Epigallocatechin-3-gallate promotes apoptosis and expression of the caspase 9a splice variant in PC3 prostate cancer cells

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Abstract. Growing evidence suggests that the flavonoid epigallocatechin-3-gallate (EGCG), notably abundant in green tea, has health-promoting properties. We examined the effect of EGCG on cell survival and apoptosis in the prostate cancer cell line PC3. Cell survival was reduced and apoptosis increased significantly with a low dose of 1 μM EGCG. The ability of the anticancer drug cisplatin to promote apoptosis was enhanced by EGCG. Furthermore, EGCG, both alone and in combination with cisplatin, promoted the expression of the pro-apoptotic splice isoform of caspase 9.

Introduction

The family of polyphenolic plant secondary metabolites known as flavonoids has attracted significant interest due to their potential health-promoting effects that include cancer prevention and chemotherapy. Among the flavonoids, the catechins are found in several land plants; and are abundant in cocoa, red wine and especially green tea (*Camellia sinensis*). Green (but not black) tea is particularly abundant in the flavonoid epigallocatechin-3-gallate (EGCG), an ester of epigallocatechin and gallic acid. EGCG has been the subject of extensive research because there is evidence that it could provide benefits in treating prostate, breast and other cancers (1-3). As well as EGCG, other polyphenolics are thought to confer health-promoting effects, including genistein, quercetin and curcumin (4).

One of the key mechanisms through which EGCG is thought to exhibit antitumour effects is through the promotion of apoptosis. Increased apoptosis caused by EGCG has been demonstrated in human bladder cancer (5); human endometrial carcinoma (6); anaplastic thyroid carcinoma (7); gastric cancer (8); melanoma (9); and prostate cancer cell lines (10). Taken together, the numerous publications on EGCG suggest that its effects are pleiotropic. There are several mechanisms through which EGCG could promote apoptosis including the generation of reactive oxygen species (ROS) (6); the inhibition of JAK/STAT3 signaling (11); the inhibition of NF-κB and Akt activation (12); the downregulation of survivin expression (8); and the activation of caspases (13).

EGCG is well known as an antioxidant; but in fact in some conditions it is a pro-oxidant and can even auto-oxidise in cell culture, generating ROS (14). A recent study suggests that EGCG-mediated ROS generation is particularly observed in cancer cells compared to normal cells (15). In other words EGCG may cause a selective increase in apoptosis predominantly in cancer cells. Thus there is a pressing need to understand the concentration of EGCG required to achieve apoptosis; its ability to synergise with widely used drugs that promote apoptosis (such as cisplatin); and the mechanisms through which it promotes apoptosis.

Recent evidence suggests that polyphenolics such as EGCG might affect alternative splicing. Resveratrol, a polyphenolic notably abundant in red wine, affects exon inclusion in the *SRSF3* and *SMN2* genes by altering the expression of the splice factors SRSF1, hnRNP A1 and HuR (16). EGCG corrects the aberrant splicing of IKAP in cells derived from patients with familial dysautonomia (17) and synergises with ibuprofen to alter the splicing of Bcl-X and Mcl-1 favouring pro-apoptotic isoforms (18). Aberrant alternative splicing is increasingly important in cancer (19) and many genes involved in apoptosis are alternatively spliced into pro- or anti-apoptotic isoforms (20). These include caspase 9, a member of the caspase family of proteases. Caspase 9 is an initiator caspase; its activity increases following DNA damage and cytochrome *c* release. The splice isoform caspase 9a is pro-apoptotic (and encodes the full length protease). Caspase 9b results from exclusion of exons 3-6; it lacks catalytic activity but retains the interaction domains and therefore acts as an endogenous inhibitor of caspase 9a. The alternative splicing of caspase 9 is dysregulated in non-small cell lung cancers (21). The reduction of caspase 9b decreases the IC_{50} of several chemotherapeutic drugs (22). It is therefore of

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interest to explore new ways in which to modify the alternative splicing of caspase 9 *in vivo*.

In this report we show that EGCG promotes apoptosis in the prostate cancer cell line PC3 even at a concentration of 1 μM; that it synergises with cisplatin to promote apoptosis and can do so at low concentrations in cell culture; and that it modifies the alternative splicing of caspase 9 favouring the pro-apoptotic isoform. The latter finding suggests that EGCG, and other polyphenolic compounds, may affect the alternative splicing of cancer-associated genes *in vivo*.

Materials and methods

Cell treatments. All materials were purchased from Sigma-Aldrich unless otherwise stated. EGCG was prepared in the solvent dimethylsulfoxide (DMSO). Cisplatin (cis-diamino-dichloroplatinum (II)) was prepared in PBS containing 0.9% (w/v) NaCl. The prostate cancer cell line, PC3 was obtained from ECACC - an androgen-independent cell line established from a bone metastasis of grade IV prostate cancer. PC3 cells were cultured in DMEM (5 mM glucose) containing 10% FBS, L-glutamine and penicillin-streptomycin. Prior to treatment with EGCG, cells were serum-starved overnight. EGCG was diluted in fresh DMEM (5 mM glucose) media to give final concentrations stated and added to cells for the indicated amounts of time. Control cells with no EGCG treatment had equal amounts of DMSO added to media to control for any DMSO-mediated effects. For cisplatin treatment, cells were serum-starved overnight and then incubated with the stated concentrations of cisplatin and EGCG. After 24 h media was removed and cells washed with fresh DMEM media to remove traces of cisplatin. Media containing EGCG was then added for a further 48 h before cells were analysed.

DNA fragmentation assay. For DNA fragmentation analysis cells were lysed in buffer (10 mM Tris-HCl, 10 mM EDTA, 0.5% (v/v) Triton X-100, pH 8.0). RNA was digested using RNase (0.1 mg/ml at 37˚C for 1 h) followed by proteinase K treatment for 2 h at 50˚C. DNA was extracted and precipitated out using phenol, chloroform and isoamyl alcohol. One microgram of DNA was then electrophoretically separated on a 1.8% agarose gel containing ethidium bromide (10 μg/ml) and visualized using a UV illuminator (Minibis, DNR Bio-Imaging Systems).

Cell proliferation analysis. Cell proliferation was measured using trypan blue. Cells were seeded in 12-well plates (70,000 cells per well) and treated with EGCG/cisplatin as stated. Cells were then trypsinised, pelleted and re-suspended in an appropriate volume of media containing equal amounts of 0.4% (w/v) trypan blue. Cells were then counted using a haemocytometer.

Acridine orange staining. Cells were seeded and grown on coverslips in 6-well plates and then treated with EGCG for 48 h as described previously. Cells were then fixed using 90% methanol for 15 min and air dried. For staining, slides were dipped in fresh phosphate buffer [0.66% (w/v) potassium phosphate monobasic + 0.32% (w/v) sodium phosphate dibasic, pH 6.4-6.5) and stained in a solution of acridine orange (0.12 mg/ml in phosphate buffer) for 2 min. Slides were washed three times in phosphate buffer, then placed on microscope slides and the morphology of the nuclei analysed and scored immediately using a fluorescence microscope (x40 objective lens, Nikon Eclipse 80i). Nuclei and micronuclei (DNA) were stained yellow/green and the cytoplasm was stained red. Relative proportions of normal, apoptotic nuclei and micronuclei were determined and recorded in a total of at least 2,000 cells per treatment. The experiment was repeated three times.

Sub-G1 cell cycle analysis. To identify and quantify the sub-G1 cell population of cells that would correspond with cells undergoing apoptosis cells were analysed using flow cytometry. Cells were trypsinized, pelleted and resuspended in PBS. Cells were then fixed in 70% ice-cold ethanol and stored at -20˚C overnight. Ethanol was then removed and cells washed with PBS before being resuspended in S-phase buffer (4 mM sodium citrate, 0.1% Triton X-100, 50 μg/ml propidium iodide, 500 μg/ml RNase A) and incubated in the dark for 20 min before being analysed on the flow cytometer (C6, Accuri). Control cells with no treatment were used to initially optimise and model collection gating conditions used to detect the sub-G1 cell population. All gates were then constant throughout subsequent analyses. Additionally during analysis the sub-G1 marker (M1) was placed based on control cells (n=16) and was kept constant for all other treated cells (n=4). Raw data were analysed and histograms constructed using Cyflogic v.1.2.1 software. The relative proportion of cells in the sub-G1 population was then recorded. Data from four independent experiments were recorded.

Reverse transcription and standard PCR. RNA was isolated using TRI reagent per manufacturer's instructions. RNA was then quantified using Nanodrop spectrophotometer (Thermo Fischer Scientific, Newark, DE, USA). One microgram of PC3 total RNA was reverse-transcribed using MMLV transcriptase (Promega) and random primers as the priming agent. After 50 min of incubation at 42˚C, the reactions were stopped by heating at 70˚C for 15 min. To evaluate the expression of endogenous caspase 9 splice variants, an upstream 5' forward primer in exon 2 of caspase 9 (5'-GCTCTTCCTTTGTTCATC-3') and a 3' reverse primer in exon 7 (5'-CATCTGGCTCGGGGTTA-3') were used. PCR was carried out using GoTaq HotStart Polymerase (Promega) as per manufacturer's recommendations and 5 ng/μl cDNA (thermal cycling conditions: 95˚C for 2 min, followed by 33 cycles of 95˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec, and a final extension step of 72˚C for 5 min. The PCR product was examined by electrophoresis using 2% (w/v) agarose gel electrophoresis. The agarose gel was then analysed using a UV illuminator (Minibis, DNR Bio-Imaging Systems). To control for potential loading differences between samples and to assess whether there was a shift in alternative splicing of caspase 9 the ratio of caspase 9a/9b was assessed by measuring the area of each PCR peak using ImageJ software. The ratio between bands was then determined and expressed for each sample.

Real-time PCR. Quantitative PCR was performed using 2x SensiFAST SYBR Hi-ROX master mix (Bioline) using primers at 300 nM concentration on StepOne Plus Real-Time PCR System (Applied Biosystems, Warrington, UK). Primers were designed to span at least one exon boundary using the Primer Express 2.0 software (Applied Biosystems) and were purchased

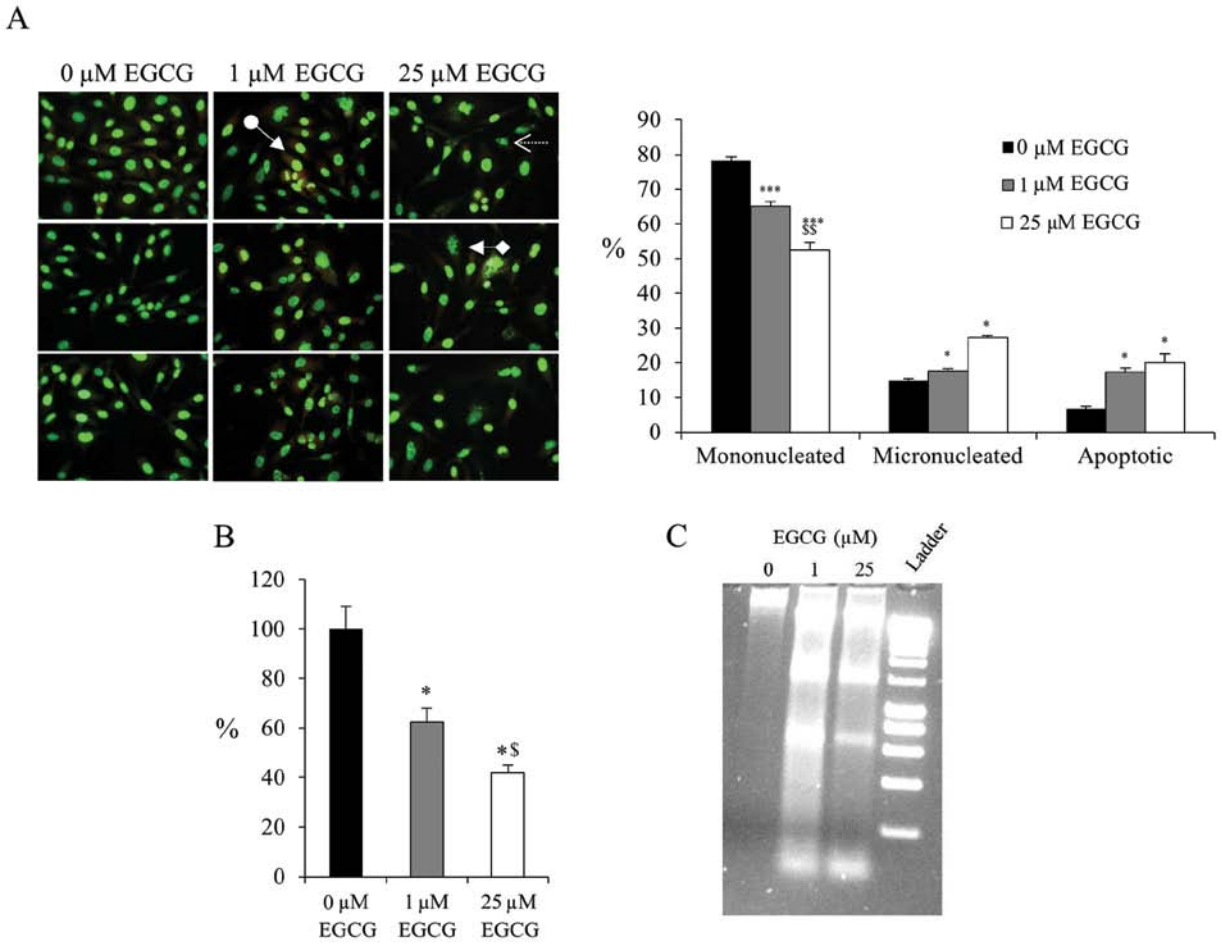

Figure 1. (A) PC3 cells were stained with acridine orange after 48 h incubation with EGCG; representative panels are shown. The arrow indicates apoptotic cells (rounded, ○; nuclear blebbing, ◇) and the arrowhead indicates the presence of micronuclei. The relative proportions of normal intact nuclei, micronuclei bodies and disorganised/apoptotic nuclei was calculated in at least 2,000 PC3 cells over three independent experiments. (B) Percentage of surviving PC3 cells identified using trypan blue after 48-h incubation with EGCG (n=3). (C) DNA fragmentation analysis showing induction of apoptosis of PC3 cells incubated with EGCG for 24 h. EGCG (0 μM) vs 1 μM/25 μM EGCG: ***p<0.001; *p<0.05. EGCG (1 μM) vs 25 μM EGCG: $^{\$\$}$p<0.01; $^{\$}$p<0.05. Data are presented as mean ± SEM (n=3 for each group).

from Sigma-Genosys (Haverhill, UK). For caspase 9a analysis a forward primer located across exon 2/3 boundary (5'-AGTGG ACATTGGTTCTGGAG) and a reverse primer located in exon 4 (5'-CTTCTCACAGTCGATGTTGG-3') were used. For caspase 9b analysis a forward primer located across exon 2/7 boundary (5'-TGGTGATGTCGAGCAGAAAG) and a reverse primer located in exon 4 (5'-CTGGTCGAAGGTCCTCA AAC-3') were used. To evaluate 18S rRNA (accession no. NR_003286.2) expression a forward primer (5'-ACCCGTTGA ACCCCATTCGTGA-3') and reverse primer (5-GCCTCACTA AACCATCCAATCGG'-3') were used. PCR reaction conditions were 20 sec at 95˚C then 40 cycles of; 3 sec at 95˚C and 30 sec at 60˚C. In addition a melting curve analysis was performed to check for multiple products and primer-dimer amplification. Melting curve analyses were conducted by a stepwise decrease in temperature from 95 to 60˚C over a 35-min period with measurement of total SYBR-Green fluorescence every 1˚C. Fold changes in expression were calculated by using a standard curve method according to established methods (23,24). Gene expression was normalised to the corresponding 18S value for each sample.

Statistical analysis. Data from experiments are presented as mean ± standard error mean (SEM), with number of replicates stated in figure legends. Statistical significance was tested using a paired two-tailed Student's t-test assuming equal variance.

Results

Several studies have used very high concentrations of EGCG, these are arguably unrealistic in terms of what could be administered to a patient. With this in mind, we decided to examine the effects of lower concentrations, 1 and 25 μM EGCG on cell survival and apoptosis in PC3 cells, a widely studied androgen-independent prostate cancer cell line. Acridine orange staining of cells treated for 48 h revealed a significant increase in the proportion of cells with micronuclei (indicative of DNA fragmentation) and of apoptotic bodies (Fig. 1A). A total of 1 μM EGCG was sufficient to cause a significant increase in the proportion of apoptotic bodies and reduced cell survival (Fig. 1B). The presence of increased DNA fragmentation, a characteristic of apoptosis, was confirmed by agarose gel electrophoresis (Fig. 1C).

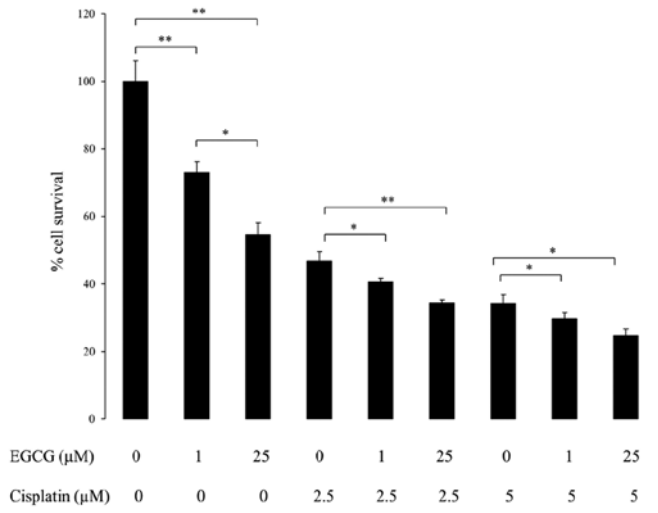

Figure 2. Percentage of surviving PC3 cells as identified using trypan blue after 24-h of cisplatin (Cis)/EGCG treatment followed by a further 48 h of EGCG treatment. $^{**}p<0.01$; $^{*}p<0.05$. Data are presented as mean ± SEM (n=3 for each group).

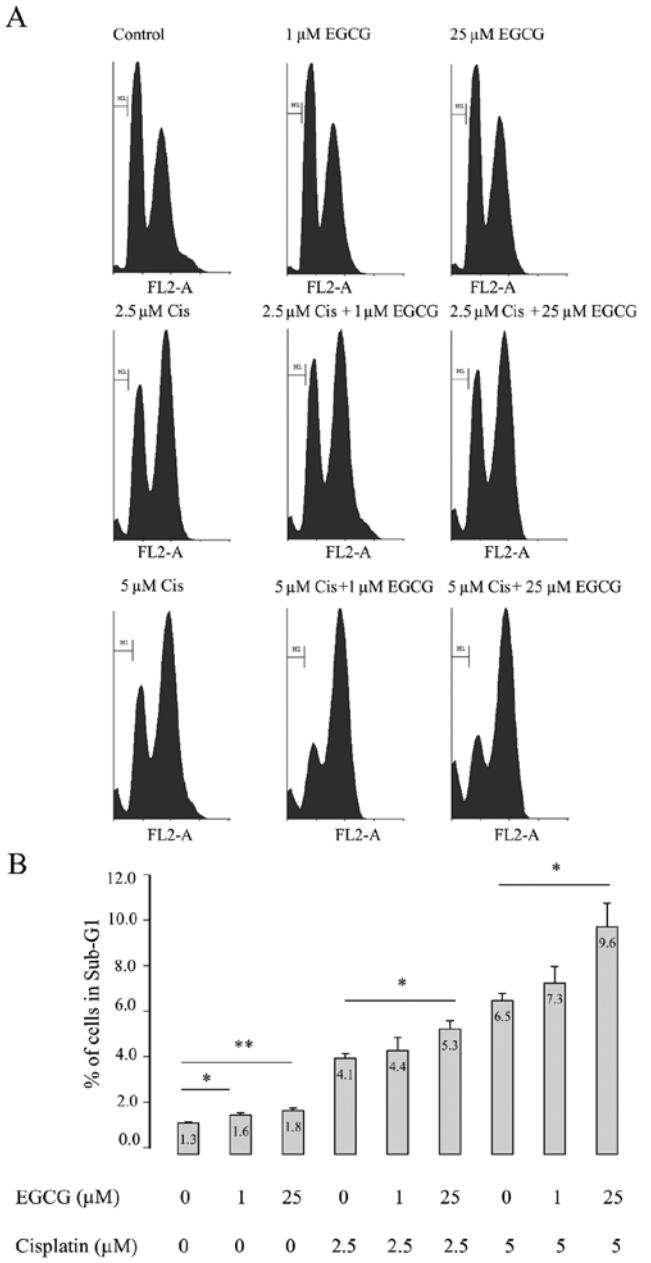

Figure 3. (A) Representative DNA histograms of cell count against FL2-A (FL2-Area: total DNA fluorescence) for propidium iodide stained PC3 cells after 24 h of cisplatin/EGCG treatment followed by a further 48-h incubation with EGCG. The marker, M1, was placed in order to determine the percentage of cells within the sub-G1 region. (B) The percentage of cells in sub-G1 phase was calculated using Cyflogic v.1.2.1 software. $^{**}p<0.01$; $^{*}p<0.05$. Data are presented as mean ± SEM (n=4 for each group).

It is important to assess the ability of EGCG to promote apoptosis in combination with a drug such as cisplatin, known as the 'penicillin of cancer' due to its widespread use. Cisplatin is a platinum-based alkylating-like drug that crosslinks DNA, causing apoptosis. PC3 cells were treated initially for 24 h with combinations of EGCG and cisplatin, and then for a further 24 h with EGCG alone (Fig. 2). A significant drop in cell survival was observed in response to the lower concentration, 1 μM EGCG. As expected, cisplatin promoted apoptosis at 2.5 and 5 μM. The effect of cisplatin was augmented when combined with EGCG treatment suggesting that EGCG synergises with cisplatin to promote apoptosis.

In order to confirm that EGCG might enhance the apoptosis promoting effects of cisplatin, flow cytometry was used in order to analyse the sub-G1 region. This method exploits the fact that after fixation, fragmented DNA leaks out of apoptotic cells. The dye propidium iodide was used to determine the proportion of cells in the sub-G1 region (Fig. 3A). The raw data were imported into the Cyflogic analysis software tool and histograms produced showing the distribution of the cells within the cell cycle. Control untreated PC3 cells were used to model gating parameters and the sub-G1 marker was set-up for all further analyses. Using the statistics tool the number and percentage of cells within the sub-G1 region was recorded for all treatments. EGCG treatment alone resulted in significantly increased percentage of cells in the sub-G1 region. As expected cisplatin treatment further increased the percentage of cells within the sub-G1 region. The combination of cisplatin and 25 μM EGCG further increased the percentage of cells within the sub-G1 region confirming that EGCG potentiates apoptosis in combination with cisplatin.

Having established that EGCG and cisplatin together promote apoptosis in PC3 cells, the alternative splicing of caspase 9 was examined using RT-PCR, initially using a forward primer in exon 2 and a reverse primer in exon 7 (Fig. 4A). Caspase 9a (full length caspase 9) expression was significantly increased with EGCG alone (even with 1 μM). Cisplatin alone also significantly increased caspase 9a; and a combination of 2.5 μM cisplatin and 25 μM EGCG increased the ratio of caspase 9a to 9b 5-fold (Fig. 4B). As standard PCR can be notoriously unreliable in terms of the quantification of splice variants, we developed qPCR primers that are specific to the caspase 9 splice isoforms. The qPCR data confirmed the findings (Fig. 4C), indicating that EGCG alone results in a shift of the alternative splicing of caspase 9 towards the pro-apoptotic isoform. The combination of 25 μM EGCG and cisplatin (2.5 and 5 μM) further increased the ratio of caspase 9a to 9b.

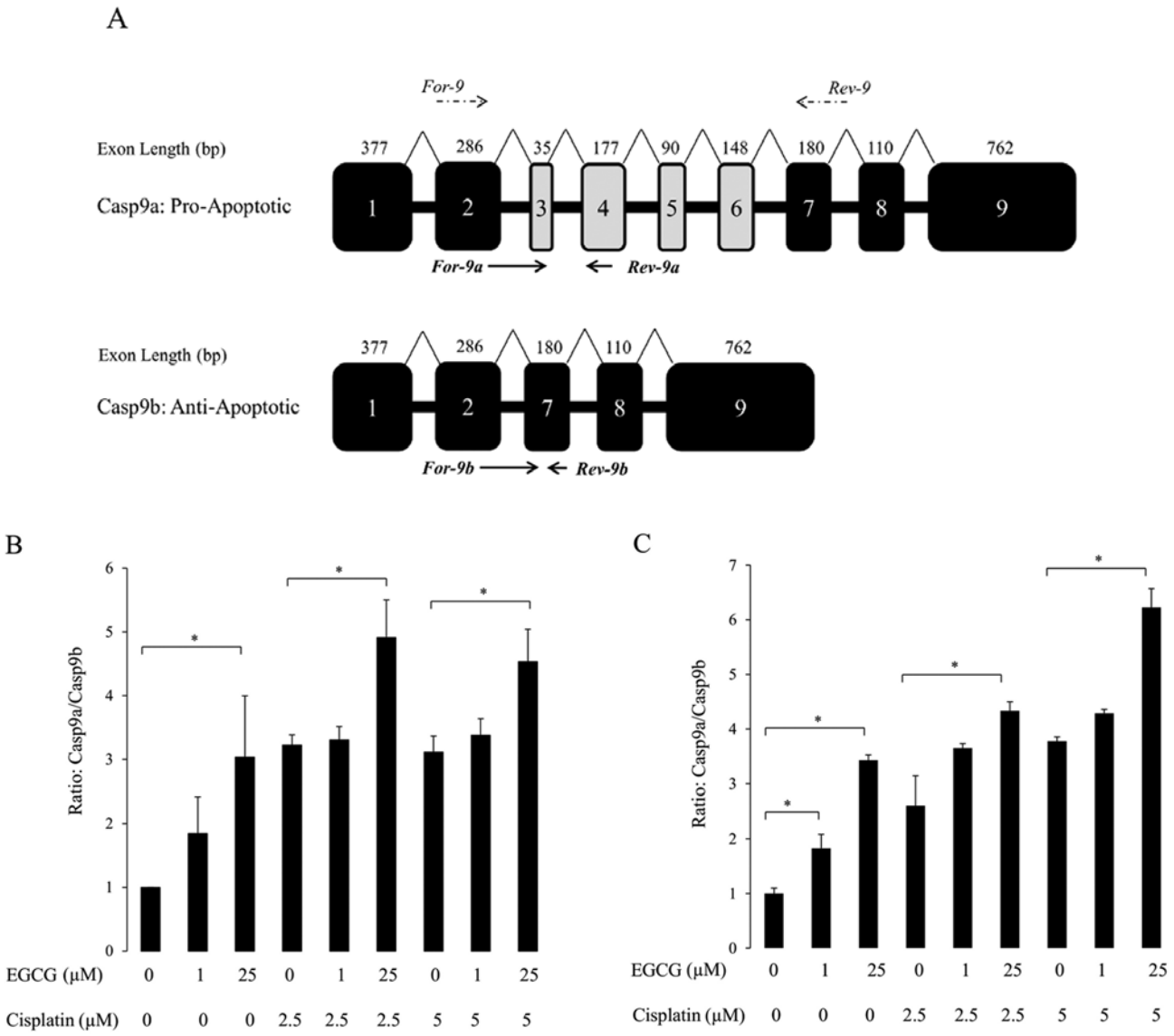

Figure 4. (A) Schematic representation of exon composition of caspase 9a and caspase 9b splice isoforms. Dashed lines indicate where primers lie for reverse transcriptase PCR and solid lines indicate where primers lie for real-time PCR analysis. (B and C) Ratio of caspase 9a to caspase 9b was determined in PC3 cells after 24-h of cisplatin/EGCG treatment followed by a further 48-h incubation with EGCG after analysis by reverse transcriptase PCR and real-time PCR, respectively. *p<0.05. Data are presented as mean ± SEM (n=3 for each group).

Discussion

The flavonoids are a class of natural compounds that have attracted considerable interest. Nutritionists estimate that the average intake of flavonoids by humans in a normal diet is 1-2 grams per day (25). Flavonoids are absorbed from the gastrointestinal tract (26,27), whereas medical flavonoids are administered directly to the diseased tissue if accessible; for example in the skin or the throat, or along a route leading immediately to the target, such as the nasal or the vascular systems (25). Therefore a compound such as EGCG is worth considering in terms of potential use in adjuvant therapy.

We find that relatively low concentrations of EGCG (as little as 1 μM) are able to induce apoptosis in the PC3 prostate cancer cell line. Other studies have demonstrated that EGCG inhibits proliferation with IC_{50} values of 39-100 μM (28-30). In this study we show that EGCG concentrations of as little as 1 μM can inhibit proliferation and that 25 μM EGCG resulted in 60% reduction in survival of PC3 cells. These concentration discrepancies may be the result of different treatment times used and general cell culture conditions, but suggest that even low doses of EGCG may have a substantial negative impact on prostate cancer cell survival. These results were further confirmed using acridine orange staining (for the detection of micronuclei and apoptotic bodies), DNA fragmentation and flow cytometry. EGCG also appeared to potentiate the apoptosis-promoting effects of cisplatin. It has been reported that plasma concentrations of cisplatin in patients that have undergone a drip infusion range from 3.0-10.3 μM (or 0.9-3.1 μg/ml) (31). Therefore, the range of cisplatin concentrations used in this study is comparable to therapeutic ranges experienced by patients. Interestingly, previous study has shown that EGCG

and cisplatin have the potential to work together to enhance apoptosis and increase cisplatin toxicity in ovarian cancer cells (32). The drug oxaliplatin, a third generation platinum drug, has also been shown to synergise with EGCG, curcumin and other phytochemicals in the context of ovarian cancer cell lines (33). In this study we show that EGCG may work synergistically with cisplatin to increase apoptosis and reduce the proportion of the anti-apoptotic splice variant caspase 9b.

As is the case with all phytochemicals, any beneficial effects of EGCG are likely to depend on its bioavailability, metabolism and dose. A recent human study demonstrated that after intake of brewed green tea EGCG was found in prostate tissue suggesting that there is bioavailability of EGCG and potential for EGCG to be active in the prostate after oral consumption (34). However it should be noted that an excessive intake of EGCG may be toxic (35). Furthermore, EGCG has been shown to interact directly with specific drugs such as sunitinib, reducing their bioavailability (36). With these caveats in mind, a recent study has shown that nanoparticle-mediated delivery of a compound such as EGCG could enhance its bioavailability while limiting any unwanted toxicity (37). These findings suggest that the administration of EGCG together with cisplatin to patients might prove to be an effective adjuvant therapy for prostate cancer.

Previous studies have indicated that polyphenolics including EGCG might cause changes in alternative splicing of several genes (16-18). In this study we confirm these observations by showing that EGCG can cause a significant alteration in caspase 9 alternative splicing, favouring the expression of the active protease. There could be several mechanisms through which EGCG might achieve this effect. One is by altering the expression of specific splice factors (16). Alternatively, EGCG might affect the expression and activity of protein kinases (such as SRPK1, Clk/Sty) and phosphatases (such as PP1) that modify the localisation and activity of splice factors (18). It is also possible that EGCG does not directly modify the machinery of alternative splicing; but rather, alternative splicing changes as a result of the initiation of apoptosis. However, the fact that EGCG also modifies the alternative splicing of genes that are not directly involved in apoptosis such as IKAP (17) would argue in favour of a more generalised effect on alternative splicing.

Further research is required to determine the precise mechanism through which EGCG modifies alternative splicing. The possibility that EGCG might modify alternative splicing directly is of interest to the alternative splicing field and warrants further research. Many other pre-mRNAs are differentially spliced in cancer and therefore EGCG could cause, in specific contexts, additional beneficial changes in splicing patterns of apoptotic and other cancer-associated genes. In the future, it is likely that highly sophisticated approaches will be used to target and manipulate the use of specific splice sites in caspase 9 and other apoptotic genes using modified antisense oligonucleotides; and small molecule inhibitors will target splice factors and splice factor kinases such as SRPK1 that are involved in the regulation of alternative splicing (38). However, at the same time, it is clearly worth exploring the potential of readily available phytochemical compounds such as ECGG to achieve therapeutically desirable changes in splice isoform expression.

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