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ORIGINAL PAPER

Epigallocatechin-3-gallate induces apoptosis and cell cycle arrest in HTLV-1-positive and -negative leukemia cells

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Abstract The objective of this study is to evaluate the efficacy of epigallocatechin gallate against ATL cells. The anti-proliferative and pro-apoptotic effects of EGCG were evaluated in HTLV-1-positive and -negative cells. EGCG exhibited a marked decrease in proliferation of ATL cells at 96 h of treatment. The results indicated that TGF-α was down-regulated whereas levels of TGF-β2 increased. Cell cycle distribution analysis revealed an increase in cells in the pre-G_1 phase which was confirmed by ELISA. The results on proteins showed an up-regulation of p53, Bax and p21 protein levels while the levels of Bcl-2α were down-regulated.

Keywords Apoptosis · Human T-cell Lymphotrophic Virus-1 (HTLV-1) · Epigallocatechin-3-gallate (EGCG) · p53 · p21 · Transforming Growth Factor (TGF)

Introduction

Green tea extract has tremendous potential in the treatment of cancer [1]. Catechins, the active component of green tea, are a special group of polyphenols with antioxidant properties [2]. The most important and most abundant catechins found in green tea is (−)-epigallocatechin-3-gallate (EGCG), an antioxidant with several physiologic modulative properties and free radical scavenging activities [3].

EGCG has a chemopreventive, anti-mutagenic, and anti-inflammatory activities attributed mainly to its pro-apoptotic effects on leukemic cells [4, 5]. EGCG was also found to induce cell cycle arrest at the G_0/G_1 phase in prostate carcinoma cells [6] and G_2/M phase in lung cancer cells [7]. EGCG's apoptotic potential involves binding to the Fas-receptor and thus inducing the cleavage and activation of caspase 8 in human monocytic leukemia [4]. The down-regulation of oncogenes Ras and Jun was also a notable effect of EGCG in transformed fibroblasts [8]. Furthermore, Li et al. [9] found that EGCG inhibited the proliferation of acute myeloblastic leukemia cells. Recently, Roy et al. [10] showed that the inhibitory concentrations tested on leukemic cells exhibited no growth inhibitory effects on normal lymphocytes. Previous studies, using EGCG as a nutrient synergy component, indicated a high anti-cancer potential of this natural compound through its effect on several cellular mechanisms critical in malignancy in various models such as breast cancer and pancreatic cancer [11–13].

Acute T-cell leukemia (ATL) is caused by the Human T-cell Lymphotrophic Virus type I (HTLV-1), the first human retrovirus to be isolated [14]. ATL is manifested by aggressive lymphoid proliferation of mature activated $CD4^+$ cells. The virus can be transmitted by sexual contact, through blood transfusions, from mother to child transplacentally, or by breast feeding [15, 16]. Only 5% of people with HTLV-I go on to have ATL after a long period of latency, usually 30–40 years [17]. Although there are treatments available against ATL, these therapies are not very effective. Therefore, the need arises for new more effective therapies. The virus has been proven to be resistant to chemotherapy and treatments using zidovudine

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(AZT) and interferon-α (IFN-α) have been effective yet not completely. Macchi et al. [18] demonstrated that AZT exerted a protective effect on mononuclear cells in vitro but was ineffective 2 weeks after infection. Zhang et al. [19] concluded that AZT concentrations from 32 to 0.5 μM inhibited the transmission of the virus and that doses as low as 0.03 μM had a protective effect. Therefore all the above methods have limitations and ATL remains without a cure [20]. This compels a thorough evaluation of the current approaches and development of new strategies in the treatment of cancer aimed at increasing efficacy of treatments as well as reducing drug toxicity.

This study was undertaken to investigate the effects of EGCG on apoptosis and proliferation in cells infected with the HTLV-1 virus and malignant cells not infected with the virus in vitro.

Materials and methods

Cell lines

Two HTLV-I-positive ATL cell lines were used: HuT-102 and C91-PL which are two ATL-derived HTLV-I-infected $CD4^+$ that upon replication constitutively express the retrovirus and two HTLV-I-negative cell lines: CEM and Jurkat. All four-cell lines were kindly obtained as a gift from A. Gessain (Institut Pasteur, Paris, France). CEM cells are human T-cells uninfected with the HTLV-I virus obtained from patients with ATL whereas Jurkat were primarily established from the peripheral blood of a patient with acute lymphoblastic leukemia (ALL). The cells were grown on RPMI 1640 media with 25 mM of Hepes, 10% heat-inactivated Fetal Bovine Serum (Gibco-BRL, Paisley, Scotland) with 100 μg/ml of Streptomycin and 100 U/ml of Penicillin in a humidified incubator at 37°C and 5% CO_2. Human mononuclear lymphocytes were obtained from healthy donors and were isolated on a Ficoll-Isopaque gradient (1.077) and used directly. Those cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} β phytohemagglutinin (PHA) (5 μg/ml, total protein concentration) were added on the first day.

EGCG

EGCG was obtained from Sigma, St. Louis, MO. Stock solutions were prepared by dissolving 50 mg of EGCG in 5 ml of RPMI 1640 media at a pH of 7.0 and stored at −20°C. On the day of the experiment, working stock solutions were used immediately. Controls received RPMI 1640 media alone.

Cytotoxicity and proliferation

Cytotoxicity of EGCG was assayed using CytoTox 96 Non-radioactive Cytotoxicity Assay (Promega, Corp., Madison, WI), a method that measures the amount of lactate dehydrogenase (LDH) released from the dead cells. Proliferation was measured using Cell Titer96^{TM} Non-radioactive Cell Proliferation kit (Promega Corp., Madison, WI) according to the instructions of the manufacturer. This method is an MTT-based method that measures the ability of metabolically active cells to convert tetrazolium salt into a colored formazan product, which is measured colorimetrically at 570 nm.

Flow cytometry

Cells treated with EGCG for 48 h and 96 h were harvested, washed with 1× phosphate buffered saline (PBS) (Gibco-BRL, Paisley, Scotland) and suspended in 70% ethanol in −20°C. The cells were later collected by centrifugation, treated with RNase and the DNA stained with propidium iodide (PI) (Molecular Probes, Eugene, Oregon). The cells were later read using a FACScan (Becton-Dickinson, San Jose, CA), which distributed the cells into their respective cell cycle phases according to their DNA content. G_0/G_1 cells were 2n, S-phase were >2n but <4n while G_2/M were 4n. DNA content was determined by CellQuest software and an increase in cells in the pre-G_1 indicated an increase in apoptosis.

Analysis of TGF mRNA using RT-PCR

RT-PCR was used to test the expression of transforming growth factors (TGF) alpha, beta 1 and beta 2. The RNA was extracted from frozen cells kept at −70°C using an RNA extraction kit, SV Total RNA Isolation Kit (Promega, Madison, WI), according to the manufacturer's instructions. The RNA of TGF-α, TGF-β1 and TGF-β2 was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) after converting RNA into its cDNA using 10 U of avian myeloblastosis reverse transcriptase, an oligo(dT) primer (50 pM), 0.2 mM each of dATP, dTTP, dCTP and dGTP, a reaction buffer consisting of 50 mM Tris–HCl of pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine and 10 mM dithiothreitol. These were added in a total volume of 50 μl for 60 min at 42°C. PCR was conducted using oligonucleotides primers designed to detect the cDNA (see Table 1) under the following conditions: cDNA was added in 100 μl of 75 mM Tris–HCl at pH 9, 20 mM $(NH_4)_2SO_4$ with 0.01% Tween 20, 1 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 U of DNA polymerase

and 50 pM of each primer. The PCR program was as follows: denaturation for 45 s at 94°C, annealing at 50°C for 45 s and elongation for 45 s at 72°C and finally a final step at 72°C for 5 min. PCR products were run on 2% agarose gel stained with ethidium bromide and the resulting bands were photographed after exposing the gel to UV light.

Apoptosis determination by cell death ELISA

Apoptosis levels were determined using a Cell Death ELISA kit obtained from Roche, Mannheim, Germany. Supernatant was obtained from lysed cells after centrifugation and used as a source of the antigen. A primary anti-histone monoclonal antibody was used to coat the wells. A secondary antibody conjugated to a peroxidase enzyme was added and then peroxidase activity was measured photometrically at 405 nm with 2,2′-azino-di (3-ethyl-benzthiazolin-sulfonate) (ABTS) as a substrate.

Protein extraction and Western blotting for protein profiling

Proteins were extracted from cells treated with various concentrations of EGCG and kept at −70°C. The method of extraction was as follows: 100 µl of lysis buffer (5 ml Tris–HCl pH 6.8, 20% glycerol, 0.4 g SDS adjusted to 10 ml by adding double distilled water and 0.002% bromophenol blue) was added to cells and then were put in boiling water for 5 min. The protein concentrations were determined and a total of 30 µg of proteins were run on 12% acrylamide gel and then blotted onto a polyvinyl difluoride (PVDF) membrane electrically overnight. The membrane was probed with primary antibodies for p21, p53, Bax, and Bcl-2α. A secondary antibody linked to horseradish peroxidase specific to the primary was then used and the reaction was initiated using a chemiluminescence system. Bands were visualized on an X-ray film developed using a Xomat. β-actin was used to ensure equal loading.

Statistical analyses

Data were analyzed by one-way analysis of variance (ANOVA). The differences between the means of treated and control groups were tested for significance using Fisher's least significant differences at $p \leq 0.05$ (Fisher PLSD). An effect was considered significant when the value (+ or −) of mean difference between groups exceeded Fisher PLSD in the one-factor ANOVA test.

Results

Effect of EGCG on cytotoxicity

Ranges of concentrations of EGCG from 0 to 400 µM were selected to evaluate its cytotoxicity. Using a cytotoxicity assay as mentioned above, the concentrations at which 50% of cells are viable (IC_{50}) of EGCG was determined at 48 and 96 h incubation with these cell lines (Fig. 1a).

As expected, the IC_{50}s at 96 h were lower than those for the same cells at 48 h. At 48 h cytotoxicity was observed at ranges between 310 µM for C91-PL and 350 µM for HuT-102. CEM cells showed the most sensitivity to EGCG at 48 h at 272 µM whereas Jurkat at around 378 µM. At 96 h of treatment with EGCG, cytotoxicity concentrations ranged from as low as 86 µM for C91-PL to as high as 378 µM for Jurkat. In activated T-lymphocytic cells, those concentrations did not have an effect on proliferation (averaging above 87%) (Data not shown). The increase in cytotoxicity compared to controls was statistically significant ($p < 0.05$). The highest tested concentration was non-cytotoxic to these cells at 24 h.

Effect of EGCG on cell proliferation

At the IC_{50}, EGCG showed a substantial inhibition of proliferation (>50%) for all cell lines except for C91-PL. Jurkat was most susceptible to EGCG showing an inhibition

Table 1 Synthetic oligonucleotides and experimental conditions used for RT-PCR analysis

Gene	Position	Size	Sequence	# of cycles	T^0 hybridization
Ribosomal phosphoprotein	344–363	486	5′-GTTCACCAAGGAGGACCTCA-3′	28	50
	850–830		3′-AGACACCTCTGCCTAATGTG-5′		
TGF-β1	731–752	661	5′-GAAGTCACCCGCGTGCTAATGG-3′	32	50
	1415–1392		3′-GGATGTAAACCTCGGACCTGTGTG-5′		
TGF-β2	1–22	192	5′-TTCGCAGGTATCGATGGCACCT-3′	37–40	50
	214–192		3′-CGTCGTATTAACGACGGAAGCGG-5′		
TGF-α	3538–3557	373	5′-ATGTTGTTCCCTGCAAGTCC-3′	30	50
	3930–3911		3′-ACTATGGAGAGGGGTCGCTT-5′		

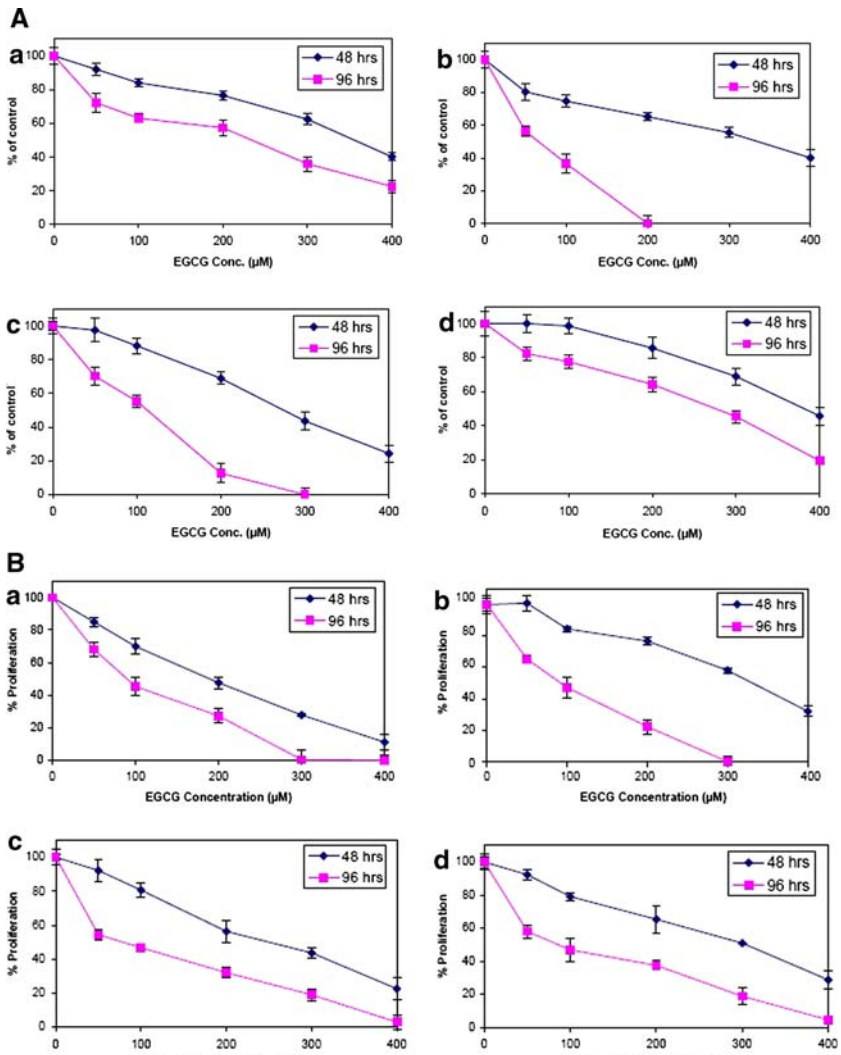

Fig. 1 (**a**, **b**) The cytotoxicity of EGCG on HTLV-I-infected and non-infected cell lines. EGCG inhibits the proliferation of HTLV-1 negative (CEM and Jurkat) (C & D), HTLV-1 positive (HuT-102 and C91-PL) (A & B). Each value is the mean ± SD of three separate experiments done in triplicate

of 72.7% at 48 h and 90.4% at 96 h. C91-PL cell line was most resistant to EGCG showing a reduction in proliferation of 41.6% at 48 h and 39.2% at 96 h (Fig. 1b). For all the consequent experiments, only non-cytotoxic concentrations of EGCG were used to obtain maximum effect with minimum side effects. There was a statistically significant decrease in proliferation in the treated versus the control at 95% confidence limits ($p < 0.05$).

Effect of EGCG on TGF mRNA levels

The expression of the transforming growth factors (TGF)-α, -β1, and -β2 at the transcriptional levels were tested using RT-PCR. A general trend of a decrease in the levels of TGF-α (a cytokine with proliferative activities) and an increase in the levels of TGF-β2 (a cytokine with apoptotic and anti-proliferative effects) was observed in all cell lines indicating a pro-apoptotic effect of EGCG on both ATL and HTLV-I. C91-PL showed the greatest response to EGCG in changing the levels of TGF-α (≈90%) and TGF-β2 (≈91%) while Jurkat exhibited the least change in TGF-α (≈69%) and TGF-β2 (75%). The levels of TGF-β1 remained unchanged (Fig. 2a, b).

Induction of apoptosis

Flow cytometry

To corroborate the results obtained using Cell Death ELISA, the flow cytometry assay was performed on the cells. As noted above, a very high increase in cells in the pre-G_1 phase was noted in CEM line up to a 97% increase in apoptotic cells at 48 h at EGCG concentration of 300 μM. Jurkat cells were similarly affected by EGCG at 48 h and 96 h (55% and 54%, respectively) at concentrations of 100 μM (Data not shown for negative cell lines). HuT-102 showed 62% apoptotic increase in apoptotic cells at 96 h at EGCG concentration of 325 μM. C91-PL was sensitive to lower concentrations of the compound and

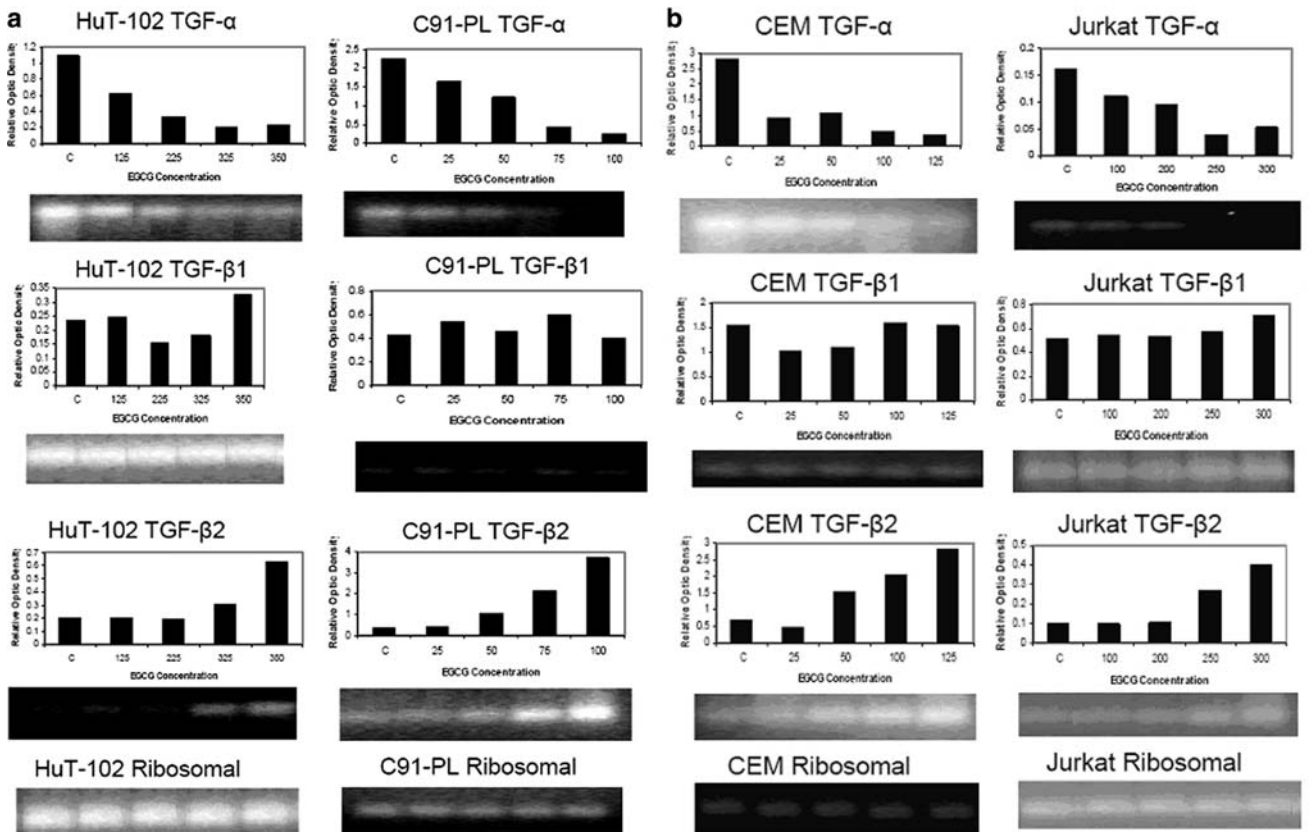

Fig. 2 (**a**, **b**) The effects of EGCG on the mRNA expression, using both HTLV-1 positive and negative cells, at 96 h of exposure. The expression of TGF α, β 1, and β 2 expression was determined using RT-PCR. The results are expressed as relative densitometry units normalized to the values of the phosphoribosomal protein mRNA used as an internal control. EF dose-dependently reduced the level of TGF-α mRNA expression and caused the up-regulation of the levels of TGFβ2 mRNA expression. However, TGFβ1 mRNA expression levels remained unchanged in HTLV-1 negative and positive leukemia cells

exhibited a maximum of 34% increase at 25 μM at 96 h and 33% at 48 h at 300 μM (Fig. 3a, b).

Cell death ELISA assay

Cell Death ELISA measures cell apoptosis by colorimetrically measuring increase in DNA histone fragments. In all four-cell lines, a significant increase in cytosolic DNA fragments was observed ($p < 0.05$). HuT-102 and CEM showed the highest increase in apoptosis followed by Jurkat and C91-PL showed the least sensitivity to treatment with EGCG (Fig. 4).

Proteins expression levels of cell cycle and apoptosis modulators

We tested the expression of four important regulators of the cell cycle and apoptosis in all cell lines. p21, an important cyclin dependent kinase inhibitor (CDKI) and p53, a pro-apoptotic protein, were up-regulated in all four cell lines. Bax, another mediator of apoptosis through damage to the mitochondria was also up-regulated. Bcl-2α, an anti-apoptotic member of the Bcl-2 family was down regulated in all cell lines (Fig. 5a–d). Densitometric analysis was done on the levels of Bcl-2 and Bax in all cell lines. The results indicate an overall increase in the Bax/Bcl-2 ratio as indicated in Fig. 5e.

Discussion

The results obtained in this study indicate that EGCG exhibits an anti-proliferative and pro-apoptotic effect in malignant T-lymphocytes infected with the HTLV-1 virus and those not infected with the virus. It is a potent antioxidant with free radical scavenging potential activities, which has been proven to be more effective than such antioxidants as vitamin C and/or vitamin E alone [21, 22].

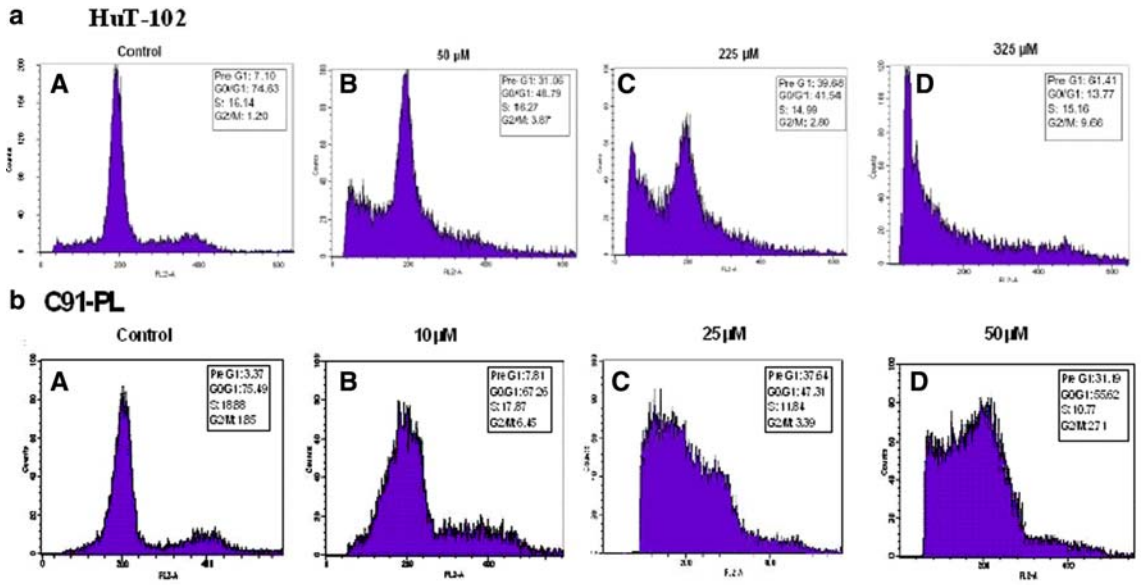

Fig. 3 (**a**, **b**) Flow cytometry data of HTLV-1 positive leukemia cells treated with EGCG shows an induction in cell cycle progression. Cells were grown under standard conditions at 50% confluence, were treated with different concentrations of EGCG for 96 h. Cells were then harvested for flow cytometric analysis of DNA content by propidium iodide staining. Distribution of cell cycle phases with different DNA contents was determined using a FACScan flow cytometer. The percentage of cells in the G_1, S, and G_2/M phases of the cell cycle were determined using Cell Quest and are indicated at the top right of each figure. The histograms shown here represent the typical result from at least two independent experiments

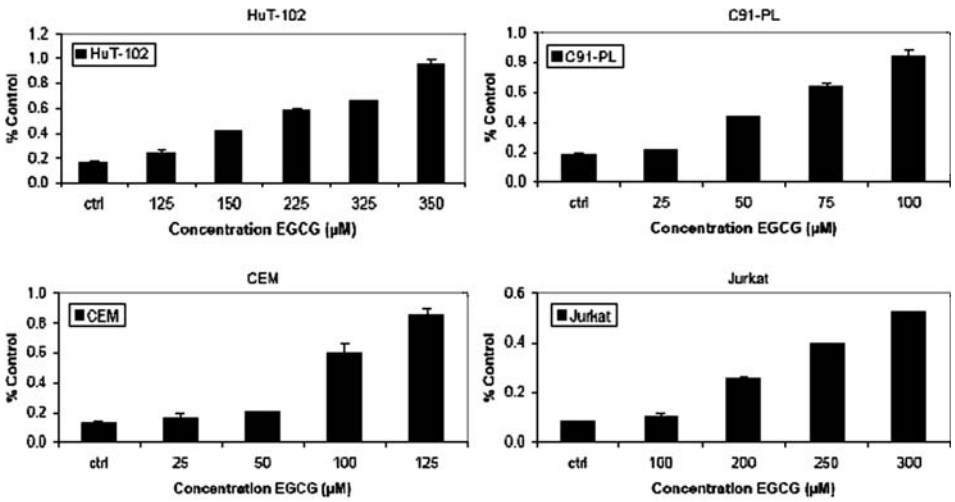

Fig. 4 EGCG induces apoptosis in HTLV-I-infected and non-infected cells. Apoptosis was assessed using an ELISA kit, which quantitatively detects cytosolic histone-associated DNA fragments, as described in ''Materials and methods''. Each value is the mean ± SD of three separate experiments done in triplicate

The concentrations used in this study were similar to those used by Hayakawa et al. [4] and Yang et al. [23] who showed that EGCG concentrations of 400 μM and 100 μM, respectively induced apoptosis in cancerous cells. EGCG was non-cytotoxic at the concentrations used in this study at 24 h. At the IC50 concentration, EGCG significantly inhibited the proliferation of both HTLV-1-positive and -negative cells. This is in accordance to what has been observed by using human and mouse leukemic cells at concentrations of around 100 μM. It was reported by that at 100 μM of EGCG, DNA synthesis was completely inhibited. Furthermore, at concentrations as low as 20 μM, EGCG completely inhibited the growth of mouse cells NFS60 cells [24].

Analysis of the cell cycle distribution at which the inhibition was taking place revealed that the cell cycle inhibition occurred at the G_0/G_1 phase and a subsequent increase in cells in the S and G_2/M stages was observed. Such results are in concordance with the work of Gupta et al. [25] on human prostate carcinoma cells. This G_0/G_1 arrest may also indicate that EGCG induces apoptosis in

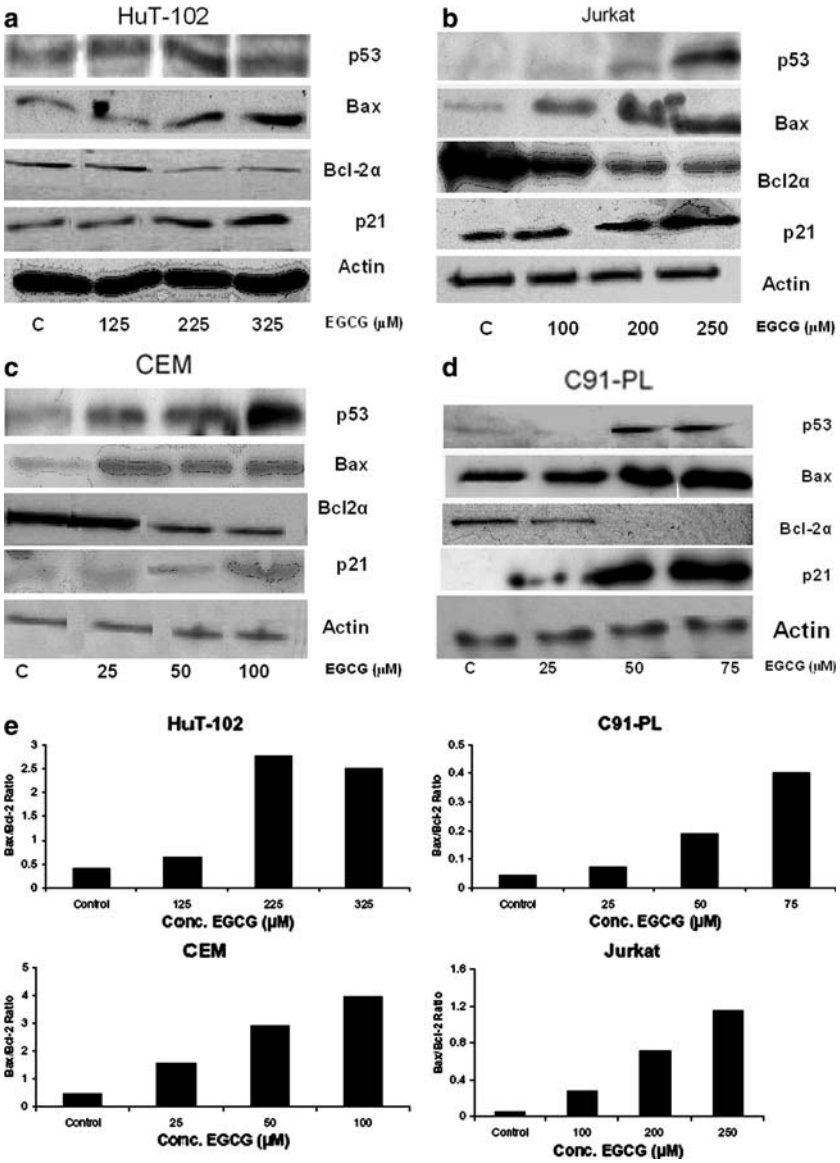

Fig. 5 Western blot analysis of p53, p21, Bax, and Bcl-2α protein expression in both (**a**–**b**) HTLV-1 positive and (**c**–**d**) negative cells treatment with EGCG for 4 days. EGCG was very effective in increasing the Bax/Bcl-2 ratio using densitometric analysis. (**e**) EGCG up-regulated the levels of p53, p21, and Bax proteins and down-regulates the levels of Bcl-2α protein expression in all the cells tested in a dose-dependent manner. β-actin has been used as a control to ensure equal loading

HTLV-1-infected and malignant cell lines. This pro-apoptotic effect of EGCG was further confirmed using Cell Death ELISA assay.

To further investigate EGCG's effect on proliferation, the levels of TGF were analyzed. EGCG caused the down-regulation of TGF-α, and the up-regulation of TGF-β2 while it had no effect on the transcriptional levels of TGF-β1. Transforming growth factors (TGF) is a large family of cytokines that have various effects on cells. Furthermore, TGF-α inhibited apoptosis in murine gastric pit cells through NF-κB pathway, which caused an up-regulation of Bcl-2 anti-apoptotic proteins [26].

Of the many functions of TGF-β are its involvements in cellular proliferation, control of mesenchymal–epithelial interactions during embryogenesis, and mediation of cell and tissue responses to injury and modulation of immune functions [27]. Many viruses besides HTLV-1 affect the TGF-β signaling pathway such as the human papilloma virus (HPV). The HPV E7 oncoprotein inhibits TGF-β signaling pathway through inhibiting its ability to activate transcription of its target genes and thus inhibit cell proliferation [28]. Saeki et al. [29] showed that TGF-β led to the induction of cell density-dependent apoptosis in human leukemia HL-60 cells. TGF-β was also shown to determine Fas-mediated apoptosis in human T-cells [30, 31]. However, in another study done using B cells TGF-β induced apoptosis through the NF-κB/Rel survival pathway [32]. This seems to be the mechanism involved in this study since up-regulation of TGF-β2 was associated with an increase in apoptosis. Contrary to this results are the findings by Inman and Allday [33] which showed that TGF-β was associated with cleavage of PARP, a DNA repair

enzyme but with no change in the levels of Bcl-2, an anti-apoptotic protein. The inhibition of proliferation is at the G_1 phase of the cell cycle through p21, which are in agreement with the results obtained in this study [34].

In this study, TGF-β1 levels were not altered by the treatment with EGCG, which may indicate that either EGCG did not affect the levels of TGF-β1 or that EGCG affected post-transcriptional modifications to the mRNA of TGF-β1. It is unique in that it has a dual role in apoptosis; it has a pro-apoptotic role in prostate epithelium and prostate cancer, ovarian carcinoma, cervical carcinoma and B- and T-lymphocytes. Importantly, TGF-β1 is known to inhibit the pro-apoptotic action of antioxidants [35]. ATL cell lines are known to produce high levels of TGF-β1 as a result of the activation of AP-1 sites on the promoter of TGF-β1 [36]. The role TGF-β1 plays in these cells is reported to be as a negative regulator of T-cell proliferation and activity [37]. Based on the above, EGCG exhibited its anti-proliferative effect through the down-regulation of TGF-α and the up-regulation of TGF-β2.

The effects of EGCG on important proteins involved in apoptosis and the cell cycle were determined. EGCG caused an up-regulation in the levels of Bax, p53, and p21 in all the tested cell lines. p21 is an important mediator of growth arrest and a regulator of CDK activity [38]. p21 inhibited cell entry into G_1 phase in response to DNA damage and blocked the re-entry of G_2 cells into S phase by blocking Cyclin E-CDK2 mediated phosphorylation of Rb. There are several factors that affect the expression of p21 some of which are TGF-β and p53 [39, 40]. The p21 protein is not only a mediator of cell cycle arrest, but can also protect cells against apoptosis [40]. p21 modulation by EGCG observed in this study may have been a result of an increase in TGF-β levels or through the increase in p53 levels or both.

p53, known as a gate keeper protein or the guardian of the genome, showed a steady increase in cells treated with EGCG. p53 can transactivate and transrepress many target genes; over 100 target genes have been identified [41]. p53 is one of the TGF-β target promoters. p53 is able to associate with Smad2 and Smad3 in a TGF-β-dependent manner. For TGF-β to function properly, expression of p53 is crucial. On the other hand, p21 over-expression increased sensitivity of T-cells to TGF-β [34]. Lack of proper levels of p53 involves a defective cytostatic response to TGF-β and the incapability to switch on the expression of the CDK inhibitor, p21 [42]. p53 can promote growth arrest in the G_1 phase through the induction of the CDK inhibitor p21WAF1 [43].

Bcl-2 and Bax are two other proteins that are regulated by p53 levels in the cell [44]. The Bcl-2-like proteins have been proposed to be involved in the development of hematopoietic and non-hematopoietic malignancies. Also, the over-expression of Bcl-2 in lymphocytes is reported to cause lymphomagenesis in mice [45]. Bax is a pro-apoptotic protein, which is reported to produce pores in the mitochondrial membrane to mediate the release of cytochrome *c*. The homozygous deletion of Bax results in viable animals that develop lymphoid hyperplasia with increased numbers of thymocytes [46]. Kuo and Lin [47] noted an increase in the levels of Bax following treatment with EGCG. Nihal et al. [48] reported an up-regulation of Bax and a down-regulation of Bcl-2 levels in melanoma cells.

This study shows the beneficial effects of EGCG in vitro both in malignant cells infected with the HTLV-1 virus and those not infected with the virus. However, the question of how this translates in vivo remains to be tackled. Previous studies argued that general antioxidative functions of tea catechins in the plasma and other tissues following ingestion are actually not strong and sometimes are not significant. There is also the matter of the low bioavailability of EGCG because it is methylated, glucuronidated, sulfated and effluxed out of the cells. Furthermore, many of the effects attributed to EGCG belong actually to oxidative and pro-oxidative reactions in the cells. H_2O_2 produced by EGCG can be a major factor and its elimination by the body may undermine EGCG's effect in vivo. The auto-oxidation of EGCG may cause the formation of radicals and quinones, which may dimerize or form thiols and this may not occur in vivo [49]. Furthermore, studies have shown that EGCG did not exhibit any antioxidant effect in lymphocytes but even caused DNA damage at concentrations ranging from 0 to 100 μM [50]. Based on the above data, EGCG should be evaluated further in clinical trials in patients with adult T-cell leukemia.

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