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Epigallocatechin-3-Gallate Delivers Hydrogen Peroxide to Induce Death of Ovarian Cancer Cells and Enhances Their Cisplatin Susceptibility

MARION M. CHAN,[1]* KENNETH J. SOPRANO,[1] KATE WEINSTEIN,[1] AND DUNNE FONG[2]

[1]Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania

[2]Department of Cell Biology and Neuroscience, Rutgers, The State University of New Jersey, Piscataway, New Jersey

The green tea polyphenol epigallocatechin-3-gallate (EGCG) has cancer chemopreventive properties against various types of cancers. The compound is known to attack various targets in transformed cells. In this report, we examined the action of EGCG on ovarian cancer cells. Eight ovarian cancer cell lines were tested (SKOV3, CAOV3, OVCAR3, OVCAR10, A2780, CP70, C30, and C200) and showed IC_{50}s for EGCG at the micromolar range, including ones that are resistant to the chemotherapeutic drug cisplatin. The ovarian cancer cells were sensitive to H_2O_2 at similar concentrations, and EGCG treatment led to enhanced intracellular H_2O_2. Neutralization with pyruvate, a scavenger of H_2O_2, suggests that the toxicity of EGCG may be mediated by oxidative stress from the free radical. Addition of Tempol, a superoxide dismutase mimetic, demonstrates that H_2O_2 might be generated endogenously from superoxide. The toxicity of cisplatin and the development of cisplatin resistance are major obstacles in treatment of ovarian cancer. We found that addition of EGCG amplified the toxicity of cisplatin. EGCG increased cisplatin potency by three to six-fold in SKOV3, CAOV3, and C200 cells, the latter being a cell line induced to have several hundred fold resistant to cisplatin above the parental line. Our findings suggest that EGCG may accentuate oxidative stress to inhibit growth of ovarian cancer cells and sensitize them to cisplatin. J. Cell. Physiol. 207: 389–396, 2006. © 2006 Wiley-Liss, Inc.

Ovarian carcinoma is one of the most common cancers of women in the United States, and is the most fatal gynecological malignancy among women in the Western world (Cancer Facts and Figures, 2005). It has high mortality rate among gynecological malignancies because 60% of patients are diagnosed with an already advanced disease and drug resistance and relapse frequently occur within 2 years of initial treatment (reviewed by Kristensen and Trope, 1997; Cvetkovic et al., 2004).

Cisplatin is a major chemotherapeutic agent for the treatment of a wide spectrum of cancers, including ovarian carcinomas. Its mechanism of action is by induction of cell death through DNA damage. However, an activated cisplatin molecule, which is strongly electrophilic, causes severe side effects induced by the oxidative stress (Matsushima et al., 1998). Cisplatin toxicity is manifested acutely as nausea and vomiting, chronically in the form of neuropathy that compromises sensory and motor responses, and cumulatively as nephrotoxicity that results in renal tubular necrosis. The latter poses the major limitation on dose. Frequently, patients develop cisplatin resistance during their course of treatment. Laboratory and clinical studies have shown that multiple mechanisms underlie tumor resistance to cisplatin. These include decrease in drug transport, increase in cellular detoxification due to increased over-production of thio-reductants, change in DNA repair efficiency and apoptotic cell death pathway, and increase in drug efflux (reviewed by Siddik, 2003). Antioxidants have been found to protect against cisplatin-induced toxicity (reviewed by Kinnula and Crapo, 2004).

Recently, in a case-control study and a prospective cohort study (for 3 years) involving 254 patients in China, Zhang et al. (2002, 2004a) concluded that increasing the frequency and duration of green tea drinking can reduce the risk of ovarian cancer, and increasing the consumption of green tea post-diagnosis may enhance ovarian cancer survival.

The polyphenolic catechin, epigallocatechin-3-gallate (EGCG), is an active principle in green tea. It has been shown to possess cancer preventive activities for stomach, esophagus, and lung tumors and arrest growth and induce apoptosis in various epithelial cancer cells (Mukhtar and Ahmad, 1999; Higdon and Frei, 2003; Lambert and Yang, 2003; Moyers and Kumar, 2004). Its action has been ascribed to its antioxidative properties, the scavenging of free radicals and prevention of mutation and DNA damage. EGCG is known to block many targets in signal transduction pathways, including AP-1 (Dong et al., 1997; Shimizu and Weinstein, 2005). Nonetheless, more recently, the compound has been found to liberate H_2O_2 (Yang et al., 2000; Sakagami et al., 2001). The free radical H_2O_2 inhibits growth of tumor cells by oxidative stress, blocking cell cycle progression, inducing apoptosis or causing necrosis in a dose and cell type dependent manner (Lambert and Yang, 2003). Yamamoto et al. (2004a) have found that susceptibility to EGCG is cell type specific, a function of whether the cells are apt to maintain oxidative/antioxidative balance. Normal human keratinocytes, for example, are capable of reducing oxidative stress and

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*Correspondence to: Marion M. Chan, Department of Microbiology and Immunology, Temple University School of Medicine, Room 534 OMS, 3400 North Broad Street, Philadelphia, PA, 19140. E-mail: marion.chan@temple.edu

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are less susceptible to damage. Whereas EGCG reduces the growth of oral epithelial tumors, immortalized breast epithelial cell lines, and prostate cancers in vitro and in vivo (Chung et al., 2001; Yamamoto et al., 2003; Roy et al., 2005), studies on ovarian cancer cells are spare. The goal of this study is to investigate whether EGCG inhibits growth of ovarian cancer cells, whether it works through production of H_2O_2 and whether EGCG modulates the response of ovarian cancer cells to cisplatin.

MATERIALS AND METHODS

Cells

Ovarian cancer cell lines CAOV3, SKOV3, OVCAR3, OVCAR10, A2780, CP70, C30, and C200 were studied (Perez et al., 1992; O'Dwyer et al., 1996; Chan et al., 2003). CAOV3 and SKOV3 were obtained from American Type Culture Collection. They were cultured in Dulbecco's modified Eagle medium (DMEM) high glucose with 10% fetal calf serum (FCS), and 2 mM L-glutamine as described previously (Wu et al., 1998). The other cell lines were obtained from Dr. Thomas Hamilton (Fox Chase Cancer Center) and grown in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine and 0.25 U/ml insulin.

Compounds

Reagents for cell treatment included H_2O_2, 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol), EGCG, sodium pyruvate and cisplatin. All compounds were prepared immediately before use. Hydrogen peroxide was prepared from 30% stock solution (Sigma) by dilution and added at 6.3, 12, 25, and 50 μM to cultures in 96-well plates. EGCG, from Cayman Chemical, was prepared by dissolving in acetone to a concentration of 20 mM, and then diluted to 4, 10, 20, 30, and 40 μM solutions in culture media containing 0.1% of the solvent (Sakagami et al., 2001; Nakagawa et al., 2004), and Tempol, from Fluka, was dissolved in water to final concentrations of 1, 1.5, 2, and 10 mM solutions. Cisplatin, from Sigma, was dissolved in water as a 0.75 or 1 mg/ml stock solution and then diluted to the desired concentration in the appropriate medium. Solutions were added at equal volumes to the cells. Sodium pyruvate was purchased from Invitrogen Gibco as a 100× stock and added at 1 mM.

Cell proliferation assay

The action of the molecules on ovarian cell lines was evaluated by comparing relative growth. Cells from subconfluent cultures were harvested by trypsin digestion, washed with phosphate-buffered saline (PBS), resuspended in culture medium and plated at concentrations of 0.5, 1, 2, 3.8, 7.5×10^3/well at 100 μl/well in 96-well plates. The compounds were added to ovarian cancer cells and at 4 days after treatment, the number of viable cells in culture samples was determined using the fluorescence indicator Alamar blue (O'Dwyer et al., 1996; Chan et al., 2003). The advantages and the validity of using Alamar blue, the newer cell proliferation indicator, have been described and established by comparison to cell counting (Chan et al., 2003). Controls with medium alone and medium with compounds at specific concentrations, but without cells, were also set up to measure any background level of Alamar blue oxidation. The degree of fluorescence was measured in a Millipore Cytofluor 2350 Fluorescence Measurement System with 530 nm excitation and 590 nm emission. Background fluorescence due to action of EGCG on Alamar blue was determined with the wells to which cells were omitted and subtracted. The degrees of fluorescence in the treated and untreated experimental samples were compared. Relative growth was calculated by the following formula: Relative growth = (Fluorescence in compound-treated wells – Fluorescence in wells with the corresponding amount of compound where cells had been omitted)/(Fluorescence in untreated wells – Fluorescence in wells with the corresponding amount of compound but without cells). It was determined using the wells that were in the log phase of proliferation. The differences were analyzed using Student's *t*-test.

Fluorometric detection of H_2O_2

For detection of H_2O_2, the cells were cultured in the presence of 15 μM EGCG, or an equal concentration of vehicle, for 16 h, harvested by trypsin digestion, and washed with PBS containing 0.1% bovine serum albumin (BSA). They were then resuspended in 200 μl of PBS with 10 μM of dichlorodihydrofluorescein diacetate (DCDHF) analog C2938 (Invitrogen Molecular Probes) in serum-free medium followed by incubation for 30 min at 37°C. DCDHF is an uncharged and cell-permeable molecule, which, once inside cells, is cleaved by nonspecific esterases to carboxydichlorofluorescein, which then can be oxidized by H_2O_2 and other intracellular peroxides (Hockenbery et al., 1993). The cells were washed of excess extracellular fluorescent dye, fixed in formaldehyde and analyzed by microscopy and cytometry (FACScan).

RESULTS

Inhibition of growth of ovarian cancer cells by EGCG

The green tea polyphenol EGCG is known to reduce the growth of various epithelial cancer cells, including those of breast and prostate cancers (reviewed in Yang et al., 2002). In this study, we examined the efficacy of EGCG to inhibit the growth of ovarian cancer cells. Eight ovarian cancer cell lines were tested: SKOV3, CAOV3, OVCAR3, OVCAR10, A2780, CP70, C30, and C200. Among them A2780 is considered very sensitive, and CAOV3 and C200 are considered relatively resistant and very resistant to cisplatin (O'Dwyer et al., 1996; Chan et al., 2003). Addition of EGCG at various concentrations, from 2 to 20 μM, inhibited their growth in a dose-dependent manner. Their IC_{50}s range from 2 to 15 μM. Figure 1 shows the relative growth inhibition in six of the cell lines. They were 2 μM for A2780, 4.5 μM for OVCAR3, 7.5 μM for OVCAR10, 15 μM for C200, which were grown at 3.8×10^3/ml in RPMI medium, and 15 μM for SKOV3 and CAOV3, which were grown at 1.9×10^3/ml in DMEM medium.

Inhibition of growth of ovarian cancer cells by hydrogen peroxide and SOD mimetic Tempol

Intracellular O_2^- is needed for proliferation of certain cancer cells but it may also be a source of H_2O_2 for inducing cell death. It has been reported that incubation with EGCG enhances the activity of manganese superoxide dismutase (MnSOD) in human hepatoma HepG2 cells and murine large intestinal cancerous epithelium (Yin et al., 1994; Murakami et al., 2002). Therefore, we compared the efficacy of H_2O_2, the oxidative metabolic product of SOD, to inhibit the growth of the ovarian cancer cell lines, OVCAR3, SKOV3, and CAOV3, which are commercially available and commonly studied by many laboratories. We observed that the IC_{50}s for H_2O_2 and EGCG were within a similar range of concentrations (Fig. 2). The IC_{50}s of SKOV3 and CAOV3 for H_2O_2 were 14 μM versus 15 μM for EGCG. The OVCAR3 cell line, which was more sensitive to EGCG than SKOV3, was also more sensitive to H_2O_2, though its IC_{50} for H_2O_2 was 10 μM, slightly higher than for EGCG, 4.5 μM. Whether this suggests that the cell line is better equipped to detoxify H_2O_2 than EGCG remains to be determined.

To verify that endogenous O_2^- can be a source of H_2O_2, the ovarian cancer cell lines were treated with the SOD mimetic, Tempol (4-hydroxy-2, 2, 6, 6-tetramethylpiperidine-1-oxyl), a low molecular weight, cyclic nitroxide

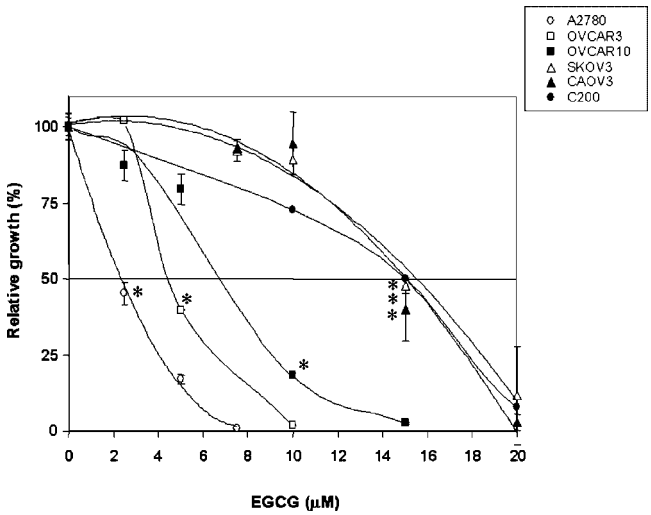

Fig. 1. Growth inhibition of ovarian cancer cells by EGCG. Cells were plated at concentrations of 0.5, 1, 2, 3.8, 7.5×10^3/well at 100 μl/well in 96-well plates. EGCG was diluted in culture media containing 0.1% acetone and then added at equal volumes to the cultures to attain the indicated concentrations. The degree of cellular proliferation was determined with Alamar blue as described in the text. Growth was determined using the wells that were in the log phase of proliferation. For A2780, OVAR3, and OVAR10, the results reported were from wells that had been inoculated with 3.8×10^3 cells/well, and SKOV3 and CAOV3 were from wells that had been inoculated with 1.9×10^3 cells/well. EGCG was also added at the indicated concentrations to a column of wells with media, acetone but not cells to determine background fluorescence due to action of EGCG. The relative growth was calculated using the formula: relative growth = (Fluorescence in compound-treated wells−Fluorescence in wells with the corresponding amount of compound where cells had been omitted)/(Fluorescence in untreated wells−Fluorescence in wells with the corresponding amount of compound but without cells). The levels of fluorescence detected at 100% growth were around 2,500–8,000 units for the three cell lines. Background fluorescence was from 200 to 800 units. Each point represents the mean + SEM of three to four samples. The asterisks indicate points, which had significance level of $P < 0.001$ in comparison to their respective untreated controls.

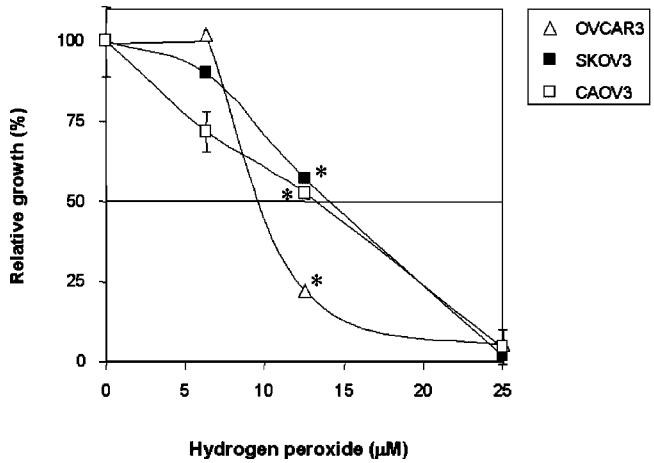

Fig. 2. Growth inhibition of ovarian cancer cells by hydrogen peroxide. Cells were plated as described for Figure 1, except that H_2O_2 was added. H_2O_2 was diluted in culture media and then added at equal volumes to the cultures to attain the indicated concentration. The results shown were representative of cultures in log phase, with cells plated at 3.8×10^3 cells/well for CAOV3, 7.5×10^3 cells/well for SKOV3 and 1.5×10^4/well for OVCAR3. Each point represents the mean + SEM of three to four samples. The asterisks indicate points, which had significance level of $P < 0.001$ in comparison to their respective untreated controls.

that can penetrate the cell membrane and convert endogenous O_2^- to H_2O_2 (Samuni et al., 1990). Using the SKOV3 and CAOV3 cell lines, we found that the SOD mimetic inhibited cell growth. The results shown in Figure 3 are representative of cultures in log phase. Addition of Tempol from 0.5 to 5 mM to SKOV3 and CAOV3 decreased the relative growth in a dose-dependent manner. Tempol inhibited SKOV3 and CAOV3 with IC_{50} of 0.5 and 1 mM, respectively. In addition, the effect of Tempol maximized beyond 2–5 mM. This concurred with the argument that H_2O_2 is mediating the growth inhibition, for it had been observed that when intracellular superoxide radicals have been totally dismutated into O_2 and H_2O_2, further increasing the amount of SOD mimic does not alter the yield of H_2O_2 (Chen et al., 2003). Furthermore, it had also been reported that if the H_2O_2 produced by Tempol was not removed by catalase, it would counteract the action of Tempol itself because the nitroxide would fail to shuttle amongst its two other states, namely the hydroxylamine and the oxoammonium cation (Offer et al., 2000).

Reversal of EGCG inhibition by the H_2O_2 scavenger sodium pyruvate

To further examine whether the toxicity of EGCG on ovarian cancer cells is mediated by H_2O_2 more directly, a neutralization study was performed using sodium pyruvate, a scavenger of H_2O_2 that can penetrate cells (Shostak et al., 2000). The cell lines, SKOV3 and CAOV3 were employed in this analysis. At 20 μM EGCG inhibited the growth of SKOV3 and CAOV3 to 10% and 19%, respectively. Addition of sodium pyruvate at 1 mM to the culture media neutralized the action of EGCG on SKOV3 by 86% and on CAOV3 by 89%, thus confirming its action as mediated mostly by H_2O_2 (Fig. 4). EGCG activated the endogenous level of H_2O_2 using the oxidative/antioxidative mechanism. FACS analyses verified that treatment with EGCG led to an increase in intracellular oxidative stress. Cleavage of C2938, which reflects a rise in the level of H_2O_2, increased when CAOV3 cells were cultured in the

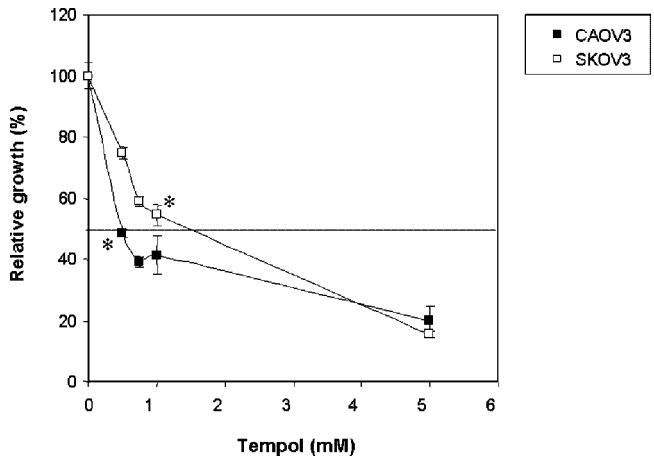

Fig. 3. Growth inhibition of SKOV3 and CAOV3 cells by Tempol. Cells were plated as described in Figure 1, except that Tempol was added. Tempol was dissolved in water, diluted in culture media and then added at equal volumes to the cultures to attain the indicated concentrations. Growth was determined using the wells that were in the log phase of proliferation. The results reported were from wells that had been inoculated at a concentration of 3.8×10^3 cells/well. Each point represents the mean + SEM of three to four samples. The asterisks indicate points that had significance level of $P < 0.001$ in comparison to their respective untreated controls.

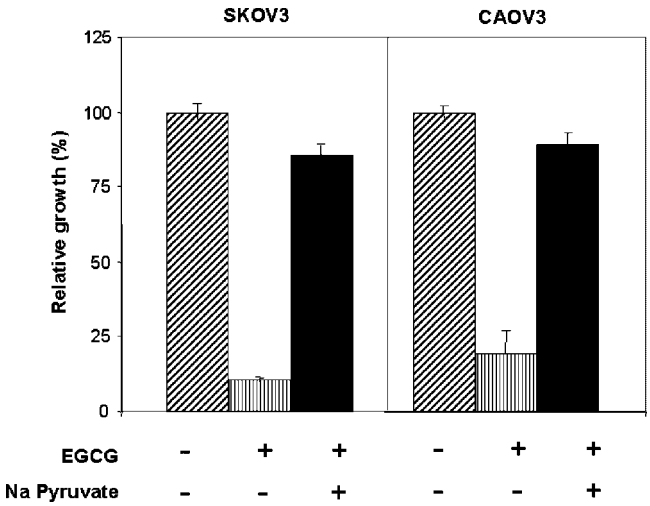

Fig. 4. Reversal of EGCG growth inhibition by sodium pyruvate. Cells were treated as described in Figure 1, except that one set of cultures were treated with sodium pyruvate at a concentration of 1 mM. The relative growth was determined with Alamar blue. Comparison was made among wells that were growing in log phase. The wells that had been inoculated with 1.9×10^3 cells/well were used.

presence of 15 μM EGCG (Fig. 5). A similar result was also observed with C200.

Enhancement of cisplatin cytotoxicity by EGCG

Next, we investigated whether EGCG modulates the toxicity of cisplatin. These experiments were conducted with three ovarian cancer cell lines, CAOV3, SKOV3, and C200, which have different degrees of cisplatin sensitivity. Pretreatment of EGCG increased the toxicity of cisplatin to these lines in a dose-dependent manner (Fig. 6). For example, 2 μg/ml cisplatin did not inhibit the growth of SKOV3 cells, unless the cells were pre-sensitized with EGCG. When EGCG was added at 7.5, 15, 22.5, and 30 μM, the degrees of growth were decreased by 8.7, 26.6, 59.8, and 90.8%, respectively. A similar sensitization was observed with CAOV3 cells. Without EGCG sensitization, 2 μg/ml of cisplatin inhibited growth by 20%, but pretreatment with 7.5, 15, 22.5, and 30 μM of EGCG enhanced the inhibitory activity of cisplatin by 20.8, 30.1, 55.3, and 69.3%. As for the strongly cisplatin-resistant C200 cells, 80 μg/ml of cisplatin inhibited cell growth by 36%, and EGCG, in the same range, increased the inhibition by 7.0, 16.0, 41.1, and 55.8%.

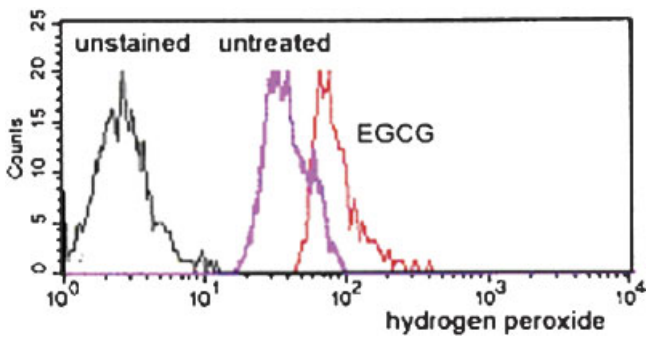

Fig. 5. Increased production of DCDHF cleavage activity of CAOV3 by EGCG. Cells were treated as described in the legend to Figure 1. After 16 h of incubation, they were harvested, stained with DCDHF analog, and FACS analyses were performed with FACScan. The figure shown is representative of two similar experiments.

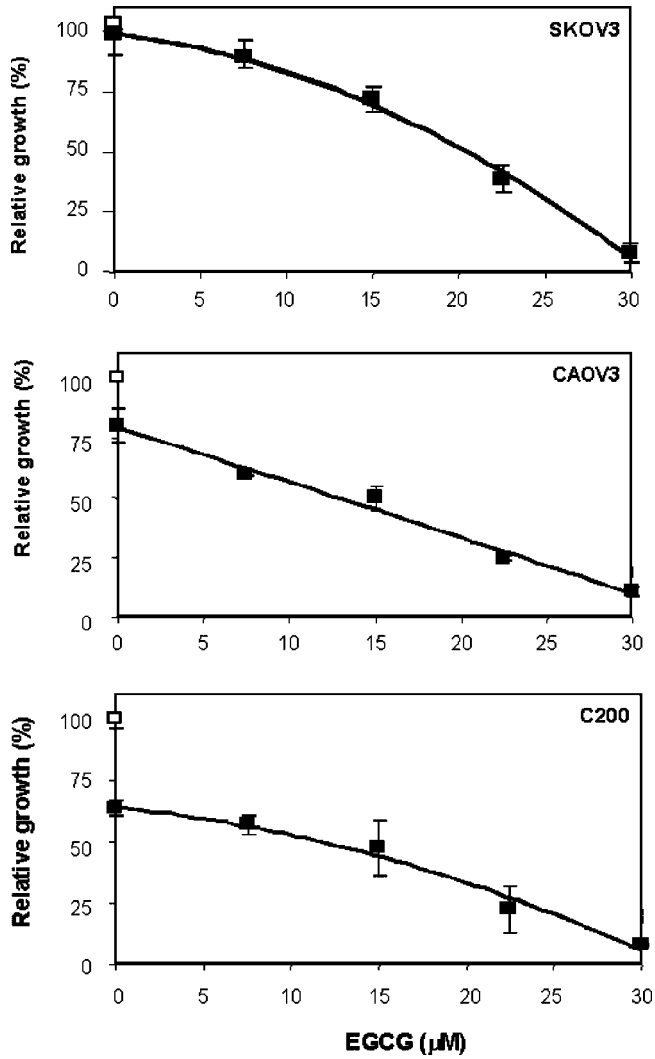

Fig. 6. Sensitization of SKOV3, CAOV3, and C200 to cisplatin by EGCG. Cells were plated at $0.75–1.5 \times 10^4$/ml and as described in legend to Figure 1. EGCG was prepared as described and added at 0, 7.5, 15, 22.5, and 30 μM to the cells. After 24 h, cisplatin was added at 80 μg/ml to C200 and 2 μg/ml to CAOV3 and SKOV3 cells (closed squares). Control wells (open squares) received equal volume of vehicle, but not cisplatin nor EGCG. Relative growth was then determined on day 4 with Alamar blue as described above. The results from the wells that had been inoculated with 1.9×10^3 cells/well were plotted. The levels of fluorescence detected at 100% growth were around 2,500–3,000 units for the three cell lines. Background fluorescence was from 300 to 600 units.

Figure 7 shows the potency of EGCG, the degree by which it can lower the amount of cisplatin needed to achieve the same amount of growth inhibition. EGCG was able to increase the potency of cisplatin by three- to six-fold for the less resistant SKOV3 and CAOV3 cells, and the more resistant C200 cells. These results are summarized in Table 1. The relative growth of C200 at 350 μg/ml of cisplatin was about 60%, that is, 40% inhibition. When 20 μM of EGCG was given, the same degree of inhibition was obtained with 50–100 μg/ml of cisplatin. With the less resistant cell line CAOV3, 10 μM of EGCG reduced the amount of cisplatin needed to inhibit cell growth from 4 μg/ml (47%) to 1 μg/ml (48%). As for the SKOV3 cell line the amount of cisplatin needed to inhibit cell growth by 40% reduced from 3 μg/ml to 0.5-1 μg/ml after sensitization with 10 μM of

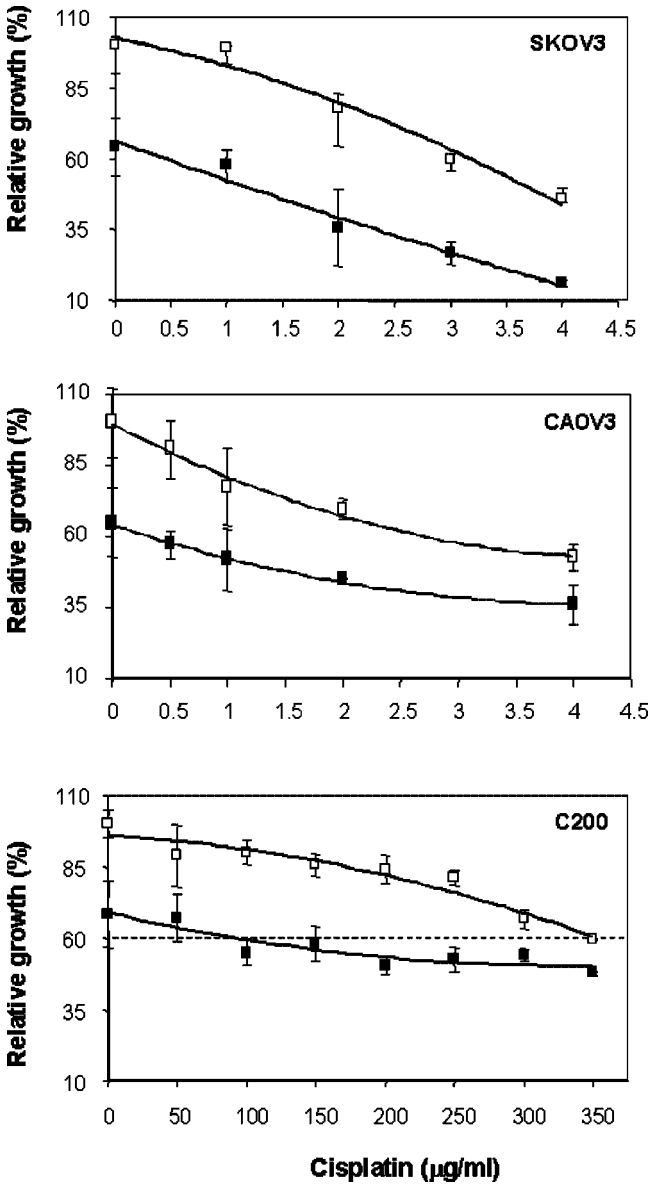

Fig. 7. Sensitization of SKOV3, CAOV3, and C200 to cisplatin by EGCG. Cells were treated at $0.75–1.5 \times 10^4$/ml as described in the legend to Figure 1. EGCG was added at 20 μM to C200 cells and 10 μM to CAOV3 and SKOV3 cells for 24 h (closed squares). The control wells to which EGCG was not added contained equal amount of the vehicle (open squares). Cisplatin was prepared by dissolving in tissue culture medium and added at 100 μl/well to attain the indicated concentrations. Relative growth was then determined with Alamar blue on day 3 for SKOV3 and day 4 for CAOV3 and C200 cells. The wells that had been inoculated with 1.5×10^4 cells/well of SKOV cells, 7.5×10^3 cells/well of CAOV3 and C200 cells, were plotted. The levels of fluorescence detected at 100% growth were around 2,500–3,000 units for the three cell lines. Background fluorescence was from 300 to 600 units.

EGCG. Similar degrees of enhancement were obtained in three or more experimental repeats.

Finally, we explored whether EGCG is susceptible to neutralization by the same drug-resistant mechanisms as cisplatin. For this investigation, we employed a set of cisplatin resistant cells, CP70, C30 and C200 that Hamilton and colleagues have generated from A2780 cells. They cover a range of cisplatin IC_{50}s with increasing levels of resistance to cisplatin (Yao et al., 1995; O'Dwyer et al., 1996). We determined the IC_{50}s of these cell lines to EGCG and found that resistance to cisplatin and to EGCG are not shared to the same extent. IC_{50}s of CP70, C30, and C200 to EGCG were 2.5, 2.5, 7.5, and 15 μM, respectively, that is, the same, three- and six-fold increase from the parental A2780 cells. These increases are very minor compared to those of cisplatin, which were 28-, 240- and 1,060-fold higher than the parental cell line (Table 2).

TABLE 1. EGCG reduced the amount of cisplatin needed to accomplish growth inhibition

	EGCG (μM)	Amount of cisplatin (μg/ml)	Relative growth (%)
SKOV3	0	3	60.0
	10	1	58.0
CAOV3	0	4	52.6
	10	1	52.0
C200	0	350	60.0
	20	100	55.0

Wells were inoculated with 1.5×10^4 cells/well of SKOV3 cells, 7.5×10^3 cells/well of CAOV3 and C200 cells. EGCG was added at 10 μM to CAOV3 and SKOV3 cells and 20 μM to C200 cells. The control wells did not receive EGCG; they only contained 0.1% acetone, the vehicle. After sensitization for 24 h, cisplatin was added at the indicated concentrations and then relative growth in the cultures was determined using Alamar blue as described in the text.

DISCUSSION

Results presented in this paper showed that EGCG inhibited the growth of eight out of eight well-studied ovarian cancer cell lines tested with IC_{50}s at the micromolar range, including ones that are resistant to cisplatin. This is in concordance with the report of Huh et al. (2004) who showed that EGCG induces cell cycle arrest, apoptosis and necrotic cell death in three of the lines, SKOV3, OVCAR3 and PA-1. Thus, the growth suppressive effect of EGCG is unlikely to be a cell line restricted phenomena.

Whereas cell cycle arrest and apoptosis have been claimed to be responsible for the effect of EGCG, how the molecule inhibits growth remains uncertain. The molecule appears to be very pleiotropic, with mechanisms of action that include working as a pro-oxidant or anti-oxidant (Hou et al., 2004). We found that H_2O_2 is a likely molecule that mediates the tumorcidal activity in ovarian cancer cell lines. Reports have shown that administration of EGCG activates production of H_2O_2 in culture medium. The data presented in this paper

TABLE 2. Cisplatin resistant cell lines were not equally resistant to EGCG

	Treatment			
	Cisplatin		EGCG	
	IC_{50}		IC_{50}	
Cells	μg/ml	Relative increase in resistance	μM	Relative increase in resistance
A2780	0.075	—	2.5	—
CP70	2.0	28X	2.5	1X
C30	18	240X	7.5	3X
C200	80	1060X	15	6X

Cells cultured at log phase in 96-well plates were treated with different concentrations of cisplatin or EGCG. Equal amounts of acetone, the vehicle, or medium were added to the control wells for EGCG and cisplatin, respectively. The degree of cellular proliferation was determined with Alamar blue as described in the text, and then the IC_{50}s for cisplatin and EGCG were determined for each cell line.

showed that ovarian cancer cells are susceptible to EGCG and H_2O_2 with similar IC_{50}s. FACS analysis showed that CAOV3 cells that have been cultured with EGCG have increased levels of intracellular H_2O_2 compared to untreated controls. Furthermore, the toxicity of EGCG was neutralized by pyruvate, a scavenger of H_2O_2.

Sugisawa and Umegaki (2002), in a cell free study, did not detect H_2O_2 when EGCG was added at 10 μM to solutions (unspecified), but did detect its production at 30 μM or higher. Our IC_{50}s for the ovarian cancer cells are between 2-20 μM. It is also lower than those reported by Huh et al. (2004) in their study of ovarian cancer cell lines in which 25-100 μM was used to attain growth inhibition. We think that this difference in efficacy occurred because we meticulously protect the EGCG from loss of potency by oxidation. The compound was purchased in small quantities and stored as aliquots in vials that had been purged with nitrogen. While the findings strongly suggest that H_2O_2 participates in EGCG-mediated inhibition of ovarian cancer cell growth, it remains possible that at the concentrations we used, EGCG might have other actions and targets, for example, on the signal transduction pathway that inhibit growth of the ovarian cancer cells (reviewed by Hou et al., 2004). EGCG might block oncogenic pathway activation by HER-2/neu, an oncogene in the EGFR tyrosine kinase superfamily, which is expressed on ovarian cancers as it has been shown to do so in breast cancer cells (Pianetti et al., 2002; Masuda et al., 2003).

Other than generating H_2O_2 in tissue culture medium, EGCG can act intracellularly. Sugisawa and Umegaki (2002) detected the presence of EGCG in cells that had been washed 5 times. Reports showed that EGCG enhances the activity of MnSOD, as observed in human hepatoma HepG2 cells and murine large intestinal cancerous epithelium (Yin et al., 1994; Murakami et al., 2002). A variety of cancer cells are known to contain significant levels of O_2^- that can be harnessed for endogenous formation of cytotoxic H_2O_2 through the action of SOD, the principal scavenger of O_2^- in cellular systems. In fact, over-expression reverses the transformed phenotype in several cancer systems and the enzyme has been regarded as a tumor suppressor gene (St. Clair et al., 2005). The finding that addition of the SOD mimetic, Tempol, inhibited growth of the ovarian cancer cells confirmed this—Tempol produces H_2O_2 during dismutation of O_2^-.

In human, 90% of ovarian cancers arise from the modified peritoneal mesothelium that covers the ovarian surface. Peritoneal dissemination is common (Piver et al., 1993). Tumor cells exofoliate from the ovarian surface. Then, they are transported by fluid in the peritoneal cavity and might implant on the peritoneal surfaces (Kristensen and Trope, 1997). About two thirds of patients at the advanced stage have intra-abdominal and peritoneal spread at diagnosis (Piver et al., 1993). Mice can tolerate 25 mg/kg of EGCG given by intraperitoneal injection. In one study, male nude mice, which are deficient in T-lymphocytes, can tolerate 40 mg/kg (Zhang et al., 2004b). Thus, intraperitoneal administration of EGCG might be an option.

Cisplatin is the frontline chemotherapy of many cancers, including ovarian tumors where it is administered intraperitoneally. Use of this compound is restricted because of its narrow therapeutic index. In vivo EGCG has been able to reverse resistance to doxorubicin in a human carcinoma KB-A-1 xenograft (Zhang et al., 2004b). EGCG can reduce the amount of cisplatin needed to inhibit ovarian cancer cells, including those that are highly resistant. Furthermore, it is known to protect against drug-induced side effects. EGCG shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts.

Mechanistically, EGCG inhibits IKK activity of NF kappa B, inhibits activity of a NADH oxidase, suppresses formation of the oxidized form of GSH, and enhances activity of MnSOD (Yang et al., 2001; Murakami et al., 2002; Morre et al., 2003). Potentially, it can disrupt the homeostatic cellular redox potential to increase sensitivity to cisplatin and sensitize ovarian cancer cells to cisplatin by increasing the amount of DNA damage that culminates in cell death. Cisplatin is a neutral inorganic, square planar complex whose mechanism of action is mainly formation of DNA adduct. However, to bind DNA, a rate-limiting step that involves the sequential replacement of the cis-chloro ligands of cisplatin with water molecules is necessary (el-Khateeb et al., 1999). The resulting monoaquated form is a highly reactive oxidizing electrophile. When cisplatin enters cells, its tumorcidal activity is vulnerable to endogenous reductants. Glutathione (glutamyl-cysteine-glycine) is a strong nucleophile with a sulfhydryl group to detoxify the molecules. In fact, besides blocking activation, glutathione competes with cisplatin for cellular targets, enhances formation of platinum-coordination complexes, as well as facilitates its efflux (Kartalou and Essigmann, 2001; Siddik, 2003). There has been evidence that associates glutathione with cisplatin resistance in ovarian cancer cells (Fracasso, 2001). The cell line C200, which has acquired resistance by in vitro exposure to cisplatin, has five-fold higher gluthathione than the parental cells. Delivering the oxidative stress through H_2O_2 from EGCG can reduce or even deplete glutathione and thus enhance the activity of cisplatin. The observation that EGCG lowers the intracellular level of GSH in human tongue squamous carcinoma and human salivary gland carcinoma cells (CAL27 and HSG1) supports this concept (Weisburg et al., 2004).

The cell type specific toxicity of EGCG—to act as a strong ROS inducer or a ROS scavenger—is a remarkable characteristic that may provide a tremendous advantage. Growing evidence suggests that cancer cells exhibit increased intrinsic ROS stress. For example, in melanoma and prostate cancers, O_2^- plays significant roles in the pathogenesis of malignancy. They appear to play an important role in supporting cellular growth and survival by controlling phosphorylation of signal molecules through activation and inactivation of phosphatases. In contrast normal cells, maintain proper cellular redox states by counterbalancing their cellular ROS with antioxidant enzymes and other redox molecules. Thus, cancer cells are more susceptible to oxidative attack, but normal cells are protected. This differential effect of ROS on cancer cells has been considered a means for therapy (reviewed in McEligot et al., 2005).

Chen et al. (1998) reported that transformed fibroblasts, but not normal fibroblasts, are susceptible to EGCG. Thus, EGCG has a differential effect on tumor and normal cells. At present, the exact reasons for the differential effect remain to be determined. However, Hsu et al. (2003) and Yamamoto et al. (2003) demonstrated that normal primary epidermal keratinocytes (NHEK) and salivary gland cells and oral epithelial cells can tolerate higher amounts of EGCG and H_2O_2

than oral squamous carcinoma cells and this effect is associated with their ability to increase catalase to a higher level in response. Ahmad et al. (2000) also showed that a much higher dose of EGCG is needed to mediate inhibition of NF kappa B in NHEK as compared to A431carcinoma cells. Not only is EGCG reported to be safe for normal cells, numerous reports showed that it protects against oxidative stress (reviewed by Lambert and Yang, 2003). With respect to cancer therapies, EGCG protects normal salivary gland cells from the effects of gamma-irradiation and the chemotherapy cisplatinum (Yamamoto et al., 2004b). With respect to nephrotoxicity, reports have shown that, in rats, EGCG protects against the oxidative stress induced by the immunosuppressive drug cyclosporine given for transplants (Mohamadin et al., 2005).

In sum, the finding that ovarian cancer cells are susceptible to the pro-oxidant activity of EGCG is potentially very important. Its capacity to differentially affect the cancerous versus and normal cells may alleviate the problem of cisplatin toxicity and resistance, a major obstacle in chemotherapy for ovarian cancer.

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