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Black tea induces tumor cell apoptosis by Bax translocation, loss in mitochondrial transmembrane potential, cytochrome c release and caspase activation

Arindam Bhattacharyya, Lakshmishri Lahiry, Debaprasad Mandal, Gaurisankar Sa and Tanya Das*

Bose Institute, P-1/12 CIT Scheme VII M, Kolkata, India

Recently the anti-cancer role of black tea has gained immense importance. Nevertheless, the signaling pathways underlying black tea-induced tumor cell death are still unknown. Previously we reported that black tea induces Ehrlich's ascites carcinoma (EAC) cell apoptosis by changing the balance between pro-and anti-apoptotic proteins. It is now well accepted that many cell death pathways converge at the mitochondria to decrease mitochondrial transmembrane potential (MTP) thereby releasing apoptogenic proteins and resulting in the activation of effecter caspases responsible for the biochemical and morphological alterations associated with apoptosis. The role of pro-apoptotic protein, Bax, in initiating mitochondrial death cascade has also been established. Here we demonstrate that in culture black tea extract induces EAC apoptosis in a dose-dependent manner – with IC_{50} at 100 μg/ml. At this dose, intracellular Bax level increases in EAC followed by its translocation from cytosol to mitochondria resulting in loss in MTP. A search for the downstream pathway further reveals that black tea induces mitochondrial cytochrome c release and activates caspases 9 and 3 by 2 pathways, a) independent of and b) dependent on MTP loss. Interestingly, Black tea-induced death signal might probably be amplified through mitochondrial membrane depolarization *via* a feedback activation loop from caspase 3. All these findings indicate that black tea initiates mitochondrial death cascade in EAC cells and thereby results in EAC apoptosis.

Key words: black tea; mitochondrial transmembrane potential; cytochrome c; caspase; apoptosis

Tea (*Camellia sinensis*) is one of the most common beverages consumed worldwide, and the possible beneficial health effects have received a great deal of attention. The inhibitory action of tea against experimental carcinogenesis has been demonstrated in many animal models, including those involving cancers of the lung, skin, esophagus, liver and stomach.[1] Interestingly, although black tea is far more widely consumed than green tea,[2] most of the studies on inhibition of carcinogenesis have been conducted with green tea and its components. Based on animal experiments and some epidemiological data, green tea has been suggested to act as a chemopreventor against various forms of cancer.[3,4] Such protective activity has generally been assumed to be due to the powerful scavenging and anti-oxidative capacity of high concentrations of unpolymerised catechins and their gallates in green tea among which epigallocatechingallate (EGCG) has been found to be the most effective one.[5,6] In contrast, only very recently attention has been focused and few sporadic studies have been conducted on the anti-tumor effects of black tea.[7] Black tea was assumed to be much less beneficial that green tea because of its lower contents of unpolymerized polyphenols particularly EGCG.[5,8] In fact, during manufacture of black tea, the ''fermentation'' process causes green tea catechins to oxidize to form oligomeric flavanols, including theaflavin, theaflavin 3′-gallate, theaflavin 3/-gallate and theaflavin 3, 3′-digallate.[9] Theaflavins account for 2–6% of the water-extractable materials from black tea,[10] whereas thearubigins are the most abundant phenolic fraction.[9] Besides, black tea also contains caffeine, theobromine and theophylline, the principal alkaloids, and phenolic acids such as gallic acids and characteristic amino acids such as theanine.[11] According to Yang *et al.*,[12] the inhibitory activity against tumorigenesis of black tea is comparable with that of green tea in some animal models. Reports also provide evidence for anti-mutagenic, anti-proliferative and anti-neoplastic effects of both black and green teas.[13] Lin[14] suggested that both green and black tea polyphenols might exert their cancer chemoprevention by blocking the mitogenic and differentiating signals. Results of Yang *et al.*[15] suggest that the gallate structure of these polyphenols is important for cell growth inhibition and apoptosis induction. However, reports are also there demonstrating higher growth-inhibitory and apoptosis-inducing activities of green tea polyphenols over black tea polyphenols in some cancer cells.[1,16–19] In another study, black tea polyphenols have been shown as better scavanger of superoxide and nitrogen species as compared to green tea polyphenols.[20] However, more detail studies with black tea are required for specific evaluation of the links and differences in black tea action to green tea polyphenols.

We have already reported black tea-induced Ehrlich's ascites carcinoma (EAC) cell apoptosis in a mice model.[21] However, the detail molecular mechanisms underlying its apoptogenic effect in cancer cells are still not clear.

Recent reports have provided evidence that mitochondrial transmembrane potential (MTP) has a key role in controlling apoptotic responses in any cell.[21] Loss of MTP changes mitochondrial permeability that triggers opening of the permeability transition pore (PT),[22] which has been implicated as a critical stage in apoptosis in isolated mitochondria and several cellular models.[23] Agents that block PT pore formation, *e.g.*, cyclosporin A, bongkrekic acid etc., have been reported to block apoptosis.[22] On the other hand, PT pore opening allows release of factors, *e.g.*, cytochrome c, that initiate the final and degradative phase of apoptosis. Thus, cytochrome c, which is normally confined in the mitochondrial inter membrane space, is found in the cytosol of cells undergoing apoptosis.[24] This released cytochrome c next binds to Apaf-1 and activates caspase 9 in the presence of dATP and the activation of this initiator caspase leads to the activation of downstream effector caspases, such as caspase 3, 6 and 7, which in turn cleave a number of cellular proteins, facilitating the final destruction of the cell.[25]

It has been well documented that Bcl-2 family proteins play a crucial role in controlling MTP.[26] In fact, this Bcl-2 oncoprotein family consists of both pro-apoptotic and anti-apoptotic proteins that regulate caspase activation mainly at the mitochondrial level.[27] Thus, changes in Bcl-2 proteins that decrease MTP, induce apoptosis, and conversely, Bcl-2 family proteins that prevent depolarization inhibit the same.[28] It has been demonstrated

Abbreviations: CsA, cyclosporin A; $DiOC_6$, dihexyl-oxacarbocyanine; EAC, Ehrlich's ascites carcinoma; MTP, mitochondrial transmembrane potential; PI, propidium iodide; PT, permeability transition.

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***Correspondence to:** Bose Institute, P-1/12 CIT Scheme VII M, Kolkata-700 054, India. Fax: +91-33-334-3886.
E-mail: tanya@boseinst.ernet.in

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that release of cytochrome c is inhibited by the anti-apoptotic Bcl-2 or Bcl-xL but is induced by the pro-apoptotic Bax, Bak, Bid or Bik.[29] Despite the fact that many death stimuli can induce cytochrome c release, the pro-apoptotic Bcl-2 family proteins are the only well-defined proteins that possess the biochemical capability to do so. Bax, one of the important pro-apoptotic members of Bcl-2 family, has been reported to translocate from the cytoplasm to mitochondria during induction of apoptosis and physically interacts with the voltage-dependent anion channel and adenine nucleotide translocator. It has been proposed that such interaction results in mitochondrial permeability transition, an event that is associated with disruption of the mitochondrial inner transmembrane potential, PT pore opening and cytochrome c release,[30–32] resulting in apoptosis.[21,33,34] However, others have not found an importance of the PT pore for the cytochrome c-releasing activity of Bax.[29] Furthermore, in both cell-free systems and in cells undergoing apoptosis, the release of cytochrome c can also occur independently of changes in MTP.[35]

Recently, new chemicals with potent MTP regulatory activity are under development in an effort to improve the effectiveness of chemotherapy on tumor cells.[36] With the ultimate aim to understand how these mechanisms are related and can be controlled in cancer cells, we investigated further and elucidated the putative action pathways of black tea-induced cancer cell death. Our results indicate that black tea induces EAC apoptosis *via* Bax translocation and MTP loss. As downstream mechanism, cytochrome c release into the cytosol and activation of caspase 9 and 3 have been found to occur *via* 2 pathways, a) independent of and b) dependent on MTP loss. Furthermore, inhibition of caspase 3 protected mitochondria from MTP loss indicating the existence of a feedback loop. These findings may add new knowledge in the field of developing therapeutic strategies for cancer in future.

Material and methods

Materials

RPMI 1640 medium, FBS, streptomycin, penicillin, RNase A and general reagents were procured from Sigma Chemical Co. (St. Louis, MO). Black tea leaves were obtained from Lipton Tea Co. (India). Dihexyl-oxacarbocyanine ($DiOC_6$) and cyclosporin A (CsA) were purchased from Merck (Germany) and Apo-Direct kit, polyclonal anti-Bax and anti-pro-caspase 9, monoclonal anti-cytochrome c and anti-pro-caspase 3 as well as HRP- and FITC-conjugated anti-rabbit, anti-mouse secondary antibodies and cell permeable caspase 3 inhibitor, z-DEVD-fmk, were obtained from Pharmingen (San Jose, CA).

Cell culture

Ehrlich's ascites carcinoma cell (EAC) were routinely maintained in RPMI 1640 medium supplemented with 10% FBS, 100 μg/ml streptomycin and 50 unit/ml penicillin at 37°C in a humidified incubator containing 5% CO_2. After 24 hr, cells were serum-starved for 24 more hr and were treated with different doses of black tea extract (brewed in hot water[20] and lyophilized).

Detection of apoptosis by TUNEL assay

The fragmented DNA of apoptotic cells was labeled by catalytically incorporating fluorescein-12-dUTP at the 3′-hydroxyl ends of the fragmented DNA using the enzyme terminal deoxynucleotidyl transferase (TdT) using Apo-direct kit. The cells were then analyzed on FACS (equipped with 488 nm Argon laser light source; 515 nm band pass filter, FL1-H, and 623 nm band pass filter, FL2-H) using CellQuest software (Becton Dickinson, San Jose, CA). Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. A total of 10,000 events were acquired and dual parameter dot plot of FL2-H (x-axis; PI-fluorescence, linear scale) *vs*. FL1-H (y-axis; FITC-fluorescence, logarithmic scale) has been shown.

Spectrophotometric determination of mitochondrial dysfunction

Mitochondrial dysfunction was measured spectrophotomertically from isolated mitochondria. After treatment, EAC cells were homogenized in homogenizing buffer, consisted of 0.25 M sucrose, 5 mM HEPES buffer and 1 mM EDTA, pH 7.2. The homogenate was centrifuged at 500*g* to pellet nuclei and the resulting supernatant was centrifuged at 12,000*g* for 5 min at 4°C to pellet the heavy membrane fraction containing mitochondria.[37] Mitochondria were further purified by resuspending heavy membrane pellets in 250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES, pH 7.4, 0.1% bovine serum albumin and layering on 30% Percoll, 225 mM mannitol, 1 mM EGTA, 25 mM HEPES, pH 7.4, and 0.1% bovine serum albumin. After centrifugation at 95,000*g*, mitochondria were recovered from the lower phase.[38] Purity of the mitochondrial fraction was assessed by assaying succinate dehydrogenase and cytochrome c oxidase activities.[39,40] Results showed that >98% activity of these enzymes was associated with the mitochondrial fractions and <2% of the total activities was with the other fractions.

To detect mitochondrial swelling due to MTP loss, the mitochondria were suspended in buffer containing 250 mM sucrose, 10 mM HEPES-NaOH, pH 7.5, 2 mM KH_2PO_4, 5 mM sodium succinate, 25 mM EGTA and 0.1 mM phenylmethylsulfonyl fluoride and absorbance at 540 nm was measured. A decreased light absorbance is consistent with an increase in mitochondrial volume indicative of MTP loss.[37]

Flowcytometric measurement of mitochondrial transmembrane potential

Loss in MTP was further verified flowcytometrically at single cell level. After black tea treatment, EAC cells were stained with the potential sensitive dye $DiOC_6$ (40 nM, 15 min at 37°C in the dark). Loss in $DiOC_6$ staining indicates disruption of the mitochondrial inner transmembrane potential.[29] The probe was excited at 488 nm and emission was measured through a 530 nm band-pass filter. Logarithmic amplification was used to detect the fluorescence of the probe.

Fluorescence imaging for determination of MTP loss

Black tea-induced loss in EAC cell MTP was reconfirmed by fluorescence imaging. For the same, cells were stained with potential sensitive dye $DiOC_6$ (green-fluorescence) and then cytospun on slides. Mitochondria was labeled using Mito-tracker red (red-fluorescence) staining. A Leica fluorescent microscope DM 900 was used to visualize the fluorescent images for $DiOC_6$ and Mito-tracker red. Digital images were captured with a cool (−25°C) CCD (Charged coupled device) camera (Princeton Instruments) controlled with the MetaMorph software.[41] Loss in MTP was assessed by comparative $DiOC_6$ staining of cells with or without black tea treatment.

Western blot analysis

Mitochondrial and cytosolic fractions from untreated and black tea-treated EAC cells were subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis the proteins were transferred to nitrocellulose membrane and the protein of interest was visualized with chemiluminescence.

Determination of sequence of events in black tea-induced EAC cell apoptosis

To map the sequence of events in black tea-treated EAC apoptosis, experiments using inhibitors of PT pore formation and caspase 3 were utilized. Cells were treated with or without CsA (25 μM) for 2 hr prior to black tea treatment for 4, 8 and 12 hr. MTP loss, cytochrome c release in cytosol and caspase 9 and 3 activation as well as EAC apoptosis were then determined at each time point. In parallel experiments, z-DEVD-fmk pre-treated cells (20 μM, 2 hr) were subjected to black tea treatment and tested for apoptosis and MTP loss.

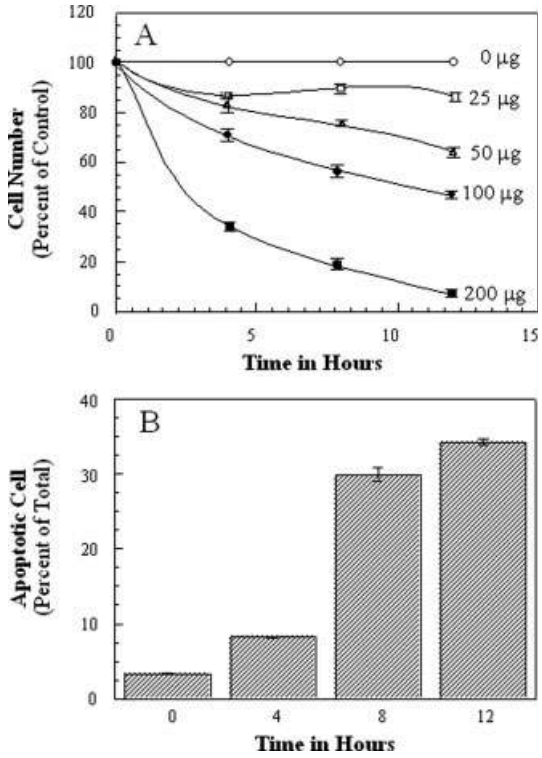

FIGURE 1 – (*a*) Effects of tea on the growth of EAC cells. Cells were seeded in tissue culture cluster. The cells were then treated with different concentrations of tea. At different time points Trypan blue-negative cells were counted. The values represent the percent change in cell number over control. Data are shown as mean ± SEM of 3 independent experiments. (*b*) EAC cells were treated with 100 μg/ml black tea *in vitro* for different time intervals and TUNEL-positive cells (apoptotic cells) were analyzed by flowcytometry and data are shown as means of 3 independent experiments; bars, ± SEM.

Results

Anti-tumor activity of black tea

We have already reported apoptogenic effect of black tea on EAC cells in mice model.[20] Here, with an aim to delineate the underlying mechanisms of black tea-induced EAC cell apoptosis, we first examined the growth inhibition of these cells in culture with different doses of black tea. Our results of Figure 1*a* indicate that black tea decreased EAC cell number in a dose-dependent manner over their control counterparts, IC_{50} being 100 μg/ml at 12 hr of incubation when from a total of 4.7×10^5 EAC cells in untreated set, 2.5×10^5 cells were found in black tea-treated group.

TUNEL assay to confirm the nature of EAC cell death by black tea

To identify the nature of black tea-induced EAC cell death *in vitro*, we utilized TUNEL assay method. Our flowcytometric data revealed that (Fig. 1*b*) in comparison to untreated set, black tea-treated (100 μg/ml dose) set showed significant increase in number of TUNEL positive (apoptotic) cells in a time-dependent manner.

Bax translocation to mitochondria up on black tea treatment

It has been reported that translocation of pro-apoptotic protein Bax to mitochondria plays important role in decreasing MTP and initiating mitochondrial death cascade.[29] In our system, Bax translocation from cytosol to mitochondria started after 4 hr of black tea treatment and reached its peak at 12 hr (Fig. 2*a*). However, the increased Bax levels in mitochondrial fraction was not accompanied with the decreased Bax level in the cytosol, suggesting that other mechanism such as upregulation of Bax may be involved. Thus, Bax expression was examined at the whole cell level in EAC after black tea treatment. Our Western blot data revealed that Bax level increased with time in EAC cell lysate (Fig. 2*a*). This explained why the cytosolic Bax was not decreased significantly because it was compensated by the increased Bax expression (Fig. 2*a*,*b*).

Since it is well accepted that increase in pro-apoptotic protein Bax and its translocation to mitochondria help in forming channels in mitochondrial membranes thereby decreasing MTP,[29] the above results raised the possibility of Bax-induced MTP loss in EAC cells as a result of black tea treatment. To confirm our hypothesis the following experiments were designed.

Black tea-induced mitochondrial transmembrane potential loss

First we studied whether black tea could induce the large amplitude swelling of mitochondria by measuring light absorbance at 540 nm. Results depicted in Figure 3*a* showed that although Bax translocation started from 4 hr after black tea (100 μg/ml) treatment, there was a decrease in absorbance at 540 nm, indicating black tea-induced EAC mitochondrial swelling after 8 h of treatment. These results together suggest that Bax translocation was followed by MTP loss in EAC cells upon black tea treatment. However, it has been suggested that permeability may not change in a synchronous manner across the mitochondrial population upon the application of low doses of induces and that the PT pore may return to the closed state after opening for a period of time. These events may result in failure to detect mitochondrial swelling by light absorbance at 540 nm. We, therefore, decided to further confirm MTP loss in EAC cells due to black tea treatment by more sensitive flowcytometric as well as fluorescence imaging techniques.

Flowcytometric results (Fig. 3*b*) revealed high level of $DiOC_6$ binding to the mitochondria of untreated EAC cells. However, a significant decrease in the fluorescence was observed in a time-dependent manner starting from 8 hr after black tea treatment. Since decrease in $DiOC_6$ binding to mitochondrial membrane is indicator of decreased integrity of the membrane, these results clearly indicate black tea-induced loss of MTP in EAC cells. These results were further supported by our fluorescence imaging data (Figs. 3*c*,*d*) where loss in $DiOC_6$ binding could clearly be seen.

Cytochrome c release in the cytosol by black tea

To check the downstream events in mitochondrial death cascade in EAC cells, next we investigated the release of cytochrome c from mitochondria to cytosol. Immunoblot analysis (Fig. 4) showed that mitochondrial cytochrome c appeared in the cytosolic fraction at as early as 4 hr after black tea treatment. However, in the untreated cells, cytochrome c was not detected in the cytosolic fraction even after 12 hr of incubation (data not shown). These results further help in mapping the mitochondrial death cascade in EAC cells as initiated by black tea.

Black tea-induced caspases 9 and 3 activation

Results of cytochrome c release and mitochondrial perturbation further tempted us to investigate the role of caspase cascade in black tea-induced EAC cell apoptosis. Western blot data (Fig. 4) revealed that unlike in untreated EAC, black tea resulted in time-dependent proteolytic processing of procaspase 9, which started 4 hr after treatment and resulted in nearly complete disappearance by 12 hr (Fig. 4), thereby signifying activation of caspase cascade by black tea in these cells.

The next experiment was designed to further map the downstream pathway of black tea-induced EAC cell apoptosis. Western blot data revealed that unlike in untreated EAC, in black tea-treated set pro-caspase 3 was cleaved to form active caspase 3 after 8 hr of black tea treatment (Fig. 4), thereby indicating its involvement in the black tea-induced death-signaling pathway in EAC cells.

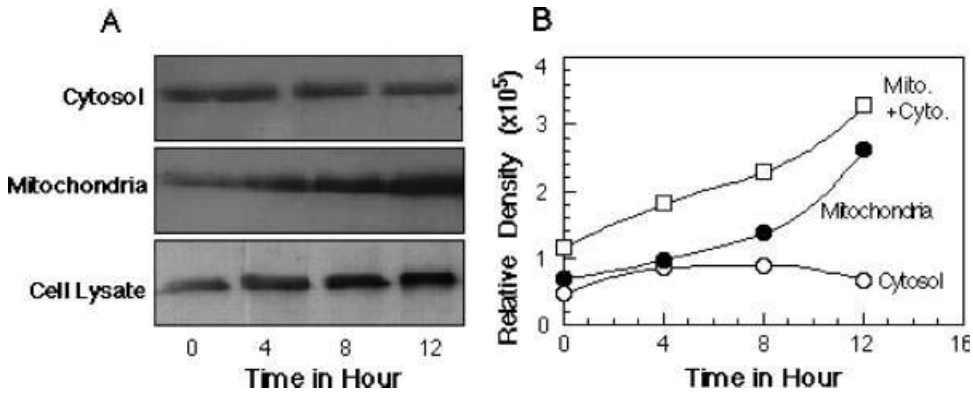

FIGURE 2 – Bax translocation to mitochondria during black tea-induced apoptosis. (*a*) Total and sub-cellular distribution Bax was detected by Western blot analysis from whole cell lysates as well as cytosolic and mitochondrial fractions of black tea-treated EAC cells. (*b*) Cytosolic and mitochondrial Bax content was estimated by quantitative Western blot analysis cytosolic and mitochondrial fractions of black tea-treated EAC cells. (open circle) Cytosolic Bax; (closed circle) mitochondrial Bax and (open square) cytosolic + mitochondrial Bax. Data shown are representative of 3 experiments performed.

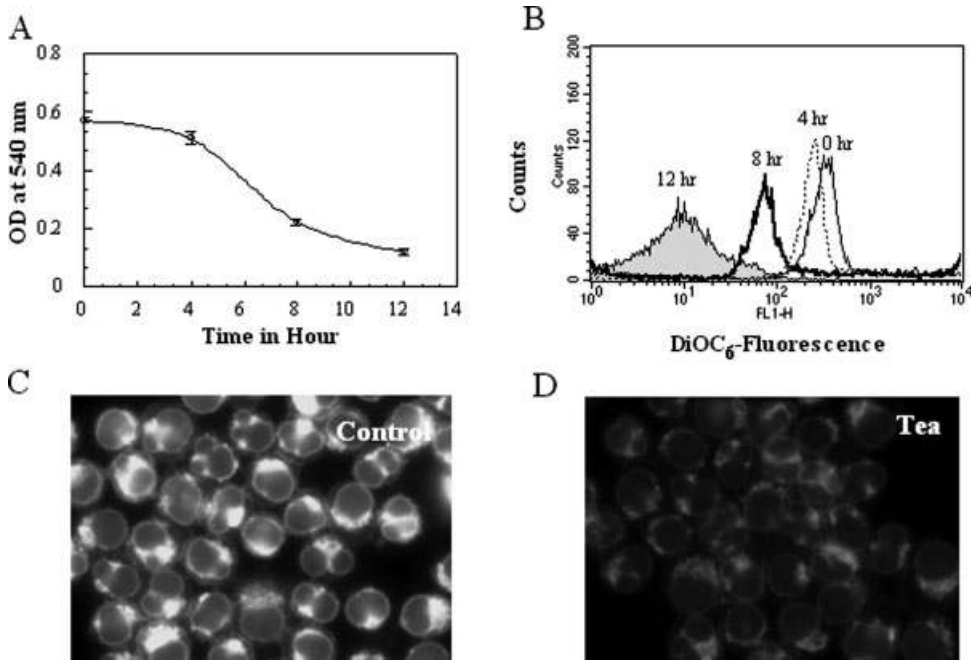

FIGURE 3 – Effect of black tea on mitochondrial membrane dysfunction. (*a*) Black tea-induced swelling of mitochondria was measured spectrophotometrically at 540 nm from isolated mitochondria. The data shown are mean ± SEM of 3 independent experiments. (*b*) Loss in MTP was analyzed flowcytometrically at single cell level using $DiOC_6$ as fluorescent probe. After black tea treatment, EAC cells were stained with $DiOC_6$, analyzed on a flowcytometer and histogram display of $DiOC_6$-fluorescence (x-axis) *vs*. counts (y-axis) has been shown in logarithmic fluorescence intensity. (*c* and *d*) Fluorescence imaging technique was used to detect mitochondrial membrane dysfunction. (*c*) Untreated or (*d*) black tea-treated EAC cells were stained with $DiOC_6$ and visualized under fluorescent microscope. The filter cube L4 was used to detect signal form $DiOC_6$. Data shown are representative of 3 independent experiments performed.

Black tea induced EAC cell apoptosis in both MTP-dependent and -independent manners

Though our data indicate that black tea induced Bax translocation, loss in MTP, cytochrome c release, caspase activation and apoptosis in EAC cells, it was unclear whether induction of downstream processes were in an MTP-dependent or -independent fashion. We, therefore, next attempted to clarify whether black tea activated cytochrome c release and pro-caspase processing in MTP-dependent or independent fashion. For the same, we first compared the time frames of black tea-stimulated events in EAC cells to understand the sequence of events in black tea-induced EAC cell apoptosis. Our results indicated that cytochrome c started releasing in the cytosol as early as at 4 hr after black tea treatment when there was no change in MTP. On the basis of these time-course studies, it seems likely that black tea may induce cytochrome c release independent of mitochondrial permeability transition at early hour but dependent at late hours.

To obtain a more definitive conclusion, we determined whether PT pore formation inhibitor has any role on cytochrome c release at different time points. Interestingly, at 4 hr, black tea-induced cytochrome c release was not blocked by cyclosporin A (CsA), the PT inhibitor, but at 8 and 12 hr, this inhibitor blocked cytochrome c release significantly (Fig. 5*a*) suggesting that its release at early time point was independent of PT pore opening but at latter time point MTP loss was responsible for further cytochrome c release, thereby causing apoptosis.

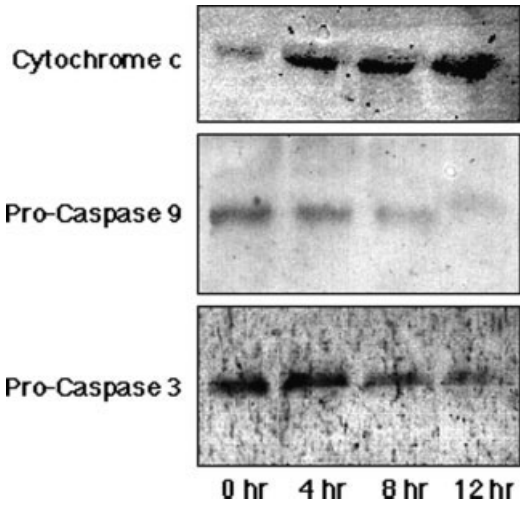

FIGURE 4 – Effect of black tea on the expression of cytochrome c and processing of caspase 9 and 3. EAC cells were treated with black tea for different time intervals. Cells were harvested and cytochrome c release into cytosolic fraction was determined by Western blot analysis. Pro-caspase 9 (middle panel) and pro-caspase 3 (lower panel) were detected by Western blot analysis form untreated or treated cellular cytosols. One representative data from 3 independent experiments has been furnished.

Similarly, upon black tea treatment, pro-caspase 9 breakdown started as early as from 4 hr. Studies with CsA showed that at this time point caspase 9 activation was independent of MTP loss but at 8 and 12 hr, CsA could partially block pro-caspase 9 breakdown by black tea (Fig. 5*b*).

As shown above (Fig. 4), Western blot data revealed that there was no pro-caspase 3 breakdown at 4 hr but the processing started from 8 hr of black tea treatment. Interestingly, CsA pretreatment inhibited caspase 3 formation partially at 8 hr and completely at 12 hr (Fig. 5*c*). These results indicate that at 8 h, black tea activated caspases 3 both *via* MTP-dependent and independent pathways but at late time point, it became completely dependent on MTP loss. All these results indicated the black tea induced EAC cell apoptosis *via* both MTP-independent and dependent pathways.

Interestingly, CsA or z-DEVD-fmk could significantly block black tea-induced EAC apoptosis though not completely (Fig. 5*d*). These results not only confirm that black tea exerts its apoptogenic activity through mitochondrial perturbation but also indicate that there may exist other pathway(s).

Existence of a feedback loop in black tea-treated EAC cell apoptosis

Some reports are there, indicating a feedback initiation of PT pore formation by caspases.[42] To check that possibility in our model system, mitochondrial membrane potential was next evaluated by exposing the cells to z-DEVD-fmk, the inhibitor of final execution caspase, *i.e.*, caspase 3. Interestingly, this inhibitor blocked MTP loss at late hours (Fig. 6), thereby raising the possibility of downstream effector caspase 3 acting as feedback activator of mitochondrial membrane perturbation.

All our data presented here indicate that black tea treatment resulted in Bax upregulation and translocation from cytosol to mitochondria as well as loss in MTP in EAC cells. In the downstream, mitochondrial cytochrome c release as well as caspase 9 and 3 activation occurred by 2 pathways, a) independent of and b) dependent on MTP loss, thereby finally resulting in EAC apoptosis. Caspase 3 in turn acted as a feedback activator of mitochondrial perturbation and thereby amplified black tea-induced death signal in these cells.

Discussion

In a previous study, we have shown that in a mice model, black tea, the second most popular beverage consumed by ~78% of world population,[2] induces apoptosis in EAC by modulating tumor cell cycle as well as the balance between pro- and anti-apoptotic proteins, *e.g.*, Bcl-2 and Bax.[20] However, the complete downstream mechanism underlying such apoptogenic effect of black tea was not unveiled so far. We thus initiated our study to investigate further and more intricately the mechanism(s) of EAC apoptosis by black tea *in vitro*.

Recent evidences have shown that mitochondria play a crucial role in apoptosis by releasing apoptogenic factors.[43] In fact, the inner membrane of mitochondria has only a limited permeability that is essential for generation of the MTP and the pH gradient across the membrane[44] and permeabilization of this membrane allows efflux of solutes, disrupting MTP and the pH gradient resulting in opening of the PT pore complex.[34] It was reported recently that pro-apoptotic protein Bax is localized in the cytoplasm and translocates to the mitochondria at the early stage of apoptosis[32] and a conformational change in Bax is responsible for MTP loss and cytochrome c release during apoptosis.[45] On the other hand, Bcl-2/Bcl-xL can inhibit such mitochondrial perturbation directly interacting with Bax.[34] Recently, new compounds, acting directly on mitochondria, are under experimental trials.[46] They may be effective in circumstances in which conventional anticancer agents fail.

With all these information in mind, we utilized EAC cell culture as our model to unveil the complete mechanism of black tea action. Although we have already reported the apoptogenic effect of black tea on EAC in a mice model,[20] it is quite logical to conceive that any agent that works *in vivo* may not be similarly effective *in vitro* if the *in vivo* action is not a direct one but through other available systems of the host, *e.g.*, immune system.[47] We, therefore, first checked whether black tea shows apoptogenic effect directly on EAC cells *in vitro* or not. Our results confirmed that black tea asserted apoptogenic insult to EAC cells also *in vitro*. Furthermore, this beverage increased intracellular Bax level in these cells thereby might be allowing free Bax to be available for mitochondrial pore formation. There are reports indicating that overexpression of Bax results in cytochrome c release from mitochondria to cytosol[48] and direct addition of recombinant Bax protein to isolated mitochondria also induces cytochrome c release.[49] On the other hand, it has also been reported that despite the increase of intracellular Bax, without its translocation from cytosol to mitochondria, there was no release of cytochrome c from mitochondria to cytosol.[50] Our results confirming translocation of Bax to mitochondria further increased the possibility of Bax-induced initiation of mitochondrial death cascade. In fact, concomitant with Bax translocation, there was a significant decrease in the absorbance at 540 nm indicating swelling of EAC mitochondria. Mitochondrial membrane perturbation was further confirmed by decrease in $DiOC_6$-fluorescence indicating lesser binding of this probe to mitochondria due to MTP loss up on 8 hr of black tea treatment.

Though our data indicate that black tea caused MTP loss and induced apoptosis, it was unclear whether it activated downstream processes in an MTP-dependent or independent fashion. Although it was originally believed that MTP loss was the root mechanism responsible for cytochrome c release in response to different cytotoxic stimuli, more recently ample evidence suggests[48,51,52] that some apoptogenic agents induce Bax expression, followed by cytochrome c release and caspase activation without generating a detectable permeability transition. To that end, report demonstrates[53] that 2 distinct pools of cytochrome c can be mobilized. The first pool is sensitive to electrostatic alterations that can be elicited by changes in ionic strength, surface-charge density or pH and thus most likely reflects cytochrome c present in the loosely bound conformation.[53] The second pool represents tightly bound cytochrome c that is detached because of disturbances in mem-

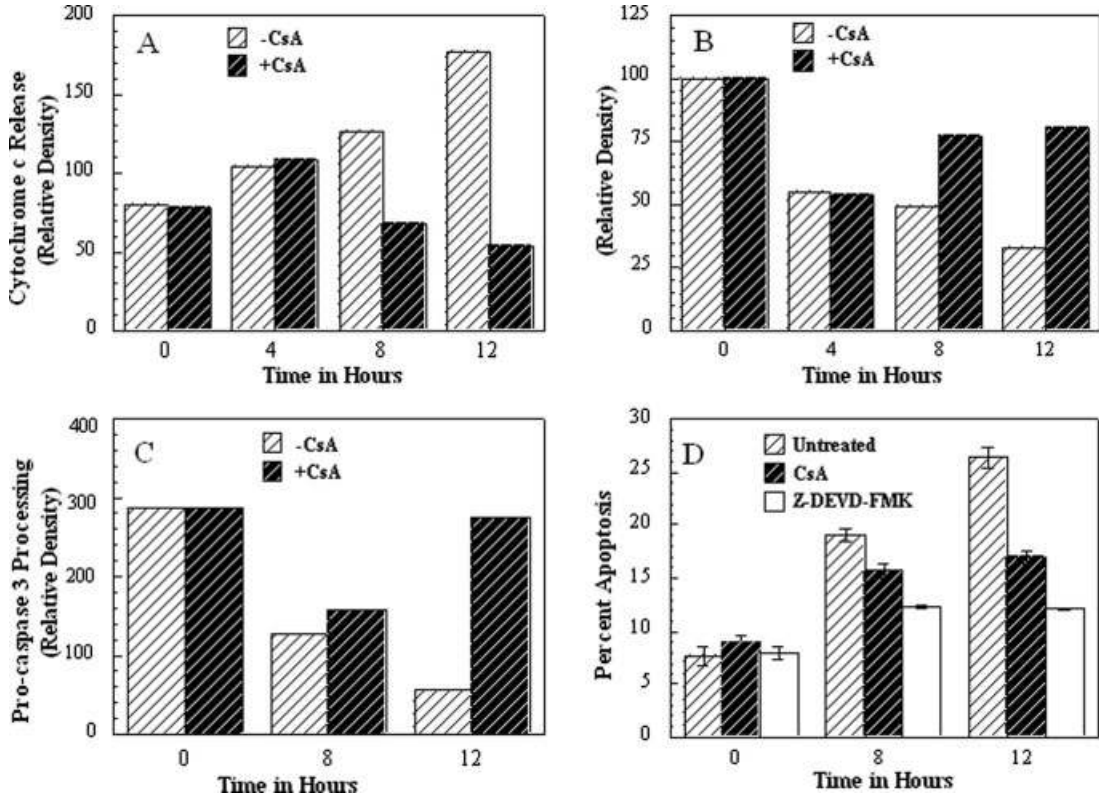

FIGURE 5 – Sequence of events in black tea induced EAC cell apoptosis. EAC cells were treated with black tea for different time period in the presence (+CsA) or absence (−CsA) of CsA or z-DEVD-fmk. (*a*) Cytochrome c release in cytosol; (*b*) pro-caspase 9 processing and (*c*) pro-caspase 3 processing. Densitometric scanning data of western blot analysis have been furnished in bar diagrams and are representative of 3 independent experiments performed. (*d*) Percent apoptosis were determined *in vitro*. Data are presented as means of three independent experiments; bars, ± SEM. Light hatch bar: without CsA; dark hatch bar: with CsA and hollow bar: with z-DEVD-fmk.

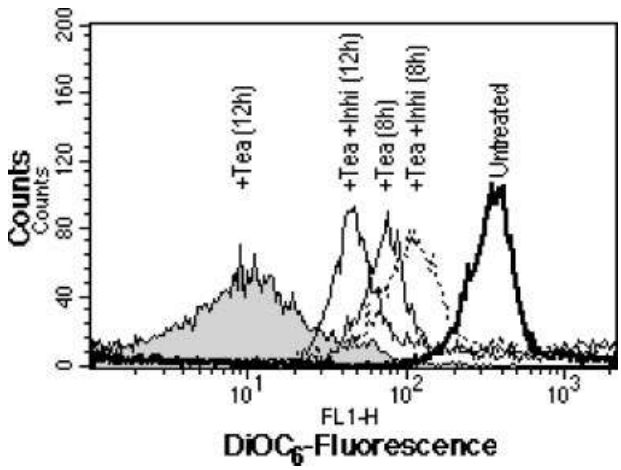

FIGURE 6 – Existence of a feedback loop in black tea-treated EAC cell apoptosis. Effect of caspase 3 inhibitor on mitochondrial membrane potential in black tea-treated EAC cells was determined flowcytometrically using $DiOC_6$ as fluorescent potential sensitive probe. z-DEVD-fmk pretreated EAC cells were treated with black tea for different time intervals, stained with $DiOC_6$, analyzed on a flowcytometer and histogram display of $DiOC_6$-fluorescence (x-axis) *vs*. counts (y-axis) has been shown in logarithmic fluorescence intensity. Data furnished are representative of 3 independent experiments.

brane structure.[53] Our studies using PT pore inhibitor cyclosporin A support the idea that at early hours, cytochrome c release occurred independent of MTP loss but at the late hours this phenomena was dependent on the decrease of mitochondrial membrane potential.

It is known that caspase 9 activation takes place along with release of cytochrome c.[54,55] In our assay system, since black tea triggered activation of caspase 9 from 4 hr in MTP-independent way, MTP independent cytochrome c release might have played a significant role in such activation to form classical ''apoptosome'' complex. Interestingly, CsA inhibited pro-caspase 9 cleavage from 8 hr in EAC cells, indicating the involvement of mitochondrial permeability in black tea-induced caspase 9 activation at later time point. On the other hand, pro-caspase 3 processing initiated at 8 hr and CsA could only slightly blocked its cleavage indicating that at this time point caspase 3 activation was mostly MTP-independent. However, at 12 hr, the inhibitor, CsA, almost completely inhibited pro-caspase 3 breakdown supporting our notion that caspase 3-activation became dependent on mitochondrial perturbation at later hour. Finally, our results also indicated that neither PT pore formation inhibitor nor caspase 3 inhibitor could block black tea-induced EAC cell apoptosis completely, thereby raising the possibility of existence of other parallel pathways. In this regard, involvements of caspase 6 and 7[56] or AIF[57] are not overruled.

Interestingly, recent observations point to an intricate cross talk between caspases and mitochondria in the apoptotic process. Reports indicate that activated caspases, in turn, can directly act on the mitochondria, thus engaging in a self-amplifying feedback loop in which changes in mitochondrial permeability lead to caspase activation and *vice versa*.[42] Our results also raised the possibility of such a feedback activation loop in black tea-induced apoptotic pathway in which activated caspase 3 again acted upstream of mitochondria. In fact, in our system caspase 3 inhibitor partially protected EAC cells from MTP loss at 8 hr. Our previous results indicating the activation of caspase 3 both by MTP-

independent and -dependent manners at this time point may have significance with this observation where only MTP-dependent caspase 3 may be taking part in the feedback pathway. Supporting this hypothesis, at 12 hr when pro-caspase 3 processing was almost completely dependent on MTP loss, caspase 3 inhibitor rendered significant protection to mitochondrial membrane. However, more detail studies are required to confirm this hypothesis.

All these observations, as well as existing knowledge, indicate that if the interplay between caspase proteases and mitochondria decides the fate of the cell during apoptosis, they may constitute useful molecular targets for specific drug designing. In this regard, black tea, with a predilection for mitochondrial membrane disruption, including pore formation on the one hand and ability to activate intracellular caspases on the other, would be an ideal candidate to induce effective apoptosis in tumor cells. However, presently, the detail mechanism underlying the antiproliferative role of black tea extracts has been delineated in case of Ehrlich's ascites carcinoma only. Thus, more work is required to find out the role of this beverage in different types of cancer cells to understand whether black tea action is cell line specific or *via* a general mechanism.

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