Green tea polyphenol epigallocatechin inhibits DNA replication and consequently induces leukemia cell apoptosis

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Abstract. Consistent with the putative role of green tea in cancer prevention, tea polyphenols have previously been shown to inhibit tumor cell proliferation by inducing G_1 or G_2/M cell cycle arrests, also documented is their ability to induce apoptosis (programmed cell death). However, it is unclear whether or not the cell cycle effects of polyphenols are related to their cell death-inducing ability. Here we report that the tea polyphenol (-)-epigallocatechin (EGC) inhibits DNA replication in three leukemia cancer cell lines, Jurkat T, HL-60 and K562. Among all the tested tea polyphenols, EGC was found to be the most potent in accumulation of S phase cells and inhibition of the S-G_2 progression. In addition, EGC-mediated inhibition of S phase progression results in induction of apoptosis, as determined by sub-G_1 cell population, breakage of endonuclear DNA, cleavage of poly(ADP-ribose) polymerase and loss of cell viability. When used in cells containing low S and high G_1 and G_2/M populations, EGC did not induce apoptosis. Furthermore, EGC did not inhibit M-G_1 transition. Our finding that EGC inhibits S phase progression that results in leukemia cell death provides a novel and plausible molecular mechanism for how green tea may inhibit the growth of rapidly proliferating neoplastic cells.

Introduction

For centuries green tea has been known for its many medicinal and prophylactic properties, and recent research has confirmed the validity of these attributes. Today, tea is a beverage consumed by more people worldwide than any other drink, save water itself. The high content of polyphenols found in green tea is considered to be the main contributors to its health benefits and cancer-preventative ability. The most abundant polyphenols found in tea consist of the following: (-)-epicatechin (EC), (-)-epigallocatechin (EGC), epicatechingallate (ECG), and (-)-epigallocatechin-3-gallate (EGCG) (Fig. 1).

Previous studies have suggested that these green tea components inhibit tumor growth in mice (1-4), and inhibit cellular proliferation (5) as well as induce apoptosis (6-8) in cell culture. Apoptosis, an active cell death program, is characterized by membrane blebbing, fragmentation of endonuclear DNA, and cleavage of many housekeeping proteins, such as poly(ADP-ribose) polymerase (PARP) and retinoblastoma protein (9-11). Recent evidence suggests that dysregulation of cell cycle progression is probably one of the important events for the initiation of apoptosis (12-16). However, whether or not the effects of green tea on cell cycle are related to cell death has not been previously demonstrated.

A few mechanisms have been suggested to explain the anti-carcinogenic properties of green tea. The molecular target proteins of tea polyphenols have not been fully identified although tea compounds affects an array of cancer-related proteins (17-24). The wide protein binding and inhibitory spectrum of tea polyphenols begs the question, which inhibitory activities are essential for the prevention of cancer? By using asynchronous human carcinoma cell lines, it has been reported that tea polyphenols, especially EGCG, can cause an arrest in G_2 (5) or G_1 (25) phase of the cell cycle. However, the observed cell cycle effects do not seem to explain well the inhibition of cellular proliferation to the extent that has been observed previously (6,7,18,26-29), nor has any blockade of S phase ever been reported.

In the current study, we found that EGC inhibits [^{3}H]-thymidine incorporation into leukemia cancer cell lines Jurkat T, HL-60 and K562. EGC was the most potent tea component among all the tested tea polyphenols. By using synchronous Jurkat cells, we found that EGC inhibited progression of S phase cells into G_2/M, and subsequently induced apoptosis. In contrast, EGC neither inhibited M to G_1 progression nor did it induced apoptosis in cells containing high G_1 and G_2/M populations. Our data suggest that the ability of green tea to block S phase progression is essential for its ability to activate the cancer cell death program in leukemia cells.

Materials and methods

Materials. Green tea polyphenols were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest grade

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Key words: EGC, EGCG, cancer prevention, cell cycle, apoptosis, tea, S-phase

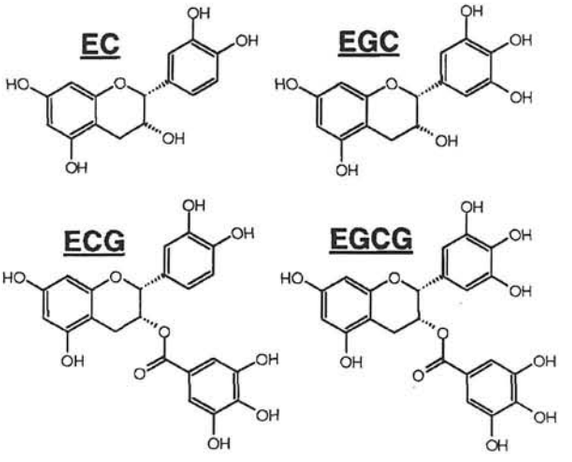

Figure 1. Structures of the four major polyphenols found in green tea.

and purity available: EC (min. 98% pure), EGC (min. 98% pure), ECG (min. 98% pure), EGCG (min. 95% pure), and polyphenon 100 (80% min. total catechins, which contained EGCG, EGC, ECG, and EC). Green tea extracts were a generous gift from Lipton. The polyphenols were freshly dissolved in de-ionized, filtered water and stored at 4°C before use. Fetal calf serum, aphidicolin, propidium iodide, RNase A, and glucose were also purchased from Sigma. RPMI 1640, penicillin and streptomycin were from Life Technologies, Inc. (Rockville, MD). Polyclonal antibody to human poly(ADP-ribose) polymerase (PARP) was from Boehringer Mannheim (Indianapolis, IN) and anti-rabbit IgG-HRP was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and cell synchronization. Human Jurkat T, HL-60 and K562 cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin and maintained in a 5% CO_2 atmosphere at 37°C. To obtain synchronous cell populations, Jurkat cells with a concentration of approximately $1x10^6$ cells/ml were incubated for 24 h with aphidicolin (30,31) at a final concentration of 3 μg/ml. After the treatment the majority of the cells were synchronized to the G_1/S boarder. To release cells from the G_1/S block and allow them to progress synchronously through the cell cycle, the cells were pelleted at 124 x g for 5 min. The media was then removed and the cells were washed one time with RPMI media. After wash the cells were incubated at 37°C in RPMI media and allowed to progress though the cell cycle. At different defined time points that correspond to different phases of the cell cycle (see figure legends), the synchronized cells were treated with tea polyphenols for additional hours. After each treatment, cells were collected, and used for measurement of cell cycle distribution and apoptotic cell death (see below).

Flow cytometry. Cell cycle analysis based on DNA content was performed as follows. Cells were harvested, counted and washed twice with PBS. $5x10^6$ cells were then suspended in 0.5 ml PBS, pipetted and fixed in 5 ml of 70% ethanol for at least 2 h at -20°C. Cells were centrifuged, resuspended in 1 ml of propidium iodide staining solution (50 μg propidium iodide, 100 units RNase A and 1 mg glucose per ml PBS) and incubated at room temperature for 30 min. The cells were then analyzed with FACScan (Becton Dickinson Immunocytometry, CA), ModFit LT and WinMDI V.2.8 cell cycle analysis software (Verity Software, Topsham, ME). The cell cycle distribution is shown as the percentage of cells containing G_1, S, G_2, and M DNA judged by propidium iodide staining. The apoptotic population (AP) is the percentage of cells with sub-G_1 DNA content.

Apoptosis assays. Apoptotic cell death was determined by increased levels of a sub-G_1 apoptotic population (as described in flow cytometry), terminal deoxynucleotidyl transferase-mediated UTP nick end labeling (TUNEL), PARP cleavage, and trypan blue incorporation. TUNEL assay was preformed with a 'Fluorescein-FragEL' DNA fragmentation Detection kit from Oncogene Research Products (Cambridge, MA). In brief, cells were fixed in ethanol at 20°C overnight and then permeabilized with Proteinase K. After permeabilization, fluorescein conjugated dNTP's and TdT enzyme (terminal deoxynucleotidyl transferase) were added to the cells. The TdT enzyme was then able to label free ends of DNA with fluorescein conjugated dNTP's that could then be detected by flow cytometry. PARP cleavage was detected by Western blot assay. A whole cell extract was prepared as we described previously (10). Equal amounts of protein (60 μg) were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a Semi-Dry Transfer System (BioRad, Hercules, CA). The membrane was blocked with 5% non-fat dry milk in PBS-Tween (v/v, 0.2%) for 1 h at room temperature and then incubated overnight at 4°C with the specific antibody to PARP (1:3000). The membrane was washed, blotted with secondary antibody conjugated with horseradish peroxidase (1:2000) at room temperature for 1 h, and then washed again. The protein bands were visualized with the ECL system (Amersham, Piscataway, NJ) according to the manufacturer's instructions. The trypan blue exclusion assay was done by injecting 10 μl of cells containing 0.2% trypan blue dye into a hemicytometer and counting. Cells that absorbed the dye and those that excluded the dye were compared.

[^{3}H]-thymidine incorporation. Jurkat T, HL-60 or K562 cells were treated simultaneously with the indicated amount of EGC and 2 μl/ml of [methyl-^{3}H]-thymidine 5'-triphosphate tetrasoduim salt [80 Ci (1.5 TBq)/mMol, NEN Boston, MA], and incubated at 37°C for 2 h. After harvesting, the cell pellet was washed with PBS, resuspended in 0.5 ml of PBS and collected on a glass microfiber filter. The filter was then washed with 5 ml/filter of PBS and the cells were disrupted with 5 ml/filter of ice-cold 0.1 N NaOH. The DNA was than precipitated on the filter with 5 ml/filter of ethanol. The filters were dried and the remaining radioactivity was measured on a scintillation counter.

Results

EGC is the responsible component in green tea extract for S-phase accumulation. Toward the goal of understanding

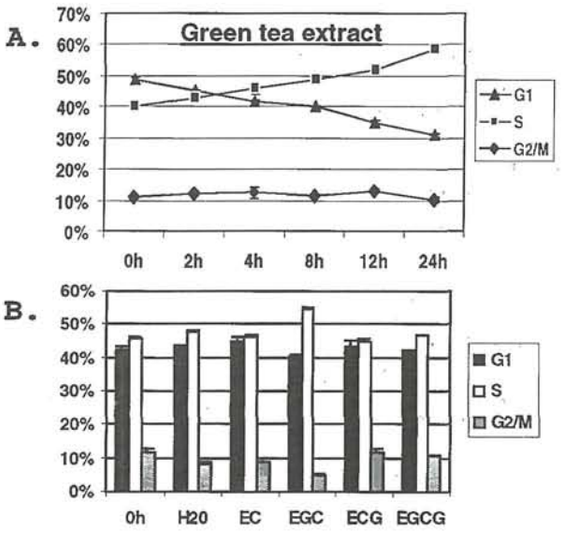

Figure 2. Cell cycle effects of green tea extract or purified tea polyphenols in asynchronous human Jurkat T cells. Asynchronous Jurkat T cells (0 h) were treated with either a green tea extract (50 μg/ml) for up to 24 h (A), or a purified tea polyphenol EC, EGC, ECG, or EGCG (50 μM) or the solvent (H_2O) for 24 h (B), followed by measurement of cell cycle distribution by flow cytometry. Standard deviations were calculated from three separate and independent experiments and were indicated by error bars; however, some error bars are so small that they reside within the data label marks. Standard deviations ranged up to ±2.03%.

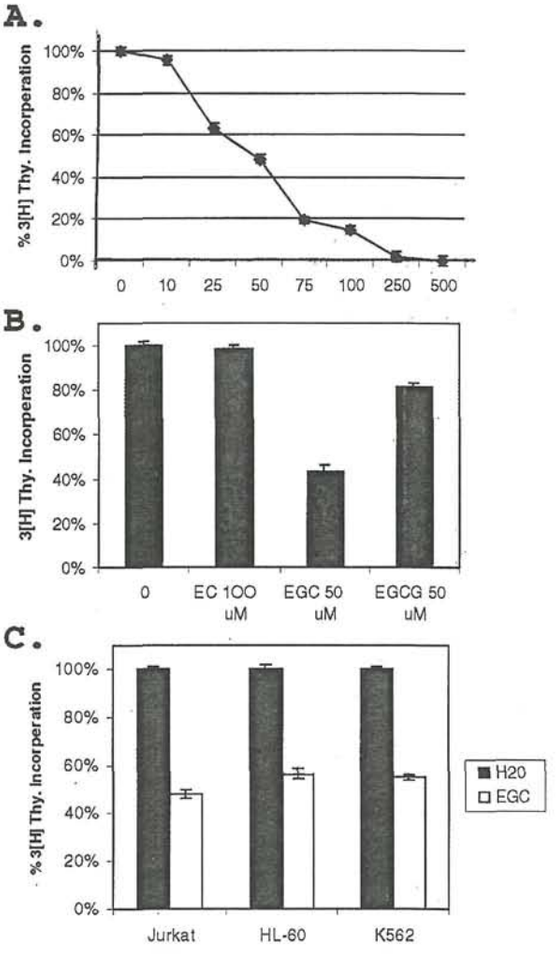

Figure 3. Inhibition of DNA replication by EGC. A, effects of EGC on [^{3}H]-thymidine incorporation assay. Asynchronous Jurkat T cells were incubated with [^{3}H]-thymidine for 2 h in the absence or presence of EGC at indicated concentrations (μM), followed by measurement of the incorporated radioactivity as described in Materials and methods. B, comparison of EGC, EGCG and EC effects. Similar to A, Jurkat cells were incubated with [^{3}H]-thymidine for 2 h in the absence or presence of 50 μM EGC, 50 μM EGCG or 100 μM EC. C, effects of EGC on three leukemia cell lines. Asynchronous Jurkat, HL-60 or K562 cells were incubated with [^{3}H]-thymidine for 2 h in the absence or presence of 50 μM EGC. Error bars are standard error, and the experiments were done three times with similar results.

molecular mechanisms for the cancer-preventative effects of green tea, we investigated how green tea affects cancer cell cycle progression. Exponentially growing human leukemia Jurkat T cells were treated with 50 μg/ml of green tea extract (polyphenon 100) for 2, 4, 8, 12 or 24 h. At each time point, cells were harvested and used for measurement of cell cycle distribution by flow cytometry (Fig. 2A). After each treatment, a decrease in the G_1 population, associated with an increase in the S phase cell population was apparent, whereas no or little change in the G_2/M population was observed (Fig. 2A). After 8 h of treatment with the tea extract, the G_1 population was decreased by 9% while the S population increased by 9%. By 24 h, an 18% decrease in G_1, associated with an 18% increase in S phase, cell population was detected (Fig. 2A). It appears that green tea extract probably accumulates cells in S phase via inhibition of the S-G_2 progression.

The four most abundant polyphenols contained in green tea extract are EC, EGC, ECG and EGCG (Fig. 1). To investigate which component(s) are responsible for the detected cell cycle effects of the tea extract, Jurkat T cells were treated for 24 h with 50 μM of EC, EGC, ECG or EGCG or an equal volume of the solvent (H_2O), followed by flow cytometry analysis. EGC was found to be the only polyphenol that was able to accumulate Jurkat cells in S phase (Fig. 2B). When used in asynchronized Jurkat cells, EGC at 50 μM induced minimal apoptosis while it at a higher concentration could induce apoptosis (Fig. 9).

EGC inhibits thymidine incorporation. To investigate whether accumulation of S phase population by EGC is due to inhibition of DNA synthesis, we measured effects of EGC on [^{3}H]-thymidine incorporation. EGC inhibited incorporation of [^{3}H]-thymidine into Jurkat T cells in a concentration-dependent manner: 5% at 10 μM, ~40% at 25 μM, and ~80% at 75 μM (Fig. 3A). The half-maximal inhibition value for EGC (IC_{50}) *in vivo* was calculated to be 48 μM. We then compared effects of EGCG and EC in this experiment. While EGC at 50 μM inhibited 56% [^{3}H]-thymidine incorporation, the same concentration of EGCG inhibited only 18% of DNA replication and 100 μM EC inhibited had no apparent inhibitory effect (Fig. 3B). This result matches the effects of these tea polyphenols on S-phase accumulation (Fig. 2).

We then tested whether EGC exerts similar inhibitory effect on DNA replication in two other leukemia cell lines, HL-60 and K562. Similar to Jurkat T cells, EGCG at 50 μM inhibited about half of [^{3}H]-thymidine incorporation into both HL-60 and K562 cells (Fig. 3C). Therefore, EGC can inhibit

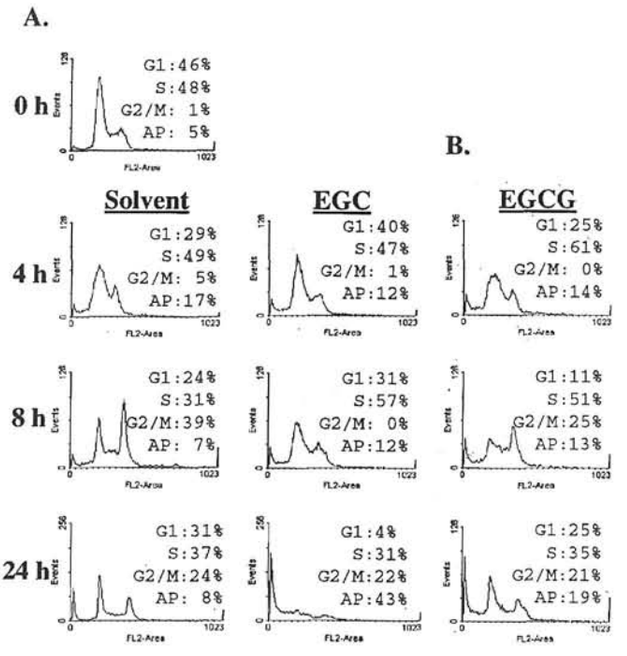

Figure 4. Effects of EGC or EGCG in Jurkat cells synchronized at G_1/S phase. Human Jurkat T cells were synchronized at G_1/S by treatment with 3 μg/ml aphidicolin for 24 h. After that, cells were washed to remove aphidicolin (0 h), and divided into three aliquots. Each aliquot of the cells was then treated with 100 μM EGC (A), 100 μM EGCG (B), or an equal volume of the solvent (H_2O; A) for an additional 4, 8 or 24 h. At each time, cells were harvested for DNA analysis. AP represents the apoptotic sub-G_1 population. Similar results were obtained in three or more experiments.

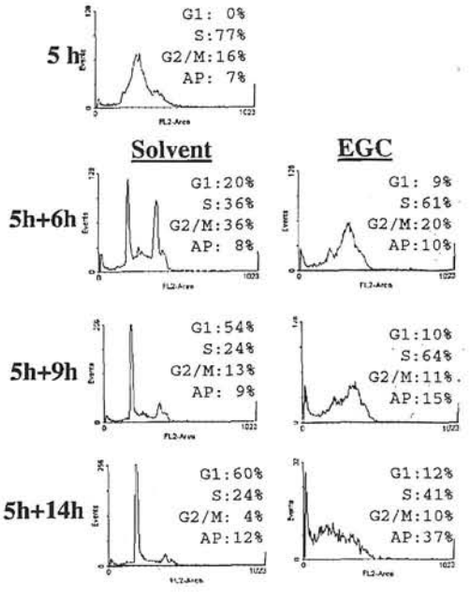

Figure 5. Effects of EGC in Jurkat cells synchronized in S phase. Human Jurkat T cells, synchronized at G_1/S by aphidicolin treatment, were released for 5 h (5 h), followed by additional incubation with either 100 μM EGC or an equal volume of the solvent for 6, 9 or 14 h. At each time, cells were harvested for DNA analysis. Similar results were obtained in three or more experiments.

[^{3}H]-thymidine incorporation in a dose-dependent manner in Jurkat cells, and can inhibit [^{3}H]-thymidine incorporation in three different leukemia cell lines.

EGC inhibits S-G_2 progression and subsequently induces apoptosis. The effects of tea extract or EGC on S phase accumulation measured in Fig. 2 were marginal (changes between 10-20%), this was probably due to the random cell cycle distribution of asynchronous cells. To further determine the effects of EGC on cell cycle progression, Jurkat T cells were synchronized on the G_1/S border by a treatment with aphidicolin, a reversible inhibitor of DNA polymerase α (30,32). Immediately after release from the aphidicolin block (0 h in Fig. 4A), cells were treated with either EGC or the solvent (H_2O; as a control) for additional 4, 8 or 24 h. As expected, the control cells progressed through the cell cycle after release (Fig. 4A). However, after the aphidicolin-pretreated cells were treated with EGC for 4 h, more G_1 and less S/G_2/M populations were observed, compared to the control cells (Fig. 4A, 4 h, EGC vs. solvent), suggesting an inhibition of G_1 to S progression by EGC. After 8 h, the control cells had progressed through S-phase and into G_2/M, the S population of the EGC-treated cells was slightly increased to 57%, compared to a decrease to 31% in the control cells. At this time, no G_2/M population was detected in the EGC-treated cells, in comparison to 39% of the control cells that progressed into the G_2/M phase (Fig. 4A). Therefore, EGC blocks progression of G_1/S phase cells into G_2 phase. A 24 h incubation with EGC resulted in the production of a significant sub-G_1 cell population (43%, Fig. 4A), indicating DNA fragmentation and cell death.

To further demonstrate that EGC inhibits the S to G_2 progression and subsequently induces apoptosis, aphidicolin-arrested cells were released for 5 h to allow most of the cells (77%) to enter into S (5 h, Fig. 5). After that, aliquots of the cells were incubated with either EGC or its solvent for additional 6, 9 or 14 h (indicated as 5 h + 6 h, 5 h + 9 h, and 5 h + 14 h, respectively, Fig. 5). The control cells again progressed from S to G_2/M and then to the G_1 phase of the next cell cycle (Fig. 5). In contrast, even with 77% of the cells in S phase, EGC almost completely halted the further progression of these cells into G_2/M phase. A 14 h incubation with EGC again induced cell death (Fig. 5). Therefore, the ability of EGC to inhibit the S to G_2 progression is tightly associated with its ability to induce apoptosis.

To confirm that EGC-induced cell death is due to apoptosis, we preformed the TUNEL assay that detects apoptosis-associated DNA strand breaks (33). When aphidicolin-synchronized Jurkat cells were treated with EGC for 12 h, fragmented DNA was detected in 56% of the cell population (Fig. 6A). In addition, the 12 h treatment with EGC also induced apoptosis-specific cleavage of PARP protein (Fig. 6B). Furthermore, a 24 h treatment with EGC resulted in loss of cell viability in 60% of the cell population as determined by Trypan blue exclusion.

Effects of EGCG and EC on cell cycle and apoptosis. We also examined the effects of EGCG and EC on cell cycle and

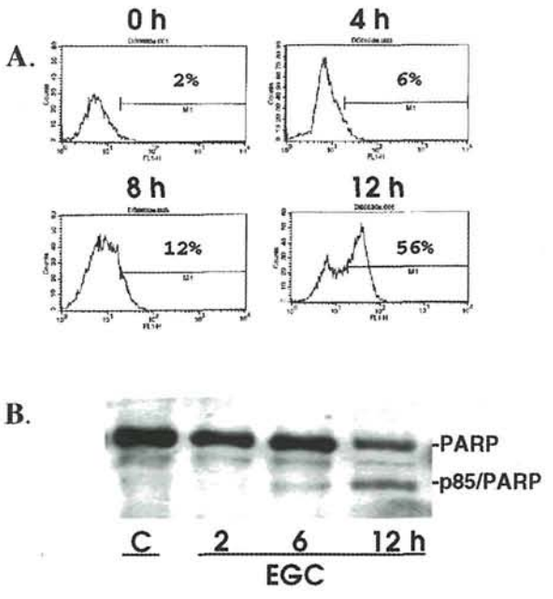

Figure 6. EGC induces apoptotic cell death in Jurkat T cells. The aphidicolin-arrested G_1/S cells (0 h; see the legend of Fig. 4) were washed and subsequently treated with 100 μM EGC or equal volume of solvent (lane C in panel B) for up to 12 h as indicated. This was followed by performance of TUNEL assay (A) and Western blot assay (B). The percentages of TUNEL-positive cell population (A) and positions of PARP protein (116 kDa) and the apoptosis-specific PARP cleavage fragment p85 (B) are indicated. Similar results were obtained in two (A) or more (B) independent experiments.

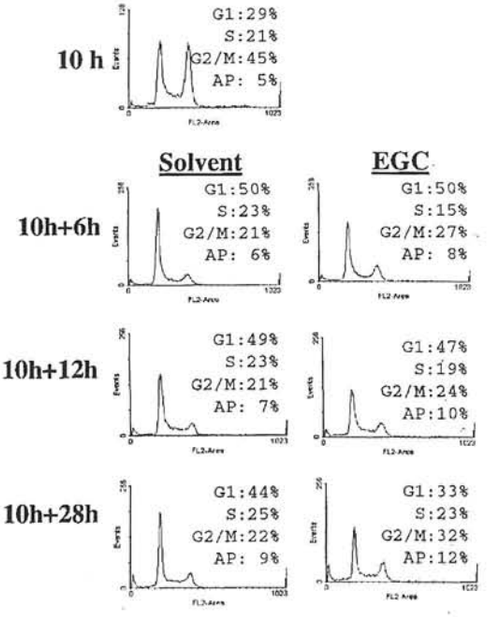

Figure 7. Effects of EGC in Jurkat cells synchronized in G_1 and G_2/M phases. Human Jurkat T cells, synchronized at G_1/S by aphidicolin treatment, were released for 10 h (10 h), followed by an additional incubation with either 100 μM EGC or an equal volume of the solvent for 6, 12 or 28 h. At each time, cells were harvested for DNA analysis. Similar results were obtained in three or more experiments.

apoptosis using synchronized Jurkat T cells. When aphidicolin-arrested cells were immediately treated with EGCG for 4 h, S phase population was dramatically increased, which was considerably more than that from the EGC treatment at this time point (compare EGCG 4 h vs. EGC 4 h in Fig. 4). After 8 h EGCG treatment, some of the S phase cells entered into G_2/M phase, in contrast to a complete blockade by EGC of the S to G_2 progression (8 h, B vs. A, Fig. 4). Therefore, EGCG slowed down the S-G_2 progression but did not block it as EGC did. In addition, EGCG was also found to be a weaker apoptosis inducer than EGC: a 24 h treatment with EGCG only increased the sub-G_1 apoptotic cell population by 11% compared to 35% by EGC (Fig. 4; normalized to solvent). Therefore, EGCG is less potent than EGC in inhibition of the S-G_2 progression and induction of apoptosis.

EC is structurally identical to EGC with the exception of one hydroxyl group (Fig. 1). When used in aphidicolin-synchronized Jurkat T cells at even 100 μM for 24 h, EC was unable to either inhibit the cell cycle progression or induce any detectable cell death (data not shown). Therefore, the order of tea polyphenols to inhibit the S-G_2 transition and to induce cell death are EGC > EGCG >> EC, which also matches their order to inhibit [^{3}H]-thymidine incorporation (Fig. 2).

EGC has little effect on cells in G_1 or G_2/M phases. Since S phase cells were sensitive to induction of apoptosis by EGC (Figs. 4-6), we hypothesized that non-S phase cells could be resistant to EGC treatment. To test this hypothesis, aphidicolin-arrested cells were released for 10 h to allow the highest percentage of cells to progress into G_2 (45%) and G_1 phase (29%) with the least percentage of the cells in S (21%) (Fig. 7). This was followed by addition of EGC or its solvent and a further incubation for up to 28 h. In the absence of EGC, the control cells progressed from G_2/M to G_1 phase, but did not synchronously enter S phase of the next cycle. However, treatment with EGC for an additional 6 or 12 h had little effect on the M to G_1 transition since similar G_1 populations were observed in both EGC- and control-treated cells (10 h + 6 h and 10 h + 12 h, EGC vs. solvent in Fig. 7). Only after 28 h treatment, a slight increase (10%) in G_2 population was observed in EGC-treated cells (Fig. 7), which is consistent with a previous report (5). This result suggests that EGC has no or little inhibitory effect on the M to G_1 transition. More importantly, treatment with EGC for even 28 h neither accumulated S phase cells nor induced apoptosis, as compared to control (Fig. 7). Therefore, the low level of S phase population is associated with the significant decrease of EGC-induced apoptosis (compare Figs. 4 and 6 to 7).

Green tea extracts also inhibit the S-G_2 progression. We found that when tested in asynchronous Jurkat cells, EGC was the only tea polyphenol that was able to accumulate S phase population (Fig. 2). In addition, when used in synchronized cells, EGC had greatest activity to inhibit the S to G_2 transition compared to EGCG and EC (Figs. 4, 5, 7). We hypothesized that green tea extract should also be able to inhibit the S to G_2 progression when applied to a synchronized cell population. To test this idea, aphidicolin-synchronized Jurkat T cells were released and immediately treated with a green tea extract at

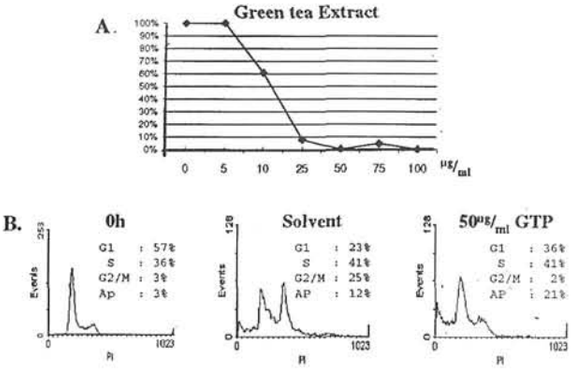

Figure 8. Green tea extract inhibits the S-G_2 transition. Jurkat T cells were synchronized to the G_1/S boarder with aphidicolin and released. At release the cells were aliquoted and treated with 0, 5, 10, 25, 50, 75, and 100 µg/ml of green tea extract (obtained from Lipton) for 10 h. The concentration of green tea extract that inhibited half the cells from progressing to G_2 was found to be approximately 13 µg/ml.

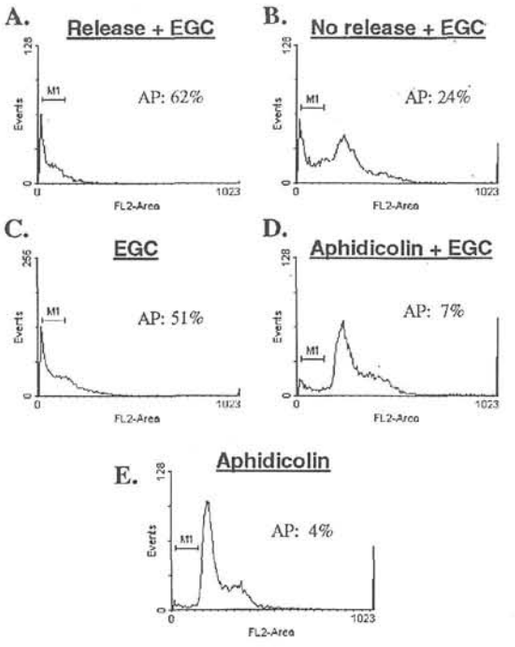

Figure 9. Aphidicolin inhibits EGC-induced cell death. A and B, aphidicolin-arrested cells were divided into two halves. One half was washed and then incubated for 100 µM EGC for 24 h (A), while another half was incubated directly with 100 µM EGC for 24 h (B). C-E, asynchronous Jurkat cells were treated for 24 h with either 100 µM EGC alone (C), 100 µM EGC plus 3 µg/ml aphidicolin (D), or 3 µg/ml aphidicolin alone. After each treatment, cells were collected for flow cytometry analysis.

different concentrations (0, 5, 10, 25, 50, 75, 100 µg/ml). After 10 h incubation, the percentage of increase in the G_2/M population, as compared to control, was measured and plotted (Fig. 8A). The progression into the G_2/M phase was inhibited significantly by the green tea extract in a concentration-dependent manner (IC_{50} 13 µg/ml; Fig. 8A). Importantly, inhibition of the S-G_2 transition by green tea extract was associated with increased cell death (Fig. 8B).

Aphidicolin blocks EGC-induced apoptosis. Our results that EGC inhibited the S-G_2 progression prior to apoptosis induction (Fig. 4A) and EGC failed to induce apoptosis in G_2 and G_1 cell populations (Fig. 7) suggested a requirement of DNA replication in the induced cell death process. Since aphidicolin inhibits DNA replication (30), we investigated whether this inhibitor could block EGC-induced apoptosis by performing the following two experiments. In the first experiment, after treated for 24 h with aphidicolin, the cells were divided into two halves. One half was washed to remove aphidicolin, followed by addition of EGC for 24 h, as described previously (see Fig. 4A), while another half was subjected directly to EGC treatment for 24 h. Aphidicolin blocked more than half of the apoptosis induced by EGC in this manner (24% vs. 62%, Fig. 9A and B).

In the second experiment, treatment of asynchronous Jurkat cells with 100 µM EGC for 24 h again induced a significant amount of cell death (51%, Fig. 9C). However, co-incubation with aphidicolin almost completely inhibited EGC-induced apoptosis in these cells (Fig. 9D). Treatment with aphidicolin alone only arrested cells at G_1/S without induction of cell death (Fig. 9E). These data suggest that EGC-induced apoptosis occurs in S phase and requires DNA synthesis.

Discussion

Cancer is an intractable disease, often characterized by abnormally high cellular proliferation. Many of today's chemotherapeutic modalities exploit this proliferative characteristic by utilizing traditional anticancer drugs that inhibit cellular proliferation (34-37). Inhibition of cancer growth in this manner typically results in apoptosis, by mechanisms not yet fully elucidated. In the current study, we have reported the following three novel findings. First, the green tea polyphenol EGC inhibits [^{3}H]-thymidine incorporation in Jurkat T, HL-60 and K562 cells and inhibits progression from S to G_2 phase in human Jurkat T cells (Figs. 2-5 and 7). Second, EGC-induced cancer cell apoptosis occurs following inhibition of S phase progression (Figs. 3-5). Third, EGC is more potent than the other tea components in accumulation of S phase in asynchronous cells (Fig. 2), in inhibition of S-G_2 progression and subsequent induction of apoptotic cell death in synchronous cells (Fig. 4 and data not shown).

A necessary concern in any drug research is the effective concentrations of a drug employed. Although tea polyphenols are present in low µM ranges in plasma and saliva of human volunteers (38,39) and in mice that had been fed with tea (39), higher concentrations of polyphenols might be necessary for studies in cell cultures. Indeed, several published important articles on tea research used effective concentrations of tea polyphenols between 30 and 100 µM (5,6,40). Our results showed that the *in vivo* IC_{50} for [^{3}H]-thymidine incorporation of EGC is 48 µM, the IC_{50} for S-G_2 progression of green tea extract is 13 µg/ml, and EGC at 100 µM completely blocks

S-phase progression. These concentrations are well within the rang of effective concentrations published by other researchers in this field (5,6,40).

Although the molecular mechanism for blockade of the S to G_2 progression by EGC is currently unknown, the following arguments suggest the involvement of inhibition of DNA replication. First, EGC inhibited [^{3}H]-thymidine incorporation into Jurkat, HL-60 and K562 cells (Fig. 3). Second, when used in Jurkat cells arrested at the G_1/S boarder by aphidicolin, EGC was able to maintain these cells at G_1/S, whereas an aliquot of the same cells progressed through the cell cycle in the absence of EGC (Figs. 4 and 5). Third, EGC-induced, S phase-associated apoptosis was inhibitable by aphidicolin (Fig. 9), suggesting the involvement of DNA polymerase α. Consistent with this idea, it has been shown that EGC and EGCG extracted from persimmon were able to inhibit the DNA polymerase α activity *in vitro* at concentrations in the low μM to nM range (41). It appears that inhibition of the S-G_2 transition by EGC could be due to direct inhibition of DNA replication, although other mechanisms could also be involved.

Our results also suggest that inhibition of the S-G_2 progression by EGC is necessary for its induction of apoptotic cell death. We found that the abilities of tea polyphenols to halt S phase cells from progression into G_2 are tightly associated with their abilities to induce apoptotic cell death. The rank of potency for inhibition of DNA replication and blockade of S-G_2 progression was EGC > EGCG >> EC (Figs. 3-5 and data not shown), which matches precisely their order for induction of cell death (Figs. 3-5 and data not shown). Moreover, when used in cells containing high percentages of G_1 and G_2/M populations, the low level of S population was associated with the failure of EGC to activate the cell death program (Fig. 7). Our results were also consistent with previous studies that demonstrated that the G_1 to S transition of the cell cycle was the most susceptible point for some cell systems to implement the apoptotic program by chemotherapeutic drugs (16,42,43). Whether or not inhibition of DNA polymerase α by EGC is responsible for the induction of apoptosis in our model has yet to be resolved, other mechanisms specific for S phase-associated apoptosis could also be responsible.

Our data also demonstrated that EGC is more potent than EGCG in inhibition of S-G_2 progression and subsequent induction of apoptotic cell death (Fig. 4), despite their structural similarities (Fig. 1). This could be due to differences in binding affinities caused by steric hindrances, due to the presence of the extra 3-gallate moiety on EGCG, or the stability of EGCG *in vivo* (38,44). Much of today's research on green tea polyphenols has centered on EGCG, most likely because it is the most abundant active component found in green tea. When tea is consumed, however, EGC is the most prevalent polyphenol found in the serum (39) and urine (45) despite the larger concentration of EGCG found in tea. Furthermore, the greatest actual percentage of contribution to antioxidant activity of EGCG is found equal to EGC (46), despite the greater antioxidant activity of EGCG than EGC. In physiological conditions it seems that the 3-gallate group of EGCG is cleaved by some type of esterase activity, giving rise to EGC (38). Taken together EGC seems to be at least as important as EGCG especially when practical and physiological conditions are considered.

Finally, a better understanding of how tea polyphenols inhibit tumor cell proliferation may help to explain the cancer-preventative effects of green tea observed previously (6,7,18, 26-29). Taking into account the several reported inhibitory properties of green tea polyphenols, it could be the combination of several of these properties at lower concentrations that gives tea its cancer preventative features in individuals who are long-term green tea drinkers. EGC-mediated, S phase inhibition-associated cancer cell death could be a part of this whole picture.

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