Roles of Catalase and Hydrogen Peroxide in Green Tea Polyphenol-Induced Chemopreventive Effects


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Received August 22, 2003; accepted October 2, 2003

ABSTRACT

The green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) possesses promising anticancer potential. Although in vivo studies unveiled the metabolic routes and pharmacokinetics of EGCG and showed no adverse effects, in vitro studies at high concentrations demonstrated oxidative stress. EGCG causes differential oxidative environments in tumor versus normal epithelial cells, but the roles that EGCG, hydrogen peroxide (H₂O₂), and intracellular catalase play in the epithelial system are largely unknown. The current study employed enzyme activity assays, reactive oxygen species quantification, and immunoblotting to investigate whether EGCG-induced differential effects correlate with levels of key antioxidant enzymes and H₂O₂. It was found that normal human keratinocytes with high catalase activity are least susceptible to H₂O₂, whereas H₂O₂ caused significant cytotoxicity in oral carcinoma cell lines. However, the EGCG-induced differential effects could not be duplicated by H₂O₂ alone. The addition of exogenous catalase failed to completely prevent the EGCG-induced cytotoxicity and rescue the EGCG-induced growth arrest in the tumor cells. The antioxidant N-acetyl-L-cysteine rescued the tumor cells from H₂O₂-induced damage only, but not from EGCG-induced mitochondrial damage. Finally, alterations in catalase or superoxide dismutase activities were not observed upon EGCG exposure. In conclusion, although endogenous catalase may play a role in response to H₂O₂-induced cytotoxicity, the EGCG-induced cytotoxic effects on tumor cells mainly result from sources other than H₂O₂.

Green tea polyphenols (GTPP), particularly (−)-epigallocatechin-3-gallate (EGCG), are strong antioxidants (Tanaka, 2000; Higdon and Frei, 2003). The ability of these compounds to scavenge reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) and superoxide radicals depends on their phenolic chemical structures (Wei et al., 1999; Zhu et al., 2000). GTPP, especially EGCG, may help to protect various cells from chemical or physical damage that leads to carcinogenesis (Wei et al., 1999; Tanaka, 2000; Katiyar and Elmets, 2001; Chen et al., 2002; Lee et al., 2003). Conversely, GTPP and EGCG induce cytotoxicity and apoptosis in many types of tumor cells (Lin et al., 1999; Roy et al., 2003). The EGCG-induced apoptosis has been reported to be associated with oxidative stress imposed on tumor cells, especially by H₂O₂ (Long et al., 1999; Yang et al., 2000; Zhu et al., 2000). EGCG-induced production of H₂O₂ was recently observed under in vitro conditions with or without the presence of cells (Long et al., 1999; Hong et al., 2002). The EGCG-induced oxidative stress triggers an apoptotic pathway that is distinct from chemical or Fas-mediated pathways and acts through activation of mitogen-activated protein kinases, c-Jun N-terminal kinase and p38, and the caspase cascade (Kong et al., 1998; Yang et al., 2000; Balasubramanian et al., 2002; Saeki et al., 2002; Chen et al., 2003a). This apoptotic pathway also involves activator protein-1 (AP-1) inactivation (Dong, 1997; Barthelman et al., 1998; Balasubramanian et al., 2002). Apoptosis induced by EGCG in certain in vitro cell models was reversed by exogenous catalase, suggesting that H₂O₂ was...
the main cause of apoptotic pathway activation (Nakagawa et al., 2002; Chai et al., 2003). It was also noted that whereas at low concentrations EGCG (<10 μM) functions as a ROS scavenger, at high concentrations it functions as a ROS producer and can cause DNA damage (100 μM and above) (Saeki et al., 2002). These observations led to a hypothesis that GTPP/EGCG-induced apoptosis under in vitro conditions is artifactual, especially when the GTPP or EGCG concentration is higher than the Cmax in the plasma (10 μM), since high levels of H2O2 cannot be achieved in vivo (Halliwell, 2003). However, it is not clear whether 1) EGCG-induced apoptosis in tumor cells is indeed due to H2O2 generated in the culture medium (Halliwell, 2003), or 2) H2O2 is irrelevant to EGCG-induced responses when the EGCG concentration is at physiological levels (Dashwood et al., 2002). Thus, it is important to determine whether H2O2 generated under in vitro experimental conditions by EGCG at concentrations greater than 10 μM could be the driving force for tumor cell apoptosis (Hong et al., 2002). We hypothesized that EGCG-induced intracellular signaling (and the subsequent effects on the cell) depends upon the combination of many factors, such as the concentration of EGCG, the origin of the cells, the culture medium used, and the intracellular antioxidant enzymatic activity/quantity of the cell population.

We previously observed that GTPP/EGCG activate different pathways depending on the cell type (Hsu et al., 2001). EGCG at concentrations significantly higher than the Cmax found in the serum activates the survival pathway associated with terminal differentiation in normal epidermal keratinocytes, and the apoptotic pathway in oral carcinoma cells (Hsu et al., 2002a, 2003a). Recently, we showed that EGCG in the 15 to 200 μM range reduced ROS/H2O2 to background levels in normal human primary epidermal keratinocytes (NHEK) and immortalized normal human salivary gland cells, whereas intracellular ROS/H2O2 levels were significantly elevated in oral carcinoma cells (Yamamoto et al., 2003). This evidence suggests that high concentrations of EGCG could still be considered physiologically and clinically relevant for certain cells/tissues since the digestive tract and the epidermis can be exposed to significant levels of GTPP from the environment. Whether the key intracellular ROS scavenging enzymes catalase and superoxide dismutase (SOD) are differentially regulated by EGCG in normal versus tumor cells, or whether EGCG-induced cytotoxicity and growth arrest in tumor cells can be reversed by catalase or antioxidant is not clear. The current study was designed to address these questions and to compare the effects of EGCG with H2O2 in normal versus tumor cells.

**Materials and Methods**

**Cell Lines.** NHEK were obtained from Cambrex Bio Science Baltimore, Inc. (Baltimore, MD) and maintained in KGM-2 medium (Cambrex Bio Science Baltimore, Inc.). The OSC-2 and OSC-4 cell lines, which were isolated from cervical metastatic lymph nodes of patients with oral squamous cell carcinoma (Yamamoto et al., 2003), were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 50/50 MIX medium (Cellgro, Kansas City, MO) and supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml hydrocortisone.

**Reagents.** Catalase, diamide, EGCG, H2O2, N-acetyl-l-cysteine (NAC), 3-amino-1,2,4-triazole (3-AT), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Dihydrofluorescein diacetate (DFDA) and SOD were obtained from Molecular Probes Inc. (Eugene, OR) and ICN Biomedicals Inc. (Aurora, OH), respectively.

**MTT Assay.** This method quantitatively measures the viability of cells when stress is introduced in cell cultures through chemical or physical means. In a 96-well microplate, 1.5 × 10^4 cells were seeded in each well. After 24 h of treatment of EGCG at indicated doses, culture medium was removed and replaced with 100 μl of 2% MTT in a solution of 0.05 M Tris, 0.5 mM MgCl2, 2.5 mM CoCl2, and 0.25 M disodium succinate as substrate (Sigma-Aldrich), and the plate was incubated at 37°C for 30 min. Then 100 μl of 0.2 M Tris-HCl (pH 7.7) containing 4% (v/v) formalin was added to each well, and the microplate was incubated for 5 min at room temperature. After the incubation, the contents in each well were aspirated, and each well was rinsed with 200 μl of H2O2 followed by the addition of 100 μl of dimethyl sulfoxide containing 6.25% (v/v) 0.1 N NaOH. Solubilized colored formazan product was measured using a Thermo MAX microplate reader (Molecular Devices Corp., Sunnyvale, CA) at a wavelength of 562 nm.

**Measurement of Intracellular ROS Levels.** The ROS assay (MTT assay) measures the accumulation of intracellular ROS levels. The non-fluorescent dye DFDA passively diffuses into cells, in which the acetates are cleaved by intracellular esterases. The metabolites are trapped within the cells and oxidized by ROS, mainly H2O2, to the fluorescent form 2′,7′-dichlorofluorescein, which can be measured by a fluorescence plate reader to reflect levels of intracellular ROS (mainly H2O2). Thus, values of the fluorescence in the cell cultures are constantly rising in this assay due to the accumulation of ROS. 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 (50–200 μM) or diamide (5 mM) for the indicated times. The intracellular ROS levels were measured by using a fluorescence plate reader (BIO-TEK FL600; Bio-Tek Instruments, Inc., Winooski, VT) at an excitation wavelength of 485 nm and emission wavelength of 530 nm.

**Caspase-3 Activity Assay.** The caspase-3 apoptosis detection kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to measure caspase-3 activity. Cells (10^5 cells/well) were plated in triplicate in a 24-well tissue culture plate. After 24-h treatments with EGCG, the cells in each well were washed with 1 ml of PBS and incubated with 100 μl of cell lysis buffer on ice for 10 min. To each well, 100 μl of 2X reaction buffer was added with 100 mM dithiothreitol. Finally, 5 μl of DEVD- AFC substrate was added to each well containing cell lysates. The reaction mixtures were incubated for 1 h at 37°C, and caspase-3 activity in each well was measured using a fluorescence microplate reader (SPECTRAFluor Plus; Tecan US, Durham, NC) at a wavelength of 405 nm for excitation and 505 nm for emission.

**Western Blotting.** After EGCG treatments, cells were washed in ice-cold PBS and lysed for 10 min in 1X PBS containing 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 10 μg/ml leupeptin, 3 μg/ml aprotonin, and 100 mM phenylmethylsulfonyl fluoride. Samples of lysates containing 25 μg of protein were loaded in each lane and electrophoretically separated on a 7.5% SDS polyacrylamide gel. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (Trans-Blot Transfer medium; Bio-Rad, Hercules, CA). The membrane was blocked for 1 h with 5% (v/v) nonfat dry milk powder in PBST (0.1% Tween-20 in PBS) and then incubated for 1 h with anti-catalase rabbit polyclonal antibody (Abcam Ltd., Cambridge, UK), anti-Mn-SOD rabbit polyclonal antibody (Upstate Biotechnology, Lake Placid, NY), and anti-actin goat polyclonal antibody (Santa Cruz Biotechnology, Inc.). The membrane was washed three times with PBST and incubated with peroxidase-conjugated, affinity-purified anti-rabbit or anti-goat IgG (Santa Cruz Biotechnology, Inc.) for 1 h. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining using Sigma-Aldrich (St. Louis, MO). Dihydrofluorescein diacetate (DFDA) and SOD were obtained from Molecular Probes Inc. (Eugene, OR) and ICN Biomedicals Inc. (Aurora, OH), respectively.
ECL Western blotting detection reagents (Amersham Biosciences Inc.).

Assays for SOD and Catalase Activities. Cells (10^5 cells/well) were incubated with or without EGCG (50 μM) in 24-well culture plates for the indicated time periods 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 μg/ml leupeptin, 10 μg/ml pepstatin A, and 100 mM phenylmethylsulfonyl fluoride by three cycles of freezing/thawing. After centrifugation at 17,000 g for 20 min at 4°C, the supernatants were used for SOD and catalase assays using the SOD assay kit-WST (Dojindo Molecular Technologies, Inc., Gaithersburg, MD) and the Amplex Red catalase assay kit (Molecular Probes, Eugene, OR), 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 per milligram protein.

Statistical Analysis. All data are reported as mean ± S.D. A one-way analysis of variance and unpaired Student’s t tests were used to analyze statistical significance. Differences were considered statistically significant at p < 0.05.

Results

Susceptibility of NHEK and OSC Cell Lines to EGCG and H2O2. After a 24 h of incubation with EGCG at various concentrations, cell viability of NHEK was not altered (Fig. 1a). However, both OSC-2 and OSC-4 cells exhibited cytotoxicity. OSC-2 was the more sensitive cell line, with cell viability declining to less than 50% of untreated control levels after incubation with 200 μM EGCG (Fig. 1a). Unlike EGCG, H2O2 induced cytotoxicity in all cell types, with noticeable differences among the cell types. For instance, cell viability of all cell types gradually declined when H2O2 concentrations increased from 100 to 500 μM. At H2O2 concentrations higher than 500 μM, cell viability in the OSC cell lines decreased more rapidly than in NHEK. Treatment with 1 mM H2O2 caused a 25% reduction of cell viability in NHEK, but only 250 μM was needed to cause the same reduction in OSC-2 and OSC-4 cells. When these cells were exposed to 1 mM H2O2 for 24 h, the cell viability was reduced to less than 20% in both cell lines (Fig. 1b).

Generation of Intracellular ROS by EGCG Compared with Exogenous H2O2 in OSC Cell Lines. We previously showed that EGCG caused differential oxidative environments in normal versus tumor cells. EGCG at concentrations of 15 to 200 μM lowered ROS to background levels in NHEK (Yamamoto et al., 2003). In contrast, the current study showed that, following a 60-min exposure to

Fig. 1. MTT assay results from NHEK, OSC-2, and OSC-4 cells treated with EGCG or H2O2. Cells were incubated with the indicated concentrations of EGCG (a) or H2O2 (b) for 24 h, followed by MTT assay. Each concentration was assayed in triplicate within an experiment. Data are expressed as percentage of untreated cells, and error bars represent one standard deviation of the mean. These figures are representative of three independent replications of the experiments, all with similar results. Different capital letters indicate statistically significant differences among cell types (ANOVA, Tukey post hoc test, α = 0.05, n = 3).
either exogenous H$_2$O$_2$ or EGCG, both OSC-2 and OSC-4 cell lines exhibited a dose-dependent accumulation of intracellular ROS, as detected by DFDA (Fig. 2). Under identical conditions, 5 mM diamide-induced ROS in OSC-2 cells was double that found in OSC-4 cells and H$_2$O$_2$ at 100 or 200 μM (Fig. 2).

**Comparison of the Effect of EGCG with NAC in OSC Cell Lines.** Two-hour pretreatment with 10 mM NAC significantly inhibited the cytotoxic effect of H$_2$O$_2$ at 250 and 500 μM in OSC-2 and OSC-4 cell lines (Fig. 3a). EGCG at 200 μM significantly reduced cell viability in both OSC-2 and OSC-4 cell lines (Fig. 1a and Fig. 3b). However, NAC not only failed to rescue both cell lines from EGCG-induced cytotoxicity, it also enhanced the cytotoxicity measured by MTT assays seen at higher EGCG levels (Fig. 3b). Treatment with exogenous catalase or 3-AT, a catalase inhibitor, had no effect on this reduction (data not shown). Moreover, NHEK did not become susceptible to EGCG cytotoxicity after pretreatment with 3-AT (data not shown).

**Impact of Catalase on EGCG-Induced Activation of Caspase-3.** Exogenous catalase partially inhibited EGCG-induced caspase-3 activation in OSC-2 and OSC-4 cells during a 24-h period (Fig. 4).

**Levels of Activity and Quantity of Endogenous Catalase and SOD in Response to EGCG Exposure.** When enzymatic activities were compared among these cells, NHEK were shown to have the highest levels of catalase activity—twice that found in OSC-4 and three times that found in OSC-2 cells (Fig. 5a). However, OSC-2 cells exhibited the highest levels of total SOD activity, twice those found in either NHEK or OSC-4 cells (Fig. 5a). EGCG had no effect on the enzymatic activity levels during the 24-h treatment period, except for the catalase activity in OSC-4 cells, which showed a slight decrease (Fig. 5a). Of the three cell types, OSC-2 cells possessed the lowest amount of endogenous catalase protein compared with NHEK and OSC-4 cells, and—consistent with the activity levels—the highest levels of Mn-SOD protein levels. Significant alteration in the protein levels of these enzymes was not observed during the 24-h period following EGCG treatment (Fig. 5b). When exposed to EGCG, NHEK showed a slight decrease in catalase protein level and an increase in Mn-SOD protein at the 24-h time point (Fig. 5b).

**Discussion**

Previous in vitro studies demonstrated that EGCG induces differential effects in normal versus tumor cells. Effects of EGCG on tumor cells include generation of intracellular ROS, induction of growth arrest, inhibition of cell invasiveness, inhibition of AP-1, nuclear factor κB, activation of caspase cascade, and induction of apoptosis through regulation of mitogen-activated protein kinase pathway (Chung et al., 1999; Liang et al., 1999; Ahmad et al., 2000; Chen et al., 2000; Dong, 2000; Gupta et al., 2000, 2003; Kong et al., 2000; Liberto and Cobrinik, 2000; Yang et al., 2000; Jung and Ellis, 2001; Hsu et al., 2002b, 2003b; Saeki et al., 2002). In normal epithelial cells, the effects of EGCG involve reduction of intracellular ROS, increase in cell viability, induction of cell differentiation associated with activation of AP-1, p57, and caspase 14 (a terminal differentiation marker for epidermal keratinocytes; Hsu et al., manuscript under review) (Hsu et al., 2001, 2002a, 2003a; Balasubramanian et al., 2002).

Based on these observations, the roles of H$_2$O$_2$ and endogenous antioxidant enzymes in EGCG-induced effects are unlikely to be identical among different cell types from various origins. For example, EGCG elevated ROS levels (especially H$_2$O$_2$) in tumor cells, but not in NHEK or immortalized normal salivary gland cells. This correlated with apoptotic pathways in the tumor cells and survival pathways in the normal cells (Yamamoto et al., 2003). In addition, elimination of H$_2$O$_2$ by addition of catalase could not prevent EGCG-induced inhibition of AP-1 and activation of c-Jun N-terminal kinase and extracellular signal-regulated kinase, suggesting that EGCG signaling might not solely rely on oxidative stress (Chung et al., 1999).

The current study further confirmed that high concentrations of EGCG damaged only tumor cells (OSC-2 and OSC-4), but not normal cells (NHEK) (Fig. 1a). This EGCG-induced differential effect was observed in coculture of OSC-2 cells and NHEK in a 50:50 mix of KGM-2 and Dulbecco’s modified Eagle’s medium/Ham’s F-12, therefore eliminating the medium as the source of the difference (data not shown). Importantly, the EGCG-induced differential effect in normal versus tumor cells could not be reproduced entirely by H$_2$O$_2$ alone (Fig. 1b). OSC cell lines showed a significant decline in cell viability at H$_2$O$_2$ concentrations of 250 μM or more, and the viable cells were reduced to less than 25% of control levels.
when the H$_2$O$_2$ concentration was increased to 1 mM (Fig. 1b). In comparison, 75% of viable NHEK cells remained when treated with 1 mM H$_2$O$_2$ for 24 h (Fig. 1B). These results demonstrated that NHEK possess a stronger ability to resist the oxidative stress from H$_2$O$_2$, whereas OSC cells are more sensitive to H$_2$O$_2$-induced cytotoxicity. In contrast, EGCG at various concentrations did not induce cytotoxicity in NHEK but caused significant cytotoxicity in OSC cells, suggesting that H$_2$O$_2$-induced effects among these cell types are quantitative, whereas EGCG-induced effects are qualitative.

Between the tumor cell lines, OSC-2 cells appeared to be more sensitive to H$_2$O$_2$-induced cytotoxicity than OSC-4 cells, as measured by the MTT assay (Fig. 1b). Consistent with this observation, when OSC-2 and OSC-4 cells were incubated with relatively high concentrations of H$_2$O$_2$ or diamide, OSC-2 cells accumulated significantly higher (approximately 2×) ROS than OSC-4 cells, indicating that OSC-2 cells possess weaker defenses against H$_2$O$_2$ (Fig. 2). In OSC-2 cells, incubation with 200 μM EGCG produced ROS equivalent to that from 50 μM H$_2$O$_2$ during the first hour (Fig. 2). The cell viability was reduced to 40% of untreated control after 24 h (Fig. 1a). In contrast, a 24-h treatment with 50 μM H$_2$O$_2$ had no effect on cell viability (Fig. 1b). Similarly, incubation of OSC-4 cells with 200 μM EGCG produced ROS equivalent to that from 100 μM H$_2$O$_2$ during the first hour (Fig. 2), and the cell viability was reduced to less than 75% of untreated control after 24 h (Fig. 1a), but 100 μM H$_2$O$_2$ had no significant effect on cell viability (Fig. 1b). Further discor-

Fig. 3. MTT assay result of OSC-2 and OSC-4 cells pretreated with NAC followed by incubation with either H$_2$O$_2$ or EGCG. OSC-2 and OSC-4 cells were pretreated with or without 10 mM NAC for 2 h prior to incubation with the indicated concentrations of H$_2$O$_2$ (a) or EGCG (b) followed by MTT assay. Each concentration was assayed in quadruplicate within an experiment. Data are expressed as percentage of untreated cells, and error bars represent one standard deviation of the mean. These figures are representative of three independent replications of the experiments, all with similar results. Different capital letters indicate statistically significant differences among conditions (ANOVA, Tukey post hoc test, α = 0.05, n = 4).

Fig. 4. Caspase-3 activity in OSC-2 and OSC-4 cells pretreated with catalase and incubated with EGCG. Cells were pretreated with 200 U/ml exogenous catalase for 5 min prior to addition of EGCG at concentrations indicated. Caspase-3 activity assay was performed immediately after a 24-h incubation with EGCG. Error bars indicate one standard deviation of the mean. Different capital letters indicate statistically significant differences among conditions (ANOVA, Tukey post hoc test, α = 0.05, n = 4).
of enzymes involved in ROS breakdown in OSC cells. EGCG did not appear to markedly regulate either catalase or SOD enzymatic activities or protein levels over a 24-h period (Fig. 5).

In conclusion, EGCG-induced ROS formation is not simply concentration-dependent, but is also cell type-dependent. Identical concentrations of EGCG (as high as 200 μM) may cause severe damage in one tumor cell line (OSC-2), less severe damage in another tumor cell line (OSC-4), but reduce ROS levels in normal epithelial cells (NHEK). These data support our hypothesis that cells in potentially frequent contact with plant-derived polyphenols, such as cells found in the epidermis, oral mucosa, and digestive tract, have developed mechanism(s) to mitigate cytotoxicity otherwise caused by the polyphenols and benefit from these compounds. However, when applied in high doses, EGCG is cytotoxic to other human cells that lack this tolerance and to cancer cells that have lost these protective mechanisms. Thus, whether an EGCG concentration is physiologically or clinically relevant is organ/tissue-dependent. In NHEK, EGCG induces a survival pathway associated with differentiation that does not appear to involve ROS. In OSC cells, EGCG induces different pathways that lead to cell death. Caspase-3 activation appears to involve EGCG-induced ROS formation, whereas cytotoxicity and growth arrest do not. Endogenous catalase plays a role in the response of cells to EGCG; cells without adequate catalase are more sensitive to EGCG-induced H₂O₂ formation, as shown in the current study and previous reports (Yang et al., 1998; Sakagami et al., 2001; Chai et al., 2003). However, H₂O₂ alone cannot reproduce the EGCG effects in other cell lines or cell types. Thus, applications of high concentrations of EGCG on epithelial tissues, especially the epidermal and digestive tract tissues, could deliver cytotoxic effects for chemoprevention purposes involving growth arrest/apoptosis signaling and oxidative stress that are clinically relevant, whereas normal epithelial cells are guided to safety by a cell differentiation pathway.

Acknowledgments

We thank Petra Lockwood and Sushma Rao for excellent technical assistance.

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