Green Tea Constituent Epigallocatechin-3-Gallate and Induction of Apoptosis and Cell Cycle Arrest in Human Carcinoma Cells

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Background and Purpose: **The polyphenolic compounds present in green tea show cancer chemopreventive effects in many animal tumor models. Epidemiologic studies have also suggested that green tea consumption might be effective in the prevention of certain human cancers. We investigated the effect of green tea polyphenols and the major constituent, epigallocatechin-3-gallate, on the induction of apoptosis (programmed cell death) and regulation of cell cycle in human and mouse carcinoma cells.** ***Methods:*** **Human epidermoid carcinoma cells (cell line A431), human carcinoma keratinocyte (cell line HaCaT), human prostate carcinoma cells (cell line DU145), mouse lymphoma cells (cell line L5178Y), and normal human epidermal keratinocytes (NHEKs) were used. Apoptosis was assessed by 1) the formation of internucleosomal DNA fragments by agarose gel electrophoresis, 2) confocal microscopy, and 3) flow cytometry after tagging the DNA fragments by fluorescence label. The distribution of cells in different phases of the cell cycle was analyzed by flow cytometry.** ***Results:*** **Treatment of A431 cells with green tea polyphenols and its components, epigallocatechin-3-gallate, epigallocatechin, and epicatechin-3-gallate, resulted in the formation of internucleosomal DNA fragments, characteristic of apoptosis. Treatment with epigallocatechin-3-gallate also resulted in apoptosis in HaCaT, L5178Y, and DU145 cells, but not in NHEK. Confocal microscopy and flow cytometry confirmed the findings. The DNA cell cycle analysis showed that in A431 cells, epigallocatechin-3-gallate treatment resulted in arrest in the G_0–G_1 phase of the cell cycle and a dose-dependent apoptosis.** ***Conclusions:*** **Green tea may protect against cancer by causing cell cycle arrest and inducing apoptosis. It needs to be evaluated in human trials.** **[J Natl Cancer Inst 1997;89:1881–6]**

The term ‘‘Chemoprevention’’ is coined for cancer prevention and cancer control by use of naturally occurring and/or synthetic compounds. The occurrence of the disease may be completely prevented, blocked, or reversed. This approach is promising because chemotherapy and surgery have not been fully effective against the high incidence or low survival rate of most of the cancers *(1)*. In this regard, the naturally occurring antioxidants present in the diet and beverages consumed by humans are receiving increasing attention *(2)*.

Tea consumption in the world is very high and ranks second to water consumption. It is prepared from the dried leaves of *Camellia sinensis.* Most tea consumed in the world can be classified in two forms, green tea (approximately 20%) and black tea (approximately 80%). Extensive studies *(3–12)* from this and other laboratories over the last 10 years have verified cancer chemopreventive effects of a polyphenol mixture derived from green tea against many animal tumor bioassay systems. In these studies, oral consumption or topical application of green tea polyphenols or its major constituent epigallocatechin-3-gallate has been shown to offer protection against all stages of multistage carcinogenesis that include initiation, promotion, and progression. A study *(13)* has also shown that green tea consumption can inhibit the growth of established skin papillomas in mice. Epidemiologic studies have not provided conclusive results but tend to suggest that green tea may reduce the risk associated with cancers of the bladder *(14)*, prostate

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See ‘‘Notes’’ following ‘‘References.’’

(15), esophagus *(16)*, and stomach *(17,18)*. Deciphering the molecular mechanism, by which green tea imparts the protective effects, is important because it may provide opportunities to interfere with cancer development through administration of purified polyphenolic derivatives. Green tea appears to be potentially an ideal agent for chemoprevention. In our judgment, an ideal agent should have the following characteristics: (*a*) little or no adverse effects, (*b*) high efficacy against multiple sites, (*c*) effectiveness at achievable dose levels, (*d*) capability for oral consumption, (*e*) a known mechanism of action, (*f*) low cost, (*g*) history of use by the human population, and (*h*) general human acceptance.

Cancer chemopreventive agents may alter the regulation of cell cycle [*(19)* and references therein]. Treatment with such agents may result in cell cycle arrest, thereby reducing the growth and proliferation of cancerous cells, and may also affect the malignant transformation. It has been suggested that tumor growth depends on evasion of normal control mechanisms that operate through a programmed deletion of cells [i.e., apoptosis *(20)*]. This, in fact, is a physiologic and pathologic process and functions as an essential mechanism of tissue homeostasis. In recent years, many cancer chemopreventive agents have been shown to induce apoptosis *(21–24)* and conversely several tumor promoters have also been shown to inhibit apoptosis *(25)*. We designed this study to investigate if green tea polyphenols and the major constituent, epigallocatechin-3-gallate, induce apoptosis and perturb cell cycle progression in carcinoma cells.

Materials and Methods

Materials

A polyphenolic fraction was prepared from green tea (hereafter referred to as green tea polyphenols) as per the method standardized in our laboratory *(3)*. Briefly, dried green leaves (100 g) were extracted twice with hot water (80 °C) and three times with 80% ethanol (700 mL each time) under nitrogen. The combined extract (3.5 L) was concentrated under vacuum to 1 L and then extracted with an equal volume of chloroform. The aqueous layer was extracted three times with ethyl acetate (800 mL each time) under nitrogen, and the total organic soluble fraction (2.4 L) was concentrated under vacuum. The residue obtained was dissolved in water (50 mL) and freeze-dried. The light-brown solid matter obtained was called green tea polyphenols. Chromatographic analysis of this mixture showed that it contains four major polyphenolic compounds (i.e., epigallocatechin-3-gallate, epicatechin-3-gallate, epigallocatechin, and epicatechin) *(3)*. Purified preparations of all of these polyphenolic compounds (>97% purity) were provided by D. A. Balentine (Lipton Tea Co., Englewood Cliffs, NJ). The cells, namely, human epidermoid carcinoma A431, human carcinoma keratinocyte HaCaT, human prostate carcinoma DU145, and mouse lymphoma L5178Y, were obtained from American Type Culture Collection (Rockville, MD). Normal human epidermal keratinocytes (NHEKs) were prepared from the human foreskin using standard procedure *(26)*. A431 and HaCaT cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD). DU145 cells were maintained in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. L5178Y cells were maintained in Fisher's medium supplemented with 10% equine serum and briefly (3 minutes) gassed with CO_2. NHEK cells were maintained in keratinocyte–serum-free medium (Life Technologies, Inc.) supplemented with L-glutamine, epidermal growth factor, and bovine pituitary extract. The cells were maintained at 37 °C and 5% CO_2 in a humid environment. Green tea polyphenols and all its individual compounds were dissolved in double distilled water for the treatment.

Detection of DNA Fragmentation Induced by Green Tea Polyphenols and Its Individual Constituents

The A431 cells were grown to about 70% confluency and treated with green tea polyphenols (40, 80, and 160 μg/mL) or its individual constituents (40 μg/mL) for 48 hours. In another study, A431, HaCaT, DU145, and L5178Y cells were treated with 80 μg/mL epigallocatechin-3-gallate for 48 hours, whereas NHEK cells were treated with 40, 80, and 160 μg/mL of epigallocatechin-3-gallate for 48 hours. Following these treatments, the cells were washed twice with phosphate-buffered saline (10 m*M* PBS, pH 7.2), suspended in 1 mL cytoplasm extraction buffer (10 m*M* Tris, pH 7.5, 150 m*M* NaCl, 5 m*M* $MgCl_2$, and 0.5% Triton X-100), left on ice for 15 minutes, and pelleted by centrifugation (14 000*g*) at 4 °C. The pellet was incubated with DNA lysis buffer (10 m*M* Tris, pH 7.5, 400 m*M* NaCl, 1 m*M* EDTA, and 1% Triton X-100) for 20 minutes on ice and then centrifuged at 14 000*g* at 4 °C. The supernatant obtained was incubated overnight with RNase (0.2 mg/mL) at room temperature and then with Proteinase K (0.1 mg/mL) for 2 hours at 37 °C. DNA was then extracted using phenol: chloroform (1:1) and precipitated with 95% ethanol for 2 hours at −80 °C. The DNA precipitate was centrifuged at 14 000*g* at 4 °C for 15 minutes and the pellet was air-dried and dissolved in 20 mL of Tris–EDTA buffer (10 m*M* Tris–HCl, pH 8.0, and 1 m*M* EDTA). Total amount of DNA was resolved over 1.5% agarose gel, containing 0.3 μg/mL ethidium bromide in 1× TBE buffer (pH 8.3; 89 m*M* Tris, 89 m*M* Boric acid, and 2 m*M* EDTA) (BioWhittaker, Inc., Walkersville, MD). The bands were visualized under UV transilluminator (Model #TM-36, UVP Inc., San Gabriel, CA) followed by polaroid photography (MP-4 Photographic System, Fotodyne Inc., Hartland, WI).

Detection of Apoptosis by Confocal Microscopy

A431 cells were cultured over round glass coverslips in a 60-mm culture dish to about 70% confluency and then treated with epigallocatechin-3-gallate (20-, 40-, and 80-μg/mL doses) for 48 hours followed by incubation with 10 μ*M* SYTO 13 (Molecular Probes, Inc., Eugene, OR) for 20 minutes in complete medium at 37 °C for labeling the nuclei. The coverslips were washed with PBS and mounted. The SYTO 13 fluorescence was excited by the argon–krypton laser with 488-nm wavelength and imaged through a 460-nm dichroic reflector and a 514–540-nm emission filter with a Zeiss 410 laser scanning confocal microscope. Apoptosis was characterized by the morphologic changes, viz., chromatin condensation, nuclear condensation, and formation of apoptotic bodies.

Quantification of Apoptosis by Flow Cytometry

The A431 cells were grown at a density of 1×10^6 cells in 100-mm culture dishes and were treated with epigallocatechin-3-gallate (20-, 40-, 80-, and 160-μg/mL doses) for 48 hours. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by use of an APO–DIRECT apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA), as per the manufacturer's protocol. The labeled cells were then analyzed by flow cytometry.

Cell Viability

The A431 cells were grown to 70% confluency and treated with epigallocatechin-3-gallate (20-, 40-, 60-, and 80-μg/mL doses) for 48 hours and the cell viability was determined by trypan blue exclusion assay.

DNA Cell Cycle Analysis

The A431 cells (70% confluent) were serum starved for 36 hours to arrest them in G_0 phase of the cell cycle, after which they were treated with epigallocatechin-3-gallate (40 or 80 μg/mL) in DMEM complete medium for 24 hours. The cells were trypsinized thereafter, washed twice with cold PBS, and centrifuged. The pellet was resuspended in 50 μL cold PBS and 450 μL cold methanol for 1 hour at 4 °C. The cells were centrifuged at 1100 rpm for 5 minutes, pellet washed twice with cold PBS, suspended in 500 μL PBS, and incubated with 5 μL RNase (20 μg/mL final concentration) for 30 minutes. The cells were chilled over ice for 10 minutes and stained with propidium iodide (50 μg/mL final concentration) for 1 hour and analyzed by flow cytometry.

Results

We studied whether the green tea polyphenols and the polyphenolic epicatechin derivatives present in it induce apoptosis in human carcinoma cells. As evident by the formation of internucleosomal DNA fragments (Fig. 1, A) compared with ve-

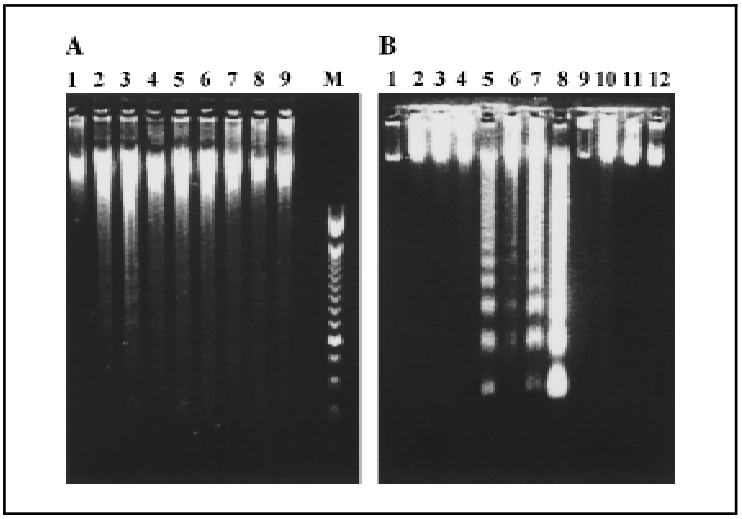

Fig. 1. DNA fragmentation by green tea polyphenols and the individual polyphenolic constituents present therein in different cell types. Cells were treated with vehicle, green tea polyphenols or its individual constituents. Forty-eight hours later, the cells were collected and cellular DNA was isolated and subjected to agarose gel electrophoresis followed by visualization of bands as described in the ''Materials and Methods'' section. Data shown here are from a representative experiment repeated three times with similar results. **A**) DNA fragmentation in A431 cells. Lane 1, vehicle only; lane 2, green tea polyphenols (40 μg/mL); lane 3, green tea polyphenols (80 μg/mL); lane 4, green tea polyphenols (160 μg/mL); lane 5, epigallocatechin-3-gallate (40 μg/mL); lane 6, epigallocatechin (40 μg/mL); lane 7, epicatechin-3-gallate (40 μg/mL); lane 8, epicatechin (40 μg/mL); lane 9, caffeine (40 μg/mL); and M, molecular weight marker. **B**) DNA fragmentation in different cell types. Lanes 1–4—A431, HaCaT, L5178Y, and DU145 cells, respectively, treated with vehicle alone; lanes 5–8—A431, HaCaT, L5178Y, and DU145 cells, respectively, treated with epigallocatechin-3-gallate (80 μg/mL); lane 9—NHEK treated with vehicle alone; and lanes 10–12—NHEK treated with a 40-, 80-, and 160-μg/mL dose of epigallocatechin-3-gallate, respectively.

hicle-treated control (Fig. 1, A, lane 1), the green tea polyphenols treatment of A431 cells resulted in an induction of apoptosis at 40-, 80-, and 160-μg/mL doses (Fig. 1, A, lanes 2–4, respectively). Among the individual constituents present in green tea polyphenols, epigallocatechin-3-gallate, epicatechin-3-gallate, and epigallocatechin resulted in the induction of apoptosis in A431 cells at a dose of 40 μg/mL, whereas epicatechin and caffeine did not show such effect (Fig. 1, A).

Since epigallocatechin-3-gallate is the major constituent present in green tea polyphenols (approximately 50%, wt/wt), we performed all of the subsequent experiments with epigallocatechin-3-gallate. We next examined if this induction of apoptosis by epigallocatechin-3-gallate is specific for A431 cells or if the other tumor and normal cell lines originating from different body sites also undergo apoptotic cell death following epigallocatechin-3-gallate treatment. As shown in Fig. 1, B, compared with vehicle-treated controls, epigallocatechin-3-gallate (80 μg/mL for 48 hours) treatment of the cells in monolayer or suspension resulted in the formation of DNA fragments in human carcinoma keratinocyte HaCaT (lane 2 versus lane 6), mouse lymphoma cells L5178Y (lane 3 versus lane 7), and human prostate carcinoma cells DU145 (lane 4 versus lane 8). Interestingly, it was important to observe that epigallocatechin-3-gallate did not result in the formation of DNA ladder in NHEK, even at twice the dose (160 μg/mL) of that used for carcinoma cell lines (Fig. 1, B, lanes 9–12).

The induction of apoptosis by epigallocatechin-3-gallate was also evident from the morphologic alterations as shown by confocal microscopy after labeling the cells with SYTO 13 (Fig. 2). Since this method can identify early apoptotic cells, we included the lower concentration of epigallocatechin-3-gallate (20 μg/mL) to assess the possibility of an early apoptotic response. The vehicle-treated control (Fig. 2, A) as well as the low dose (20 μg/mL) of epigallocatechin-3-gallate (Fig. 2, B) did not cause any alteration in the nuclear morphology of A431 cells. At the 40-μg/mL dose of epigallocatechin-3-gallate, several cells displayed early apoptotic morphology, such as peripheral aggregation of nuclear chromatin (Fig. 2, C). However, the plasma

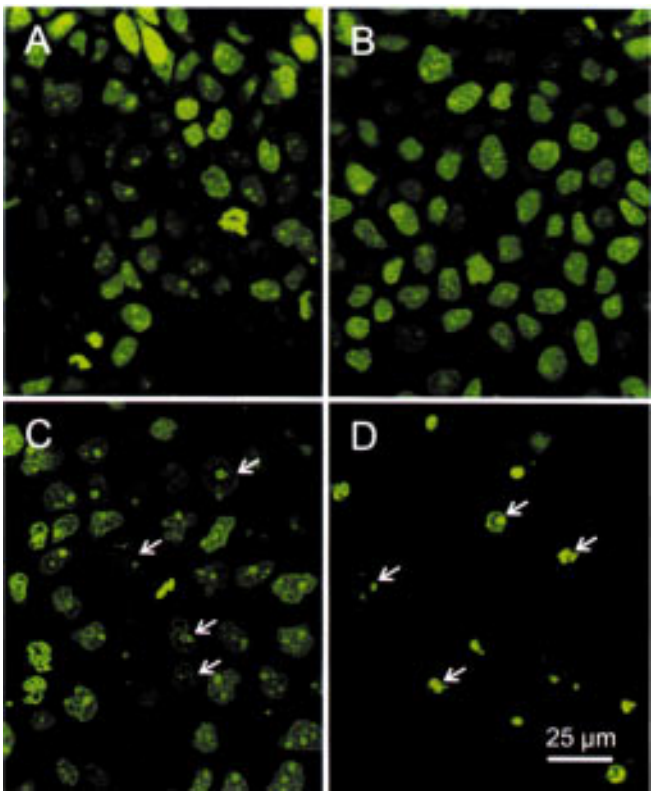

Fig. 2. Morphologic changes in A431 cells following epigallocatechin-3-gallate treatment as evident by confocal microscopy. **A**) vehicle only; **B**–**D**), 20-, 40-, and 80-μg/mL dose of epigallocatechin-3-gallate, respectively, for 48 hours. Morphologic changes denoting apoptotic cells are shown by arrows. Bar represents 25 μm. Data shown here are from a representative experiment repeated four times with similar results.

membrane in the epigallocatechin-3-gallate-treated cells did not take up propidium iodide showing that the plasma membrane was still intact. At the highest dose of epigallocatechin-3-gallate (80 μg/mL) that could be tested in this experiment, nearly all cells were found to be in the late stage of apoptosis as evident from advanced chromatin condensation, nuclear condensation, and formation of apoptotic bodies (Fig. 2, D). In addition, the number of cells that could be observed on the coverslip was markedly diminished at 40- and 80-μg/mL doses of epigallocatechin-3-gallate, indicating that the treatment has also resulted in a detachment of the apoptotic cells. We could not assess the extent of apoptosis at 160-μg/mL dose of epigallocatechin-3-gallate because this dose resulted in a total detachment of the cells from the coverslip.

We next quantified the extent of apoptosis by flow cytometric analysis of the cells labeled with deoxyuridine triphosphate and propidium iodide. Since this is a quantitative procedure to measure the extent of apoptosis, for this experiment we selected epigallocatechin-3-gallate treatment at doses of 20, 40, 80, and 160 μg/mL for 48 hours. Such treatments resulted in 6.3%, 27.7%, 58.9%, and 80.9% of apoptotic cells, respectively (Fig. 3, A). While the induction of apoptosis was almost negligible at the lowest dose (20 μg/mL), the highest dose (160 μg/mL) resulted in a massive apoptosis and a drastic decline in number of cells as determined by flow cytometry. When we assessed the effect of epigallocatechin-3-gallate on the viability of A431 cells by dye-exclusion technique, there was no loss in cell viability at the 20-μg/mL dose but a considerable dose-dependent loss of viability was observed at concentrations of 40, 60, and 80 μg/mL (Fig. 3, B).

Since the induction of apoptosis may be mediated through the regulation of cell cycle, we also examined the effect of epigallocatechin-3-gallate on cell cycle perturbations. Compared with vehicle-treated controls, the epigallocatechin-3-gallate treatment resulted in an appreciable arrest of A431 cells in G_0–G_1 phase of the cell cycle after 24 hours of the treatment. The treatment caused an arrest of 51% cells in G_0–G_1 phase of the cell cycle at a 40-μg/mL dose, which further increased to 74% at the higher dose of 80 μg/mL (Fig. 4), which did not change further at 160 μg/mL (data not shown). This increase in G_0–G_1 cell population was accompanied with a decrease of cell number in S phase. However, the G_2–M cell population remained essentially unchanged. At earlier time points and at a low dose of 20 μg/mL, there was no change in distribution of cells in the cell cycle following treatment with epigallocatechin-3-gallate (data not shown).

Discussion

As a part of our ongoing large-scale program on cancer chemoprevention through dietary constituents, the main aim of this study was to elucidate the mechanism of antitumorigenic effect of epigallocatechin-3-gallate, the major polyphenolic agent present in green tea, and to determine if it directly affects the cell cycle regulation and apoptosis. Apoptosis, in recent years, has become an important issue in biomedical research. The life spans of both normal and cancer cells within a living system are regarded to be substantially affected by the rate of apoptosis. In addition, apoptosis is a discrete

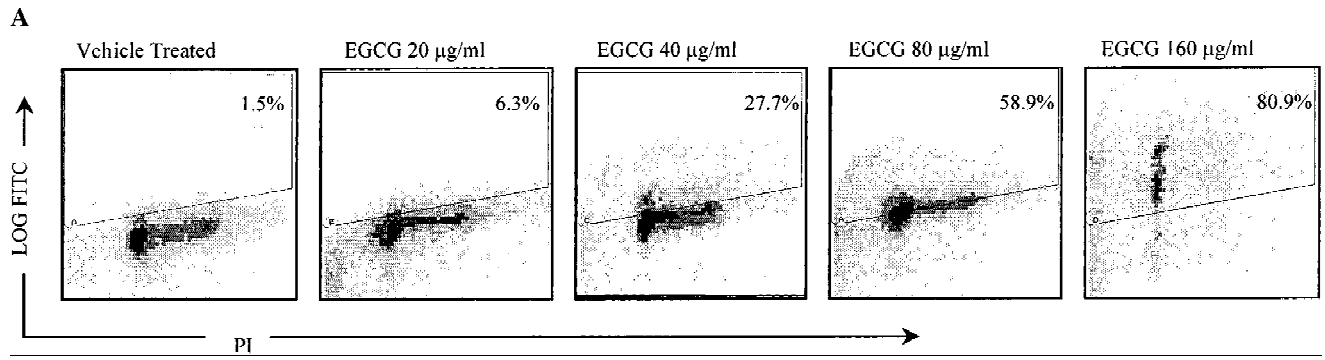

Fig. 3. A) Apoptosis by flow cytometry. A431 cells were treated with epigallocatechin-3-gallate (20, 40, 80, and 160 μg/mL for 48 hours), labeled with deoxyuridine triphosphate using terminal deoxynucleotide transferase and propidium iodide and analyzed by flow cytometry. Results are displayed using the dual-parameter method where the *X* and *Y* axes represent DNA and deoxyuridine triphosphate fluorescence, respectively. Cells showing deoxyuridine triphosphate fluorescence above that of the control population, as indicated by the line in each histogram, are considered as apoptotic cells and their percent population is shown at the right top in each box. Data shown here are from a representative experiment repeated three times with similar results. **B**) Viability of the A431 cells following epigallocatechin-3-gallate treatment. A431 cells were incubated with epigallocatechin-3-gallate (20, 40, 60, and 80 μg/mL for 48 hours) and the number of viable cells was counted by trypan blue exclusion assay. Cell viability is represented as percent viable cells, where the vehicle-treated cells are regarded as 100%. Data shown are mean values ± standard errors determined from three independent experiments.

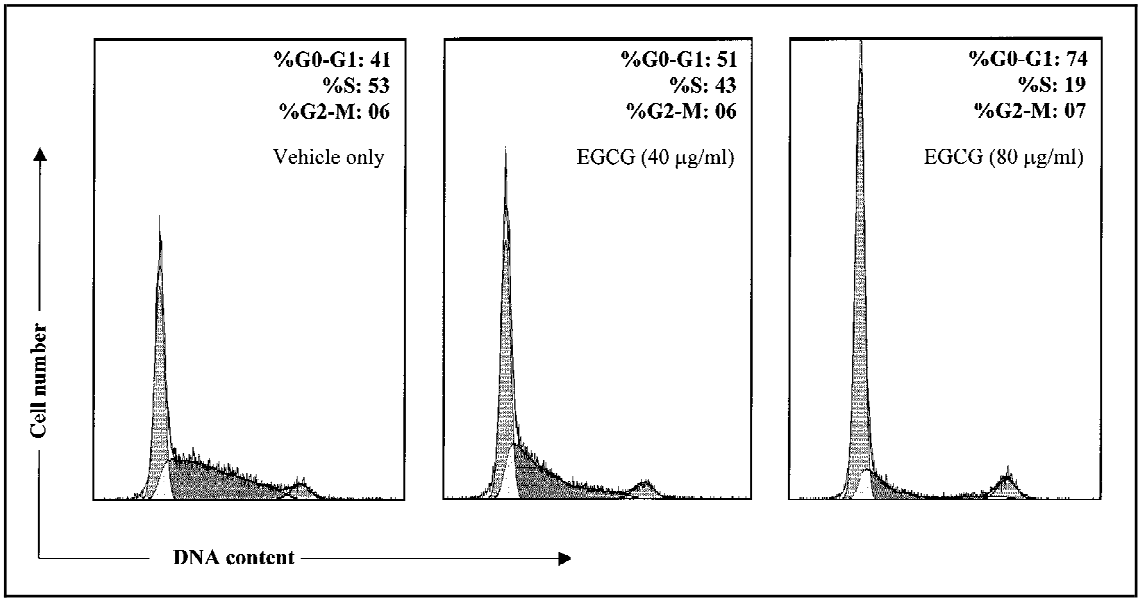

Fig. 4. DNA flow cytometric analysis. A431cells were treated with vehicle or epigallocatechin-3-gallate (40 and 80 μg/mL) for 24 hours and analyzed by flow cytometry. Percentages of cells in G_0–G_1, S, and G_2–M phase were calculated using Cellfit computer software and are represented within the histograms. Data shown here are from a representative experiment repeated two times with similar results.

way of cell death different from necrotic cell death and regarded to be an ideal way of cell elimination. Thus, the chemopreventive agents, which can modulate apoptosis, may be able to affect the steady-state cell population that are often useful targets in the management and therapy for cancer.

In this study, we have shown that green tea polyphenols and their constituents, viz., epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate resulted in an induction of apoptosis in A431 cells. Epigallocatechin-3-gallate possesses two triphenolic groups in its structure, which are reported to be important for its stronger biologic activity *(27)*. We selected this polyphenol for the detailed study because it is the major polyphenol of the green tea polyphenols and is primarily responsible for the green tea effect. It is important to note that epigallocatechin-3-gallate resulted in the induction of apoptosis in all carcinoma cells but not in the normal cells we examined. This selectivity, if it can be observed *in vivo* at the desirable doses, will be of great therapeutic importance. A vast variety of the chemotherapeutic agents, currently used in cancer therapy, are thought to kill the cells by the mechanisms other than apoptosis. This may not always be a preferable way of cancer management. Our results showing the induction of apoptosis by epigallocatechin-3-gallate is of importance because epigallocatechin-3-gallate treatment of A431 cells did not cause any necrosis as evident from the confocal microscopy. The cells were stained with SYTO 13 that is indicative only of apoptosis. The cells did not take up propidium iodide, which also stains the necrotic cells. Moreover, the four different carcinoma cells used in our study have origins from different body sites. This showed that epigallocatechin-3-gallate may be useful against many, if not all, types of cancers. The demonstration of induction of apoptosis by epigallocatechin-3-gallate is also important because green tea is a well-consumed beverage worldwide and has shown promising cancer chemopreventive effects in both laboratory experiments and human epidemiologic studies.

The effect of epigallocatechin-3-gallate in arresting the A431 cells in G_0–G_1 phase of the cell cycle suggests the possibility that green tea may also be useful for the control of cancer growth. Most of the cancer types known to date have defects in one or more cell cycle checkpoints. For example, a number of studies *(28–30)* have shown an implication of p53-mediated induction of WAF1/Cip1/p21, resulting in a G_1 cell cycle arrest. The loss of cell cycle checkpoints results in the selection of cells that have a growth advantage and a predisposition for acquiring more chromosome aberrations. This may also result in drug resistance, invasion, and metastasis. The G_0–G_1 arrest shown by epigallocatechin-3-gallate, therefore, suggests that this agent may slow down the growth of cancer cells by artificially imposing the cell cycle checkpoint. The exact mechanism(s) of apoptosis and cell cycle deregulation by epigallocatechin-3-gallate needs a further exploration of genetic and signal transduction pathways. It is, however, tempting to speculate that cyclin kinase inhibitor(s), cyclin dependent kinase(s) and their regulatory cyclin(s) proteins operating in G_1 phase of the cell cycle may be involved in the epigallocatechin-3-gallate-mediated apoptosis and cell cycle arrest. The involvement of more than one independent or interdependent pathways for apoptotic and cell cycle deregulatory response of epigallocatechin-3-gallate is a possibility that needs to be further explored. In summary, based on our findings reported here and on the extensive amount of laboratory and epidemiology data available, we suggest that clinical trials with epigallocatechin-3-gallate are needed in a population with high cancer risk.

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Notes

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