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Tea Polyphenols Induce Apoptosis Through Mitochondrial Pathway and by Inhibiting Nuclear Factor-κB and Akt Activation in Human Cervical Cancer Cells

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Phytochemicals present in tea, particularly polyphenols, have anticancer properties against several cancer types. However, studies elucidating the role and the mechanism(s) of action of tea polyphenols in cervical cancer are sparse. In this study, we investigated the mechanism of antiproliferative and apoptotic actions exerted by tea polyphenols on human papilloma virus-18-positive HeLa cervical cancer cells. Treatment of green tea polyphenol (−)-epigallocatechin gallate (EGCG) and black tea polyphenol theaflavins (TF) in HeLa cells showed a marked concentration- and time-dependent inhibition of proliferation and induced sub-G_1 phase in a dose-dependent manner after 24 h. There was an attenuation of mitochondrial membrane potential with the increase of reactive oxygen species generation, p53 expression, Bax/Bcl-2 ratio, cytochrome-c release, and cleavage of procaspase-3 and -9 and poly(ADP-ribose)-polymerase, indicating the participation of a mitochondria related mechanism. In addition, EGCG as well as TF inhibited activation of Akt and nuclear factor-κB (NF-κB) via blocking phosphorylation and subsequent degradation of inhibitor of κBα and κBβ subunits, thereby downregulating cyclooxygenase-2. Additionally, the protein level of cyclin D1, a transcriptional target of NF-κB, was also reduced significantly. Thus, we can conclude that tea polyphenols inhibit the growth of cervical cancer cells by inducing apoptosis and regulating NF-κB and Akt.

Key words: Tea polyphenols; Cervical cancer; Apoptosis; Nuclear factor-κB (NF-κB); Akt; Cyclooxygenase-2

INTRODUCTION

Worldwide, cervical cancer comprises approximately 12% of all cancers in women and is a major global public health problem affecting socioeconomically deprived populations (1). Cervical cancer is caused by infection of specific types of human papillomaviruses (HPVs). Current treatment strategies for locally advanced cervical cancer consist of cisplatin-based chemotherapy and concurrent radiation therapy. This combination is often associated with an increased probability of dose-limiting toxicities like gastrointestinal and hematological toxicities and 5 years of overall survival rates are about only 50% (2). Taking into consideration these limitations, more effective and less toxic anticancer agents are needed to reach the goal of cervical cancer treatment.

Plant-derived products have been demonstrated for their anticancer effects (3) and some of them have also been proved in clinical trials (4). Tea (*Camellia sinensis*, family Theaceae), second only to water as the most consumed beverage in the world, has incredible potential as a chemopreventive agent. There are evidences from laboratory studies that the tea phytochemicals possess obvious proapoptotic, antiproliferative, antiangiogenic, and antimetastatic activities against a variety of cancers (5–7). These experimental studies together with several epidemiologic studies have suggested that tea polyphenols harbor anticancer and cancer preventive effects in human cancers (8). However, little is known regarding the anticancer effects of tea phytochemicals in cervical cancer.

Among the positive and negative regulators of apoptosis p53 is an important defense molecule as it suppresses tumor growth through cell cycle regulation and apoptosis (9). Bax, the proapoptotic member of the Bcl-2 family, is a p53 target and is transactivated in a number of systems during p53-mediated apoptosis (10). The upregulation of Bax expression and downregulation of Bcl-2 leads to caspase-3 activation, the key executioner of apoptosis (11).

Several studies have demonstrated that constitutive overexpression of nuclear factor-κB (NF-κB, belonging to the rel family of transcriptrion factors) in human cancers plays mitogenic and antiapoptotic roles (12,13). Ac-

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tivation of NF-κB, which is sequestered in the cytoplasm, requires phosphorylation of IκB at two specific serine residues at the N-terminus of IkB (14,15). This allows free NF-κB to translocate into the nucleus to bind with DNA for the activation of genes such as cyclin D1 and cyclooxygenase-2 (Cox- 2) (16,17). Involvement of both NF-κB and Cox-2 has been implicated with the development cervical cancer (18,19). It is reported that HPV E6/E7-regulated gene products involved in the expression of proteins necessary for innate immunity, apoptosis, and cell proliferation are target genes of NF-κB (20). Akt is well known to regulate NF-κB activation and Cox-2 expression (21). Therefore, inhibiting Akt and NF-κB activation by chemopreventive agents could be one logical approach to prevent and/or treat cervical cancer.

In this study, we attempted to untie the anticancer effects of black tea polyphenol, theaflavins (TF), and green tea polyphenol, (−)-epigallocatechin gallate (EGCG), in human cervical cancer HeLa cells, containing high-risk HPV subtype 18 and underlying molecular mechanisms.

MATERIALS AND METHODS

Chemicals

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium-bromide (MTT), 5,5′,6,6′,-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide (JC-1), 2′,7′-dichlorofluorescein diacetate (DCF-DA); 5-chloromethylfluorescein diacetate (CMF-DA), 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI), propidium iodide (PI), and β-actin (clone AC-74) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified TF and EGCG (>98% pure) were also procured from Sigma-Aldrich. Caspase-3, caspase-9, cytochrome c, poly(ADP-ribose)polymerase (PARP), p-NF-κB, p-IκBα, p-IκBβ, p53, cyclin D1, Cox-2, Bcl-2, and p-Akt antibodies was procured from Cell Signaling Technology (Beverly, MA, UDA), while Bax antibody was procured from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The rabbit anti-mouse or goat anti-rabbit horseradish peroxidase conjugate secondary antibodies were obtained from Bangalore Genei (Bangalore, India). The polyvinylidene fluoride (PVDF) membrane was obtained from Millipore (Bedford, MA, USA). Annexin-V/PI FITC apoptosis detection kit was from BD Pharmingen (San Diego, CA, USA). Caspase-3 inhibitor z-DEVD-fmk was purchased from Calbiochem (Boston, MA, USA). The rest of the chemicals were of analytical grade and were procured locally.

Cell Culture and Treatment

HeLa cells were obtained from National Centre for Cell Science (Pune, India) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1% penicillin streptomycin (Gibco Lifetech, Karlsruche, Germany) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Cell Proliferation Assay

The effect of TF and EGCG treatments on the viability of HeLa cells was determined by MTT assay (22). In brief, cells were treated with various concentrations of TF and EGCG ranging from 5 to 50 μg/ml in 96-well microtiter plates for 24, 48, and 72 h. After completion of incubation periods MTT dye (5 mg/ml) was added 20 μl/well and after 3 h of incubation 100 μl DMSO was added to dissolve the crystals. Absorbance was recorded on a microplate reader (Biotek Instruments Inc., Winooski, VT, USA) at 530-nm wavelengths. The IC_{50} value was determined from a plot of percentage of survival versus EGCG and TF concentrations where cells with no treatment were considered 100% viable. For trypan blue dye exclusion assay, 5×10^3 cells were plated in six-well plates and allowed to attach overnight. The medium was replaced with fresh medium containing desired concentrations of TF and EGCG and incubated for 24 h. Both floating and adherent cells were collected and pelleted by centrifugation at $700 \times g$ for 5 min. Cells were resuspended in 25 μl of PBS, mixed with 5 μl of 0.4% trypan blue solution, and counted using a hemocytometer under an inverted microscope.

Cell Cycle Analysis

The effect of TF and EGCG on cell cycle distribution was determined by analysis of DNA content of cells following staining with PI. After treating with desired concentrations of TF and EGCG, the cells were washed with PBS and fixed in 70% ethanol overnight at 4°C. The cells were then treated with DNase-free RNaseA and PI (50 μg/ml) for 30 min in the dark at 4°C, and analyzed using a flow cytometer (7).

Determination of Apoptosis

Apoptosis induction in control and tea polyphenol-treated cells was assessed by (i) quantification of cells with sub-G_0/G_1 DNA content by flow cytometry following staining with PI, (ii) annexin-V/PI staining, (iii) DAPI staining. For annexin-V/PI staining 1×10^5 HeLa cells were seeded in six-well plates, allowed to attach, and treated with desired concentrations of TF and EGCG for 24 h. Both attached and floating cells were harvested, washed in PBS, and further processed as per manufacturer's instructions. Cells' positivity for annexin-V/PI was analyzed using a flow cytometer equipped

with CellQuest software (7). Apoptotic cells with condensed or fragmented nuclei were observed following staining with 10 ng/ml DAPI and captured using fluorescence microscope, Olympus IX51 (Olympus America Inc., Center Valley, PA, USA), at excitation wavelength 350 nm and software Image Pro Express (23).

Measurement of Intracellular Reactive Oxygen Species Generation and Glutathione Content

Intracellular level of reactive oxygen species (ROS) and glutathione (GSH) content was determined by using oxidation-sensitive fluorescent probes DCF-DA and CMF-DA, respectively (24). For microscopic detection of ROS formation, cells were plated in black bottom 96-well microtiter plates and treated with various concentrations of TF and EGCG for 6-, 12-, and 24-h durations. At the end of treatment durations cells were incubated with DCF-DA (10 μM) for 30 min at 37°C in the dark. After incubation the living cells were kept on ice and immediately fluorescence was measured at 485 nm excitation and 535 nm emissions using a microplate fluorescence reader (Biotek Instruments Inc. Winooski, VT, USA).

For estimation of GSH content, after completion of 24-h treatment period with different concentrations of TF and EGCG, cultured cells were incubated with CMF-DA (25 μM) for 30 min at 37°C in a CO_2 incubator and subjected to flow cytometry for the estimation of CMF fluorescence, which is directly related to intracellular GSH level.

Measurement of Mitochondrial Membrane Potential ($\Delta\Psi m$)

The loss of mitochondrial potential (MMP) by treatment with TF and EGCG was determined using mito-

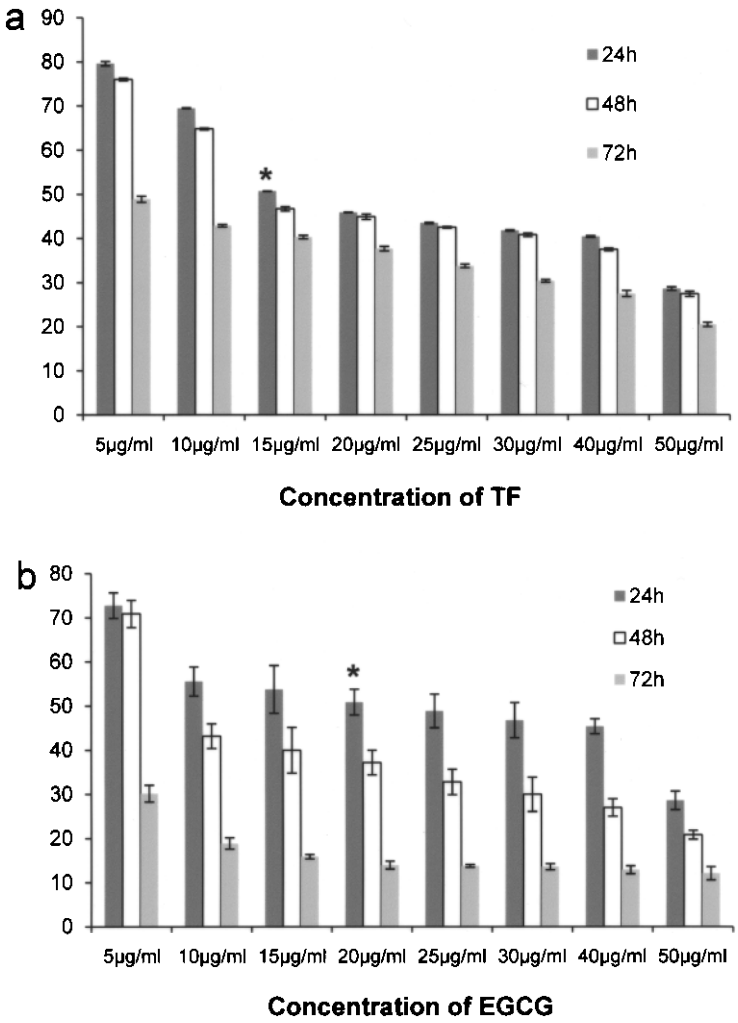

Figure 1. Effects of tea polyphenols on proliferation of HeLa cells determined by MTT assay. (a) TF, (b) EGCG. *IC_{50} concentration.

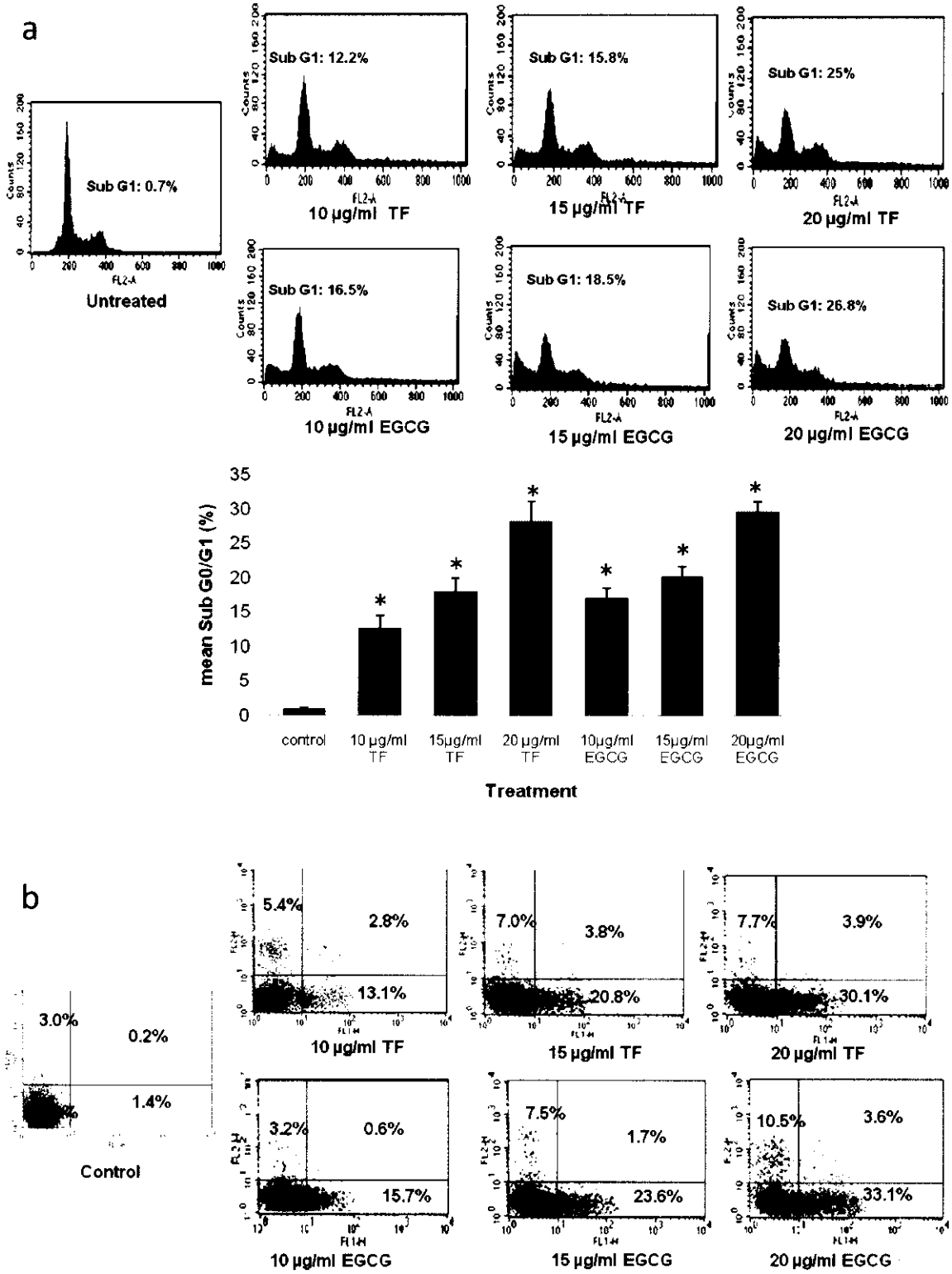

Figure 2. Effects of tea polyphenols on apoptosis induction assessed by (a) flow cytometric analysis of HeLa cells with sub-G_1 DNA content. Representative histograms from control and TF- and EGCG-treated cells (10, 15, and 20 µg/ml for 24 h). Bar diagram shows mean ± SD of three independent experiments ($p < 0.05$). (b) Annexin and PI dual staining. Representative dot plots from control and TF- and EGCG-treated cells (10, 15, and 20 µg/ml for 24 h). (c) DAPI staining (40×) of HeLa cells following treatment with TF and EGCG or untreated (24 h).

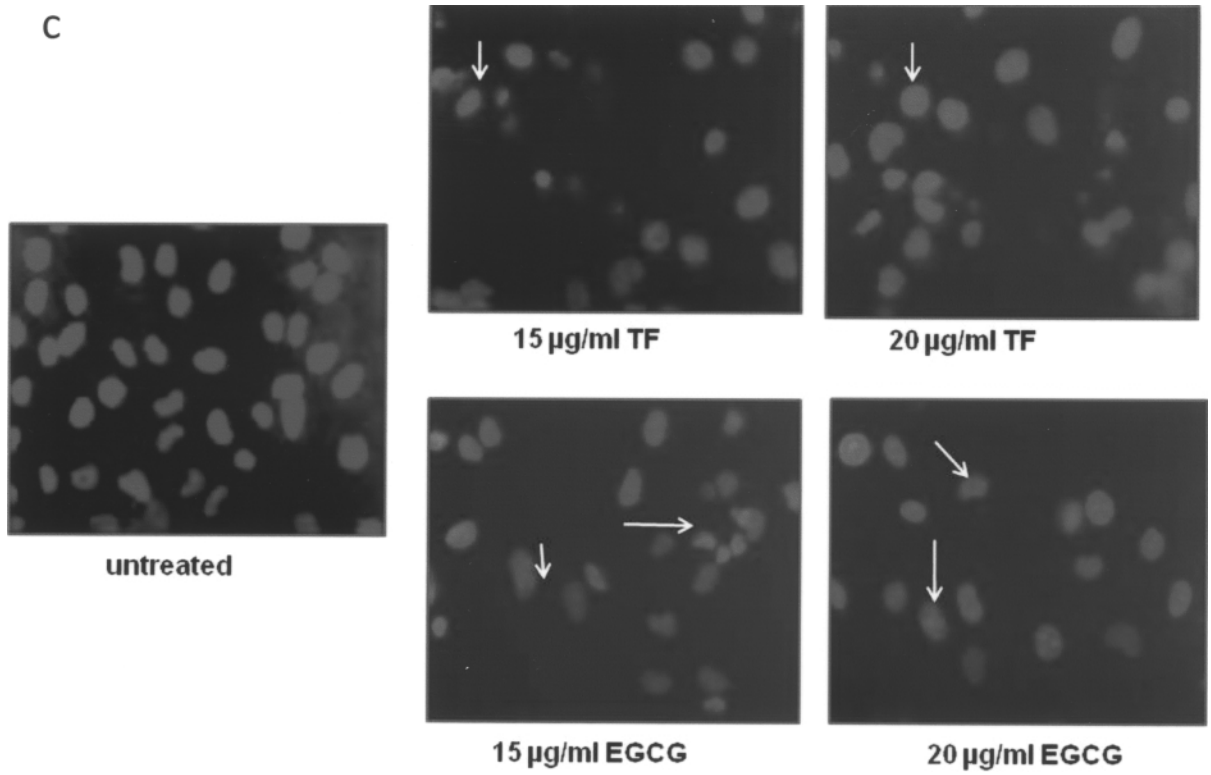

Figure 2. Continued.

chondrial potential sensor JC-1. In brief, cultured cells were incubated with JC-1 (2.5 µg/ml) for 30 min at 37°C in the dark and then washed with PBS. ΔΨm was assessed by comparing two fluorescence [i.e., red (Ex/Em 580/590 nm)/green (Ex/Em 510/527 nm)] using flow cytometer (BD-LSR) and analysis was performed on Cell Quest software (25).

Western Blotting

Treated and untreated cells were washed with PBS, resuspended in ice-cold lysis buffer (50 mM Tris-HCl, 150 nM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, pH 7.4) at 4°C for 30 min and then centrifuged at 12,000 $\times g$ for 10 min at 4°C to obtain total cell lysates and stored at −80°C until further use (26). Total/nuclear/cytosolic extracts were prepared and protein concentration was estimated (27). Cytosolic fraction (for caspase-3, caspase-9, cytochrome c, PARP, p-IκBα, p-IκBβ, p53, cyclin D1, Cox-2, Bcl-2, Bax, p-Akt, and β-actin) and nuclear fraction (for NF-κB) were used. For Western blotting, proteins (50 µg) were resolved on 10–15% SDS-polyacrylamide gels followed by electrotransfer onto an immobile PVDF membrane. The blots were blocked overnight with 5% nonfat dry milk, probed with respective primary antibodies, and detected by horseradish peroxidase conjugated anti-rabbit or anti-mouse IgG using chemiluminescence kit from Millipore and visualized by Versa Doc Imaging System (BioRad Model 4000, Hercules, CA, USA). The intensity was given in terms of relative pixel density for each band normalized to band of β-actin. The intensity of the bands was measured using software UNSCAN-IT automated digital system version 5.1 (Orem, USA).

Statistical Analysis

Statistical significance of difference was determined by Student's *t*-test. A value of $p < 0.05$ was considered significant.

RESULTS

Tea Polyphenols Inhibit Proliferation of HeLa Cells

Both TF and EGCG (5–50 µg/ml) treatments resulted in concentration- and time-dependent inhibition of HeLa cell proliferation with an IC_{50} of 15 and 20 µg/ml for TF and EGCG at 24 h, respectively (Fig. 1). On the bases of MTT assays 10, 15, and 20 µg/ml doses of both TF and EGCG were selected for further mechanistic studies.

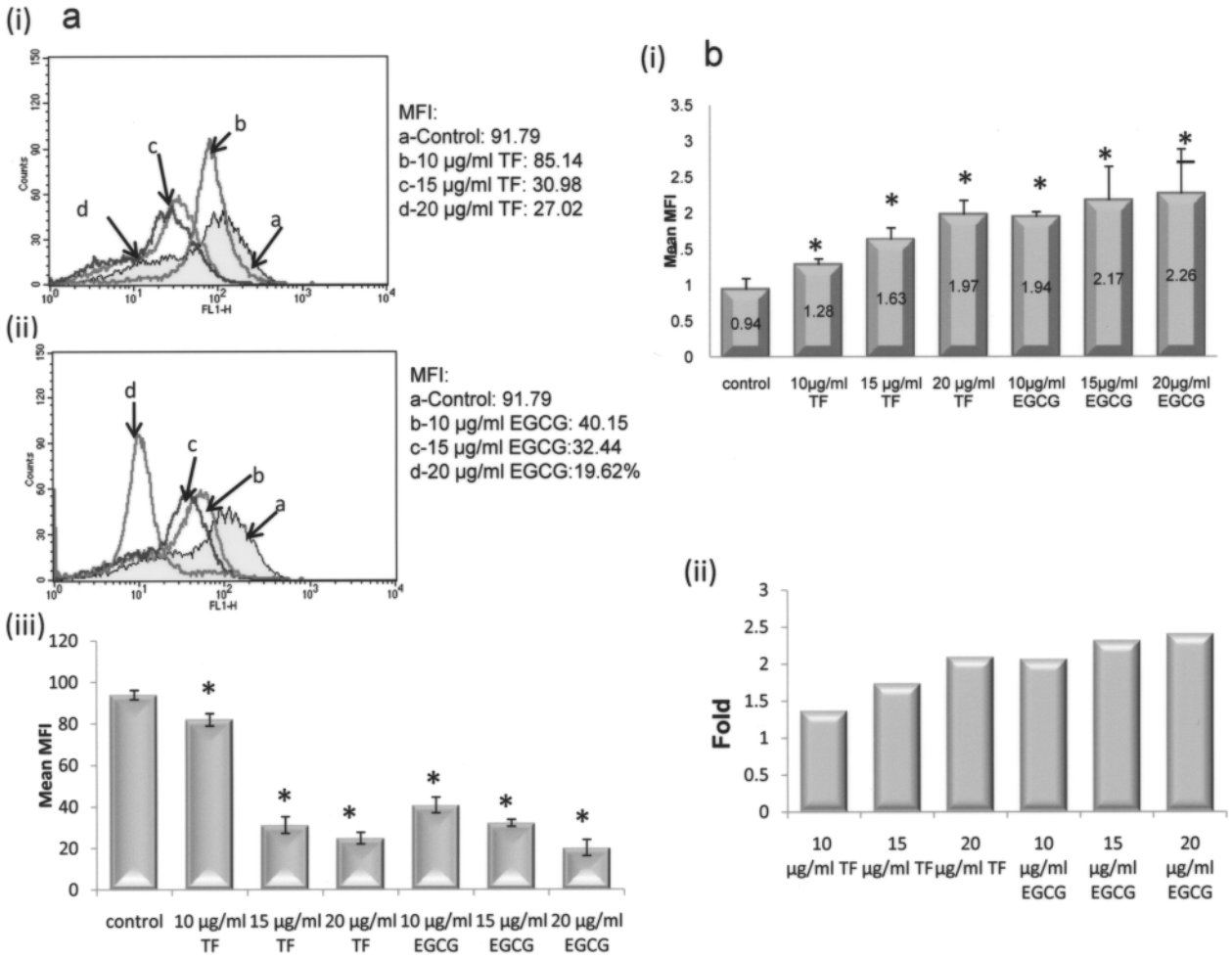

Figure 3. (a) HeLa cells treated with (i) TF and (ii) EGCG (10–20 µg/ml) for 24 h. For determination of GSH content, CMF-DA (25 µM) was added, incubated for 30 min, and its fluorescence measured using a flow cytometer with FL-1 filter (log). Results expressed as representative histogram of three individual experiments. Bar diagram (iii) shows mean ± SD of three independent experiments ($p < 0.05$). (b) For determination of intracellular ROS untreated and TF- and EGCG-treated HeLa cells (10–20 µg/ml for 24 h) were stained with DCF-DA (10 µM), incubated for 30 min, and its fluorescence measured. Bar diagram shows (i) mean ± SD and (ii) fold change of three independent experiments ($p < 0.05$).

Tea Polyphenols Induce Apoptosis in HeLa Cells

The apoptosis-inducing potential of TF and EGCG was examined by (i) quantifying percentage of cells with sub-G_0/G_1 DNA content, (ii) annexin V/PI staining, and (iii) analysis of cells with condensed nuclei. Exposure of HeLa cells to TF and EGCG for 24 h resulted in a statistically significant increase of cells with sub-diploid DNA content, which was 12.6 ± 1.9%, 17.9 ± 2.0%, and 28.2 ± 2.8% in TF-treated cells and 17.0 ± 1.4%, 20.1 ± 1.50%, and 29.5 ± 1.5% in EGCG-treated cells by 10, 15, and 20 µg/ml doses, respectively ($p < 0.05$) (Fig. 2a). Annexin-V/PI staining was performed to determine apoptotic (early and late) and necrotic population following TF and EGCG (24 h) treatment to the cells. Percentage of early and late apoptosis (Annexin-V stained) cells were found to be significantly increased in TF-treated cells (17.93 ± 2.25, 26.57 ± 3.35, and 33.30 ± 4.9 by 10, 15, and 20 µg/ml doses, respectively) and EGCG-treated cells (17.5 ± 1.11, 28.33 ± 2.78, and 39.37 ± 2.92 by 10, 15, and 20 µg/ml doses, respectively) in a dose-dependent manner ($p < 0.05$) (Fig. 2b). Figure 2c shows nuclear condensation in HeLa cells treated with TF and EGCG as determined by DAPI staining.

Inhibition of GSH of HeLa Cells by Tea Polyphenols

Glutathione is the key regulator of intracellular redox status, scavenging ROS directly and indirectly (28). Whether TF and EGCG exacerbate oxidative stress by causing depletion of intracellular glutathione was inves-

tigated in this study. Flow cytometric analysis revealed reduction in cellular GSH content in a dose-dependent mode by both TF (13%, 67%, and 73% by 10, 15, and 20 μg/ml doses, respectively) and EGCG (57%, 66%, and 79% by 10, 15, and 20 μg/ml doses, respectively) ($p < 0.05$) (Fig. 3a).

Tea Polyphenols Induce ROS Generation in HeLa Cells

We investigated whether generation of intracellular ROS is part of the mechanism by which tea polyphenols induce apoptosis in HeLa cells. Treatment of HeLa cells increased the DCF fluorescence intensity, which was 106% by 10 μg/ml, 130% by 15 μg/ml, and 141% by 20 μg/ml EGCG doses compared with untreated control. Increase in intensity was also noted in cells treated with selected concentrations of TF (36% by 10 μg/ml, 73% by 15 μg/ml, and 109% by 20 μg/ml TF) compared with untreated cells (Fig. 3b).

Tea Polyphenols Induce Mitochondrial Damage in HeLa Cells

The functional alterations occur in mitochondria due to changes in MMP ($\Delta\Psi m$). Thus, to elucidate the possible involvement of MMP in tea polyphenol-induced apoptosis, we evaluated $\Delta\Psi m$ by using JC-1. A significant ($p < 0.05$) (Fig. 4) dose-dependent decrease in MMP was observed by TF treatment (4.4-fold at 10 μg/ml,

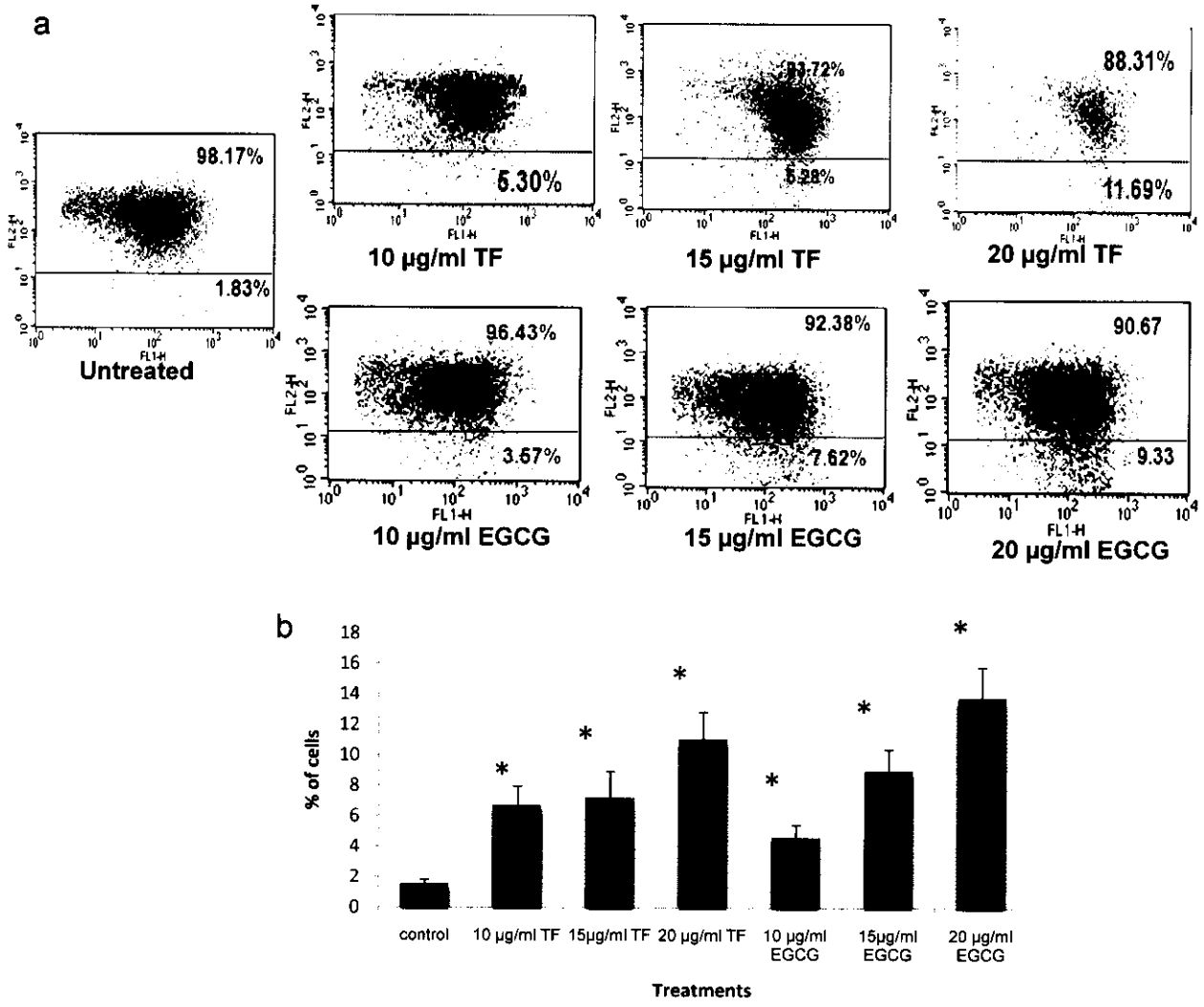

Figure 4. HeLa cells treated with TF and EGCG (10–20 μg/ml) for 24 h. For determination of mitochondrial membrane potential, JC-1 (2.5 μg/ml) was added, incubated for 30 min, and its fluorescence measured using a flow cytometer with FL-1 filter. (a) Results expressed as representative dot plot of three individual experiments. (b) Bar diagram shows mean ± SD values of three individual experiments ($p < 0.05$).

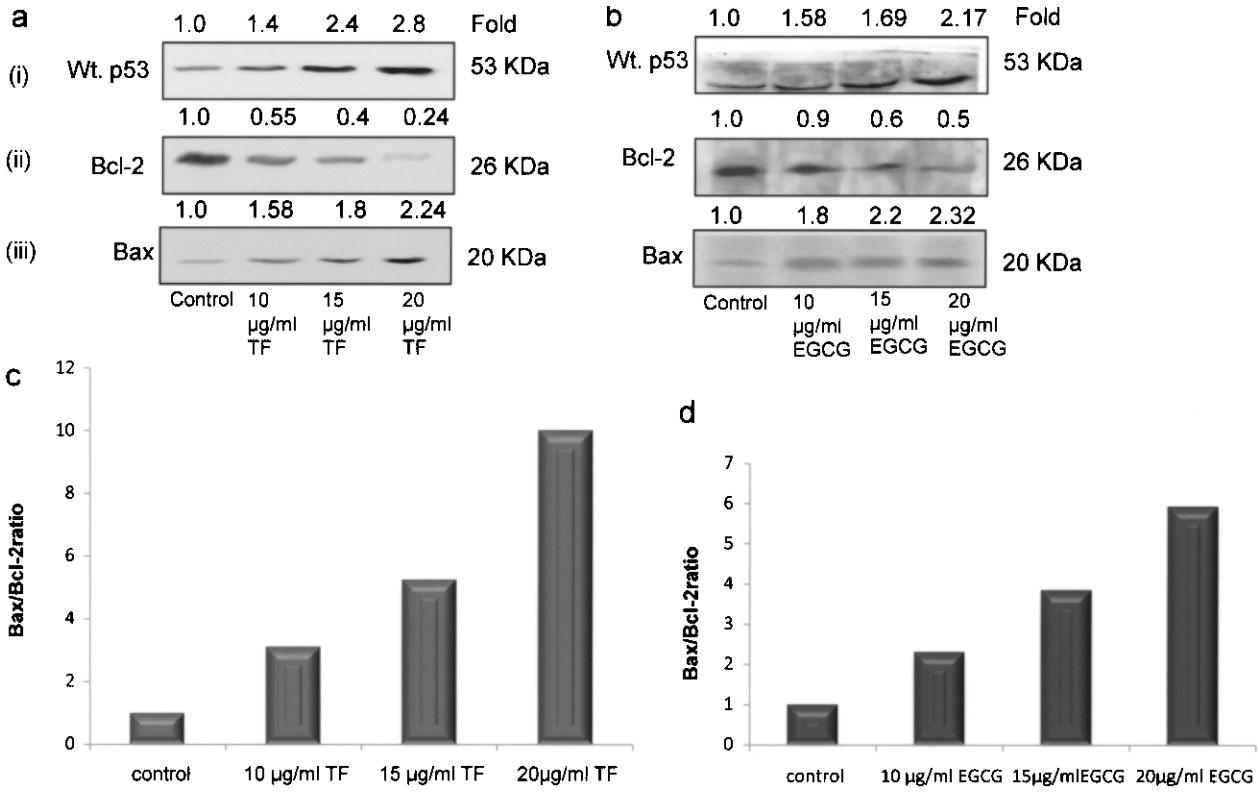

Figure 5. Representative immunoblots showing effect of (a) TF and (b) EGCG treatment on levels of (i) p53, (ii) Bcl-2, and (iii) Bax. For immunoblotting HeLa cells were treated with indicated concentrations (10–20 µg/ml) of tea polyphenols for 24 h prior to preparation of lysates. Intensities of the immunoreactive bands were quantified by densitometric scanning. A change in the protein level is indicated at the top of the band following normalization to control. Bar diagrams summarizing the effect of (c) TF and (d) EGCG on Bax/Bcl-2 ratio.

4.7-fold at 15 µg/ml, and 7.3-fold at 20 µg/ml dose for 24 h) and EGCG (3.0-fold at 10 µg/ml, 5.8-fold at 15 µg/ml, and 9.0-fold at 20 µg/ml doses for 24 h) in HeLa cells in comparison to untreated cells.

Tea Polyphenols Modulate the Levels of Proapoptotic and Antiapoptotic Proteins

p53 is known to play a key role in cell cycle regulation and apoptosis (9) and is involved in positive regulation of bax and negative regulation of bcl-2 gene expression (10). Bax and bcl-2 genes play an important role in the regulation of apoptosis. Treatment of HeLa cells for 24 h with increasing concentrations of TF and EGCG caused a dose-dependent increase in p53 and Bax proteins level (Fig. 5a, b). In contrast, the level of Bcl-2 protein was reduced significantly upon treatment with TF and EGCG in a dose-dependent manner (Fig. 5a, b). Further, the ratio of Bax to Bcl-2 increased after treatment in a dose-dependent manner, indicative of the apoptosis process (Fig. 5c, d).

Tea Polyphenols Induce Release of Cytochrome c and Cleavage of Caspase-3 and -9 in HeLa Cells

The release of cytchrome c from the mitochondria into the cytosol is a key event in mitochondria-dependent apoptosis (29) and Bax has been reported to induce cytchrome c release and subsequent activation of caspase-9 (30). Results of the study showed that there was a substantial increase in release of cytochrome c in HeLa cells upon exposure to TF and EGCG (24 h) in a dose-dependent manner (Fig. 6a, b). Further dose-dependent increase in 37 kDa caspase-9, corresponding to the active form, was detected in both TF- and EGCG-treated HeLa cells (24 h), but not in untreated cells (Fig. 6a, b). The activation of caspase-3, as evidenced by the appearance of cleavage intermediates, was observed in cells after TF and EGCG treatments (Fig. 6a, b). To determine whether activation of caspase-3 contributed to cell death by tea polyphenols, effect of z-DEVD-fmk (a specific inhibitor of caspase-3, 50 µM/L; 1 h prior) on TF- and EGCG-induced pro-caspase-3 cleavage was deter-

mined. It revealed that TF- and EGCG-induced cleavage of pro-caspase-3 was fully blocked in the presence of z-DEVD-fmk (data not shown), confirming involvement of caspase-3 in tea polyphenol-mediated cell death. In conformity of these annotations, the growth inhibitory effect of tea polyphenols against HeLa cells were also significantly attenuated upon treatment with z-DEVD-fmk (Fig. 6c, d), confirming involvement of caspase-3 in TF- and EGCG-mediated cell death.

Tea Polyphenol Treatment Causes PARP Cleavage in HeLa Cells

Caspase-3 is an executioner caspase whose activation leads to the cleavage of key cellular proteins including DNA repair enzyme PARP (31). Treatment of HeLa cells with increasing concentrations of TF and EGCG for 24 h resulted in cleavage of PARP, which was evidenced by appearance of the 85-kDa cleaved intermediate (Fig. 6a, b). Cleavage of PARP by tea polyphenols was blocked when the cells were pretreated with z-DEVD-fmk prior (1 h) to TF and EGCG treatment (data not shown).

Tea Polyphenols Inhibit Activation of NF-κB and Akt and Expression of Cox-2

Increase in NF-κB activity has been documented to have antiapoptotic effects (13). NF-κB is retained in the cytoplasm in association with inhibitor protein IκB (14,15). Upon phosphorylation on serine residues, degraded IκBα allows NF-κB to translocate to the nucleus and activate transcription of genes responsible for cell growth such as cyclin D1. NF-κB binds with the consensus sequence located in the Cox-2 gene promoter, thereby regulating Cox-2 protein expression (17). The results showed that treatment of TF and EGCG (10–20 μg/ml doses, 24 h) in HeLa cells decreased the phosphorylation of IκBα, which in turn caused inactivation and inhibition of nuclear translocation of NF-κB (Ser536) (Fig. 7). A significant decrease in Cox-2 protein expression levels was also noted in cells following

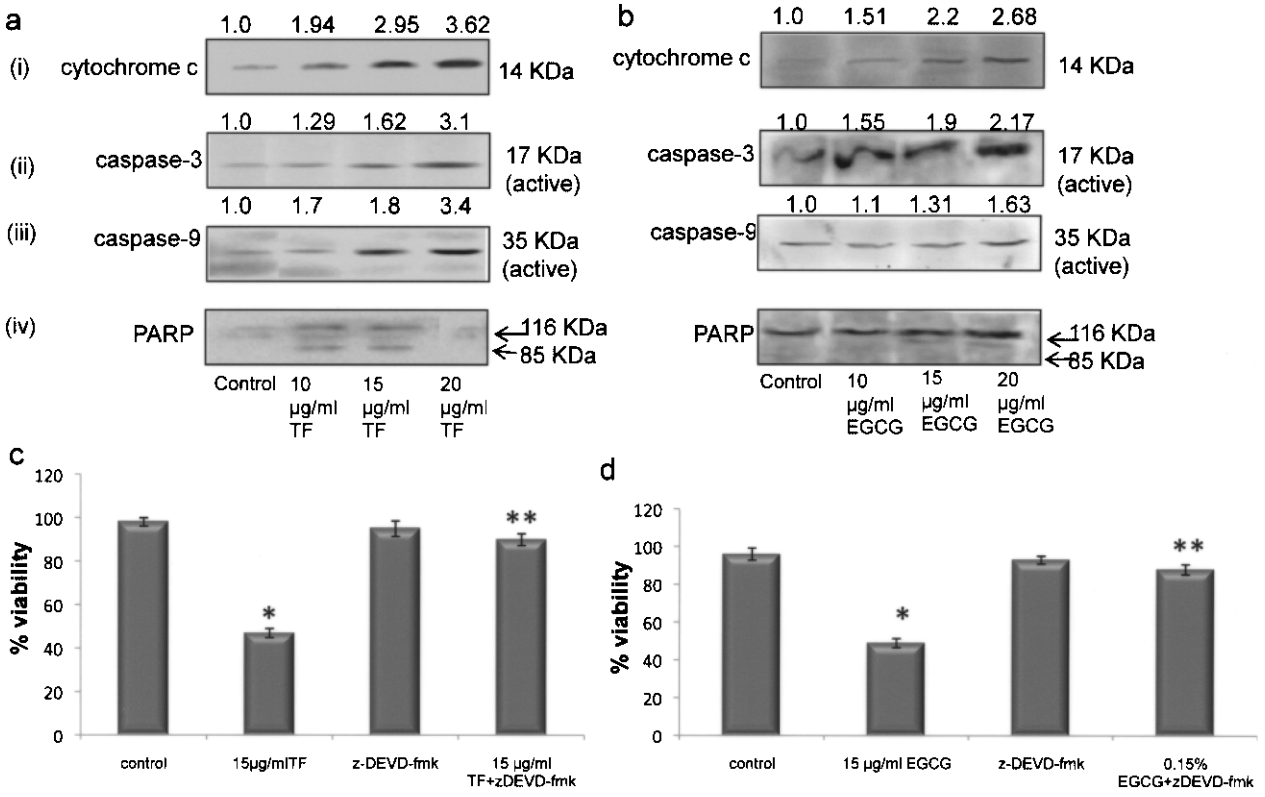

Figure 6. (a, b) Immunoblotting for the effect of TF and EGCG, respectively, on (i) cytochrome c, (ii) caspase-3, (iii) caspase-9, and (iv) PARP. HeLa cells were treated with 10–20 μg/ml of tea polyphenols (24 h). A change in the protein level is indicated at the top of the band following normalization to control. (c, d) Effect of caspase-3-specific inhibitor on survival of HeLa cells in presence or absence of (c) TF and (d) EGCG as determined by trypan blue dye exclusion assay. Data are mean ± SD of two independent assays. *Significantly different from control ($p < 0.05$), and **significantly different compared with TF or EGCG (15 μg/ml) alone group ($p < 0.05$).

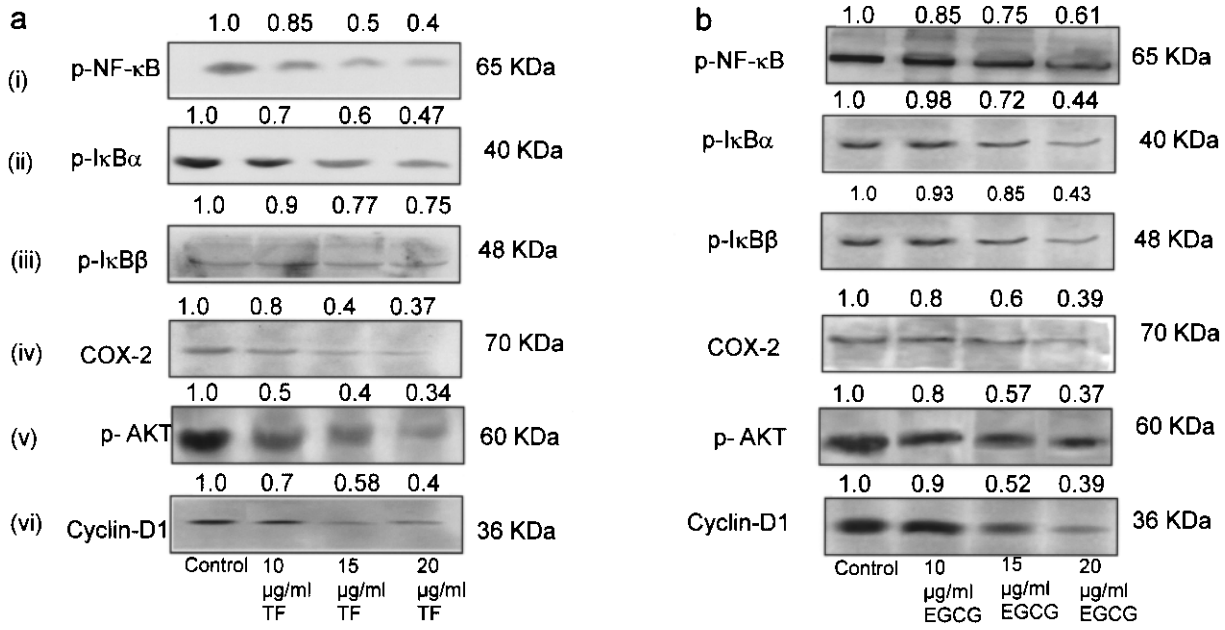

Figure 7. Immunoblotting for the effect of (a) TF and (b) EGCG (10, 15, and 20 μg/ml) on (i) NF-κB protein level in the nuclear extract, (ii) p-IκBα, (iii) IκBβ, (iv) Cox-2, (v) p-Akt, and (vi) cyclin D1 using lysates from HeLa cells treated with varying doses of tea polyphenols for 24 h. Intensities of the immunoreactive bands were quantified by densitometric scanning. Protein level relative to control is indicated at the top of each band.

the treatment of tea polyphenols (Fig. 7). Because Akt is well known to regulate NF-κB activation and Cox-2 expression (21), we also studied the role of Akt in mediating the inhibitory effects of TF and EGCG. Decreased expression of pAkt (Ser473) was observed in TF- and EGCG-treated HeLa cells compared to untreated cells (Fig. 7).

Tea Polyphenols Treatment Reduces Level of Cyclin D1 Protein

Cyclin D1, transcriptionally activated by NF-κB, has been shown to be overexpressed in cervical cancers (32). Treatment for 24 h with 10, 15, and 20 μg/ml of both TF and EGCG individually in HeLa cells resulted in reduction of the cyclin D1 protein level (Fig. 7), which is consistent with a decrease in NF-κB activity.

DISCUSSION

Polyphenols, present in green tea and black tea, considered important sources of antioxidants, demonstrate anticancer effects via induction of apoptosis, activation of p53, and inhibition of angiogenesis (7,33,34). The present study showed that both TF and EGCG exert anticancer activity against HeLa cells by inducing sub-G_1 phase of the cell cycle, thereby causing apoptosis (Fig. 2a), which is mediated in part by inhibition of NF-κB expression. It also revealed that mitochondrial dysfunction and increase of ROS generation are the early events involved in apoptotic cell death induced by TF and EGCG treatment (Fig. 3b). To the best of our knowledge, the present study is the first report to document the novel activity of tea polyphenols as an inhibitor of the NF-κB signaling pathway in human cervical cancer cells.

Mitochondria, which play a pivotal role in apoptosis, are major sites of ROS generation. Excessive generation of ROS can lead to opening of the mitochondrial permeability transition pore with decline in $\Delta\Psi m$ and consequent release of cytochrome c from the intermembrane space into the cytosol, culminating in activation of the caspase cascade and apoptotic cell death pathway (35). In the tea polyphenol-treated HeLa cells, the $\Delta\Psi m$ level loss occurred after 24 h of treatment (Fig. 4), indicating that decrease in MMP was a critical event in TF- and EGCG-induced apoptosis. Simultaneously, significant increase in ROS generation together with decline in GSH content of cells treated with TF and EGCG was also noted (Fig. 3a). Phytochemicals are reported to induce apoptosis through increasing intracellular ROS and GSH depletion (36,37). A rapid collapse of $\Delta\Psi m$ is found in some anticancer compounds, including tea polyphenol-induced apoptosis in cancer cells (7,34,36,37). This onset of apoptosis was further confirmed by the expression of membrane phosphatidylserine and chromatin condensation as observed in tea polyphenol-

treated HeLa cells (Fig. 2b, c). Taken together, these results suggest that tea polyphenols induce apoptosis via mitochondrial perturbation pathway.

We further extended this work to achieve insight into the signaling network and interaction points modulated by TF and EGCG via determining their possible role, if any, in modulation of the proteins involved in the mitochondrial pathway of apoptosis. It is well established that when a cell would be committed to apoptosis partly depends on the balance between proteins that mediate cell death (e.g., p53, Bax) and proteins that promote cell viability (e.g., Bcl-2) (38). It is known that p53 may induce genes in response to stress signals, thereby contributing to apoptosis. Bax is an apoptosis-promoting member of the Bcl-2 protein family and is also considered to be one of the primary p53 targets (39). Bcl-2 protein is known to form heterodimers with the Bax protein in vivo and the molar ratio of Bcl-2 to Bax determines whether apoptosis is induced or inhibited in cells (40). In this study, we found that the expression of p53 and Bax was increased and Bcl-2 was decreased on tea polyphenol treatment. Increased expression of Bax can induce apoptosis by suppressing the activity of Bcl-2 (11), confirming that the ratio of Bcl-2 and Bax is crucial for the apoptosis induced by anticancer agents. Interaction among the Bcl-2 family proteins (Bax, Bak, Bcl-2, Bcl-X, etc.) stimulates the release of cytochrome c and leads to the formation of apoptosome with Apaf1, which in turn activates executioner caspases to orchestrate apoptosis (39). PARP is a protein involved in a number of cellular processes involving mainly DNA repair and programmed cell death (41). The ability of PARP to repair damaged DNA is prevented through its cleavage by executioner caspases (42). The present study showed the upregulation of the cytochrome c and cleavage of caspase-9, caspase-3, and PARP in tea polyphenol-exposed cells (Fig. 6). Therefore, it implied the proapoptotic efficacy of tea polyphenols in cervical cancer cells via activation of caspase-3, which was mediated by mitochondrial pathway involving release of apoptosis-promoting factors (e.g., cytochrome c) from mitochondria to the cytosol, leading to recruitment and activation of caspase-9.

NF-κB exists as a heterodimer of p50 and p65 subunits, and is sequestered in the cytoplasm as an inactive complex bound to an endogenous inhibitor IκB (14,15). Following cellular stimulation, IκB proteins are phosphorylated at two specific serine residues at the N-terminus IκBα (serine 32/36) or IκBβ (Ser19/23) by IκB kinase. The phosphorylation of IκB promotes its ubiquitination and degradation through the 26S proteasome, thereby allowing NF-κB to translocate to the nucleus (14,15). NF-κB activation in cancer cells correlates with resistance to apoptosis and increased levels of antiapoptotic Bcl-2 family proteins (14,15). NF-κB also activates genes involved in cell proliferation (cyclin D1), angiogenesis (vascular endothelial growth factor), and metastasis, thereby promoting tumor growth and metastasis (14–16). Inhibition of NF-κB by tea polyphenols has been reported in in vitro and in vivo conditions (43,44). It is becoming increasingly clear that compounds that block NF-κB activation could be highly useful for the treatment of cancers. Data presented herein indicated that TF and EGCG treatment significantly inhibited NF-κB activation by reducing the level of p-IκBα. Expression of Cox-2 and activation of Akt at Ser473 in treated HeLa cells was significantly decreased (Fig. 7). Akt has been shown to be activated in cancers (45) and promotes cell survival by activating the NF-κB signaling pathway (46). Increased expression of Cox-2 is associated with tumor invasion (47) and resistance to apoptosis in cancer cells (48). Tea polyphenols are reported to suppress activation of Akt and expression of Cox-2 (44). We also found that tea polyphenols treatment reduced the level of cyclin D1 protein, which again confirmed that TF and EGCG inhibited NF-κB activation because cyclin D1 is a downstream target of NF-κB. Interestingly, a recent study indicated correlation between HPV-induced cervical cancer and overexpression of cyclin D1 (49).

In conclusion, the present study showed that both TF and EGCG exert anticancer activity against HeLa cells by inducing sub-G_1 phase of the cell cycle, thereby causing apoptosis, which is mediated in part by inhibition of NF-κB expression. It also revealed that mitochondrial dysfunction and increase of ROS generation are the early events involved in apoptotic cell death induced by TF and EGCG treatment. To the best of our knowledge, the present study is the first report to document the novel activity of tea polyphenols as an inhibitor of the NF-κB signaling pathway in human cervical cancer cells. Taken together, these data suggest that tea polyphenols treatment induced mitochondrial dysfunction, release of cytochrome c into the cytosol, and then activation of the cascade of caspases. Tea polyphenols individually have concurrent effect on important transcription factors p53 and NF-κB, causing a change in the ratio of pro- and antiapoptotic proteins, namely Bax/Bcl-2, and thus leading to apoptosis. The study also confirms that attenuation of NF-κB and Akt activation may be an important mechanism for growth-suppressive activity of tea polyphenols against HPV-positive human cervical cancer cells.

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