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

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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 background 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 (H₂O₂), 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 H₂O₂, 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. H₂O₂ 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 H₂O₂, 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.

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 \(\mu\)M) 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 \(\mu\)M EGCG induced apoptosis 24 to 96 h after treatment (Hsu et al., 2002b). Although the apoptosis-inducing factor(s) in these normal cells (isare) 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 those found in the epidermis, oral mucosa, and digestive tract, have developed mechanisms(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 (\(C_{\text{max}}\)) 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 \(\mu\)g/ml streptomycin, and 5 \(\mu\)g/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., 1991).

**Reagents.** EGCG, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), catalase, and diamide were purchased from Sigma-Aldrich (St. Louis, MO). Dihydorfluorescein 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 \(\text{H}_2\text{O}_2\), to the fluorescent form, 2’,7’-dichlorofluorescein, which can be measured by fluorescent plate reader to reflect levels of intracellular ROS (mainly \(\text{H}_2\text{O}_2\)). Thus, values of the fluorescence in the cell cultures are constantly rising in this assay. Cells (1.5 \(\times\) 10\(^5\) cells/well) were incubated with Hallam’s physiological saline (HPS) containing DFDA (10 \(\mu\)M) 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 \(\mu\)M) or diamide (5 mM) for the indicated time periods. The intracellular ROS levels were measured by using a fluorescent 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 (Onogene Research Products, San Diego, CA). Briefly, cells (1 \(\times\) 10^6 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 \(\times\) 10^5 cells/well) were seeded in a 96-well microplate at 3 \(\times\) 10^4 cells/well and treated with the indicated doses of EGCG for 24 h. After the treatment, the cells in each well were washed with 200 \(\mu\)l of phosphate-buffered saline, incubated with 100 \(\mu\)l 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 \(\mu\)l of 4% formalin in 0.2 M Tris (pH 7.4), 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 \(\mu\)l of water and cells were solubilized by the addition of 100 \(\mu\)l 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 592 nm. Experiments were repeated three times with triplicate samples for each experiment.

**Assays for SOD and Catalase Activities.** Cells (1 \(\times\) 10^6 cells/well) were incubated with or without EGCG (50 \(\mu\)M) 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 \(\mu\)l of 10 mM Tris-HCl (pH 7.4) containing 0.1% (v/v) Triton X-100, 10 \(\mu\)g/ml leupeptin, 10 \(\mu\)g/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 (Dojinjo Molecular Technologies, Gaitersburg, 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 (UV1006 cells).

**Statistical Analysis.** All data are reported as mean ± 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 $\mu$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 $\mu$M EGCG inducing higher levels of ROS than 50 $\mu$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 $\mu$M EGCG-treated cells had ROS levels identical to those of the control cells, whereas ROS in 200 $\mu$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 $\mu$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 106 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 $\mu$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 $\mu$M EGCG for 24 h. However, when EGCG concentration increased to 200 $\mu$M, OSC-4 cells demonstrated significantly reduced SDH activity and DNA synthesis. In comparison to previously reported SDH activity and DNA synthesis in...
EGCG-treated OSC-2 cells (Hsu et al., 2003b), where 50 μM 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$_2$O$_2$, 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 μM) failed to induce ROS formation in normal epidermal keratinocytes cultured in growth media. In contrast, intracellular ROS 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 μM). 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$_2$O$_2$ produced by environmental factors, such as radical-producing agents and ultraviolet...
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 H₂O₂ 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 H₂O₂ but not superoxide, possibly propelled by H₂O₂-triggered Ca²⁺ release (Shen et al., 2003). Human embryonic kidney 293 cells also respond to EGCG with H₂O₂ production in a dose-dependent pattern (Dashwood et al., 2002). The evidence suggested that formation of H₂O₂ 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 due to the formation of EGCG-induced ROS in the tumor cells, mainly in the form of H₂O₂, 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, hypoca
talasemic 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 re
don-sensitive transcription factors (i.e., nuclear factor-aB, activa
tor protein-1, and nuclear factor E2 p45-related factor 2) is altered by changes in Mn-SOD levels (Kinningham and St. Clair, 1997). GTPPs belong to the phenolic flavonoid class of antioxidants, which recently have been proposed to act as

**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, α = 0.05).
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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 μM) 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 Cmax do not produce H2O2 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 therapy or 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 μM, would successfully provide protection against oxidative stress, especially H2O2, in such tolerant cells.

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References


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