Apoptosis (2006) 11:2267–2276
DOI 10.1007/s10495-006-0198-2

Epigallocatechin gallate induced apoptosis in Sarcoma180 cells in vivo: Mediated by p53 pathway and inhibition in U1B, U4-U6 UsnRNAs expression

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Published online: 12 October 2006
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Abstract The aim of this study was to understand the mode of action of tea polyphenol epigallocatechin gallate (EGCG) in vivo. Swiss albino mice were treated i.p. with EGCG at two different doses i.e. 12-mg/kg body weight and 15-mg/kg body weight, for 7 days prior to inoculation of Sarcoma180 (S180) cells and continued for another 7 days. The growth of the S180, harvested 7 days after inoculation, was significantly reduced due to treatment with EGCG. The flowcytometric analysis of S180 cells, showed significant increase in apoptosis and reduction in the number of cells in G2/M phase of cell cycle due to treatment with EGCG. The induction of apoptosis has also been confirmed by the TUNEL and DNA fragmentation assays. Both RT-PCR and Western blot analysis showed significant up-regulation of p53 and bax, and down-regulation of bcl-2 and c-myc due to EGCG treatment. No changes in the expression pattern of p21, p27, bcl-xl, mdm2 and cyclin D1 were seen. Interestingly, there was significant down-regulation of spliceosomal uridylic acid rich small nuclear RNAs (UsnRNAs) U1B and U4-U6 due to EGCG treatment. This indicates that these UsnRNAs may be involved in the apoptosis process. Taken together, our study suggests that *in vivo* EGCG could induce apoptosis in S180 cells through alteration in G2/M phase of the cell cycle by up-regulation of p53, bax and down-regulation of c-myc, bcl-2 and U1B, U4-U6 UsnRNAs.

Keywords Epigallocatechin gallate · Chemoprevention · In vivo S180 · Apoptosis · Uridylic acid rich small nuclear RNAs

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Introduction

Among the tea polyphenols, epigallocatechin gallate (EGCG) has been shown to possess inhibitory activity against tumorigenecity in different animal model systems at different organ sites like lung, colon and skin [1–4]. This anti-tumor activity of EGCG might be due to the induction of cell cycle arrest and apoptosis as well as inhibition of cellular proliferation as evidenced in different animal models and in vitro tumor cell lines [1, 4–7]. Jia et al. reported that tea polyphenols are effective in preventing the precancerous liver lesions in rats, and modulation of cell cycle by regulating cell cycle regulators p21, cyclin D1 and cyclin-dependent kinase (CDK4) [8]. Gupta et al. also reported that oral infusion of green tea inhibit prostate cancer by inhibiting serum insulin-like growth factor-1, restoration of insulin–like growth factor binding protein-3 levels and also by reducing the proliferating cell nuclear antigen (PCNA) in the prostate [9]. Similarly, Bhattacharyya et al. demonstrated that black tea extract could induce apoptosis in Ehrlich's ascites carcinoma (EAC) *in vivo* by increasing the expression of p53 and bax/bcl-2 ratio [10].

EGCG could modulate different cell signaling pathways in various in vitro system, particularly c-Jun N-terminal kinase (JNK), Ras-MAP kinase and nuclear factor-κB (NF-κB) which ultimately inhibit cellular proliferation and induce apoptosis [11, 12]. It has been demonstrated that EGCG could induce the expression of p53 [13] and different cell cycle inhibitors like p16, p21 and p27, and inhibit the expression of c-myc, c-jun, c-fos, cyclin D1 and cdk2 as well as modulate the expression of bcl-2 family genes [14–18]. It was suggested that the activation of p53 blocks the cell cycle progression either in the G1/S or G2/M checkpoints and induces apoptosis by tilting the ratio of bax and bcl-2 [19–22] as well as by affecting the

RNA splicing through inhibition of spliceosomal uridylic acid rich small nuclear RNAs (UsnRNAs) U1 and U6 transcription [23–25]. Interestingly, the modulatory effect of EGCG in the metabolism of spliceosomal UsnRNAs during lung carcinogenesis by benzo(a)pyrene has also been reported [26]. Quantitative alterations in the metabolic pattern of the UsnRNAs have been observed during induced cellular proliferation, differentiation, tissue regeneration, and embryonic development and also in tissue specific distribution/expression in fetal/neonatal stages, indicating the importance of the genes in these cellular processes [27–31]. More over, the embryonic and adult variants of some UsnRNAs like U1, U3 and U4 have been identified [32, 33] and suggested to have some specific function in the cellular proliferation and differentiation. However, the role of the UsnRNAs during apoptosis has not yet been studied in detail, though Degen et al. showed the cleavage of both U1 snRNA and U1–70 K protein during apoptosis [34].

The mechanism of action of EGCG in in vitro cell lines and in vivo system is often varied which may account for the differences in response to EGCG treatment [35]. We had earlier reported the anti-proliferative and pro-apoptotic action of tea polyphenols during lung carcinogenesis [36]. Our study in vitro using a human lung cancer cell line NCI-H460 has shown reduction of cell proliferation and induction of apoptosis [37]. Analysis of p53, bcl-2, c-myc and H-ras expression revealed up regulation of p53, down regulation of bcl-2 and no significant change on H-ras and c-myc expression by the tea compounds, which is different from the observation of our in vivo study [38] where down regulation of H-ras and c-myc was noted.

In the present study, the effect of EGCG on cellular proliferation and apoptosis was analyzed in more detail on an acsites tumor cell line in vivo-Sarcoma180 (S180). EGCG was administered at different concentrations for 7 days before and after intra-peritoneum inoculation of S180 cells. The cell cycle progression of the S180 was checked by fluorescence-activated cell sorter (FACS) analysis and expression of different cellular proliferation and apoptosis associated genes as well as the metabolism of the major UsnRNAs (U1-U6) were analyzed. It is likely that EGCG induced apoptosis of the S180 cells noted is mediated by p53 pathway and also by alterations of UsnRNAs level particularly U1B (one U1 variant) and U4-U6.

Materials and methods

Mice and tumor models

Swiss albino mice obtained from animal colony of Chittaranjan National Cancer Institute, Kolkata, were maintained in standard condition and provided with drinking water and food pellet (Lipton India Ltd., India) ad libitum.

Swiss mice were randomly divided into three groups (∼20 g each; 5 mice in each group) i.e. (i) one tumor-bearing group, which were intra-peritoneally injected with 1×10^5 exponentially grown S180 cells and (ii) two EGCG-treated tumor-bearing groups. One of this group received EGCG at a dose of 12 mg/kg body weight and the other was treated with a higher dose of 15 mg/kg body weight. EGCG was administered intra-peritoneally, starting 7 days prior to inoculation of S180 cells and continued for another 7 days post-inoculation, before sacrifice. For better exposure of S180 cells to EGCG i.p injection of EGCG was preferred in this study. Dose selection was based on the basis of our previous dose response studies. The selected doses were inhibitory but not toxic. Control and treated mice were sacrificed at the end of the experimental period, tumor cells collected from the peritoneal cavity washed in PBS before further processing. Viable cell counts were made and recorded for all groups.

Detection of apoptosis by flowcytometry

The analysis of cell cycle phase distribution of S180 cells was done according the method of Holmes et al. [39] with slight modification. In short 1×10^6 cells were permeabilized by 70% ice cold ethanol followed by staining with propidium iodide (PI, 20 μl of 1 mg/ml stock). Cell cycle phase distribution of nuclear DNA was determined on FACS Calibur fluorescence-activated cell sorter (FACS), fluorescence detector equipped with 488 nm argon-ion laser light source using CellQuest software (Becton Dickinson, USA). A total of 10000 events were acquired for each sample analyzed. A histogram of DNA content (x-axis, PI fluorescence) versus counts (y-axis) has been displayed. The percentage of apoptotic cells was determined by measuring the fraction of nuclei that contained a sub-diploid DNA content.

TUNEL assay

Apoptosis in S180 cells was determined by the terminal deoxyneucleotidyl transferase (TdT)-mediated dUTP–biotin nick end labeling (TUNEL) method using *in situ* cell detection kit (Roche Molecular Biochemiocals, Germany). Methanol fixed S180 cells were incubated with 50 μl TUNEL reaction mixture (TdT and fluoresccin–dUTP) at 37°C, for 60 min in humid atmosphere. Next cells were incubated with converter AP (Anti-fluorescein antibody, Fab fragment from sheep, conjugated with alkaline phosphatage [AP]) for 30 min and analyzed under light microscope after color development, achieved by incubation for 5 min at room temperature with a substrate solution containing Nitroblue Tetrazolium (NBT) and X-phosphatase.

Table 1 Primer sequences used in RT-PCR analysis

Gene of interest	Sequences of primer	References
p53:	FP 5′ATG ACT GCC ATG GAG GAG TCA CAG T 3′ RP 5′GTG GGG GCA GCG TCT CAC GAC CTC C 3′	[42]
c-myc:	FP 5′TCC TGT ACC TCG TCC GAT TC 3′ RP 5′ATT GAT GTT ATT TAC ACT TAA GGG T3′	[43, 44]
bcl-xl:	FP 5′TGG ATC CTG GAA GAG AAT CG 3′ RP 5′AGA TCA CTG AAC GCT CTC CG 3′	[43]
bax:	FP 5′AAG CTG AGC GAG TGT CTC CGG CG 3′ RP 5′GCC ACA AAG ATG GTC ACT GTC TGC C 3′	[45]
bcl-2:	FP 5′CTC GTC GCT ACC GTC GTG ACT TCG 3′ RP 5′CAG ATG CCG GTT CAG GTA CTC AGT C 3′	[45]
mdm2:	FP 5′CGA CTA TTC CCA ACC ATC G 3′ RP 5′CTA GTT GAA GTA ACT TAG CAC AAT 3′	[46]
cyclin D1:	FP 5′AAC ACC AGC TCC TGT GCT GCG AA 3′ RP 5′GTC TCC TTC ATC TTA GAG GCC ACG 3′	[47]
p21:	FP 5′AAT CCT GGT GAT GTC CGA CC 3′ RP 5′AAA GTT CCA CCG TTC TCG G 3′	[48]
p27:	FP 5′GAG GGC AGA TAC GAG TGG CAG 3′ RP 5′CTG GAC ACT GCT CCG CTA ACC 3′	[48]
β-actin:	FP 5′GTG GGC CGC TCT AGG CAC CAA 3′ RP 5′CTC TTT GAT GTC ACG CAC GAT TTC 3′	[48]
hprt:	FP 5′GCT GGT GAA AAG GAC CTC T 3′ RP 5′CAC AGG ACT AGA ACA CCT GC 3′	[44]

DNA fragmentation assay

DNA extraction from S180 cells was performed as described by Yoshida et al. [40]. PBS washed S180 cells was resuspended in DNA extraction buffer (50 mM Tris, pH 8.0, 20 mM Na-EDTA, 10 mM NaCl, 1% [w/v] SDS, and 20 μg/ml RNase A and incubated at 37°C for 1 h, followed by proteinase K treatment to a final concentration of 100 μg/ml at 65°C for 1 h. The DNA was extracted once with equal volume of buffer saturated phenol and once with chloroform:isoamyl alcohol mixture [24:1] followed by ethanol precipitation. Then DNA was dissolved in TE (10 mM Tris [pH 7.4], 1 mM EDTA) and analyzed by 2% agarose gel electrophoresis [41].

Analysis of gene expression by semi-quantitative RT-PCR

Expression of the genes was analyzed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from S180 cells with TRIzol Reagent (Roche, Mannheim, Germany). The cDNA was synthesized from the total RNA using SuperScript III Reverse Transcriptase (Invitrogen, USA) according to the manufacturer protocol. One microgram of RNA after treatment with DNase1 was taken in a 20 μl reaction volume containing 200 ng of random hexamer, 40 units of RNaseOUT (Invitrogen, USA), 1x reverse transcription buffer (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$), 0.005 M DTT, 0.5 mM of each deoxynucleoside triphosphates (dNTPs; Gibco-BRL, USA) and 200 units SuperScript III. The reaction was at 50°C for 60 min followed by 75°C for 15 min.

To analyze the expression of specific gene, 2 μl of the cDNA was taken in a 20 μl PCR mixture containing 1 × PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25°C), 0.01% Tween-20), 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 3 pmol of each gene specific primer and 0.3 units Taq polymerase (Bioline, London, UK) enzyme. p53, bax, bcl-2, bcl-xl, c-myc, mdm2, and cyclin D1 genes were co-amplified with the hypoxanthine phosphorybosile transferase (hprt) or β-actin gene as control. Primers used in this experiment are listed in Table 1. The PCR cycle conditions were as follows: 95°C for 3 min, then 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 60 s with a final extension step of 7 min at 72°C in a thermal cycler (Applied BioSystem, USA). The PCR products were electrophoresed in 2% agarose gel, stained in ethidium bromide, visualized in UV transilluminator and then photographed. Intensity of the bands were analyzed by densitometric scanning in densitometric scanner (Model: CS-9000, SHIMADZU, Japan). In each case the intensity of the band was normalized with respect to the control band of hprt/β-actin.

Analysis of protein expression by Western blotting

Western blotting was done according to Herold et al. [49]. The cells (1×10^7) were lysed in 1 ml 2 × ice cold sample buffer (2 mM phenylmethyle-sulfonyl fluoride (PMSF), 4% SDS, 4% DTT, 20% glycerol, 0.01% bromophenol blue, 2 M urea, 0.01 M Na-EDTA, 0.15 M Tris-HCl (pH 6.8), containing 1 mM, 1 μg/ml aprotinin, 0.1 mM leupeptin). DNA was sheared by pipetting up and down for 3 min at room temperature and suspension boiled at 95°C for 15 min, centrifuged

Table 2 Effect of i.p administration of EGCG on S180 growth

Groups	No. of mice	Mean of total cell count	Percent of inhibition
(i) S180	5	$(13.712 \pm 0.916) \times 10^8$	–
(ii) S180 + EGCG (12 mg/kg body weight)	5	$(8.358 \pm 0.622) \times 10^{8a}$	39
(iii) S180 + EGCG (15 mg/kg body weight)	5	$(7.837 \pm 0.454) \times 10^{8a}$	43

[a]Significantly different from control (S180) group by One Way ANOVA ($p < 0.01$)

at 14000 r.p.m for 10 min. The supernatant was aliquoted at 40 μl volume and kept at -20°C until further use. After addition of bromophenol blue (0.01%) a 40 μl aliquot was electrophoresed on 10–15% SDS-polyacrylamide gel according to Laemmli [50]. Electrophoretically resolved proteins were transblotted onto polyvinylidine difluoride (PVDF) membrane and blocked with blocking buffer (100 mM Tris-HCl and 150 mM NaCl and 0.1% Tween 20). The membrane was then incubated with specific primary antibody [mouse anti-P53, anti-Bcl-2, anti-Bax, anti-c-Myc and β-Actin (used as a control for protein loading) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a dilution of 1:250 for over night at 4°C followed by incubation with HRP-conjugated specific secondary anti mouse IgG (1:500 dilution) for 1 h. The target protein band was then visualized using luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) followed by exposure with Kodak XOMAT film. The band intensity was quantified densitometrically using densitometric scanner (Model: CS-9000, SHIMADZU, Japan). Relative protein level was calculated as the ratio of the optical density of P53, Bcl-2, Bax or c-Myc and that of the β-Actin.

UsnRNA analysis

The major UsnRNAs (U1–U6) were analyzed electrophoretically according to the method of Choudhury et al. [51]. Total RNA was dissolved in 80% deionized formamide, heated to 85°C for 3 min, chilled immediately in ice and electrophoresed on 12% polyacrylamide-7M urea gel. The gel was stained with ethidium bromide, photographed in UV-transilluminator. The intensity of UsnRNA bands was scanned densitometrically in densitometric scanner (Model: CS-9000, SHIMADZU, Japan).

Identification of UsnRNA bands on the scanning profile was accomplished by comparing their mobility with that of known cytoplasmic marker RNAs namely 4S, 4.5S, 5S and 5.8S as well as by direct comparison to UsnRNAs isolated from whole nuclei. Relative abundance of each UsnRNA was calculated by normalizing the areas of the peak of each UsnRNA with respect to that of corresponding 5S RNA in the corresponding lane.

Statistical analysis

All data were expressed as mean $\pm$ SD. Statistical analyses were performed using One Way ANOVA followed by Paired *t*-test with the help of critical difference (CD).

Results

Effect of EGCG treatment on S180 growth

Treatment of tumor bearing mice using EGCG at different doses revealed that at the lower concentrations no significant changes occurred (data not shown). Viable cell count was found to be significantly reduced by 39% and 43% ($p < 0.01$) at 12 mg/kg body weight and 15 mg/kg body weight respectively (Table 2).

Effect of EGCG on cell cycle progression and apoptosis

Effect of EGCG on cell cycle progression of S180 cells was analyzed by FACS using PI. Initially we treated the mice with EGCG with 4 mg/kg body weight and 8 mg/kg body weight. In FACS analysis we did not see any significant difference compared to untreated control (data not shown). Therefore we enhanced the dose of EGCG to 12 mg/kg body weight and 15 mg/kg body weight to check if there was any difference in the cell cycle progression. It was evident from Fig. 1 that treatment with EGCG at 12 mg/kg body weight and 15 mg/kg body weight resulted in significant increase (2.08 and 2.69 fold: $p < 0.01$) of apoptotic cells in the subG1 phase of the cell cycle and significant decrease (23% and 29%: $p < 0.01$) in number of cells in G2/M phase of the cell cycle compared to that in the untreated control.

Apoptosis in S180 cells was detected by TUNEL and DNA fragmentation assays to support the observation made by FACS. TUNEL assay on tumor cells showed increased presence of apoptotic cells in the EGCG treated groups compared to the untreated control (Fig. 2A). DNA fragmentation analysis indicated increased apoptotic cell death in the treated groups as shown by the ladder formation (Fig. 2B).

Effect of EGCG on some proliferation and apoptosis associated genes

In cell cycle progression c-myc, cyclinD1 and p27 are important cell cycle regulators particularly in the G1/S phase [52, 53]. On the other hand, p53 has been suggested to cause cell cycle arrest, mediated by p21 and apoptosis through transactivation of bax gene, the elevated expression of which induces apoptosis, and repression of anti-apoptotic protein Bcl-2, Bcl-xl, another Bcl-2 family protein, and Mdm2, the negative regulator of p53 also play important roles in apoptotic

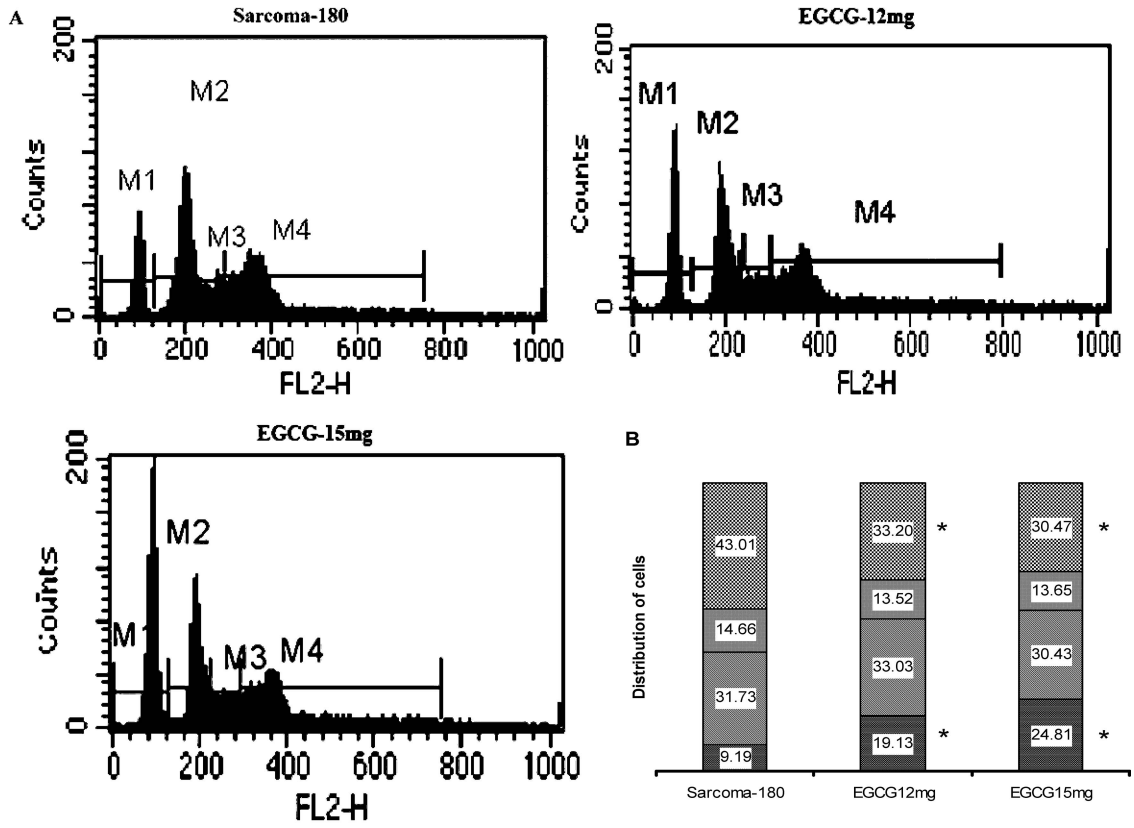

Fig. 1 EGCG induced apoptotic cell death in S180 cells. (A) Flow-cytrometric analysis of S180 cell cycle phase distribution. (B) Histogram display of DNA content (*x*-axis, PI fluorescence) versus counts (*y*-axis) has been shown. Results are mean ± SE. $^{*}p < 0.01$. (▩) SubG1; (▨) G1; (▩) S; (▩) G2-M

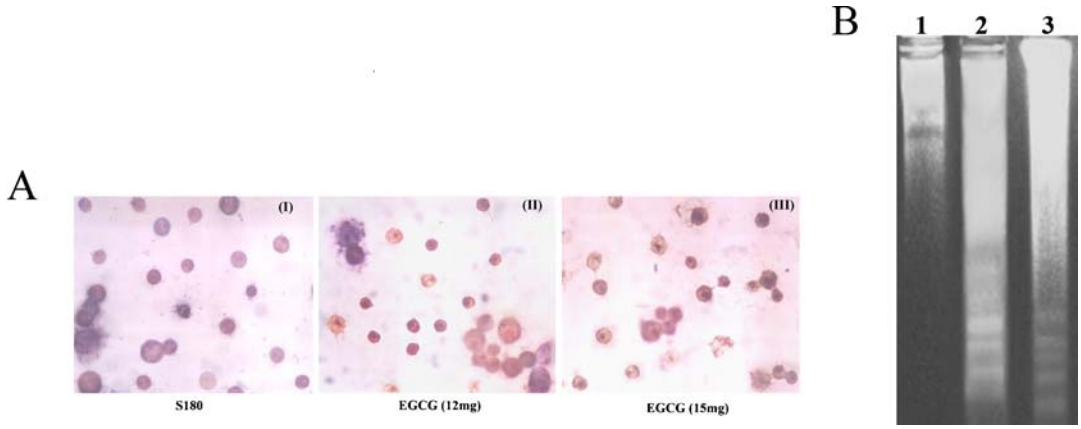

Fig. 2 (A) Induction of apoptosis in S180 by EGCG was detected by TUNEL staining. As shown in (II) and (III), the cells with morphologically condensed nucleus were TUNEL positive, indicating the existence of fragmented DNA compared to the control S180 cells in (I), which maintained normal feature. (B) DNA laddering. Cellular DNA from S180 cells was extracted and resolved by agarose gel electrophoresis. Lane 1. S180; lane 2. EGCG (12 mg/kg body wt.) treated S180; lane 3. EGCG (15 mg/kg body wt.) treated S180

pathway. Mdm2 participate in an autoregulatory loop with p53 and has been shown to function as an ubiquitin ligase that targets p53 for degradation [20, 21, 54]. To elucidate whether the level of these critical determinants of apoptosis and cell cycle were changed in EGCG induced apoptosis, we examined the transcript level by semi-quantitative RT-PCR analysis.

It can be seen from Fig. 3 that EGCG differentially altered the expression of proliferation and apoptosis associated genes. Semi-quantitative RT-PCR analysis revealed c-myc

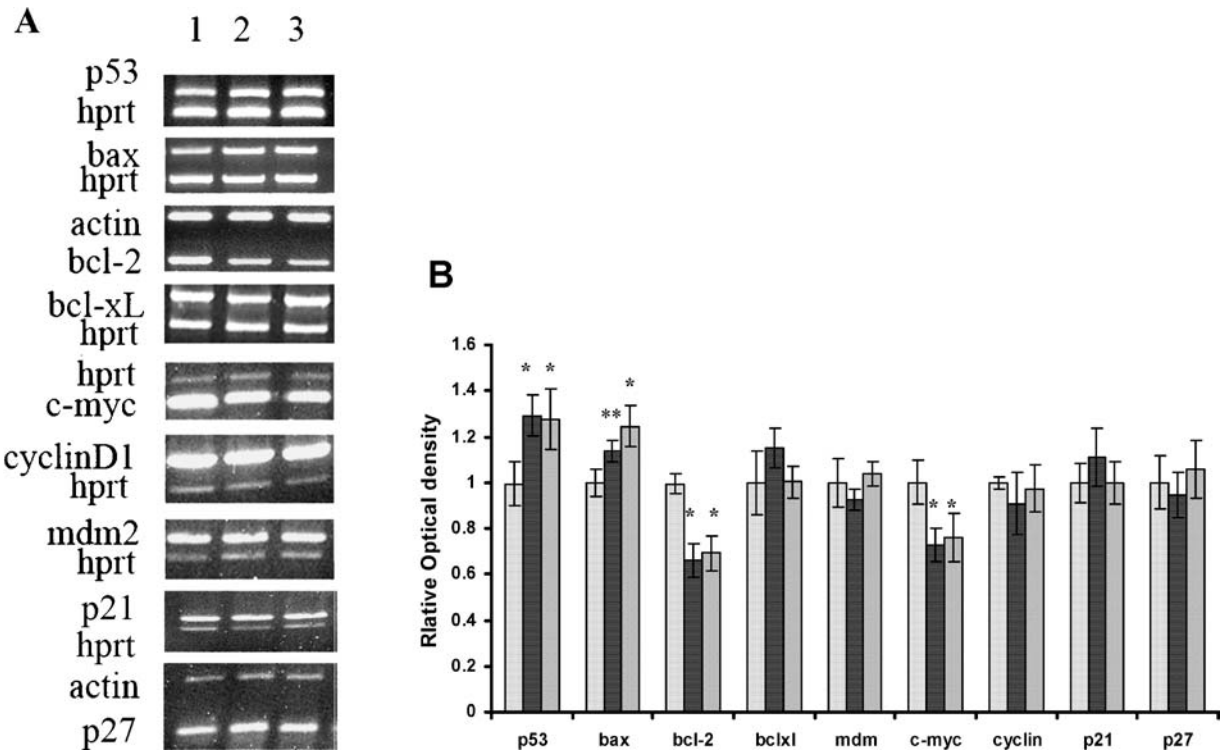

Fig. 3 Effect of EGCG on the mRNA level of apoptotic and cell cycle regulatory genes. The genes were co-amplified with either β-actin or hprt. (A) Representative photographs of ethidium bromide stained 2% agarose gels under UV. Lane 1. S180; lane 2. EGCG (12 mg/kg body wt.) treated S180; lane 3. EGCG (15 mg/kg body wt.) treated S180. (B) Histogram display of quantitative analysis of mRNA level of the genes. Results are mean $\pm$ SE. $^{**}P < 0.05$; $^{*}p < 0.01$. (▨) S180; (▩) EGCG (12 mg); (▧) EGCG (15 mg)

and bcl-2 expressions were significantly reduced by 27% and 34% ($p < 0.01$) compared to treatment group at the dose of 12 mg/kg body weight respectively. The inhibitory effect of EGCG was more or less same at 15 mg/kg body weight. On the other hand, the expression of p53 was significantly increased nearly 29% ($p < 0.01$) at both the low and high doses of EGCG. Whereas, the expression of bax was increased significantly by 14% ($p < 0.05$) and 25% ($p < 0.01$) with low and high doses of EGCG treatment respectively. However, the expression of other genes i.e. cyclin D1, mdm2, bcl-xl, p21 and p27 did not change significantly due to EGCG treatment.

Next we looked for P53, Bax, Bcl-2 and c-Myc protein expressions in EGCG treated S180 cell using Western blot analysis. The level of c-Myc was reduced by nearly 36% ($p < 0.01$) due to the treatment with EGCG only at 15 mg/kg body weight, whereas the level of Bcl-2 was reduced nearly 30% ($p < 0.05$) at the low and high doses of EGCG (Fig. 4). On the other hand EGCG increased the level of P53 protein by about 47% ($p < 0.05$) with similar effect at both concentrations. Like RNA, the protein level of Bax significantly increased by 32% ($p < 0.05$) and 46% ($p < 0.01$) following EGCG treatment at the low and high doses respectively. Interestingly, the Bax/Bcl-2 ratio has been increased by 2 fold. Western blot analysis confirms RT-PCR data and supported the notion that as a result of EGCG treatment the balance between positive and negative regulators of apoptosis was shifted towards cell death.

Effect of EGCG on the level of major UsnRNAs

Metabolism of major UsnRNAs (U1–U6) are altered during proliferation, regeneration, differentiation and development. But their behavior in apoptosis is not clear. Quantitative alteration of those UsnRNAs might have some role in apoptosis. To understand the role of major UsnRNAs in tumorigenesis and their modulation by EGCG, expression pattern of the UsnRNAs in S180 cells was analyzed.

It is evident from Fig. 5 that there was significant reduction in the level of U4, U5 and U6 UsnRNAs due to treatment with EGCG compared to untreated control. The level of U4, U5 and U6 was reduced by 26%, 51% and 45% ($p < 0.01$) respectively at 12 mg/kg body weight EGCG. The level of the UsnRNAs was more or less constant even at 15 mg/kg body weight EGCG. However, the level of U1B was reduced by nearly 28% ($p < 0.05$) only at the higher dose (15 mg/kg body weight). The level of the other UsnRNAs did not change considerably due to the treatment.

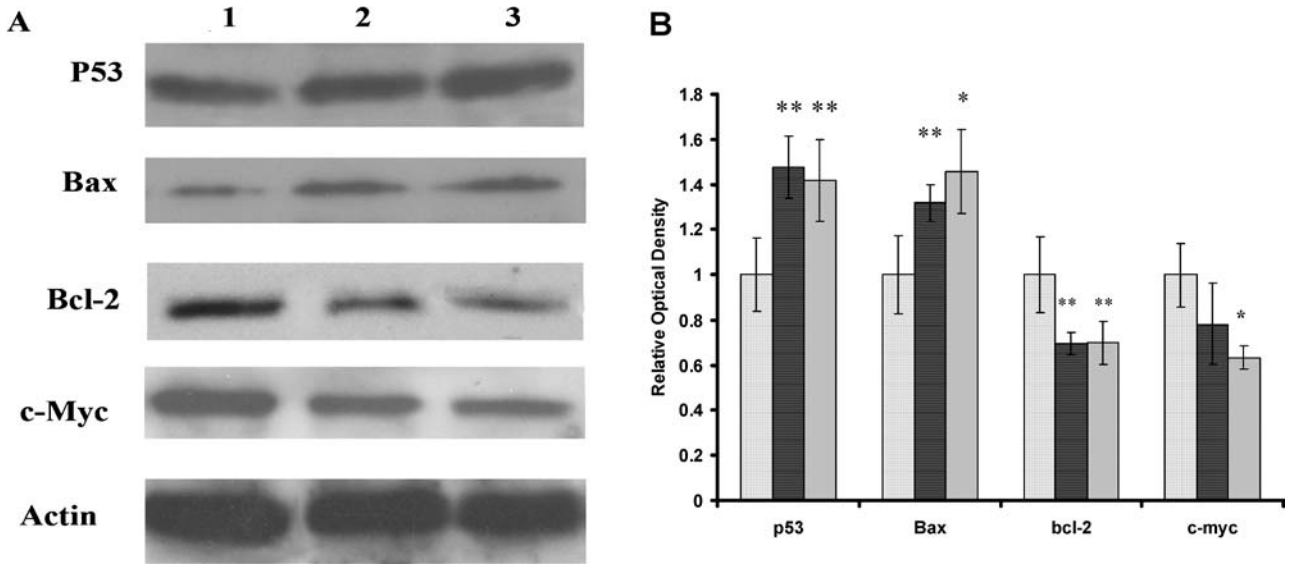

Fig. 4 Effect of EGCG on P53, Bax, Bcl-2 and c-Myc protein level. (A) Representative Western blot analysis of the genes. Lane 1. S180; lane 2. EGCG (12 mg/kg body wt.) treated S180; lane 3. EGCG (15 mg/kg body wt.) treated S180. (B) Histogram display of quantitative analysis of protein level. Results are mean ± SE. $^{**}P < 0.05$; $^{*}p < 0.01$. (▨) S180; (▩) EGCG (12 mg); (▧) EGCG (15 mg)

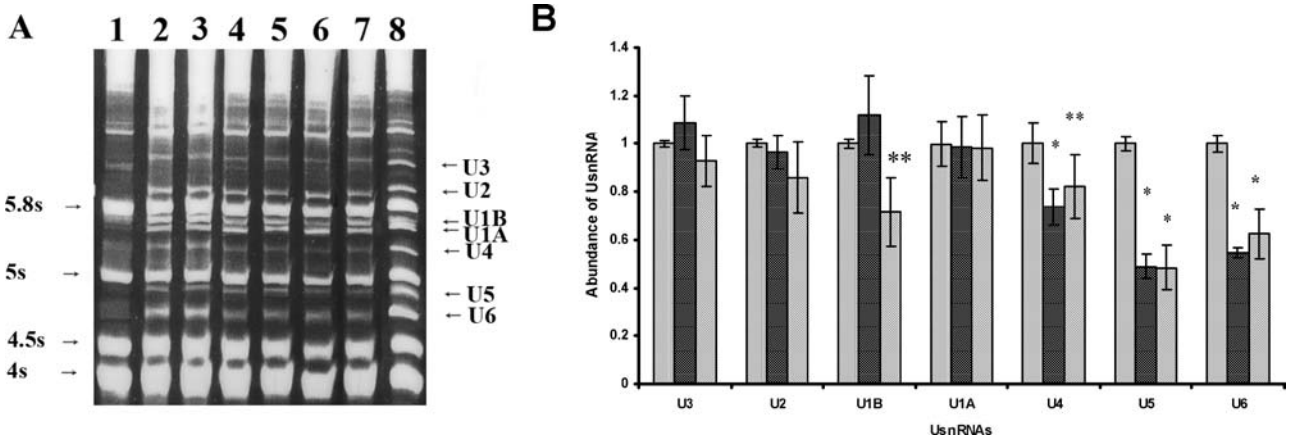

Fig. 5 (A) Representative photograph of RNA gel electrophoresed in 12% acrylamide-7 M Urea gel and stained in ethidium bromide. Thirty microgram of whole cellular RNA isolated from S180 cells was loaded per lane. Lane1: Cytoplasmic RNAs as marker; lanes 2, 3: RNAs of untreated S180, lanes 4, 5: RNAs of S180 treated with 12 mg/kg body wt EGCG; lanes 6, 7: RNAs of S180 treated with 15 mg/kg body wt EGCG; lane 8: Nuclear RNAs as marker. (B) Abundance of major UsnRNAs in S180. Results are ± SE. $^{**}p < 0.05$; $^{*}p < 0.01$. (▨) S180; (▩) EGCG (12 mg); (▧) EGCG (15 mg)

Discussion

In this study we have tried to understand the effect of EGCG, the major polyphenolic agent present in green tea, on S180 grown in the peritoneum of Swiss albino mice in an attempt to explore its molecular mechanism of action. EGCG was found to produce significant induction of apoptosis in S180 cells by increasing subG1 population. This finding was confirmed by TUNEL and DNA fragmentation assays. EGCG also significantly reduced the number of cells in the G2/M phase. It seems that EGCG did not block the cells in the G2/M phase, rather it induces apoptosis in this phase and as a result the number of apoptotic cells are increased in the subG1 population. Cytotoxic effect of black tea extract has been observed in EAC cells *in vivo*, where induction of apoptosis due to block in the G0/G1 of the cell cycle has been suggested [10]. In that study the cells were harvested from the stationary phase of the cell growth i.e. after 21 days of inoculation. But in our case we have studied the effect of EGCG at the exponentially growing stage of the ascitic tumor. In different *in vitro* tumor cell line systems EGCG could induce apoptosis differentially i.e. either in the G0/G1 or G2/M phase of the cell cycle [6, 55–57]. This difference in the action of EGCG in different phases of the cell cycle might be due to the difference in the metabolism of EGCG in different cell systems. Thus, the effect of EGCG, inducing

apoptosis of S180 cells in the present study, seems to be specifically at the G2/M phase of the cell cycle.

To analyze the mechanism of apoptosis in S180 cells we have analyzed the expression of different proliferative and apoptosis associated genes. The significant up-regulation of p53 and down-regulation of c-myc in S180 cells due to treatment with EGCG indicates that the apoptotic process might be regulated by both p53 and c-myc. The increase of Bax/Bcl-2 ratio from the control value, due to up-regulation of Bax and down-regulation of Bcl-2, following treatment with EGCG has also supported this data. The up-regulation of bax might be directly associated with p53 activation whereas, the down-regulation of bcl-2 might be by both p53 and c-myc [20, 21, 58]. In different in vitro tumor cell lines, EGCG induced apoptosis was mainly due to the induction of p53, bax, p21, p27 genes and repression of c-myc, cyclin D1, cyclin E and different cdks [59]. But in our in vivo S180 system, EGCG failed to produce any alteration in the expression of different cell cycle check point genes like cyclinD1, p21, p27 and mdm2 genes as well as the anti-apoptotic gene bcl-xl. This indicates that the G1/S checkpoint of the cell cycle might not be the only target of EGCG and the apoptotic process mediated through bax/bcl-2.

It appears from our study that the apoptotic process is associated with down regulation of U1B and U4-U6 UsnRNAs. To the best of our knowledge the association of UsnRNAs with apoptosis has not yet been reported except U1 snRNA [34]. Degen et al. suggested that the cleavage of U1 snRNA during apoptosis might be due to the activation of specific ribonuclease [34]. On the other hand, it has also been shown that p53 could down-regulate U1 and U6 UsnRNA by interacting with its promoter element [23–25]. The differential metabolism of the U1-U6 UsnRNAs during the cellular proliferation, differentiation, development, and lung carcinogenesis has been reported [26–31]. The association of U5 in in vitro transformation [60] and reduced expression of UsnRNAs due to UV exposure have also been seen [61–63]. This indicates that, the alterations in the expression of U1B and U4–U6 UsnRNAs during EGCG induced apoptosis in S180 cells in vivo might have some effect on this phenomenon.

Conclusion

It can be concluded from this study that EGCG could induce apoptosis in S180 cells grown in vivo through alteration in G2/M phases of the cell cycle. While EGCG treatment induced the expression of pro apoptotic genes p53 and bax, the expression of c-myc and anti apoptotic gene bcl-2 was reduced. It appears that EGCG exploits p53 and c-myc regulated apoptotic pathways. Experimental demonstration of the specific reduction in the abundance of U1B and U4–U6 snRNAs during the apoptotic process of S180 cells induced by EGCG is probably the first report to show the involvement of the UsnRNAs in apoptosis.

Acknowledgments Authors are grateful to the Director, Chittaranjan National Cancer Institute, Kolkata, for kind interest in this work. We thank Dr. R. Baral for his help in FACS analysis and Dr. S Mandal for his assistance in statistical analysis. This work was supported by grants to S. M. [9/30(29)/2002-EMR-I]; to S. D. and C. K. P. [37(1151)/03/EMR-II] from CSIR, New Delhi.

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