounds are tolerant to, and possibly benefit from, GTPPs in high concentrations. One potential mechanism might be the association of GTPP/EGCG sensitivity to the loss of the ability of a tumor cell to differentiate, regardless of the origin of the tumor.

Results from the catalase activity assay demonstrated that the NHEKs possess the highest levels of catalase activity per cell among the cell types examined, and EGCG had no effect on this activity (Fig. 3). This high level of catalase activity could be part of a defense system specific to the epithelial cells designed to eliminate H_2O_2 produced by environmental factors, such as radical-producing agents and ultraviolet

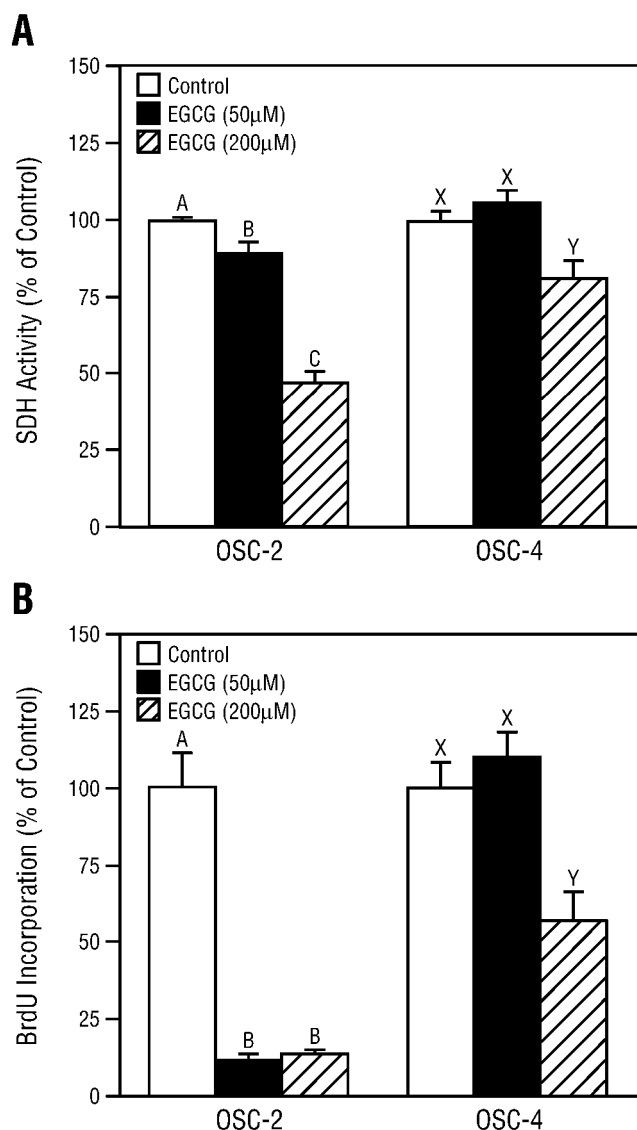


Fig. 5. Comparison of MTT assay results and BrdU incorporation rates in OSC-2 cells and OSC-4 cells following EGCG treatment for 24 h. Data are presented as percentage of control. A, OSC-2 cells demonstrated higher sensitivity to EGCG in mitochondrial tricarboxylic acid cycle enzyme SDH than did OSC-4 cells. B, OSC-2 cells were even more sensitive in BrdU incorporation, a measurement of new DNA synthesis, than OSC-4 cells. Experiments were repeated three times. Error bars represent standard deviations ($n = 3$). Letters in each series denote statistical groupings (ANOVA, Tukey, $\alpha = 0.05$).

light, in this case, diamide (Fig. 1C). In the tumor cell lines, endogenous catalase activity in OSC-2 cells was the lowest. This observation correlated with the high ROS levels produced by EGCG both initially and sustained in OSC-2 cells (Fig. 1A). The cause for the low activity of catalase in OSC-2 cells may be due to low catalase protein produced by these cells. In this regard, it is expected that OSC-2 cells would be more sensitive to oxidant-induced DNA damage, mutation, or apoptosis, since catalase is a major scavenger for H_2O_2 . OSC-4 cells showed moderate levels of catalase activity (Fig. 3) and produced less ROS than did OSC-2 cells (Fig. 1, A and B). The protein levels of catalase in each cell type are consistent with the activity measurements (data not shown). This result may explain why OSC-4 cells are more resistant to GTPP/EGCG-induced cytotoxicity when compared with

OSC-2 cells, as reflected by the reduced effect of these agents on mitochondrial SDH activities and BrdU incorporation (Fig. 5). In contrast, identical conditions of EGCG treatment did not significantly alter levels of the SDH activity or BrdU incorporation in NHEKs (Hsu et al., 2003a).

OSC-2 cells possess a defective p53 pathway due to a gene mutation (Yoneda et al., 1999), which may contribute to their susceptibility to GTPP/EGCG-induced apoptosis (Hsu et al., 2001, 2002a). It was reported previously that H_2O_2 is able to induce apoptosis in certain tumor cells, and addition of exogenous catalase completely eliminated this apoptotic effect (Yang et al., 1998). Interestingly, normal rat aorta responded to EGCG by phasic contraction, which was triggered by EGCG-induced H_2O_2 but not superoxide, possibly propelled by H_2O_2 -triggered Ca^{2+} release (Shen et al., 2003). Human embryonic kidney 293 cells also respond to EGCG with H_2O_2 production in a dose-dependent pattern (Dashwood et al., 2002). The evidence suggested that formation of H_2O_2 occurs when cells from internal organs are exposed to EGCG.

Inhibition of SOD in tumor cells was reported in human promyelocytic leukemia HL-60 cells, which was associated with apoptosis (Zhang et al., 2002). On the other hand, activation of SOD was found in normal large intestine of GTPP- or EGCG-fed rat (Yin et al., 1994), suggesting that the EGCG effect on SOD activity is cell type-specific. In the current study, all three cell types showed moderate levels of SOD activities (Fig. 4). Compared with catalase activity, SOD activity appeared to be a relatively insignificant factor in ROS scavenging capacity when the cells were incubated with EGCG for 30 min. This may be due to the formation of EGCG-induced ROS in the tumor cells, mainly in the form of H_2O_2 , which depends on catalase for its elimination. Nevertheless, whether EGCG differentially regulates catalase and SOD on transcription/translation levels in epithelial cell systems remains to be investigated.

Many studies suggest that antioxidant systems are critical in protecting against tumor-promoting agents, and that one or more components of these systems are deficient in many forms of cancer (for review see Mates and Sanchez-Jimenez, 2000). This observation is logical, given the fact that DNA is a major target of oxidative stress and accumulation of DNA damage contributes to tumor formation. Both catalase and manganese SOD (Mn-SOD) appear to be particularly important in this regard. Several studies found catalase deficiencies in a variety of tumors, as well as in cells derived from patients with the DNA repair-defective disease xeroderma pigmentosa (Vuillaume et al., 1992). In addition, hypocatalasemic mice were protected against breast tumor formation by vitamin E supplementation, supporting an oxidative component in mammary tumor development (Ishii et al., 1996). We previously showed that ROS-induced apoptosis in tumor cells could be rescued by Mn-SOD (Ueta et al., 1999, 2001). Likewise, overexpression of Mn-SOD can reduce oxidative DNA damage and alter transcription regulation, leading some to propose it as a new type of tumor suppressor. The mechanism responsible for this suppressor function remains unclear, but several studies report that activation of redox-sensitive transcription factors (i.e., nuclear factor- κB , activator protein-1, and nuclear factor E2 p45-related factor 2) is altered by changes in Mn-SOD levels (Kiningham and St. Clair, 1997). GTPPs belong to the phenolic flavonoid class of antioxidants, which recently have been proposed to act as

electrophiles that can activate mitogen-activated protein kinase pathways through an electrophilic-mediated stress response and activate the phase 2 gene-inducing transcription factor, nuclear factor E2 p45-related factor 2 (Rushmore and Kong, 2002). Thus, EGCG may serve as an important modulator of certain transcription factors to regulate intracellular redox status.

EGCG is rapidly absorbed through the oral mucosa in humans and secreted back into the oral cavity by saliva, suggesting that salivary glandular cells may be tolerant of high concentrations of EGCG (Yang et al., 1999). The current study supports this concept by data from incubating various concentrations of EGCG (15–200 μ M) with a SV40-immortalized normal human sublingual salivary acinar cell line (Fig. 2). Consistent with data obtained from human epidermal cells (NHEKs), EGCG, regardless of the concentration, reduced the ROS to background levels in these cells. We further tested the mitochondrial SDH activity in NS-SV-AC cells and two other immortalized normal human salivary glandular cell lines. The results suggested that these salivary glandular cells were tolerant to high concentrations of EGCG with accelerated energy expenditure (data not shown; T. Yamamoto, manuscript in preparation).

The current study identified two novel observations: 1) EGCG differentially affects oxidative status and can act as either a ROS inducer or a ROS suppressor depending upon the cell type; and 2) EGCG concentrations higher than plasma $C_{\max}$ do not produce H_2O_2 in cells derived from the normal epidermis and oral cavity (and possibly digestive tract), but rather protect these cells by decreasing ROS production. Mechanisms responsible for the differential effects of EGCG could rely on distinctive signal pathways activated by EGCG in a tissue-specific manner that requires further investigation. Nevertheless, knowledge gained from this study should lay a foundation for the potential future use of high concentrations of GTPPs in combination with chemo/radiation therapies in the epidermis, oral cavity, and digestive tract, to simultaneously enhance tumor cell death rate and protect normal cells from chemo/radiation-induced oxidative stress. In addition, topical and oral administration of GTPPs, even at low concentrations such as 15 μ M, would successfully provide protection against oxidative stress, especially H_2O_2 , in such tolerant cells.

Acknowledgments

We thank David Hardy for technical assistance and Sharon Barnes for the artwork.

References

- Azuma M, Tamatani T, Kasai Y, and Sato M (1993) Immortalization of normal human salivary gland cells with duct-, myoepithelial-, acinar-, or squamous phenotype by transfection with SV40 ori- mutant deoxyribonucleic acid. *Lab Invest* **69**:24–42.
- Bors W, Michel C, and Stettmaier K (2000) Electron paramagnetic resonance studies of radical species of proanthocyanidins and gallate esters. *Arch Biochem Biophys* **374**:347–355.
- Bravo L (1998) Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutr Rev* **56**:317–333.
- Chai PC, Long LH, and Halliwell B (2003) Contribution of hydrogen peroxide to the cytotoxicity of green tea and red wines. *Biochem Biophys Res Commun* **304**:650–654.
- Dashwood WM, Orner GA, and Dashwood RH (2002) Inhibition of beta-catenin/Tcf activity by white tea, green tea and epigallocatechin-3-gallate (EGCG): minor contribution of H_2O_2 at physiologically relevant EGCG concentrations. *Biochem Biophys Res Commun* **296**:584–588.
- Higdon JV and Frei B (2003) Tea catechins and polyphenols: health effects, metabolism and antioxidant functions. *Crit Rev Food Sci Nutr* **43**:89–143.
- Hong J, Lu H, Meng X, Ryu JH, Hara Y, and Yang CS (2002) Stability, cellular uptake, biotransformation and efflux of tea polyphenol (–)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. *Cancer Res* **62**:7241–7246.
- Hsu S, Bollag WB, Lewis J, Huang Q, Singh B, Sharawy M, Yamamoto T, and Schuster GS (2003a) Tea polyphenols induce differentiation and proliferation in epidermal keratinocytes. *J Pharmacol Exp Ther* **306**:29–34.
- Hsu S, Lewis JE, Borke JL, Singh B, Dickinson DP, Caughman GB, Athar M, Drake L, Aiken AC, Huynh CT, et al. (2001) Chemopreventive effects of green tea polyphenols correlate with reversible induction of p57 expression. *Anticancer Res* **21**:3743–3748.
- Hsu S, Lewis J, Singh B, Schoenlein P, Osaki T, Athar M, Porter AG, and Schuster G (2003b) Green tea polyphenol targets the mitochondria in tumor cells inducing caspase 3-dependent apoptosis. *Anticancer Res* **23**:1533–1539.
- Hsu S, Singh BB, Lewis JB, Borke JL, Dickinson DP, Drake L, Caughman GB, and Schuster GS (2002a) Chemoprevention of oral cancer by green tea. *Gen Dent* **50**:140–146.
- Hsu S, Yu FS, Lewis J, Singh B, Borke J, Osaki T, Athar M, and Schuster G (2002b) Induction of p57 is required for cell survival when exposed to green tea polyphenols. *Anticancer Res* **22**:4115–4120.
- Huang MT, Ho CT, Wang ZY, Ferraro T, Finnegan T, Lou YR, Mitchell JM, Laskin JD, Newmark H, Yang CS, and Conney AH (1992) Inhibitory effect of topical application of a green tea polyphenol fraction on tumor initiation and promotion in mouse skin. *Carcinogenesis* **13**:947–954.
- Ishii K, Zhen LX, Wang DH, Funamori Y, Ogawa K, and Taketa K (1996) Prevention of mammary tumorigenesis in acatalasemic mice by vitamin E supplementation. *Jpn J Cancer Res* **87**:680–684.
- Katiyar SK, Afaq F, Azizuddin K, and Mukhtar H (2001a) Inhibition of UVB-induced oxidative stress-mediated phosphorylation of mitogen-activated protein kinase signaling pathways in cultured human epidermal keratinocytes by green tea polyphenol (–)-epigallocatechin-3-gallate. *Toxicol Appl Pharmacol* **176**:110–117.
- Katiyar SK, Afaq F, Perez A, and Mukhtar H (2001b) Green tea polyphenol (–)-epigallocatechin-3-gallate treatment of human skin inhibits ultraviolet radiation-induced oxidative stress. *Carcinogenesis* **22**:287–294.
- Kiningham KK and St Clair DK (1997) Overexpression of manganese superoxide dismutase selectively modulates the activity of Jun-associated transcription factors in fibrosarcoma cells. *Cancer Res* **57**:5265–5271.
- Lambert JD and Yang CS (2003) Cancer chemopreventive activity and bioavailability of tea and tea polyphenols. *Mutat Res* **523–524**:201–208.
- Lee SF, Liang YC, and Lin JK (1995) Inhibition of 1,2,4-benzenetriol-generated active oxygen species and induction of phase II enzymes by green tea polyphenols. *Chem Biol Interact* **98**:283–301.
- Liberto M and Cobrinik D (2000) Growth factor-dependent induction of p21^{CIP1} by the green tea polyphenol, epigallocatechin gallate. *Cancer Lett* **154**:151–161.
- Mates JM and Sanchez-Jimenez FM (2000) Role of reactive oxygen species in apoptosis: implications for cancer therapy. *Int J Biochem Cell Biol* **32**:157–170.
- Milton K (1999) Nutritional characteristics of wild primate foods: do the diets of our closest living relatives have lessons for us? *Nutrition* **15**:488–498.
- Nepka C, Asprodingi E, and Kouretas D (1999) Tannins, xenobiotic metabolism and cancer chemoprevention in experimental animals. *Eur J Drug Metab Pharmacokin* **24**:183–189.
- Osaki T, Tatamoto Y, Yoneda K, and Yamamoto T (1994) Tumorigenicity of cell lines established from oral squamous cell carcinoma and its metastatic lymph nodes. *Eur J Cancer B Oral Oncol* **30B**:296–301.
- Ruch RJ, Cheng SJ, and Klaunig JE (1989) Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis* **10**:1003–1008.
- Rushmore TH and Kong AN (2002) Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. *Curr Drug Metab* **3**:481–490.
- Sakagami H, Arakawa H, Maeda M, Satoh K, Kadofuku T, Fukuchi K, and Gomi K (2001) Production of hydrogen peroxide and methionine sulfoxide by epigallocatechin gallate and antioxidants. *Anticancer Res* **21**:2633–2641.
- Shen JZ, Zheng XF, Wei EQ, and Kwan CY (2003) Green tea catechins evoke a phasic contraction in rat aorta via H_2O_2 -mediated multiple-signalling pathways. *Clin Exp Pharmacol Physiol* **30**:88–95.
- Stoner GD and Mukhtar H (1995) Polyphenols as cancer chemopreventive agents. *J Cell Biochem Suppl* **22**:169–180.
- Sugisawa A and Umegaki K (2002) Physiological concentrations of (–)-epigallocatechin-3-O-gallate (EGCG) prevent chromosomal damage induced by reactive oxygen species in WIL2-NS cells. *J Nutr* **132**:1836–1839.
- Ueta E, Yoneda K, Kimura T, Tatamoto Y, Doi S, Yamamoto T, and Osaki T (2001) Mn-SOD antisense upregulates in vivo apoptosis of squamous cell carcinoma cells by anticancer drugs and γ -rays regulating expression of the BCL-2 family proteins, COX-2 and p21. *Int J Cancer* **94**:545–550.
- Ueta E, Yoneda K, Yamamoto T, and Osaki T (1999) Manganese superoxide dismutase negatively regulates the induction of apoptosis by 5-fluorouracil, peplomycin and γ -rays in squamous cell carcinoma cells. *Jpn J Cancer Res* **90**:555–564.
- Vuillaume M, Daya-Grosjean L, Vincens P, Pennetier JL, Tarroux P, Baret A, Calvayrac R, Taieb A, and Sarasin A (1992) Striking differences in cellular catalase activity between two DNA repair-deficient diseases: xeroderma pigmentosum and trichothiodystrophy. *Carcinogenesis* **13**:321–328.
- Wei H, Zhang X, Zhao JF, Wang ZY, Bickers D, and Leibold M (1999) Scavenging of hydrogen peroxide and inhibition of ultraviolet light-induced oxidative DNA damage by aqueous extracts from green and black teas. *Free Radic Biol Med* **26**:1427–1435.
- Yang CS, Lee MJ, and Chen L (1999) Human salivary tea catechin levels and catechin esterase activities: implication in human cancer prevention studies. *Cancer Epidemiol Biomarkers Prev* **8**:83–89.
- Yang GY, Liao J, Kim K, Yurkow EJ, and Yang CS (1998) Inhibition of growth and induction of apoptosis in human cancer cell lines by tea polyphenols. *Carcinogenesis* **19**:611–616.
- Yang GY, Liao J, Li C, Chung J, Yurkow EJ, Ho CT, and Yang CS (2000) Effect of black and green tea polyphenols on c-jun phosphorylation and H_2O_2 production in trans-

- formed and non-transformed human bronchial cell lines: possible mechanisms of cell growth inhibition and apoptosis induction. *Carcinogenesis* **21**:2035–2039.
- Yin P, Zhao J, Cheng S, Zhu Q, Liu Z, and Zhengguo L (1994) Experimental studies of the inhibitory effects of green tea catechin on mice large intestinal cancers induced by 1,2-dimethylhydrazine. *Cancer Lett* **79**:33–38.
- Yoneda K, Yokoyama T, Yamamoto T, Hatabe T, and Osaki T (1999) p53 gene mutations and p21 protein expression induced independently of p53, by TGF- β and γ -rays in squamous cell carcinoma cells. *Eur J Cancer* **35**:278–283.

- Zhang W, Hashimoto K, Yu GY, and Sakagami H (2002) Decline of superoxide dismutase activity during antioxidant-induced apoptosis in HL-60 cells. *Anticancer Res* **22**:219–224.

Address correspondence to: Dr. Stephen Hsu, Department of Oral Biology and Maxillofacial Pathology, AD1443 School of Dentistry, Medical College of Georgia, Augusta, GA 30912-1126. E-mail: shsu@mail.mcg.edu
