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The green tea polyphenol, epigallocatechin-3-gallate inhibits telomerase and induces apoptosis in drug-resistant lung cancer cells

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Abstract

Epidemiological studies on humans and investigations in animal models suggest that consumption of green tea has anti-cancer effects. Small-cell lung carcinoma (SCLC) has a poor prognosis, particularly due to the development of drug resistance. We investigated the effects of the green tea polyphenol, epigallocatechin-3-gallate (EGCG) on human SCLC cells. EGCG had similar effects (IC_{50} of ~70 μM) on drug-sensitive (H69) and drug-resistant (H69VP) SCLC cells, indicating that it is not part of the drug resistance phenotype expressed in these cells. In both cell lines, incubation in EGCG at 1× IC_{50} for 24 h resulted in 50–60% reduced telomerase activity as measured by a PCR-based assay for telomeric repeats. Colorimetric assays of cells treated for 36 h with EGCG demonstrated a reduction in activities of caspases 3 (50%) and 9 (70%) but not caspase 8, indicating initiation of apoptosis. DNA fragmentation as measured by ELISA occurred within cells treated with EGCG and this was confirmed by TUNEL staining. Flow-cytometric analysis of SCLC cells incubated for 36 h in EGCG indicated a cell-cycle block in S phase. These data indicate the potential use of EGCG, and possibly green tea, in treating SCLC.

Keywords: Lung cancer; Drug resistance; Green tea; EGCG; Telomerase; Apoptosis

Small-cell lung carcinoma (SCLC) accounts for about 15% of all lung cancer and has a poor prognosis, with 5-year survival at diagnosis rarely exceeding 15% [1]. Chemotherapy for SCLC typically involves combinations with etoposide, doxorubicin, vincristine, paclitaxel, and platinum-based regimens [2,3]. However, multi-drug resistance usually develops, making chemotherapy ineffective [4]. Thus treatment of lung cancer and SCLC in particular, presents a significant clinical challenge.

Tea is an aqueous infusion from the dried leaves of *Camellia sinensis* and is one of the most widely used beverages throughout the world. Green tea, which is prepared with minimal oxidation of polyphenols, has been shown in animal studies and human epidemiological studies to prevent cancer, including lung cancer [5–8]. The major anti-cancer components of teas are catechins [9], and the most abundant catechin in green tea, epigallocatechin-3-gallate (EGCG) has strong anti-proliferative and anti-tumor effects [10–12].

Studies of the molecular mechanism of the anti-cancer effects of green tea in general and EGCG in particular have focused on anti-oxidative and anti-inflammatory effects [13]. Other mechanisms investigated have focused on molecular pathways of the cell cycle, angiogenesis, invasion, and growth factor signaling [14,15]. Telomerase has an important role in cancer and inhibition of telomerase activity is a potential target for cancer treatment [16]. Telomerase is expressed in lung cancer cells [17] and this prevents apoptosis [18]. Thus, inhibition of telomerase might be a useful approach in stimulating lung cancer cell death [19].

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In this report, we describe studies on the effects of EGCG on drug-sensitive and multi-drug-resistant human SCLC cells. We show that EGCG treatment was not part of the drug resistance phenotype and resulted in telomerase inhibition. Subsequently, the cells showed the molecular characteristics of apoptosis.

Materials and methods

Cell growth. H69 human small-cell lung carcinoma cells were grown in suspension in AIM-V serum-free medium (Invitrogen-Gibco, Carlsbad, CA) in 5% CO_2 atmosphere at 37 °C. Culture medium was changed by 1:5 dilution every 4–5 days. Multi-drug-resistant H69VP cells were selected in etoposide [20] and grown as above. These cells were resistant to etoposide (9-fold), doxorubicin (11-fold), and vincristine (15-fold) [21].

Cytotoxicity. EGCG (Sigma, St. Louis. MO) dissolved in DMSO was added to logarithmically growing SCLC cells (generally 3 days after new medium was added) in 1 ml suspensions at 10^4 cells/ml. After 4 days of continuous exposure, cells were counted in a Coulter Z-1 counter. Cell counts were validated by staining with Trypan blue and estimation of live cells microscopically by hemocytometer. All experiments were done in triplicate and repeated at least three times. IC_{50} was calculated compared to solvent controls.

Telomerase assay. Logarithmically growing SCLC cells (10-ml cultures, 2×10^5 cells/ml) were incubated in EGCG for 24 h. For each experiment (done in triplicate), 10^6 cells were harvested by centrifugation and assayed immediately for telomerase activity (Roche Applied Science, Indianapolis, IN). Briefly, this assay involved telomerase in cell extracts (10 μg protein) catalyzing the addition of telomeric repeats to a biotinylated primer, followed by amplification of the reaction products by PCR. The amplified products were then denatured and hybridized to a digoxigenin-labeled probe specific for the telomeric repeats. The probe was affixed to a streptavidin-coated microtiter plate. The PCR product was then quantitated by an anti-digoxigenin antibody coupled to peroxidase, with colorimetric assay of peroxidase activity.

Caspase assays. Ten-milliliter cultures of SCLC cells (2×10^5 cells/ml) were incubated in 70 μM EGCG in AIM-V for 36 h. Following lysis, capases 3, 8, and 9 were assayed colorimetrically in cell extracts (20 μg protein) (Biovision, Mountain View, CA). The assays involved the use of peptide substrates coupled with *p*-nitroanilide, which is released upon substrate cleavage and can be detected spectrophotometrically at 405 nm. Means of triplicate experiments were compared statistically by the use of two-tailed *t*-test.

DNA fragmentation test. The mechanism of cytotoxicity of EGCG was investigated by detection of DNA fragmentation (Roche Applied Science kit, Indianapolis, IN). Briefly, triplicate 5-ml cultures of SCLC at 2.5×10^5 cells/ml were incubated in AIM-V with 10 μM BUdR for 15 h. The cells were washed and resuspended in fresh medium. Then, triplicate 200-μl cultures were incubated in AIM-V with 70 μM EGCG. After the incubation period, the cells were pelleted by centrifugation and 100 μl of the culture medium was removed for ELISA of DNA fragments; this is a measure of cell death by necrosis. The cells were lysed and DNA fragments from inside the cells were measured by ELISA; this is a measure of apoptosis.

TUNEL analysis for apoptosis. Ten-milliliter cultures of SCLC cells with 10^5 cells/ml were incubated with 70 μM EGCG in AIM-V for 36 h and then washed twice in PBS and fixed in 1% paraformaldehyde at room temperature for 1 h. After two additional washes in PBS, the cells were resuspended in permeabilization solution (0.1% Triton X-100, 0.1% Na-citrate) for 2 min at 4 °C. Following washing with PBS, the cells were resuspended in 50 μl TUNEL mixture containing TdT and fluoresecin-dUTP (Roche Applied Science, Indianapolis, IN). Following incubation in the dark for 2 h, the cells were counterstained with 3 ml of 50 μg/ml propidium iodide in 3.8 mM Na-citrate, 10 μg/ml RNase A for 1 h and examined by flow cytometry.

Cell-cycle analysis. Ten-milliliter cultures of SCLC cells at a density of about 10^5 cells/ml were incubated in 70 μM EGCG for 36 h. After washing in PBS, the cells were fixed in cold 75% ethanol for 1 h at 4 °C. After an additional wash in PBS, the cells were stained in propidium iodide as above and analyzed by flow cytometry.

Results

Cytotoxicity of EGCG

EGCG had similar cytotoxicities in H69 drug-sensitive and H69VP drug-resistant SCLC cells (Fig. 1), with an IC_{50} of about 70 μM in both cases. The time-course for cytotoxicity showed reductions in cell counts beginning at 72 h and peaking at 96 h, where they remained. Cell deaths in untreated cells began to appear after this time, so 96 h was selected for further cytotoxicity studies.

Telomerase activity

Incubation of both drug-sensitive and drug-resistant SCLC cells in EGCG resulted in a dose-dependent reduction in telomerase activity (Fig. 2). Although H69VP cells had higher basal telomerase activity than H69 cells, the dose-dependent response to EGCG was the same in both cell lines. Maximal reduction in enzyme activity was achieved at 70 μM EGCG, the IC_{50} concentration. Pretreatment of cell extracts with 5 μg/ml DNase-free RNase (Sigma) for 20 min at 37 °C, led to no activity in the assay.

Caspase activities

Compared to untreated controls, ECGC incubation for 36 h of both H69 and H69VP cells resulted in an increase in the activities of caspase 3 and caspase 9, but not of caspase 8 (Table 1) . The activities were linear with enzyme protein concentration up to 50 μg protein and they were abolished by pre-boiling of the cell extracts.

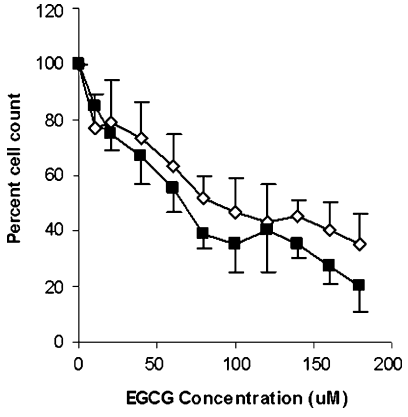

Fig. 1. Cytotoxicity of EGCG on SCLC cells. Triplicate cultures of H69 drug-sensitive (closed squares) and H69VP drug-resistant (open diamonds) cells were incubated in EGCG in DMSO for 96 h. Mean cell counts (±SD) were compared with vehicle-treated controls: H69, 3.4×10^5 cells/ml; H69VP, 2.7×10^5 cells/ml).

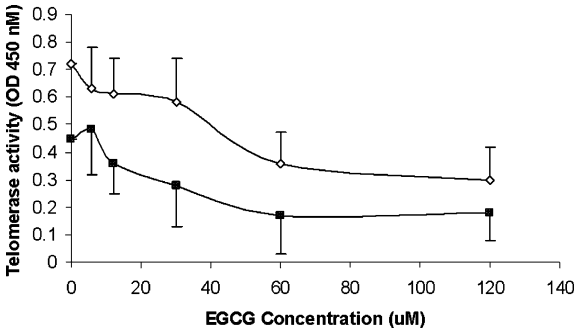

Fig. 2. Effect of EGCG incubation on telomerase activity in SCLC cells. Triplicate 10-ml cultures of 2×10^6 cells of H69 drug-sensitive (closed squares) and H69VP drug-resistant (open diamonds) cells were incubated in EGCG for 24 h. Telomerase activity (means ± SD) was measured by TRAP-ELISA.

Table 1
Caspase activities in SCLC cells treated with EGCG (OD at 405 nm)

Cells/treatment	Caspase 3	Caspase 8	Caspase 9
H69-sensitive	0.06	0.04	0.14
H69 + EGCG	0.26*	0.05	0.79*
H69VP-resistant	0.08	0.05	0.21
H69VP + EGCG	0.50*	0.04	0.50*

Cells were incubated with or without 70 μM EGCG for 36 h. Caspases were assayed from extracts of 0.8×10^6 cells.
* $p < 0.05$ compared to untreated cells.

DNA fragmentation and TUNEL assays

To determine the mechanism of cytotoxicity, we treated H69VP SCLC cells with EGCG and then measured DNA fragmentation by ELISA. Over time, more fragments were formed within the cells, since the bulk of the ELISA-positivity was on cell lysates as opposed to the medium surrounding the cells, where fragments would come from necrotic cells. (Fig. 3). Similar data were obtained with H69 cells (data not shown). DNA breaks due to apoptosis were measured directly by the TUNEL method (Fig. 4 and data not shown). Both H69 and H69VP SCLC cells treated with EGCG showed considerable DNA breaks. In two separate experiments, untreated control cells were 3% and 11% apoptotic, treated H69 cells were 63% and 72% apoptotic and treated H69VP cells were 57% and 62% apoptotic.

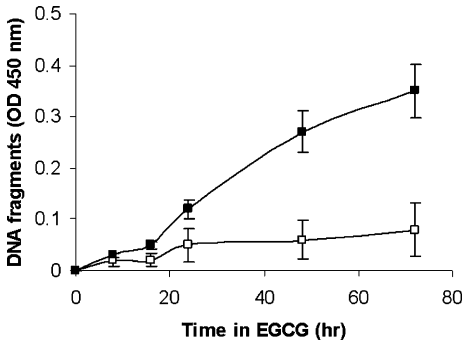

Fig. 3. Kinetics of DNA fragmentation in H69VP cells exposed to EGCG. Triplicate 5-ml cultures, 2×10^5 cells/ml were incubated in 70 μM EGCG. DNA fragments were measured in the medium (open squares) and in cell lysate (closed squares) by ELISA (mean ± SD).

Cell-cycle analysis

Flow-cytometric analyses showed that EGCG treatment of H69VP cells resulted in changes in cell-cycle distribution of the cell population (Fig. 5). Of the non-apoptotic cells, untreated cells had the following distribution: G_1: 70%; S: 19%; and G_2/M: 8%. Treated cells were: G_1: 59%; S: 28%; and G_2/M: 9%. With H69 cells, the patterns were similar. Untreated cells were G_1: 72%; S: 20%; and G_2/M: 7% and treated cells were G_1: 62%; S: 29%; and G_2/M: 7%.

Discussion

This is the first report of the effects of green tea polyphenols on SCLC cells, and in particular on drug-resistant tumor cells. EGCG had similar cytotoxicity in both drug-sensitive and drug-resistant SCLC cells (Fig. 1). This indicates that it is not part of the drug resistance phenotype that occurs in SCLC [4].

The molecular mechanism for EGCG cytotoxicity in SCLC appears to involve telomerase inhibition, followed by apoptosis. Incubation of both drug-sensitive and drug-resistant cells in EGCG for 24 h led to pronounced reduction in telomerase activity (Fig. 2). This confirms previous reports showing such inhibition in osteosarcoma cells [22] and breast cancer cells [23]. The treated SCLC cells underwent apoptosis, rather than necrosis, as indicated by release of DNA fragments from cell lysates as opposed to generalized release into the medium (Fig. 3). This conclusion is strengthened by TUNEL analysis (Fig. 4), which showed a pronounced increase in DNA breaks after 36 h of EGCG treatment.

Apoptosis in mammalian cells can occur by the extrinsic or intrinsic pathway. The latter occurs after DNA damage, typified in cells treated with chemotherapeutic drugs [24,25]. In both drug-sensitive and drug-resistant SCLC cells, EGCG incubation led to increases in the activities of caspase 3 and caspase 9, which occur in the intrinsic pathway; the activity of capsase 8, which occurs in the extrinsic pathway, was unchanged (Table 1). This indicates that the cellular response to EGCG is similar to that to chemotherapeutic drugs.

Cell-cycle analysis showed that ECGC incubation led to an accumulation of SCLC cells in S phase. This differs from an accumulation in G_1 in breast cancer cells [26] and epidermoid carcinoma cells [27] and G_2–M accumulation in non-small-cell lung cancer cells [10]. Telomere DNA appears to be important in the S phase cell-cycle checkpoint [28] and so telomerase inhibition might be expected to result in an accumulation in S phase.

The concentration of EGCG for telomerase inhibition, apoptosis and cytotoxicity in our studies (70 μM) was similar to that reported in other types of tumor cells such as

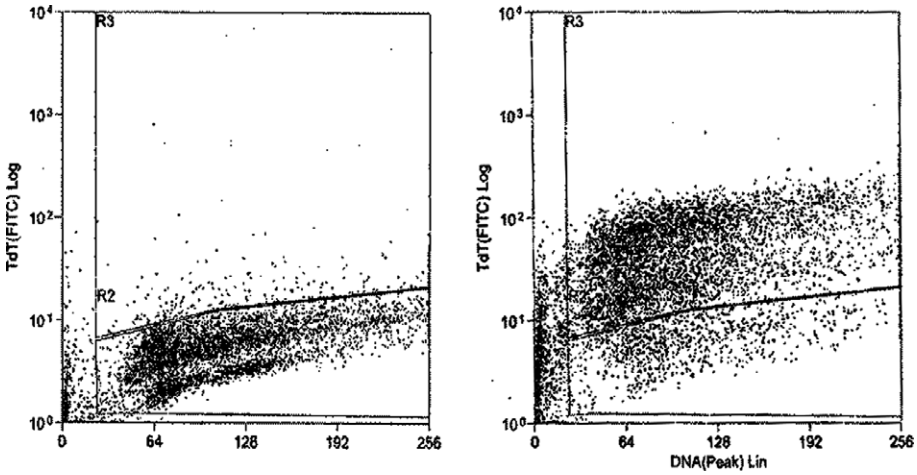

Fig. 4. Apoptosis of H69VP cells treated with EGCG. Ten-milliliter cultures of 10^6 cells were exposed to 70 μM EGCG for 36 h and then fixed, permeabilized and stained with a fluorescent TUNEL reaction. Following counterstaining with PI, the cells were analyzed by flow cytometry.

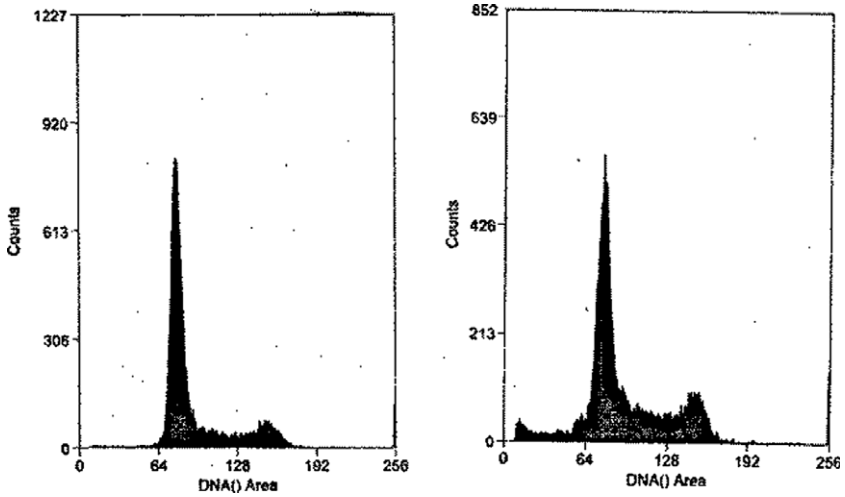

Fig. 5. Cell-cycle analysis of H69VP cells treated with EGCG. Ten-milliliter cultures of 10^6 cells were incubated in 70 μM EGCG for 36 h and then fixed and stained with PI. The cells were immediately analyzed for DNA content by flow cytometry.

non-small-cell lung (50 μM – Ref. [10]), breast (100 μM – Refs. [23,26]) and epidermoid (100 μM – Ref. [27]). This is similar to the concentration used for cancer prevention in animal studies. However, this is significantly higher than the concentration of EGCG in humans in vivo. For example, in a pharmacokinetic study, the peak plasma concentration of EGCG after drinking the equivalent 2 cups of green tea was 0.2 μM [29]. EGCG is somewhat unstable in neutral or slightly basic solutions such as the culture medium used in our studies and it may be degradation products rather than native EGCG that are responsible to the effects observed in cell studies such as ours and others [22]. Therefore, caution is required in extrapolating our conclusions as to mechanism to the known in vivo beneficial effects of EGCG.

Taken together, our data indicate the potential usefulness of the green tea polyphenol, EGCG, in treating both drug-sensitive and drug-resistant SCLC.

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