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Apoptogenic effects of black tea on Ehrlich's ascites carcinoma cell

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Next to water, tea is the most ancient and widely consumed beverage in the world. Epidemiological studies have suggested a cancer protective effect, but the results obtained so far are not conclusive. In the current study, mechanisms of the apoptogenic effect of black tea extract were delineated. Black tea administration to Ehrlich's ascites carcinoma (EAC)-bearing Swiss albino mice caused a significant decrease in the tumor cell count in a dose-dependent manner. Flowcytometric analysis showed an increase in the number of cells in the sub-G_0/G_1 population signifying tumor cell apoptosis by black tea. These results were further confirmed by nuclear staining that demonstrated distinct morphological features of apoptosis. Our data also revealed an increase in the expression of pro-apoptotic protein p53 in EAC. It is known that upon p53 induction, multiple downstream factors contribute to the decision making between growth arrest and apoptosis. Among those, pro-apoptotic gene Bax is up regulated during p53-mediated apoptosis. On the other hand, p53-mediated growth arrest involves p21 as a major effecter. In our system, increase in p53 expression was followed by moderate expression of p21/Waf-1 and high expression of Bax at protein levels. Interestingly, anti-apoptotic protein Bcl-2 was down regulated resulting in decrease in Bcl-2/Bax ratio. All these observations together signify that black tea-induced apoptogenic signals overrode the growth-arresting message of p21, thereby leading the tumor cells towards death.

Introduction

Cancer chemoprevention can be defined as the prevention, inhibition or reversal of carcinogenesis by administration of one or more chemical entities, either as individual drugs or as naturally occurring constituents of the diet. Next to water, tea is the most ancient and widely consumed beverage in the world. Epidemiologically green tea has been shown to possess cancer chemopreventive effects in a wide range of target tumors in rodents (1,2). Black tea has also been shown to inhibit tumorigenesis in animal model systems, including lung (3), colon (4) and skin (5). However, besides some scattered reports and supporting epidemiological evidences on the protective role of black tea, the detailed molecular mechanisms underlying its anticancer effect are still unclear and inconclusive although the worldwide production and consumption of black tea far exceeds that of green tea (6).

It is now well recognized that apoptosis is a mode of cell death used by multi-cellular organisms to eradicate cells in diverse physiological and pathological settings. Recent evidence also shows that suppression of apoptosis by tumor-promoting agents in pre-neoplastic cells is an important mechanism in tumor promotion (7). In this context, it is noteworthy that apoptosis-inducing ability seems to have become a primary factor in considering the efficacy of chemo-preventive agents.

Among the positive and negative regulators of apoptosis, p53, the tumor suppressor gene, is an important defence against cancer as it suppresses tumor growth through two mechanisms, cell cycle arrest and apoptosis (8–10), each of which operates in a distinct context. An important function of wild-type p53 is to act as a transcription factor by binding to a p53-specific DNA consensus sequence in responsive genes, which would be expected to increase the synthesis of p21/Waf-1 (11). p21 is a potent inhibitor of cell cycle regulatory cyclin-dependent kinases (12), and thus up-regulation of p21 results in the inhibition of cell by blocking cell cycle progression. Bax, the pro-apoptotic member of Bcl-2 family, is a p53 target and is transactivated in a number of systems during p53-mediated apoptosis (13). The up-regulation of Bax expression and down-regulation of Bcl-2 have been well demonstrated during apoptosis (14). All these reports indicate that a cross talk between these pro- and anti-apoptotic proteins is one of the important factors deciding the fate of a cell. However, the role of black tea in regulating the balance between these pro- and anti-apoptotic factors in tumor cells is not yet revealed.

Here we report the dose-dependent apoptogenic effect of black tea on Ehrlich's ascites carcinoma (EAC) cells as grown in the peritoneal cavity of Swiss albino mice. In the present study, we addressed the relevance of p53 and its downstream effectors p21, Bax and Bcl-2 in black tea-induced EAC cell apoptosis. We suggest that such a multi-marker analysis of apoptosis pathways could be useful for individualization of therapeutic strategies in the future.

Materials and methods

Materials

RPMI 1640 medium, fetal bovine serum, streptomycin and penicillin were purchased from Gibco BRL (Gaithersburg, MD). RNase A, 4′,6′-diamidino-2-phenylindole (DAPI), and general reagents were purchased from Sigma (St Louis, MO). Black tea leaf was obtained from Lipton Tea Co., India. Cycle TEST PLUS DNA reagent kit, AnnexinV-fluorescein isothiocyanate (FITC) and Apo-Direct kit were procured from Becton Dickinson Immunocytometry system (San Jose, CA). Polyclonal anti-Bax, anti-Bcl-2, anti-p21, anti-p53, anti-CD3/CD14/CD19/CD56 antibodies, HRP- and FITC-conjugated goat anti-rabbit antibodies were obtained from Pharmingen (San Jose, CA). The remaining chemicals were purchased from local firms (India) and were of highest purity grade.

Black tea preparation

Black tea leaves were brewed in hot water (w/v) to obtain black tea extract. Animals were given either drinking water or black tea extract as sole source

Abbreviations: DAPI, 4′,6′-diamidino-2-phenylindole; EAC, Ehrlich's ascites carcinoma; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; MNC, mononuclear cell; PI, propidium iodide.

of drinking fluid at doses of 0.625, 1.25, 2.5, 3.75 and 5% (w/v) 1 week before tumor inoculation until death.

Mice and tumor models

All animal experiments were performed following 'Principles of laboratory animal care' (NIH publication No. 85-23, revised in 1985) as well as specific Indian laws on 'Protection of Animals' under the prevision of authorized investigators. Swiss albino mice (~20 g each; 10 mice in each group) were randomly divided into different groups including: (i) normal set (non-tumor-bearing); (ii) tumor-bearing set (which were intra-peritoneally injected with 1×10^5 exponentially grown EAC; and (iii) black tea-treated tumor-bearing set. Untreated mice received drinking water instead of black tea. Lymphocytes from spleen and EAC cells from peritoneal cavity were collected, freed from adherent cells and viable cells were counted by Trypan Blue exclusion test. The non-adherent splenic cell populations were subjected to Ficol-hypaque density gradient separation to obtain mononuclear cell (MNC)-rich population (15,16).

Isolation of EAC from mice peritoneal cavity

The EAC cells were isolated from the peritoneal cavity of tumor-bearing mice (control or treated). Two to three milliliters of sterile PBS was injected into the peritoneal cavity of the mice and the peritoneal fluid containing the tumor cells was withdrawn, collected in sterile Petri dishes and incubated at 37°C for 2 h. The cells of macrophage lineage adhered to the bottom of the Petri dishes. The non-adherent population was aspirated out gently and washed repeatedly with PBS. EAC cells were then separated from other non-adherent contaminating cells by FACS (fluorescence-activated cell sorter). More than 98% of this separated cell population was CD3 (T cells)/CD14 (macrophages)/CD19 (B cells)/CD56 (NK cell) negative as was determined by flowcytometer. Moreover, these cells were morphologically characterized as EAC by Wright staining (17) and viability was assessed to be >95% by Trypan Blue dye exclusion. The viable EAC cells were processed for further experiments.

Detection of apoptosis by flowcytometry

For the determination of cell cycle phase distribution, EAC cells harvested from tumor-bearing mice were permeabilized and nuclear DNA was labeled with propidium iodide (PI) using Cycle TEST PLUS DNA reagent kit. Cell cycle phase distribution of nuclear DNA was determined on FACS, fluorescence detector equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using CellQuest software (Becton Dickinson). A total of 10 000 events was acquired and analysis of flowcytometric data was performed using ModFit software. A histogram of DNA content (*x*-axis, PI-fluorescence) versus counts (*y*-axis) has been displayed.

To distinguish between apoptosis and necrosis, in a double labeling system, EAC cells (1×10^6 cells in each case) from untreated- or black tea-treated tumor-bearing mice were harvested and PI and Annexin V Fluos were added directly to the medium. The mixture was incubated for 15 min at 37°C. Excess PI and Annexin V Fluos were then washed off, cells were fixed and then analyzed on flowcytometer (equipped with 488 nm argon laser light source; 515 nm band pass filter for FITC-fluorescence and 623 nm band pass filter for PI-fluorescence) using CellQuest software. Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. A total of 10 000 events were acquired and the cells were properly gated for analysis.

To confirm the nature of tumor killing by black tea, EAC cells were fixed, permeabilized and incubated with TdT enzyme and FITC-Br-dUTP. Cells were washed, incubated with PI/RNase solution and analyzed on FACS. Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. A dot plot of PI-fluorescence (*x*-axis) versus FITC-fluorescence (*y*-axis) has been displayed (15,16).

Oligonucleosomal fragmentation

For the assessment of chromatin condensation and nuclear blebbing, EAC cells were fixed and nuclear DNA was stained with DAPI (0.2 mg/ml for 15 min at room temperature). A Lica model DM 900 fluorescent microscope was used to visualize apoptotic cells. The filter cube A was used to detect the signal from DAPI. Digital images were captured with cool (–25°C) CCD camera controlled with MetaMorph software (Universal Imaging, PA, USA) (18).

Flowcytometric analysis of expression of pro- and anti-apoptotic proteins

For the determination of the expression of pro-apoptotic proteins p53, p21 and Bax or anti-apoptotic protein Bcl-2, EAC cells from mice were fixed and permeabilized as described earlier. Cells (1×10^6) from each group were incubated either with polyclonal anti-p53 or anti-p21 or anti-Bcl-2 or anti-Bax (1 μg/ml) antibody for 1 h at room temperature, and then with FITC-conjugated goat anti-rabbit antibody. Cells were washed thoroughly and analyzed on a Flowcytometer equipped with 488 nm Argon laser light source and a 515 nm band pass filter for FITC-fluorescence. A total of 10 000 events were acquired for analysis using CellQuest software and histogram plot of FITC-fluorescence (*x*-axis) versus counts (*y*-axis) has been shown in logarithmic fluorescence intensity.

Western blotting

For western blot analysis of p53, p21, Bax and Bcl-2, EAC lysate was loaded into a 10% SDS–polyacrylamide gel. After electrophoresis the gel was transferred to nitrocellulose membrane and blocked with non-fat milk in PBS containing Tween-20 prior to antibody treatments. The protein of interest was visualized with chemiluminescence. The blot was then stripped and after extensive washing, the blot was used again for probing the next molecule. Equal loading of protein in each lane was confirmed by probing with α-actin antibody.

Results

Effect of black tea on tumor and splenic cell number

We evaluated the effect of black tea on the numbers of EAC cells in black tea-treated or untreated tumor-bearing mice. Mice who have been given black tea as the sole source of drinking fluid showed a decrease in the number of tumor cells over their control counterparts. It was observed that black tea lessened the tumor burden considerably in a dose-dependent manner showing significant effect at 2.5% (w/v). At day 21 a total of 460×10^6 EAC cells were measured in the peritoneal fluid of untreated mice, whereas in 2.5% dose black tea-treated group only 80×10^6 EAC cells were found (Table I). Black tea restored the depressed splenic MNC number in a dose-dependent manner showing maximum effect at 2.5%. Interestingly, at doses above these, a simultaneous decrease in splenic cell number was again observed. These results signified that beyond the dose 2.5%, black tea extract is either imparting some immunotoxic effects in host by itself or is failing to ameliorate the tumor-related immunosuppression. Therefore, further studies were performed using 2.5% black tea extract.

Flowcytometric analysis of EAC cell cycle phase distribution

To find out the mechanism of tumor cell killing by black tea extract we exploited FACS and analyzed tumor cell cycle phase distribution. The FACS data described the effect of black tea extract on cell cycle phase distribution of EAC DNA. On day 21 after EAC inoculation, at 2.5% dose, the content of hypoploid DNA (8.1% before treatment, Figure 1A) was increased (45.8%, Figure 1B), DNA content in G_0/G_1 phases (38.2 versus 19.3%, Figure 1A versus B) as well as in S and G_2/M (31.2 versus 12.8%, Figure 1A versus B) phases decreased. These results suggested the breakdown of EAC DNA resulting in tumor killing. The obvious ramifications were the growth arrest of EAC.

Black tea induces apoptosis, not necrosis

To understand the nature of cell death, we utilized double labeling techniques using Annexin V Fluos/PI to distinguish between apoptotic and necrotic cells. In the early stages of apoptosis, while the cell membrane is still intact and impermeable to PI, the DNA-binding dye, phosphotidylserine to which Annexin V binds specifically, is translocated to the extra-cellular leaflet of the membrane. In contrast, during necrosis, because the cell membrane is ruptured, these cells take up both the fluorochrome. Our flowcytometric data revealed that, in comparison with control untreated EAC cells (Figure 1C), black tea-treated unfixed EAC cells showed Annexin V-FITC-binding but no PI staining (Figure 1D) indicating that the mode of cell death is apoptosis but not necrosis. These results indicated that black tea could increase

Table I. Effect of black tea on EAC and splenic MNC numbers in tumor-bearing mice

Doses of black tea (w/v)	EAC number ($\times 10^6$)		Splenic MNC number ($\times 10^6$)	
	1st week	2nd week	3rd week	3rd week
0.00%	110.02 ± 7.51	312.00 ± 15.34	460.00 ± 38.51	81.2 ± 8.2
0.625%	90.06 ± 7.32	188.04 ± 8.94	230.00 ± 12.10	103.3 ± 10.4
1.25%	77.03 ± 5.96	164.60 ± 12.38	146.00 ± 4.40	125.2 ± 12.3
2.50%	52.60 ± 4.88	94.06 ± 4.58	80.00 ± 3.04	153.0 ± 8.9
3.75%	44.06 ± 8.63	82.04 ± 11.26	57.00 ± 10.12	130.0 ± 22.4
5.00%	31.08 ± 9.46	60.28 ± 7.34	46.00 ± 2.10	107.8 ± 12.2

Swiss albino mice were intra-peritoneally injected with 1×10^5 EAC. After 1 week, various doses of black tea were administered orally. Control mice received drinking water as carrier vehicle. At the end of each week viable EAC cells and splenic MNCs were counted. Each group contained 10 mice and the values are mean ± SEM. Normal mice contained 164 ± 10.2×10^6 and black tea-treated normal mice contained 158 ± 5.6×10^6 splenic MNCs.

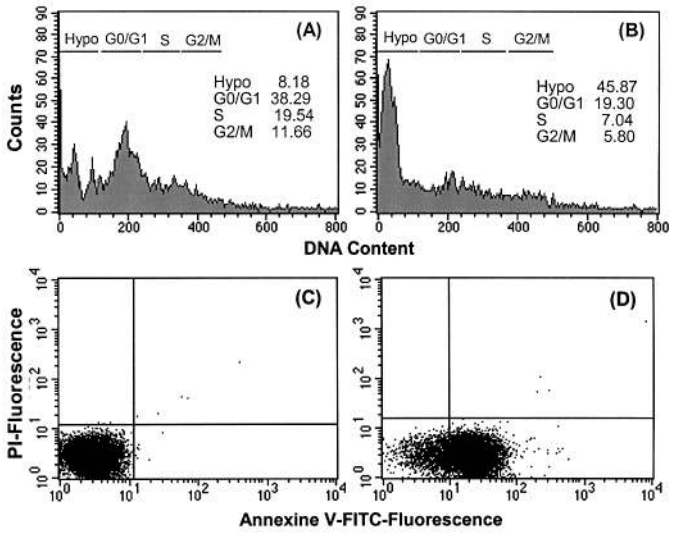

Fig. 1. Flowcytometric detection of EAC apoptosis. (**A** and **B**) Analysis of EAC cell cycle phase distribution. EAC cells from tumor-bearing mice orally fed with drinking water as carrier vehicle (A) or black tea (B) were fixed and nuclear DNA was labeled with PI. Cell cycle phase distribution of EAC nuclear DNA was determined by single label flowcytometry. Histogram display of DNA content (*x*-axis, PI-fluorescence) versus counts (*y*-axis) has been shown. (**C** and **D**) Distinguish between apoptosis and necrosis. In a double label system, unfixed EACs from control (C) or black tea-treated (D) tumor-bearing mice were labeled with PI and Annexin V Fluos and then fixed and analyzed on a Flowcytometer. Dual parameter dot plot of FITC-fluorescence (*x*-axis) versus PI-fluorescence (*y*-axis) has been shown in logarithmic fluorescence intensity. Quadrants: lower left, live cells; lower right, apoptotic cells; upper right, necrotic cells.

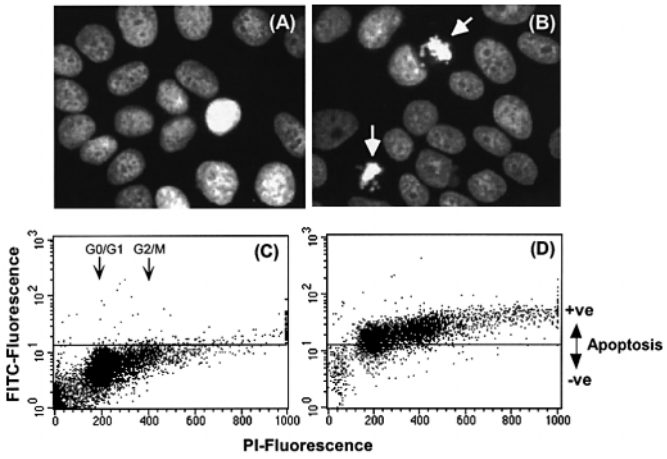

Fig. 2. Detection of black tea-induced EAC apoptosis. For the determination of oligonucleosomal fragmentation and nuclear blebbing (arrowhead) EAC cells of (**A**) drinking water- or (**B**) black tea-treated mice were stained with DAPI and visualized under microscope. In a double label system, EAC nuclear DNA of (**C**) drinking water- or (**D**) black tea-treated mice was labeled with FITC-conjugated dUTP and PI. Apoptotic cells (FITC-dUTP positive) were analyzed flowcytometrically. Dot plot display of PI-fluorescence (*x*-axis; linear scale) versus FITC-fluorescence (*y*-axis; logarithmic scale) has been displayed.

apoptosis in EAC cells. It also suggested that necrosis was not the cause of cellular killing in EAC.

Black tea induces tumor killing by apoptosis

Phenotypically, apoptosis is characterized by cell shrinkage, chromatin compaction, plasma membrane blebbing, DNA fragmentation and collapse of the cell into small intact fragments (apoptotic bodies). We observed that black tea treatment caused DNA fragmentation (Figure 2A and B) in EAC indicating that black tea induces apoptosis in EAC. To confirm the nature of cell death, we utilized TUNEL assay method in which FITC-conjugated dUTP was incorporated in to the DNA strand breaks due to apoptosis by terminal deoxynucleotidyl transferase. Our flowcytometric data revealed that, in comparison with control untreated EAC cells (Figure 2C), the tumor cells from black tea-treated mice underwent appreciable amount of apoptosis (Figure 2D). These findings confirmed that black tea-induced EAC killing is due to apoptosis.

Effect of black tea on the expression of pro- and anti-apoptotic proteins

After confirming that black tea induces EAC apoptosis, we next attempted to unveil the mechanism of EAC killing. As it is well recognized that various pro- and anti-apoptotic proteins play a crucial role in programmed cell death, we examined whether or not black tea has any effect on the expression of growth-arresting and pro-apoptotic proteins, p53, p21 and Bax as well as anti-apoptotic protein, Bcl-2, in our mice model. Using flowcytometric technique, we observed that in EAC, the levels of p53 and Bax expression increased significantly (Figures 3A and 4B) and the level of p21 increased moderately (Figure 3B) after black tea treatment. Interestingly, the level of Bcl-2 decreased upon black tea treatment (Figure 4A) thereby resulting in a decrease in Bcl-2/Bax ratio. Western blot analysis (see inset) further confirmed flowcytometric data and supported the notion that as a result of black tea treatment the balance between positive and negative regulators of apoptosis was shifted towards cell death.

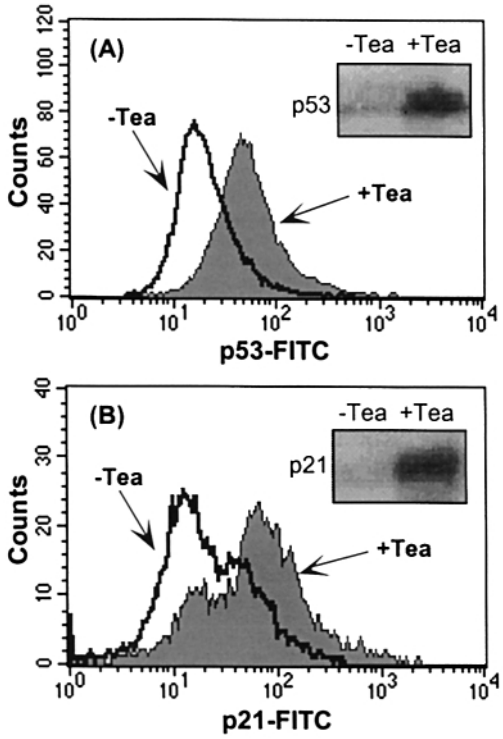

Fig. 3. Effect of black tea on the expression of p53 and p21 in EAC. EAC cells from untreated and black tea-treated tumor-bearing mice were fixed. (**A**) One part of the cells from each group was incubated with anti-p53 antibody and then with FITC-conjugated second antibody. (**B**) Other part of the cells of each group was incubated with anti-p21 antibody/FITC-conjugated second antibody. Cells were then analyzed on a flowcytometer and histogram display of FITC-fluorescence (*x*-axis) versus counts (*y*-axis) has been shown in logarithmic fluorescence intensity. (Inset) EAC lysates (untreated or black tea-treated) were subjected to western blot analysis with either anti-p53 or anti-p21 antibody and visualized with chemiluminescence.

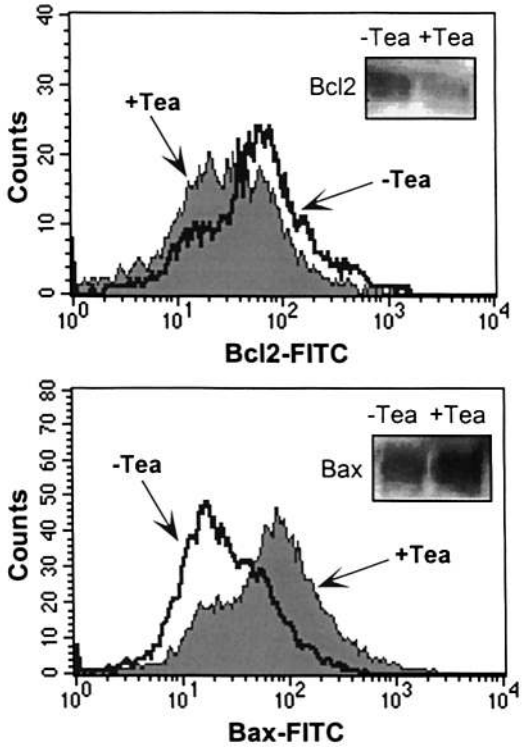

Fig. 4. Effect of black tea on the expression of Bcl-2 and Bax in EAC. EAC cells from untreated or black tea-treated tumor-bearing mice were fixed. (**A**) One part of the cells from each group was incubated with anti-Bcl-2 antibody and then with FITC-conjugated second antibody. (**B**) The other part of the cells from each group was incubated with anti-Bax antibody/FITC-conjugated second antibody. Cells were then analyzed on a flowcytometer and histogram display of FITC-fluorescence (*x*-axis) versus counts (*y*-axis) has been shown in logarithmic fluorescence intensity. (Inset) EAC lysates (untreated or black tea-treated) were subjected to western blot analysis with either anti-Bcl-2 or anti-Bax antibody and visualized with chemiluminescence.

Discussion

Epidemiological surveys and experimental studies have provided evidence that environmental factors, including dietary substances play a major role in the regression of cancer (19). Various findings suggest that tea and its polyphenolic components possess anticancer effect. Tea polyphenols, e.g. epigallocatechin gallate and theaflavins, are the key active components for the inhibition of NF-kappaB activation and thereby account for the antitumor promotion effects of tea in JB6 mouse epidermal cell line (20). On the other hand, a role for different forms of MAPKs in the antitumor effect of green tea polyphenols, especially EGC, in EAC has been reported (21). Black tea extract has been found to effectively inhibit the onset of 7,12-dimethyl-benzanthracene-induced tumorigenesis, cumulative number of tumors and average number of tumors per mouse (22). Moreover, black tea extracts of different cultivars of black tea, viz orthodox, CTC and dust, have been found to increase the life span of EAC-bearing animals (22). Cytotoxic effect of various doses of all three cultivars of black tea was also observed *in vitro* on EAC cells (22). However, unlike green tea, limited studies have been carried out to assess the detailed mechanisms of antitumor effect of black tea. The present set of investigations were therefore initiated to study the antitumorigenic potential of aqueous black tea extract in Swiss albino mice as well as the molecular mechanisms of such effect. Results of the present study suggest that black tea treatment has apoptogenic effect on EAC cells grown in the peritoneal cavity of Swiss albino mice.

A search for a safe agent that enhances the levels of expression of tumor suppressor proteins is a worthwhile but relatively under-explored approach towards cancer chemoprevention. It is nowadays well recognized that apoptosis is a form of cell death characterized by active suicide of cells. Our studies have shown that black tea was effective in imparting growth inhibition, cell cycle deregulation and apoptosis in EAC cells. It is now well recognized that whether a cell becomes committed to apoptosis partly depends upon the balance between proteins that mediate growth arrest and cell death, e.g. p53, p21, Bax and proteins that promote cell viability, e.g. Bcl-2 (23,24). Among these proteins, p53 has been found to facilitate apoptosis in various cell types. In our study black tea increased the p53 expression. It is known that p53 may induce two sets of genes in response to stress signals thereby contributing to the decision making between growth arrest and apoptosis. This tumor suppressor protein may up-regulate the pro-apoptotic protein Bax on the one hand, and/or mediate growth arrest involving p21 as a major

effecter on the other (25). In fact, p53-dependent induction of p21 prevents entry of the cells into S phase (26,27). Our results indicated that p21 level increases after black tea treatment. The other set of gene products downstream to p53, e.g. Bax, induces apoptosis (13). Bax, being a Bcl-2 family member, not only promotes apoptosis but also counters the protective effect of Bcl-2 (28). In fact, over-expression of Bax, an effect that is associated with the formation of Bax/Bax homodimers, has been shown to accelerate the cell death of murine FL5.12 cells after interleukin-3 withdrawal (29). In our system, black tea treatment decreased the expression level of Bcl-2 and increased Bax concentration thereby decreasing the Bcl-2/Bax ratio in these cells. Also, it was evident from various studies, that up-regulation of cell growth-regulating genes, upon p53 induction, may block the cell cycle but increased expression of pro-apoptotic factors can override the growth-arresting message and thereby ultimately leads to apoptosis. In many tumor cell lines, p53 has been found to induce an increased expression of p21 and decreased expression of Bcl-2 during peak of the apoptosis (30). In HNSCC, p53 expression significantly increased the expression of both p21 and Bax, and as the resultant effect, growth arrest and apoptosis took place in these cells (31). It has also been shown that cells that are functionally deficient in p53 or that express elevated levels of Bcl-2, are relatively resistant to chemotherapy-induced apoptosis (32). Here we have demonstrated that black tea induced apoptogenic signal in a p53-dependent pathway in which over-expression of Bax led to tumor cell apoptosis by overriding the growth-arresting message of p21.

All these observations establish the relationship between p53 status, p21 induction, Bcl-2/Bax ratio, cell cycle deregulation and apoptosis in black tea-treated tumor cells. To conclude, these results imply an apoptosis enhancing capability of black tea in EAC that was affected by modulating the tumor cell cycle as well as the balance between pro- and anti-apoptotic factors. The most notable implication of our work is that oral infusion of black tea could result in significant inhibition in progression of cancer in animal model that emulates human disease.

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