Molecular mechanism of black tea polyphenols induced apoptosis in human skin cancer cells: involvement of Bax translocation and mitochondria mediated death cascade

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Theaflavins (TF) and thearubigins (TR) are the most exclusive polyphenols of black tea. Even though few previous reports showed the anticancerous effects of TF through apoptosis, the potential effect of TR has not been appraised. This study investigated the induction of apoptosis in human skin cancer cells after treatment of TF and TR. We report that both TF and TR could exert inhibition of A431 (human epidermoid carcinoma) and A375 (human malignant melanoma) cell proliferation without adversely affecting normal human epidermal keratinocyte cells. Growth inhibition of A375 cells occurred through apoptosis, as evident from cell cycle arrest at G0/G1 phase, increase in early apoptotic cells, externalization of phosphatidylserine and DNA fragmentation. In our pursuit to dissect the molecular mechanism of TF- and TR-induced apoptosis in A375 cells, we investigated whether cell death is being mediated by mitochondria. In our system, Bax translocation to mitochondria persuaded depolarization of mitochondrial membrane potential, cytochrome c release in cytosol and induced activation of caspase-9, caspase-3 and poly (ADP-ribose) polymerase cleavage. Our intricate investigations on apoptosis also explained that TF and TR augmented Bax:Bcl2 ratio, up-regulated the expression of p53 as well as p21 and inhibited phosphorylation of the cell survival protein Akt. Furthermore, TF and TR elicited intracellular reactive oxygen species generation in A375 cells. These observations raise speculations that TF as well as TR might exert chemopreventive effect through cell cycle arrest and induction of apoptotic signals via mitochondrial death cascade in human skin cancer cells.

Introduction

Polyphenols present in food have been demonstrated to decrease various types of experimental carcinogenesis. In recent years, identification of effective chemopreventive polyphenols in diets or dietary supplements for human use is of much interest. Treatments with such polyphenols result in cell cycle arrest (1), thereby reducing the growth and proliferation of cancerous cells through apoptosis or programmed cell death (2). Tea (Camellia sinensis) polyphenols exert their potent anticancer activity and appear to be the ideal agents for chemoprevention. During the fermentation process, tea catechins are polymerized by polyphenol oxidase (released from crushed tea leaves) to form theaflavins (TF) and thearubigins (TR). TF and TR account for 3–6% and 12–18% of dry weight of black tea, respectively (3). Black tea has been shown to be potent in inhibiting tumorigenesis in animal systems, including lung (4), colon (5) and skin (6). Some reports provide evidence that black tea significantly inhibits proliferation and enhances apoptosis in established mouse skin tumor (7). Black tea polyphenols, especially TF exert cancer chemopreventive activity by inducing apoptotic signals (7,8). However, besides some sporadic reports on the protective role of black tea and its derivatives, the detailed molecular mechanisms underlying their anticancerous effect are inconclusive.

Apoptosis triggered by various stimuli, is characterized by a series of distinct biochemical and morphological changes (9), including increase in reactive oxygen species (ROS) level (10), activation of caspases, cell shrinkage, chromatin condensation and nucleosomal degradation (11). One of the most significant events in apoptosis is mitochondrial dysfunction. Loss of mitochondrial transmembrane potential (MTP) elicits the release of cytochrome c from mitochondria to cytosol (12). After release, it activates caspase-9 and subsequently activates downstream caspase-3 (13). Activated caspase-3 cleaves intracellular protein poly (ADP-ribose) polymerase (PARP), which is an important marker of apoptosis (14).

It has been evident that the proapoptotic protein Bax plays an essential role for the onset of MTP changes and induces cytochrome c release, which is inhibited by the antiapoptotic protein Bcl2 (12). Studies have also shown that Bax:Bcl2 ratio increases during apoptosis (15). Bax is a p53 target and is known to be transactivated in a number of systems during p53-mediated apoptosis (16). p53 is known to mediate cell cycle arrest (17) and also acts as a transcription factor that binds to a p53-specific DNA consensus sequence in response genes such as p21 (18). Up-regulation of p21 functions as the inhibitor of cell cycle kinetics (19). Previous reports showed that Akt phosphorylation confers cell survival signals through up-regulation of antiapoptotic protein Bcl2 and down-regulation of proapoptotic factors, such as caspase-9 (20,21).

All these above reports indicate that the balance between the proapoptotic and antiapoptotic proteins, dysfunction of mitochondria and activation of caspases are the important factors deciding apoptotic cell death of tumor cells. Previously we have reported the antimutagenic and anticalcogenetic effects of TF and TR in vivo and in vitro in multiple test systems (22,23). Although the content of TR is much higher than that of TF in black tea, interestingly there are no such reports available on potential anticancerous effects of TR till date.

In the present study, we have investigated further and elucidated the putative pathways of TF- and TR-induced cancer cell death specially emphasizing on TR. The effect of different doses of TF and TR on the growth of A431 and A375 cells was examined and the efficacy of these polyphenols as a growth inhibitor on these cell lines was compared. A375 cells were selected because they were much more susceptible than A431 cells toward the treatment of different doses of TF and TR. In the current study, we determined the role of the mitochondrial death cascade in TF- and TR-induced apoptosis in A375 cells. Bax:Bcl2 ratio along with the tumor suppressor protein p53 and p21, which are important regulators of mitochondria-mediated apoptosis, was also evaluated. We also addressed the relevance of phosphorylated Akt in apoptosis.

Materials and methods

Reagents

Primary antibodies (Bcl2, Bax, p21, p53, Akt-ser, PARP, cytochrome c, β-actin) and polyclonal secondary antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cycle TEST PLUS DNA reagent kit and JC-1 kit from BD Biosciences (San Diego, CA), AnnexinV-FITC Apoptosis Detection Kit was obtained from BD PharMingen (San Diego, CA), Apo-direct TUNEL ASSAY KIT, caspase protease assay kit, were purchased from Chemicon International Corporation. 4’, 6-diamidino-2-phenylindole (DAPI), FITC-AnnexinV, bromophenol blue, EGTA, NaF, Na3VO4, NP 40, PMSF, aprotinin, leupeptin, Pepstatin A, catalase, HEPES, 3-(4-dimethylthiazol-2-71)-2,5-diphenyl tetrazolium bromide (MTT), H2O2, digitonin, molecular
grade BSA, Tween-20, Nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine (NBT/BCIP), Tris–HCl, DMSO and pifithrin-α (P35 inhibitor) were from Sigma-Aldrich (St Louis, MO). 2′, 7′-dichlorofluorescein diacetate from Molecular Probes Inc. (Eugene, OR). Mitochondria/Cytosol fractionation kit from BioVision (Mountain View, CA). Caspases fluorometric assay kit obtained from Chemicon International Corporation. All caspase inhibitors Z-VAD-FMK (pan-caspase inhibitor), DEVD-CHO (caspase-3-specific inhibitor), LEHD-CHO (caspase-9-specific inhibitor), LY294002 (PI3K/Akt inhibitor) were purchased from Calbiochem (La Jolla, CA). Bio-Rad Protein Assay Kit from Bio-Rad Laboratories, Hercules, CA.

**Cell culture**

A431 (human epidermoid carcinoma cells) and A375 (human malignant melanoma cells) were purchased from National Centre for Cell Science, Pune, India, and were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen Corporation, Grand Island, NY). Normal human epidermal keratinocytes (NHEK) from Clonetics® and grown in Keratinocyte growth medium (KGM™ from Clonetics® Cambrex Bioscience Walkersville, Inc., Walkersville, MD). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 inside a CO2 incubator.

**Extraction of TF and TR from black tea**

Both TF and TR were extracted from the black tea (Tata Tea Gold) as described in previous papers (22, 23). TF and TR were extracted according to the method of Xie et al. (24). Extraction was carried out in the Medicinal Chemistry Department of our Institute. In brief, initially black tea (10 g) was extracted by boiling water (250 ml), then the aqueous fraction was further extracted with chloroform to remove caffeine and successively with ethyl acetate and n-butanol by liquid–liquid partition (24). Ethyl acetate fraction contains TF, whereas the n-butanol fraction contains TR (25). We have also estimated the total TF and TR content of our sample, which were calculated to be 2.3% and 12.5%, respectively, of the dry weight of the sample.

**MTT assay**

Effect of TF and TR (0–125 µg/ml) on the viability of A375, A431 and NHEK cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following the method of Mosmaan (26). Briefly, ~5000 of A431, A375 and NHEK cells per well were plated in 96-well plates and treated with TF or TR (0, 15, 25, 50, 75, 100, 125 µg/ml) for 24 or 48 h. At the end of stipulated time following TF and TR treatment, the medium was aspirated and MTT (50 µl of 5 mg/ml stock solution in PBS) was added into each well and incubated at 37°C for 2 h. After shaking, the purple-colored precipitate of formazan was dissolved in 150 µl of DMSO (Sigma-Aldrich). The absorbance of each well was recorded at 540 nm with a Bio-Rad microplate reader with a reference serving as blank. Then, IC50 value of TF and TR was calculated (27).

Detection of apoptosis by flow cytometry

A375 cells (1 × 10⁶ in each case) were treated with different concentrations of TF or TR (25, 50, 75, 100 µg/ml) for 24 h. For the determination of cell cycle phase distribution, A375 cells were harvested, 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 (Becton Dickinson, San Diego, CA), fluorescence detector equipped with 488 nm argon laser light source and 623 nm band-pass filter. The fragmented DNA of apoptotic cells was labeled using Apo-direct TUNEL assay 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). A total of 10 000 events were acquired and analyzed.

**Measurement of MMP**

The loss of mitochondrial membrane potential (MMP) is a hallmark for apoptosis. It is an early event preceding phosphatidylserine externalization and coincident with caspase activation. MMP was measured using JC-1 kit following the protocol submitted by the supplier. The MMP detection kit uses a unique fluorescent cationic dye, JC-1 (5,5′,6′,6′-tetrachloro-1,1′,3′,3′-tetraethylbenzimidazolylcarbocyanine iodide) (excitation at 488 nm and emission at 525 nm), to signal the loss of MMP.

Cells were harvested at 24 h of treatment with different concentrations (0, 25, 50, 75, 100 µg/ml) of TF or TR. Mitochondrial permeability transition was determined by the staining of the cells with JC-1 as described. Briefly, equal numbers of cells (1 × 10⁶) were incubated with JC-1 at 2.5 µg/ml in 1 ml PBS for 30 min at 37°C with moderate shaking. Cells were then centrifuged at 300 g at 4°C for 5 min, washed twice with ice-cold PBS and finally resuspended in 200 µl PBS. Mitochondrial permeability transition was subsequently quantified on FACS (Becton Dickinson). Data were given in percentage of cells with altered MMP.

Preparation of cytosolic and mitochondrial fractions

A375 cells were harvested after treatment with 0, 25, 50, 75, 100 µg/ml concentrations of TF or TR for 24 h. Isolation of a highly enriched mitochondrial fraction and cytosolic fraction of cells was performed using a Mitochondria/Cytosol fractionation kit. Briefly, A375 cells (5 × 10⁶) were centrifuged at 600g for 5 min at 4°C, resuspended in ice-cold PBS and centrifuged at 600g for 5 min at 4°C. Then the cells were resuspended in 1.0 ml of cytosol extraction buffer mix containing DTT and protease inhibitors and incubated on ice for 10 min. The cells were homogenized on ice. The homogenate was centrifuged at 700g for 10 min at 4°C and the supernatant was collected and centrifuged at 10 000g for 30 min at 4°C. Then, the supernatant was collected as the cytosolic fraction. The pellet was resuspended in 0.1 ml mitochondrial extraction buffer mix containing DTT and protease inhibitors, vortexed for 10 s, and saved as the mitochondrial fraction. These isolated cytosolic and mitochondrial fractions were subjected to western blot analysis for cytochrome c release assay and Bax translocation assay. Cytochrome c expression was analyzed with anti-cytochrome c polyclonal antibody and Bax expression was determined by anti-Bax polyclonal antibody. An alkaline phosphatase-conjugated goat anti-rabbit secondary antibody was used for this experiment.

Measurement of intracellular ROS level

A375 cells were seeded into 100-mm dishes (5 × 10⁶ cells per dish) overnight. Cells were treated with 0, 25, 50, 75, 100 µg/ml of TF or TR for 24 h and then collected for fluorometry. 200 µl of cell suspension (100 µg/ml) was added to 800 µl of PBS and was incubated with 2′, 7′-dichlorofluorescein diacetate at 10 µM concentration for 15 min. H2O2 (25 µM) and TF or TR (0, 25, 50, 75, 100 µg/ml) were added in the culture and the incubation was continued for an additional 20 min at 37°C. The production of intracellular H2O2 was measured using fluorometer (excitation at 365 nm and emission at 430 nm). Another set of experiment was performed simultaneously, with same treatments and conditions, but catalase (50 U/ml) was added to the cell culture medium 5 min before the addition of different concentrations of TF and TR.

Activity of caspases

Caspase-3 and caspase-9 were assayed using caspases fluorometric assay kit designed for each caspase by the suppliers. A375 cells (1 × 10⁶ per well) were placed in triplicate in a 24-well tissue culture plate and treated with TF or TR (0, 25, 50, 75, 100 µg/ml) for 24 h. The activity of caspases was determined fluorometrically (excitation at 400 nm and emission at 505 nm). Different concentration of TF or TR treatment was done for 24 h either alone or in combination with Z-VAD-FMK (pan-caspase inhibitor), DEVD-CHO (caspase-3-specific inhibitor), LEHD-CHO (caspase-9-specific inhibitor).

**PARP cleavage**

The intact PARP molecule (116 kDa) is activated by breaks in DNA and is cleaved by caspases to yield a p85 fragment that is a characteristic marker of apoptosis. For the detection of PARP cleavage, A375 cells were treated with TF or TR (0, 25, 50, 75, 100 µg/ml) for 24 h and subjected to western blotting.
An anti-PARP rabbit polyclonal antibody recognizes both 116-kDa and the 85-kDa cleavage fragment. An alkaline phosphatase-conjugated goat anti-rabbit secondary antibody was used for this experiment.

Western blot analysis

Western blot analysis was done to determine the expression of different proteins. A375 cells were treated with different concentrations of TF or TR (0, 25, 50, 75, 100 µg/ml) for 24 h. Cells were harvested, washed with cold PBS (pH 7.4) and lysed with ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5% NP-40, 1 mM PMSE, 10 µg/ml aprotinin and 10 µg/ml leupeptin, pH 7.4 for 30 min and centrifuged at 12,000g for 30 min at 4°C. The protein concentration of the clear supernatant was collected by means of centrifugation and was evaluated using Bio-Rad protein assay kit. Aliquots of equal amounts of proteins from the cells were subjected to SDS–PAGE. Thereafter, proteins were electrophoretically transferred to nitrocellulose membrane and non-specific sites were blocked with 5% skimmed milk in 1% Tween-20 (Sigma-Aldrich) in 20 mM TBS (pH 7.5) and reacted with a primary polyclonal antibody, Bax, Bcl2, Akt, Akt-ser, p21, p53, cytochrome c, PARP, cytochrome c and β-actin for 4 h at room temperature. After washing the tris-buffered saline containing 0.1% Tween-20, the membrane was then incubated with alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. The protein bands were visualized using NBT–BCIP.

Statistical analyses

GraphPad Instat software was used for statistical analysis. All data are expressed as the mean ± SD of three independent experiments. The differences between the control and the treatment groups were determined by one-way ANOVA and posttests were done using Dunnett’s multiple comparison test to determine the significant levels.

Results

TF and TR treatment inhibits cell viability in A431 and A375 cells

The cytotoxic effect of TF and TR on NHEK, A431 and A375 cells was determined with varying concentrations of TF and TR (0, 15, 25, 50, 75, 100, 125 µg/ml) for 24 h (Figure 1A I and A II) and 48 h (Figure 1B I and B II) by MTT assay. Treatment of lower doses of TF and TR did not exhibit any significant cytotoxic effects on the cells at 24 h of incubation. Our study revealed that significant reduction in cell viability commenced at 75 and 100 µg/ml of TF treatment on A375 and A431 cells, respectively (Figure 1A I), whereas significant diminution of viable cell count instigated at 100 and 125 µg/ml of TR treatment on A375 and A431 cells, respectively (Figure 1A II). However, during 48 h of incubation, inhibition of cell viability of A375 and A431 cells were increased in dose-dependent manner (Figure 1B I and B II), which were statistically significant (P < 0.05, P < 0.01). Treatment with TF and TR appeared to display inhibitory activities in A431 cells with estimated IC50 was 99.66 µg/ml, whereas in A375 cells estimated IC50 was 50.02 µg/ml at 48 h of treatment. In case of NHEK cells for both TF and TR, IC50 value (data not shown) was >125 µg/ml at 48 h of treatment. Treatment of <25 µg/ml concentrations of TF and TR did not produce any statistically significant reduction in cell proliferation. Based on cell viability assay in A431 and A375 cells after treatment with different doses of TF and TR, we have selected A375 cells and 0, 25, 50, 75, 100 µg/ml doses for our further studies.
Induction of apoptosis in A375 cells by TF and TR treatment

We thought that the inhibition of cell viability by TF and TR in A375 cells might be mediated through the initiation of apoptotic cascade. Accordingly, we extended our study to examine the induction of apoptosis and the arrest of cell cycle kinetics by TF and TR using A375 cells. The cells were treated with different concentrations of TF or TR (0, 25, 50, 75, 100 µg/ml) for 24 h and analyzed for cell cycle distribution by means of flow cytometry. The flow cytometric data depicted the effect of TF and TR on cell cycle phase distribution of the skin cancer cells. Compared with the vehicle-treated controls (0 µg/ml), the cultivation with different concentrations of TF and TR increased the population of cells in the G0/G1 phase with a reduction of cells in the S phase. The TF treatment caused an arrest of 50% cells in G0/G1 phase of cell cycle at a 25 µg/ml dose and 56%, 65%, 78% arrest caused at the higher doses of 50, 75, 100 µg/ml, respectively (Figure 2A I), whereas in case of TR treatment the arrest of cell cycle kinetics was 47%, 57%, 68%, 75% at the doses of 25, 50, 75, 100 µg/ml, respectively (Figure 2A II). These results indicated that TF as well as TR led to cell cycle arrest at the G0/G1 phase followed by apoptosis in dose-dependent manner. Then, we confirmed TF- and TR-induced apoptosis by means of AnnexinV/PI double-staining method. In case of TF, induction of apoptosis ranged from 8.7% to 59%, whereas in case of TR the range was 7.5–41% (Figure 2B II). We also examined whether the induction of apoptosis was specific for cancer cells or normal cells following TF and TR incubation. Interestingly, any dose of both TF and TR did not induce significant apoptosis (0.02–4.7%) for TF-treated cells and in case of TR treatment it was 0.02–2.7% in NHEK cells (Figure 2B I), even at the highest dose (100 µg/ml) of that used for carcinoma cell lines. This could be explained by the fact that TF and TR induced externalization of phosphatidylserine at very early in apoptosis before any nuclear changes have occurred. All these results together indicated that the different dose of TF and TR (25, 50, 75, 100 µg/ml) rapidly induced apoptosis in A375 cells.

Next, we quantified the extent of apoptosis by DNA fragmentation by means of flow cytometric analysis of the cells labeled with fluorescent-tagged dUTP. As is evident from Figure 2C I, significant DNA fragmentation augmented at higher dose that is 75 µg/ml of both TF and TR treatment for 24 h in A375 cells. However, in case of 48 h of treatment statistically significant increment of DNA fragmentations in A375 cells initiated even at lower dose that is 25 µg/ml of both TF and
TR (Figure 2C II). At the doses, 25–100 μg/ml of TF showed 17.67–57.98% of apoptotic cells, whereas 12.76–47.76% was observed during the treatment of TR at the dose ranged 25–100 μg/ml (Figure 2C II).

The induction of apoptosis by TF and TR was also evident from the morphologic alteration as shown by confocal microscopy after labeling the cells with DAPI- and FITC-tagged AnnexinV. Even at the lower doses of TF and TR, several cells displayed early apoptotic morphology (Figure 3).

**Bax translocation to mitochondria upon the TF and TR treatment**

It has been reported that translocation of proapoptotic protein Bax to mitochondria plays an important role in decreasing MMP and initiating mitochondrial death cascade. In our system, Bax translocation from cytosol to mitochondria was measured by western blot at 24 h of incubation in A375 cells. Our observation revealed that Bax level in mitochondria increased by 1.8- to 3.5-fold for TF and 1.6- to 3.5-fold for TR in dose-dependent manner (Figure 4A). The above results raised the possibility of Bax-induced MMP loss in A375 cells as a result of TF and TR treatment.

**Effect of TF and TR treatment on MMP**

Loss of MMP is associated with mitochondrial dysfunction leading to apoptosis. Consequently, we next evaluated the effect of TF and TR on MMP. Our results showed that these two polyphenols significantly (P < 0.01) decreased MMP in A375 cells (Figure 4B).

**Cytochrome c release in the cytosol by TF and TR treatment**

To check the downstream events of mitochondrial death cascade in A375 cells, we investigated the release of cytochrome c from mitochondria to cytosol. Western blot analysis showed that mitochondrial cytochrome c appeared in the cytosolic fractions after 24 h of incubation with TF or TR. Cytosolic cytochrome c increased by 2- to 4.6-fold for TF (25–100 μg/ml) and 1.8- to 3.5-fold for TR (25–100 μg/ml) (Figure 4C).

**Effects of TF and TR on the generation of intracellular ROS**

The prevailing notion implicates intracellular ROS as signaling intermediates that are involved in signal transduction pathways of apoptosis. So, furthermore we investigated whether TF and TR treatment resulted in the generation of ROS in the skin cancer cell. A statistically significant increase (P < 0.01) of intracellular H2O2 level was detected after treatment with TF and TR, which was attenuated by pretreatment with catalase (Figure 4D). Our data also revealed that the preincubation with the catalase prevented the TF- and TR-induced apoptosis in A375 cells (Figure 4E).

**Effect of TF and TR on caspases activity and PARP cleavage**

Results of cytochrome c release and mitochondrial perturbation further led us to investigate the role of caspase cascade in TF- and TR-induced A375 cell apoptosis. Caspases are believed to play a central role in mediating various apoptotic responses. We examined whether specific caspases were involved in signaling pathway for the induction of apoptosis in A375 cells after treatment with TF and TR for 24 h. Both the polyphenols induced statistically significant (P < 0.05 and P < 0.01) increase in the caspase-3 (Figure 5A) and caspase-9 (Figure 5B) activation in A375 cells. We confirmed this result by using respective caspase inhibitors to elucidate the functional role of caspases in A375 cells. TF- and TR-induced apoptosis was completely blocked by treatment with the respective blockers of caspase-9 and caspase-3, which confirmed that TF- and TR-induced apoptosis is associated with the activation of caspase-3 (Figure 5A) and caspase-9 (Figure 5B). We have also studied the impact of catalase on TF- and TR-induced caspase-3 and caspase-9 activation. Exogenous catalase inhibited TF- and TR-induced caspase-3 (Figure 5B) and caspase-9 (Figure 5C) activation in A375 cells during the 24-h period. Then, we conducted experiments to investigate whether TF and TR induced PARP cleavage as a hallmark sign of caspase-3 activation. Our results revealed that the PARP cleavage was increased in dose-dependent manner in A375 cells (Figure 5E).

**Expression of apoptosis-associated proteins**

After confirming that TF and TR induce apoptosis in A375 cells, we next attempted to unveil further the mechanism of A375 killing. It is well recognized that various proapoptotic and antiapoptotic proteins play a crucial role in mitochondria-mediated programmed cell death. So to examine the role of mitochondrial pathway in TF- and TR-treated apoptosis in A375 cells, we used the western blot analysis (Figure 6A I and A II) to measure the antiapoptotic (Bcl2) and proapoptotic proteins (Bax, p53). We observed that different doses of TF

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**Fig. 3. Representative confocal pictures of TF- or TR-induced apoptotic cells (A375).** The upper most row of the figure showing control cells (0 μg/ml). The doses of TF and TR (25 and 50 μg/ml) are mentioned at the extreme right side of the figure. The first (extreme left) column showing only DAPI (blue) labeled cells, the second (middle) column showing only FITC-tagged AnnexinV (green) labeled cells and the third column showing merged (DAPI and FITC-AnnexinV) pictures. Arrows indicate apoptotic cells. Data shown here are from a representative experiment repeated three times with similar results.
Fig. 4. Effect of different concentrations of TF and TR on Bax translocation, MMP, cytochrome c release, ROS production in A375 cells. (A) Translocation of Bax from cytosol to mitochondria during TF- and TR-induced apoptosis. Cells were treated with different concentrations of TF or TR (0, 25, 50, 75, 100 μg/ml) for 24 h. Relative density of bands obtained from western blot analysis was quantified and represented here as fold change. The Bax level in cytosol and mitochondria in control (0 μg/ml) was adjusted to 1-fold and the change of Bax level in the treated groups was represented as fold of control. Columns, mean of experiments done in triplicate; bars, SD. (B) Effect of TF and TR on MMP in A375 cells. Cells were treated with different concentrations (0, 25, 50, 75, 100 μg/ml) of TF or TR for 24 h. TF- and TR-induced cells were labeled with JC-1 probe and the altered MMP was measured flow cytometrically. Columns, mean of experiments done in triplicate; bars, SD. * * P < 0.01 is the TF- or TR-treated group compared with the control (0 μg/ml). (C) Release of cytochrome c in cytosol from mitochondria in A375 cells after treatment with TF or TR in dose-dependent manner. Cells were treated with TF or TR (0, 25, 50, 75, 100 μg/ml) for 24 h. Cells were harvested and cytochrome c level in cytosolic and mitochondrial fractions was determined by western blot. Relative density of the bands of cytochrome c was quantified and represented here as fold change. The cytochrome c level in cytosol and mitochondria in control (0 μg/ml) was adjusted to 1-fold and the changes of cytochrome c level in the treated groups were represented as fold of control. Columns, mean of experiments done in triplicate; bars, SD. (D) Estimation of H2O2 in A375 cells after treatment with different concentrations (0, 25, 50, 75, 100 μg/ml) of TF or TR for 24 h; 50 U/ml catalase can suppress H2O2 production in TF- and TR-treated cells; 25 μM H2O2 was used as a positive control. H2O2-induced fluorescence was analyzed fluorometrically. Each value is expressed as mean ± SD (n = 3). **P < 0.01 is the TF- or TR-treated groups compared with the control (0 μg/ml). (E) Effect of catalase on TF- and TR-induced apoptosis. Catalase (50 U/ml) was added to cell culture medium 5 min before the addition of different concentrations of TF and TR. Apoptosis was determined by flow cytometrically following 24 h of TF and TR exposure. Each value is expressed as mean ± SD (n = 3). ** P < 0.01 is the TF- or TR-treated group compared with the control (0 μg/ml).

(Figure 6A I) or TR (Figure 6A II) up-regulated the expression of Bax and p53 and down-regulated the expression of Bcl2, thereby increasing Bax:Bcl2 ratio by 0.5- to 6-fold for TF (25–100 μg/ml) and 0.4- to 5-fold for TR (25–100 μg/ml) (Figure 6B). Interestingly, the level of p21 (cell cycle regulatory protein) was increased upon TF and TR treatment. We further investigated the expression of the cell survival protein Akt. Expression of phosphorylated Akt (Akt-ser) was down-regulated upon TF and TR treatment. We made an effort to find out whether expressions of p21 and Akt proteins depend on the p53 and also if there was any relationship between up-regulation of Bcl2 protein and the activation of Akt. In order to investigate the effect of TF (Figure 6C I) and TR (Figure 6C II) on the expression of p53, p21 and Bax in A375 cells preincubated with p53 inhibitor, we decided to block the activation of p53-responsive genes by pifithrin-α. Pifithrin-α has been demonstrated to block p53-dependent transcriptional activation and apoptosis. In the absence of pifithrin-α, p53 expression was found to be dose dependent in A375 cells for TF (Figure 6A I) and TR (Figure 6A II) treatment. Upon addition of pifithrin-α, p53 expression was reduced during TF (Figure 6C I) and TR (Figure 6C II) incubation when compared with the cells not treated with p53 blocker.

Similar to p53, the expression of p21 and Bax protein also substantially declined in TF-treated (Figure 6C I) and TR-treated (Figure 6C II) A375 cells, preincubated with p53 inhibitor, as compared with cells not treated with pifithrin-α. The inhibition suggests that pifithrin-α effectively blocked the transactivation potential of p53. Next our endeavor was to look into the effect of TF and TR on the expression of Akt phosphorylation and Bcl2 in A375 cells preincubated with Akt kinase inhibitor, LY294002. Akt phosphorylation was noticeably inhibited in TF-treated (Figure 6D I) or TR-treated (Figure 6D II) A375 cells preincubated with LY294002 as compared with cells treated with TF (Figure 6A I) or TR (Figure 6A II) alone. But the reduction of Bcl2 expression was more about the same in case of TF-treated (Figure 6D I) and TR-treated (Figure 6D II) cells preincubated with LY294002 when compared with the cells treated with TF (Figure 6A I) and TR (Figure 6A II) alone.

Discussion

Apoptosis plays a crucial role in eliminating the mutated hyperproliferating cells from the system. Thus, induction of apoptosis in tumor
cells may be considered as a protective mechanism against development and progression of cancer. The inhibitory activity of green tea catechins and black tea polyphenols on tumor growth has been reported, both in vitro and in vivo (7,28,29). However, information is very limited on the antitumor activity of TF and there is no such report on TR. Previously, we have reported the antimutagenic and anticlastogenic effects of TF and TR in multiple test systems (22,23). So our endeavor was to investigate the intrinsic molecular mechanism of apoptosis in vitro and the probable signaling cascade of chemoprevention induced by TF and TR in skin cancer cells, with special emphasis on TR. Our study on growth inhibition demonstrated that A375 cells were more susceptible than A431 cells toward the treatment of different concentrations of TF and TR. Our findings are in agreement with other studies demonstrating the growth inhibitory

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Fig. 5. Activation of caspase-3 and caspase-9 after treatment of different concentrations of TF or TR in A375 cells. Cells were treated with TF or TR (0, 25, 50, 75, 100 μg/ml) for 24 h and subjected to measurement of caspase activity by means of cleavage of color substrate. (A) DEVD-AFC for caspase-3 and (B) LEHD-AFC for caspase-9. Effects of caspase-3 (A) and caspase-9 (B) inhibitors on TF- or TR-treated A375 cells were estimated in a coculture with caspase inhibitors. Cells were incubated with each caspase inhibitor for 2 h before addition of TF or TR. Z-VAD-FMK, pan-caspase inhibitor, DEVD-CHO, caspase-3 inhibitors, LEHD-CHO caspase-9 inhibitor. Each value is expressed as mean ± SD (n = 3). *P < 0.01 is the TF- or TR-treated group compared with the control (0 μg/ml). (C and D) Effects of TF and TR on activity of caspase-3 and caspase-9 in A375 cells pretreated with catalase. Cells were pretreated with 50 U/ml exogenous catalase for 5 min prior to addition of TF and TR at concentration indicated. (C) Caspase-3 and (D) caspase-9 activity assay was performed immediately after 24 h incubation with TF or TR. (E) PARP cleavage was determined by western blotting using a rabbit polyclonal PARP antibody. A375 cells were treated with 0, 25, 50, 75, 100 μg/ml of TF or TR for 24 h.

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Fig. 6. Western blot analysis of Bcl2, Bax, p21, p53 and Akt-ser and Akt protein levels upon treatment with TF and TR in different doses. (A I and A II) A375 cells were treated with 0, 25, 50, 75, 100 µg/ml of (A I) TF or (A II) TR for 24 h. Protein from the total cell lysate was subjected to SDS–PAGE and western blot using Bcl2, Bax, p21, p53, Akt-ser and β-actin antibody. Representative blot from three independent experiments was with identical results. Relative intensity of each band after normalization with the intensity of β-actin in a blot (below each western blot) was measured. (B) The ratio of Bax and Bcl2 protein expression was determined from three separate experiments by comparing the relative intensities of protein bands. Each value expressed as mean ± SD (n = 3). (C I and C II) Western blot analysis of (C I) TF- and (C II) TR-induced p53, p21 and Bax protein expression in A375 cells in the presence of p53-inhibitor, pifithrin-α (30 µmol/l). A375 cells were preincubated with pifithrin-α at a concentration of 30 µmol/l for 1.5 h before the treatment of TF and TR. Then, the cells were treated with 0, 25, 50, 75, 100 µg/ml of TF or TR for 24 h. Protein from the total cell lysate was subjected to SDS–PAGE and western blot using p53, p21, Bax and β-actin antibody. Representative blot from three independent experiments was with identical results. Relative intensity of each band after normalization with the intensity of β-actin in a blot (below each western blot) was measured. (D I and D II) Western blot analysis of (D I) TF- and (D II) TR-induced Akt, Akt-ser and Bcl2 protein expression in A375 cells in the presence of PI3k/Akt-inhibitor, LY294002. A375 cells were preincubated with LY294002. Then, the cells were treated with 0, 25, 50, 75, 100 µg/ml of TF (D I) or TR (D II) for 24 h. Protein from the total cell lysate was subjected to SDS–PAGE and western blot using Akt, Akt-ser and β-actin antibody. Representative blot from three independent experiments was with identical results. Relative intensity of each band after normalization with the intensity of β-actin in a blot (below each western blot) was measured.
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effect of TF on various cancer cell lines such as H-ras-transformed mouse epidermal JB-6 cells (30) and mouse fibroblast NIH3T3 cells (31). Our observations indicate that TF exhibits more antiproliferative activity in comparison with that of TR in a dose-dependent manner.

Based on this investigation, next we decided to extend our series of experiments further in A375 cells to find out the induction of apoptosis and its intrinsic mechanism. Previous studies have anticipated that the black tea TF were capable of inducing apoptosis in varieties of human cancer cell lines (32). We observed that TF and TR treatment resulted in augmentation of apoptosis in A375 cells, thus suggesting that the inhibition of cell viability might be, in part, due to the induction of apoptosis. Nuclear DNA fragmentation, a classical feature of apoptotic cell death, was clearly shown in skin cancer cells with different doses of TF and TR.

Eukaryotic cell cycle is regulated by signal transduction pathways mediated by a series of cell cycle regulators. One of those is p21 that has been shown to induce tumor cell growth arrest and apoptosis (33,34). In this study, we determined the inhibition of A375 cell growth at different concentrations of TF and TR, accompanied with a G0/G1 cell cycle arrest.

Apoptosis is modulated by antiapoptotic and proapoptotic effectors that involve a large number of proteins. Therefore, to gain insight into mechanisms controlling apoptosis, we looked at the effect of TF and TR on antiapoptotic and proapoptotic proteins of the Bcl2 family. Bcl2 protein functions as a suppressor of apoptosis (35). Bax is a proapoptotic protein and its predominance over Bcl2 promotes apoptosis (36). In the present study, we have found that treatment with different concentrations of TF and TR in A375 cells resulted in reduction of Bcl2 protein expression, whereas increased the expression of Bax, indicating that the increased ratio of Bax:Bcl2 proteins may be responsible for the induction of apoptosis.

It is known that p53 may induce two sets of genes in response to stress signals thereby contributing to the decision-making growth arrest and apoptosis. This tumor suppressor protein may up-regulate the proapoptotic protein Bax and is known to mediate growth arrest involving p21 as a major effector (37). In fact, p53-dependent induction of p21 prevents entry of cells into S phase (38). In our present set of investigation, we have found increased expression of p53 and p21, suggesting that TF and TR treatment could inhibit cell cycle kinetics at G0/G1 phase thus inducing apoptogenic signal in A375 cells. In order to investigate whether the up-regulation of p21 and Bax is correlated with the elevation of p53 level in TF- and TR-treated A375 cells, we decided to block p53 by pifithrin-α, a known p53 inhibitor. Our results exhibited that abolition of p53 transactivation potential by pifithrin-α reduced the expression level of p21 and Bax in A375 cells after treatment of TF and TR. So, it could be postulated that there might be a relationship between p53 activation and the up-regulation of p21 and Bax in TF- and TR-treated A375 cells. All these elucidations corroborated the relationship between p53 status, p21 induction and Bax:Bcl2 ratio in cell cycle deregulation and apoptosis in TF- and TR-treated A375 cells. However, the results of our study also illustrated that inhibition of Bcl2 expression was not changed substantially upon the treatment with PI3K/Akt inhibitor, LY294002, in TF- and TR-treated A375 cells as compared with the cells not treated with Akt blocker. It could be hypothesized that Bcl2 expression might not depend on the Akt phosphorylation in TF- and TR-treated A375 cells.

Translocation of Bax protein causes change in MTP that plays an important role in the induction of apoptosis (12). The mitochondrial membrane has only a limited permeability that is essential for formation of the MTP (39). Permeabilization of this membrane allows release of cytochrome c and activation of subsequent downstream caspases (12,13,40). Activated caspase-3 cleaves intracellular proteins such as PARP, which are vital to cell survival and growth, and PARP cleavage has been used as an important marker of apoptosis (14). In our present study, we have found that the treatment of TF and TR induced mitochondria-mediated apoptosis by translocation of Bax to mitochondria in A375 cells. This intracellular movement coincided with the MTP change, release of cytochrome c into cytosol, subsequent activation of downstream caspase-9, caspase-3 and PARP cleavage.

In a variety of cell types, the apoptosis-triggering effects of ROS were noted in vitro and in vivo (41,42). TF and TR treatment elevated intracellular ROS levels and also induced apoptosis in A375 cells. Furthermore, pretreatment of A375 cells with catalase could prevent TF- and TR-induced intracellular ROS generation and apoptosis induction. This observation corroborated with the previous finding that oxidative stress induces apoptotic pathways in the tumor cells (43). In addition, we noted a substantial prevention of TF- and TR-mediated caspase-3 and caspase-9 activation in A375 cells pretreated with exogenous catalase. From these observations it could be suggested that TF and TR-derived intracellular ROS might have a role in caspase-3 and caspase-9 activation and apoptosis induction in A375 cells.

In conclusion, our study indicates that both TF and TR induce apoptosis via mitochondria-mediated pathway and involvement of proapoptotic and antiapoptotic proteins, thus providing a molecular basis for understanding the chemopreventive effect of TF and TR that might be ideal candidates to induce effective apoptosis in human skin cancer cells. However, presently the detailed mechanism underlying the antiproliferative role of TF and TR in different types of cancer cells to understand whether the action of these polyphenols is cell line specific or via a general mechanism. These observations will add new light in the field of developing therapeutic strategies for cancer in near future. To our knowledge, this is the first report of the induction of apoptosis and its mechanism is induced by TR, an important polyphenol exclusively present in black tea.

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