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Green Tea Constituent (–)-Epigallocatechin-3-Gallate Inhibits Hep G2 Cell Proliferation and Induces Apoptosis through p53-Dependent and Fas-Mediated Pathways

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Key Words
EGCG · p53 · p21/WAF1 · Fas/APO-1 · Fas ligand, apoptosis

Abstract
(–)-Epigallocatechin-3-gallate (EGCG) is a polyphenolic compound found in green tea. It has been reported to possess a wide range of pharmacological properties, and is one of the most promising chemopreventive agents for cancer. To provide a better understanding of the preventive effect of EGCG on liver cancer, we examined EGCG for its effect on proliferation and cell cycle progression in a human liver cancer cell line, Hep G2. The results showed that EGCG inhibited the proliferation of Hep G2 by inducing apoptosis and blocking cell cycle progression in the G1 phase. ELISA showed that EGCG significantly increased the expression of p53 and p21/WAF1 protein, and this contributed to cell cycle arrest. An enhancement in Fas/APO-1 and its two form ligands, membrane-bound Fas ligand (mFasL) and soluble Fas ligand (sFasL), as well as Bax protein, was responsible for the apoptotic effect induced by EGCG. Taken together, our study suggests that the induction of p53 and the activity of the Fas/FasL apoptotic system play major roles in the antiproliferative activity of EGCG in Hep G2 cells.

Introduction

Recently, natural herbs and pure compounds that are present in the human diet have attracted extensive attention as cancer chemopreventive agents. Green tea *(Camellia sinensis)* has been demonstrated to display cancer chemopreventive effects in different systems due to its striking inhibition of diverse cellular events related to cancer development [5, 40]. These effects are associated with antioxidative properties, blocking angiogenesis, carcinogenesis, metastasis and proliferation of tumor cells [1, 6, 11, 15, 39, 43]. The main active constituents of green tea are polyphenolic compounds, which consist of (–)-epigallocatechin-3-gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin-3-gallate (ECG) and (–)-epigallocatechin (EC) [2]. Among them, EGCG is the most abundant ingredient. Many studies have reported that EGCG possesses direct tumor growth inhibition ability in

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several types of tumor cells, including human liver cancer [8, 26, 31, 32, 37], which is one of most common and the most lethal malignant diseases in the world [25]. However, the precise antitumorigenic mechanisms of EGCG in hepatoma cells remain largely unknown. In this study, we determined the antiproliferative activity of EGCG in the human liver cancer cell line, Hep G2, and examined its effect on cell cycle distribution and apoptosis in Hep G2 cells. Furthermore, to establish the anticancer effects of EGCG, we assayed the levels of p53, p21/WAF1, Fas ligand and Fas/APO-1 receptor, which are strongly associated with the signal transduction pathway of apoptosis and affect the chemosensitivity of tumor cells to anticancer agents.

Materials and Methods

Materials

Fetal calf serum (FCS), penicillin G, streptomycin and amphotericin B were obtained from Gibco BRL (Gaithersburg, Md., USA). EGCG, dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), ribonuclease (RNase), and propidium iodide (PI) were purchased from Sigma (St. Louis, Mo., USA). XTT and p53 pan ELISA kits were obtained from Roche Diagnostics GmbH (Germany). Nucleosome ELISA, WAF1 ELISA and Fas/APO-1 ELISA kits were purchased from Calbiochem (Cambridge, Mass., USA).

Preparation of EGCG

EGCG was dissolved in DMSO and stored at −20°C. For all experiments, final concentrations of the tested compound were prepared by diluting the stock with DMEM (Sigma). Control cultures received the carrier solvent (0.1% DMSO).

Cell Line and Culture

Hep G2 cells [American Type Culture Collection (ATCC) HB8065] were maintained in DMEM supplemented with 10% FCS, 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B in a CO_2-air mixture.

Assay for Cell Proliferation Inhibition

Cell proliferation inhibition was assessed by XTT assay [sodium 3′-(1-(phenylamino-carbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulfoic acid hydrate]. Briefly, the cell line was subcultured into a 96-well plate with 1×10^4 cells per well in 100 μl medium. After 24 h of incubation, the medium in the 96-well plate was discarded and replaced by 90 μl of DMEM, and 10 μl of EGCG was then added to each well to final concentrations of 10, 50, 100, and 200 μ*M* each in triplicate. After the addition of the sample, the plates were incubated in a 37°C humidified incubator with 5% CO_2 for 12, 24, 48, and 72 h. At the end of the incubation, the medium containing the various concentrations of EGCG was discarded. The cells were then washed once with PBS. Fifty microliters of XTT test solution was prepared by mixing 5 ml of XTT-labeling reagent, and 100 μl of electron-coupling reagent was then added to each well. After 6 h of incubation at 37°C in a 5% CO_2 incubator, the absorbance of test samples was measured with an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm [12, 27].

Assay for Cell Cycle Distribution

Hep G2 cells were cultured in triplicate in 6-well plates at a concentration of 5×10^5 /well. After 24 h of incubation, cells were treated with 50, 100, and 200 μ*M* EGCG were analyzed in parallel with cells grown in the absence of green polyphenol compounds to determine the effects on cell cycle distribution. Cells were collected and fixed with 70% ethanol. Cell pellets were suspended in 2 μl of 10 μg/ml RNase containing 0.5% Triton (J.T. Baker, Inc.) plus the same volume of 20 μg/ml PI (Sigma), followed by incubation in the dark at room temperature for 30 min. Cell suspensions were filtered through a 60-μm mesh filter (Spectrum Medical Industries, Calif., USA). Data acquisition and analysis were performed on an EPICS flow cytometer (Coulter Electronics), and data from 10,000 cells were collected for each data file. Cell cycle analysis was performed with Multicycle software (Phoenix Flow Systems, San Diego, Calif., USA) [30].

Assay for Apoptosis by Nucleosome ELISA

For the quantification of apoptotic cells, we used the Nucleosome ELISA kit, operated according to the manufacturer's protocol. Briefly, Hep G2 cells were treated with 50, 100, and 200 μ*M* of EGCG for 6, 12, 24, and 48 h. Samples of the cell lysate and the biotinylated detector antibody were placed (triplicated) in the 96-well plate that were coated DNA binding protein, with final cell counts of 1×10^6 per well. The assayed mono- and oligonucleosomes were captured on plates that were pre-coated with DNA binding proteins. Antihistones 3 (H3) biotin-labeled antibody was then bound to the histone component of captured nucleosomes and was detected following incubation with streptavidin-linked horseradish peroxidase (SA-HRP) conjugate. HRP catalyzed the conversion of colorless tetramethylbenzidine to blue. The addition of stop solution changed the color to yellow, the intensity of which was proportional to the number of nucleosomes in the sample. Absorbance was measured with an ELISA reader at a test wavelength of 450 nm. By comparing the absorbance obtained from a sample containing an unknown amount of nucleosomes with that obtained from the standards, one can assign a nucleosome unit value to the unknown sample. In addition, one can assign an apoptotic index (ratio) to an unknown sample when the absorbance of the treated (apoptotic) sample is divided by that of the untreated (control) sample [18, 38].

ELISA Assay of p53, p21/WAF1, Fas Ligand and Fas/APO-1 Receptor

For the detection of p53, p21/WAF1, Fas ligand and Fas/APO-1 receptor, we used the p53 pan ELISA, WAF1 ELISA, Fas Ligand and Fas/APO-1 ELISA kits. Briefly, Hep G2 cells were treated with 50, 100, and 200 μ*M* of EGCG for 6, 12, 24, and 48 h. The samples of cell lysate and the biotinylated specific detector antibody were placed (triplicated) in 96-well microtiter plates coated with monoclonal antibodies with final cells counts of 1×10^6 per well, and were incubated for 1 h (Fas/APO-1), 2 h (p53 or p21/WAF1) or 3 h (FasL) at room temperature. Particularly, the soluble Fas ligand in the cell culture supernatant also needed to be determined by using an enzyme-linked immunosorbent assay (Fas Ligand ELISA). After removing the unbound material by washing with PBS, horseradish-peroxidase-conjugated streptavidin was added to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of chromogenic substrate

(tetramethylbenzidine) to a colored solution with a color intensity proportional to the amount of protein (p53, p21/WAF1, Fas L or Fas/APO-1) present in the sample. The absorbance of each well was measured at 450 nm and concentrations of p53, p21/WAF1, FasL, and Fas/APO-1 were determined by interpolating from standard curves obtained with known concentrations of protein [7, 22, 35, 36].

Assay for Western Blotting

Cells treated with 50, 100, and 200 μ*M* EGCG for 24 h were lysed and the protein concentration was determined by using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif., USA). For Western blotting, 50–100 μg of total cell lysates were subjected to SDS-PAGE. The protein was transferred to PVDF membranes using transfer buffer (50 m*M* Tris, 190 m*M* glycin, and 10% methanol) at 100 V for 2 h. The membranes were incubated with blocking buffer (50 m*M* Tris, 200 m*M* NaCl, 0.2% Tween 20, and 3% BSA) overnight at 4 °C. After washing three times with washing buffer (blocking buffer without 3% BSA) for 10 min each, the blot was incubated with Bcl-2 or Bax antibody for 2–15 h, followed by horseradish peroxidase-labeled secondary antibody for 1 h. The membranes were washed again, and detection was performed using the enhanced chemiluminescence Western blotting detection system (Amersham, USA) [30].

Results

Effect of EGCG on Hep G2 Cell Proliferation

We first tested the antiproliferative effect of EGCG in a liver cancer cell line, Hep G2. As shown in figure 1, the growth-inhibitory effect of EGCG was observed in a dose-dependent manner and with an IC_{50} value of 147.26 ± 5.3 μ*M*.

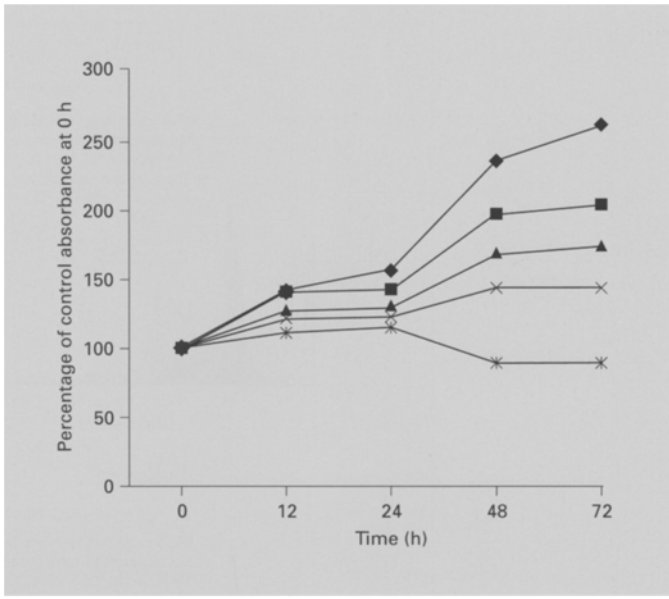


Fig. 1. Growth inhibition of Hep G2 cells by EGCG. Adherent cells that proliferated in 96-well plates (10^4 cells/well) were incubated with different concentrations of EGCG for various time intervals. Cell proliferation was determined by XTT assay. Results are expressed as percent of cell proliferation of control at 0 h. The data shown are the mean from three independent experiments each with triplicate wells. Standard deviations are less than 10%.

EGCG-Induced Cell Cycle Arrest and Apoptosis in Hep G2 Cells

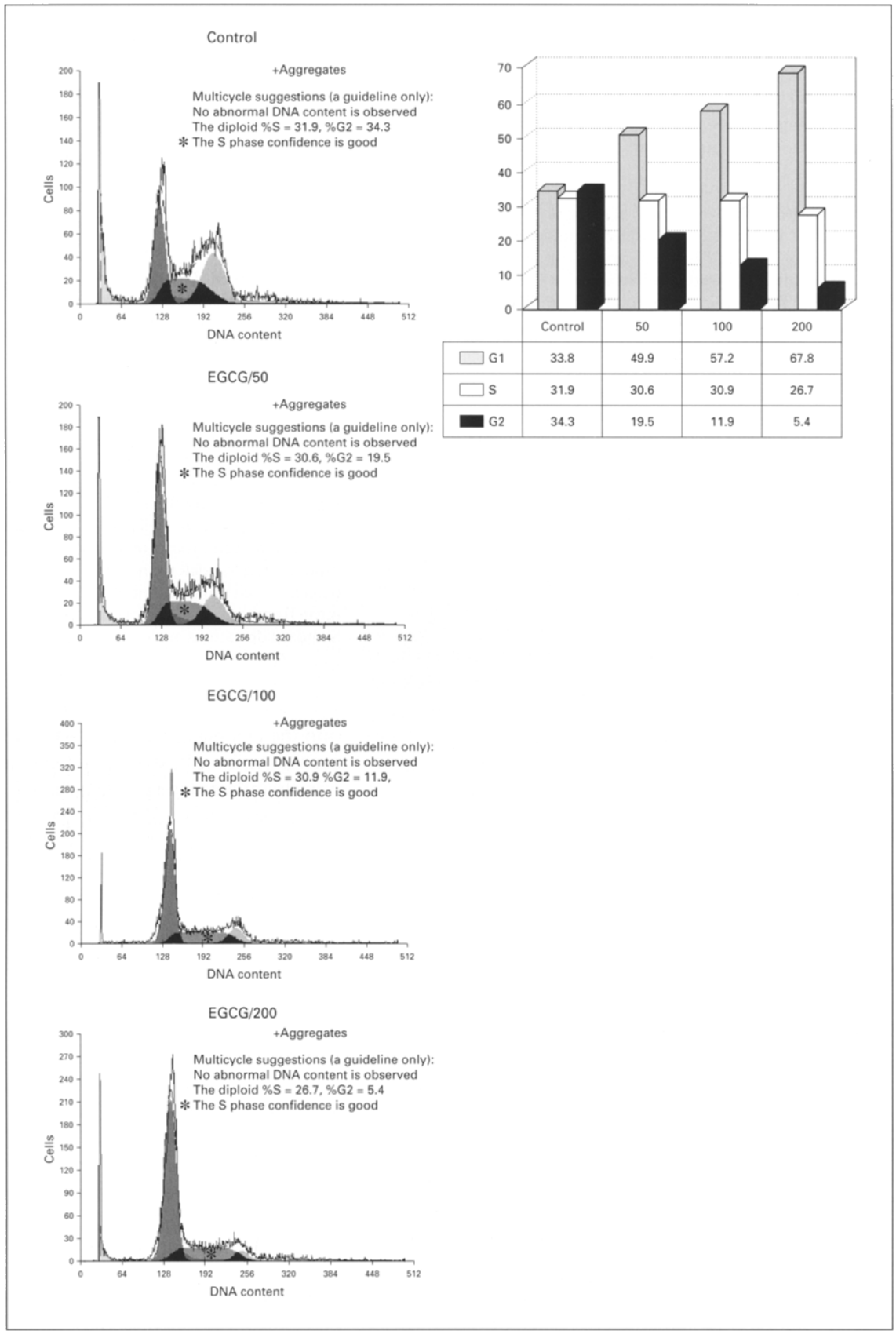
The effect of EGCG on cell cycle progression of Hep G2 was determined by flow cytometric analysis 24 h after its addition. As shown in figure 2, the results indicated that, compared with the control, EGCG increased the population in the G1 phase. At the dose of 100 μ*M* EGCG, the percentage of cells in the G1 phase increased from 33.8% to 57.2%, and those in the G2 phase decreased from 34.3 to 11.9%. These effects were enhanced when Hep G2 cells were treated with 200 μ*M* EGCG. Our data suggest that EGCG arrests the cell cycle of Hep G2 cells at the G1 phase.

Apoptosis is an active process of cell death. It is characterized by nuclear condensation, DNA fragmentation and blebbing of the plasma membrane. Activation of endonucleases during the process of apoptosis leads to the fragmentation of chromatin into about 180-bp oligonucleosomal fragments, with subsequent releases of nucleosomes into the cytoplasm. By contrast, necrotic cell death often occurs with extensive tissue damage, resulting in an intense inflammatory response. The main characteristic of necrosis is the swelling of organelles and cells. Later, the cell loses membrane integrity and releases randomly digested DNA fragments. Thus, the quantification of nucleosomes in the cytoplasm by a nucleosome ELISA assay can precisely measure apoptotic response [28].

Figure 3 shows the time course of DNA fragmentation in continuous treatment with 50, 100, and 200 μ*M* EGCG. DNA fragmentation of Hep G2 was noted at 6 h and maximized at 48 h after addition of EGCG. In contrast to the controls, when cells were treated with higher concentrations of EGCG, the number of cells undergoing apoptosis was further enhanced from about 5-fold at 100 μ*M* EGCG to 9-fold at 200 μ*M* at 48 h.

EGCG Increases the Expression of p53 and p21/WAF1 Proteins in Hep G2 Cells

To determine whether tumor suppression factor p53 is involved in the EGCG-mediated antiproliferation of Hep G2 cells, three concentrations of EGCG (50, 100, and 200 μ*M*) were added to Hep G2 cells, then the levels of



	Control	50	100	200
G1	33.8	49.9	57.2	67.8
S	31.9	30.6	30.9	26.7
G2	34.3	19.5	11.9	5.4

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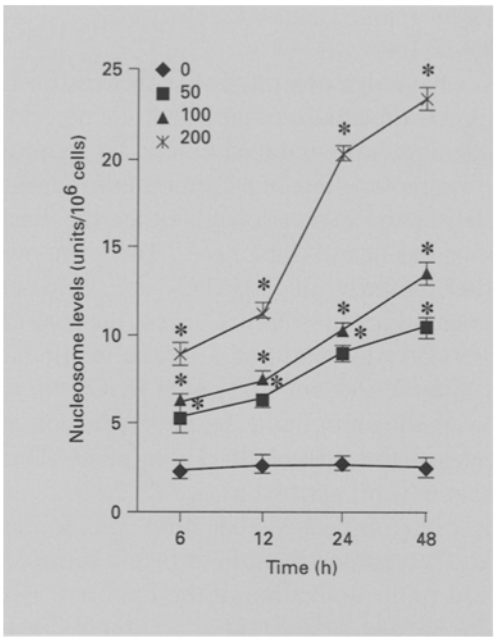

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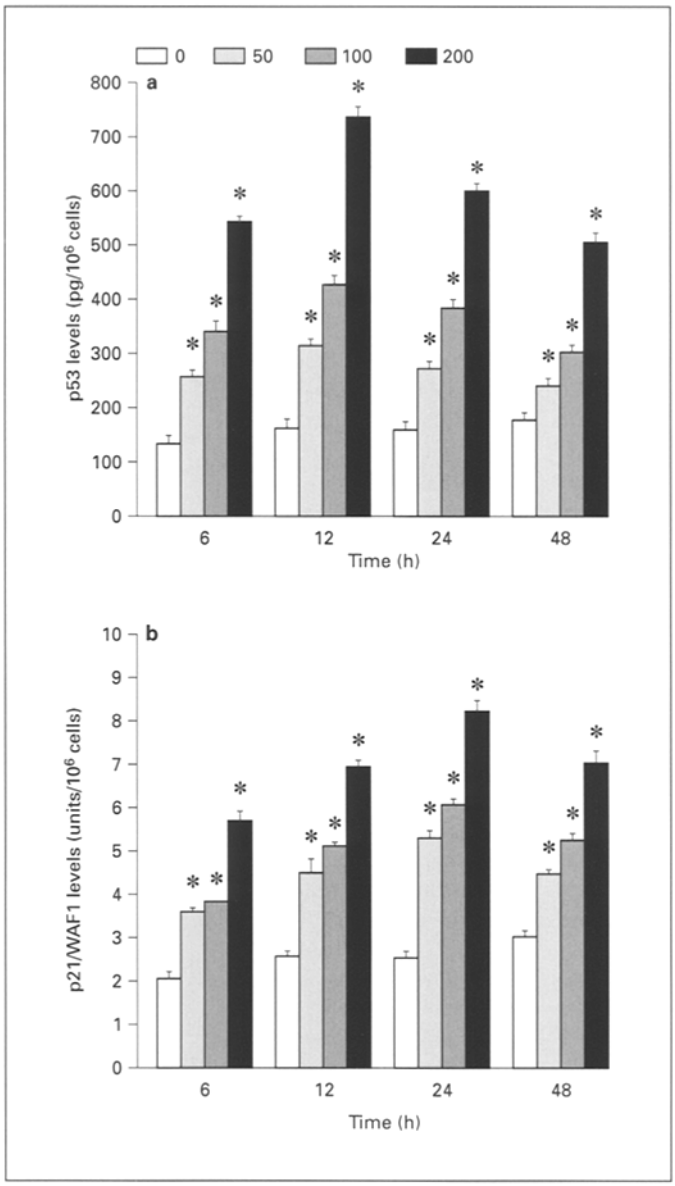

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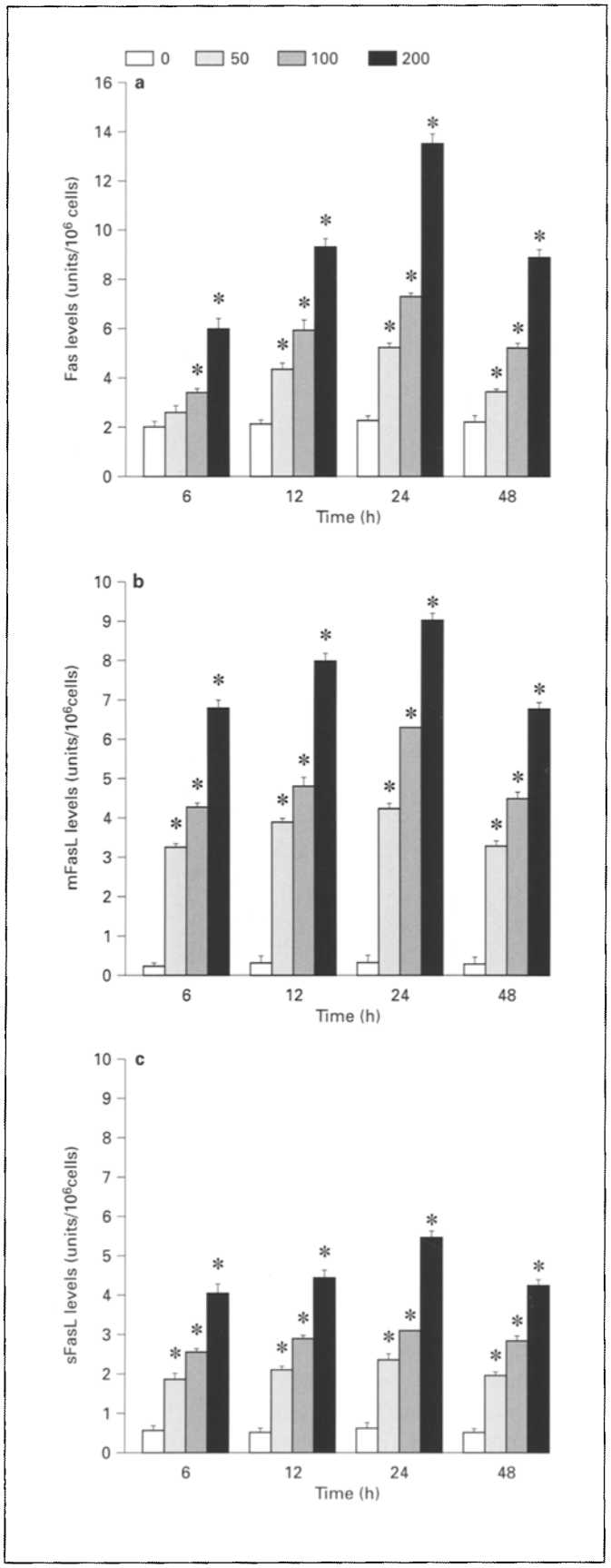
Fig. 3. Induction of apoptosis in Hep G2 by EGCG. Hep G2 cells were cultured with 50, 100, and 200 μ*M* EGCG for 6, 12, 24, and 48 h. Cells were harvested and lysed with lysis buffer. Cell lysates that contained cytoplasmic oligonucleosomes of apoptotic cells were analyzed in the Nucleosome ELISA. The data shown are the mean ± SD of three independent experiments each with triplicate wells. The asterisk indicates a significant difference between control and EGCG-treated cells, * $p < 0.05$.

Fig. 4. Effects of EGCG on protein expression of p53 (**a**) and p21/WAF1 (**b**) in Hep G2 cells. Hepatoma cells were treated with 50, 100, and 200 μ*M* EGCG. Lysates were prepared from these cells and p53 and p21/WAF1 levels were determined by p53 pan ELISA and WAF1 ELISA kits, respectively. The detailed protocol is described in 'Materials and Methods'. The data shown are the mean ± SD of three independent experiments each with triplicate wells. The asterisk indicates a significant difference between control and EGCG-treated cells, * $p < 0.05$.

p53 protein were assayed by ELISA. Marked induction of p53 protein was observed in a dose-dependent manner (fig. 4a). The upregulation of p53 by EGCG started to increase 6 h after treatment with EGCG, and reached maximum expression at 12 h.

Fig. 2. Inhibition of cell cycle progress in Hep G2 cells by treating with EGCG. Cell cycle analysis of Hep G2 cells following treatment with 50, 100, and 200 μ*M* EGCG for 24 h. Cells were fixed and stained with propidium iodide. Cell cycle distribution was analyzed by flow cytometry.

p21/WAF1 protein is first characterized as a downstream target of p53, and is thought to be responsible for G1 cell cycle arrest [21]. Thus, we also assessed the p21/WAF1 expression of p53-expressing Hep G2 cells using a WAF1 ELISA assay. Figure 4b shows that an increase in p21/WAF1 protein was apparent at 6 h and reached maximum induction at 24 h in EGCG-treated Hep G2 cells. Moreover, p21/WAF1 was induced in a dose-dependent manner. Based on these data, we suggest that EGCG-mediated cell cycle arrest operates through the induction of p21/WAF1 protein in a p53-dependent event in Hep G2 cells.



Fas/FasL System Is an Essential Pathway of EGCG-Mediated Apoptosis

Recent studies have demonstrated that the Fas/FasL system is an important mediator of the apoptotic process, particularly in chemotherapy-induced cancer cell apoptosis [16, 24]. The apoptotic effect of a chemotherapy agent acts by upregulating the expression of either the Fas/APO-1 receptor or Fas ligand (FasL) [23]. Therefore, we wondered whether the levels of Fas/APO-1 and FasL in Hep G2 cells were modulated by EGCG. First, the level of Fas/APO-1 expression was measured after the treatment of Hep G2 cells with 50, 100, and 200 μ*M* of EGCG for 6, 12, 24, and 48 h. As shown in figure 5a, the expression of Fas/APO-1 increased following EGCG treatment. This change occurred at 6 h and reached its peak at 24 h.

Recent reports have indicated that both soluble Fas ligand (sFasL) and membrane-bound Fas ligand (mFasL) might be involved in the activation of the Fas/FasL system [29, 34]. Thus, we also assayed the alteration of sFasL and mFasL in Hep G2 cells with treatments of 50, 100, and 200 μ*M* EGCG. At the mFasL level, ligand accumulation appeared 6 h after EGCG treatment and progressively increased up to 24 h (fig. 5b). A similar result was observed for sFasL. Six hours after the addition of EGCG, sFasL was significantly induced and remained at a high level for 12–24 h (fig. 5c). However, the increase in mFasL with EGCG was greater than the increase in sFasL at 24 h (30-fold vs. 9-fold in Hep G2 cells treated with 200 μ*M* EGCG). Interestingly, the induction of Fas/APO-1, mFasL as well as sFasL showed slight differences between cells treated with 50 and 100 μ*M* EGCG, but the differences greatly increased when Hep G2 cells were treated with 200 μ*M* EGCG.

EGCG Treatment of Hep G2 Cells Results in an Increase in Proapoptotic Protein Bax

Bcl-2 family proteins are important regulators in the apoptotis signaling pathway. These proteins, including Bax and Bcl-2, are regulated by p53 [21]. Here, we exam-

Fig. 5. The Fas/FasL system was involved in EGCG-mediated apotopsis. Hepatoma cells were incubated with 50, 100, and 200 μ*M* EGCG for the time indicated. **a** Amount of Fas/APO-1 receptor in Hep G2 cells. **b** Amount of mFasL in Hep G2 cells. **c** Amount of sFasL in Hep G2 cells. The data shown are the mean ± SD of three independent experiments each with triplicate wells. The asterisk indicates a significant difference between control and EGCG-treated cells, * $p < 0.05$.

ined the effect of EGCG on Bax and Bcl-2 protein expression (fig. 6). After 24 h of exposure to 50, 100, and 200 μ*M* of EGCG, the amount of Bax protein increased in a dose-dependent manner. In contrast, we were unable to detect Bcl-2 protein expression in Hep G2 cells in the absence or presence of EGCG. This result was similar to that of a previous report which indicated that Hep G2 cells did not express Bcl-2 [33].

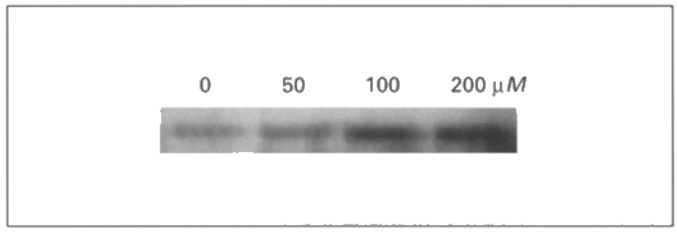


Fig. 6. Effect of EGCG on protein expression of Bax. As detailed in 'Materials and Methods', the cells were treated with 50, 100, and 200 μ*M* of EGCG. The cells were harvested after 24 h. Total cell lysates were separated, and 50 μg of proteins were separated via SDS-PAGE followed by Western blot analysis and chemiluminescence detection.

Discussion

In recent years, natural antioxidants that are present in food and beverages consumed by humans have earned extensive attention because of their cancer chemopreventive effects. EGCG, a polyphenolic compound present in green tea, is a potent antioxidant with tumor-preventive effects [1, 5, 40]. Many studies have demonstrated that EGCG has an important antiproliferative effect against certain cancers, including lung, bronchial, colon, prostate, breast, liver and leukemia cancer cells [2, 11, 15, 32, 39, 43]. The mechanism of action of EGCG on tumor cell growth inhibition is related to the induction of apoptosis and the blockade of cell cycle progression. EGCG inhibited the activities of several key G1 regulation proteins by arresting cells in the G1 phase [19]. EGCG can also induce apoptosis by several molecular mechanisms, including inhibition of growth-related signal transduction pathways that lead to the activation of an important transcription factor activator protein (AP-1) [10, 20, 42], triggering apoptosis by directly binding to Fas receptor [13], and blocking the activity of topoisomerase I [3]. Moreover, a recent study showed that the synergistic cancer-preventive effects occurred when EGCG was combined with EGC, sulindac or tamoxifen [32]. In contrast to its antioxidant effect, EGCG may also act as a prooxidant through H_2O_2 production to induce apoptosis in human lung cancer H661 cells [41, 42]. In the study by Uesato et al. [37], EGCG exhibited cell growth inhibition in hepatoma cell lines, but the precise mechanism against liver cancer was not reported. In our study, we demonstrated that ECGC restores Hep G2 cell apoptosis sensitivity and arrests cells in the G1 phase. Moreover, this effect was found to be mediated through a p53-dependent- and Fas/FasL-system-mediated cell death pathway.

Normal p53 function has been found to play a crucial role in the induction of apoptosis and triggering of cell cycle checkpoints in human and murine cells following DNA damage [21]. This notion has further been supported by findings that p53 is the most commonly mutated tumor suppressor gene and a lacking p53 function is related to an enhanced risk of carcinogenesis [9]. Moreover, the chemosensitivity of cancer cells to chemotherapy agents is greatly influenced when the function of p53 is abrogated [4]. Our results indicated that p53 plays a key role in EGCG-mediated antiproliferation in Hep G2 cells. p53 cannot only arrest the cell cycle in the G1 phase, but also induce apoptosis. This hypothesis is supported by the following results: (a) Flow cytometric analysis indicated that EGCG could arrest Hep G2 cells in the G1 phase. The blockade of cell cycle progression was attributed to the amount of enhancement of p21/WAF1 protein that is induced in a p53-dependent manner. A similar result was found in EGCG-treated breast carcinoma cells [19]. (b) Both proapoptotic downstream targets of p53, Fas/APO-1 and Bax proteins were increased by EGCG. Moreover, the expression levels of these proteins were greatly increased (at 24 h) after maximal accumulation of p53 protein (at 12 h) in Hep G2 cells.

The Fas/FasL system is a key signaling transduction pathway of apoptosis in cells and tissues. Binding of Fas ligand to Fas/APO-1 induces receptor oligomerization and formation of death-inducing signaling complex (DISC), followed by activation of a series caspase cascades resulting in apoptotic cell death [14]. FasL is a TNF-related type II membrane protein. Cleavage of membrane-bound Fas ligand (mFasL) by a metalloprotease-like enzyme results in the formation of soluble Fas ligand (sFasL) [17]. Both mFasL and sFasL can bind to Fas/APO-1, and subsequently trigger the Fas/FasL system, but sFasL has been reported to be a weaker inducer of apoptosis than mFasL [29]. Our study indicated that both Fas ligands, mFasL and sFasL, were increased in EGCG-treated hepatoma cells. Moreover, the level of Fas/APO-1

was simultaneously enhanced in FasL-upexpressing Hep G2 cells. Thus, we demonstrated that the Fas/FasL system participates in EGCG-mediated apoptosis.

In summary, our study demonstrates that EGCG can effectively inhibit Hep G2 cells proliferation by two different cellular responses. Firstly, EGCG can block cell cycle progression and cause a G1 arrest via p21/WAF1 activation. Secondly, the apoptotic sensitivity of Hep G2 cells was restored by a p53- and Fas/FasL-dependent pathway. Our study has clearly demonstrated that EGCG might be a promising chemopreventive agent for liver cancer, even when absorbed solely by means of diet.

Acknowledgements

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