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Role of p53 and NF-κB in epigallocatechin-3-gallate-induced apoptosis of LNCaP cells

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We have recently shown that oral consumption of green tea polyphenols inhibits prostate carcinogenesis in transgenic mouse model of prostate cancer and suggested that induction of apoptosis in prostate cancer cells is responsible for these effects. Much of the chemopreventive effects of green tea are attributed to its major polyphenolic constituent (−) epigallocatechin-3-gallate (EGCG). In the present study, we report that EGCG-induced apoptosis in human prostate carcinoma LNCaP cells is mediated via modulation of two related pathways: (a) stabilization of p53 by phosphorylation on critical serine residues and p14^{ARF}-mediated downregulation of murine double minute 2(MDM2) protein, and (b) negative regulation of NF-κB activity, thereby decreasing the expression of the proapoptotic protein Bcl-2. EGCG-induced stabilization of p53 caused an upregulation in its transcriptional activity, thereby resulting in activation of its downstream targets p21/WAF1 and Bax. Thus, EGCG had a concurrent effect on two important transcription factors p53 and NF-κB, causing a change in the ratio of Bax/Bcl-2 in a manner that favors apoptosis. This altered expression of Bcl-2 family members triggered the activation of initiator capsases 9 and 8 followed by activation of effector caspase 3. Activation of the caspases was followed by poly (ADP-ribose) polymerase cleavage and induction of apoptosis. Taken together, the data indicate that EGCG induces apoptosis in human prostate carcinoma cells by shifting the balance between pro- and antiapoptotic proteins in favor of apoptosis.
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Keywords: epigallocatechin-3-gallate; prostate cancer; p53; NF-κB; Bax, Bcl-2; caspases; apoptosis

Introduction

Green tea polyphenols, particularly its major constituent epigallocatechin-3-gallate (EGCG), has shown remarkable chemopreventive and chemotherapeutic effects in several tumor model systems (Katiyar and Mukhtar, 1996; Conney *et al*., 1999; Weisburger, 1999; Yang *et al*., 2000; Gupta *et al*., 2001). Recent studies from our laboratory and other groups have shown that green tea and EGCG are capable of selectively inhibiting cell growth and inducing apoptosis in cancer cells without adversely affecting normal cells (Ahmad *et al*., 1997; Chen *et al*.,1998; Otsuka *et al*., 1998; Yang *et al*., 1998; Gupta *et al*., 2001). Further, extending this work, we showed that this differential response of normal cells versus cancer cells may be due to inhibition of constitutive expression and activation of NF-κB in human epidermoid carcinoma A431 cells, but not in normal human epidermal keratinocytes (NHEK) at a similar concentration of EGCG. Moreover, EGCG-mediated NF-κB inhibition was found to occur at much higher dose in NHEK as compared to A431 cells (Ahmad *et al*., 2000). It is now widely appreciated that agents capable of inducing apoptosis in cancer cells can potentially lead to the development of mechanism-based prevention and treatment approaches for cancer. Therefore, a complete understanding of the mechanism(s) of EGCG action is important for its development for cancer prevention and/or therapy.

We have earlier shown that EGCG induces G1 phase arrest and apoptosis in human prostate carcinoma cells via p21/WAF1, irrespective of p53 status (Gupta *et al*., 2000). The tumor suppressor gene p53 plays a pivotal role in inhibiting tumorigenesis (Agarwal *et al*., 1998; Vogelstein *et al*., 2000; Vousden, 2002). This transcription factor regulates cell cycle progression, checkpoint activation (El-Deiry *et al*., 1994; Budram-Mahadeo *et al*., 2002), apoptosis (Miyashita and Reed, 1995; Budram-Mahadeo *et al*., 2002; Wu and Deng, 2002) and repair of DNA damage (Vogelstein *et al*., 2000). Manipulating p53-mediated pathway has, thus, been an ongoing focus for the development of effective anticancer agents (Vogelstein and Kinzler, 2001). Increase in p53 levels is observed in response to a variety of stimuli such as irradiation, drug treatment, growth factor withdrawal and is followed by transactivation of p53 targets p21/Waf1, Bax, murine double minute 2 (MDM2), etc. (Vogelstein *et al*., 2000). p21/Waf1 is an

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inhibitor of cyclin-dependent kinases 2, 4 and 6 and its upregulation causes the cells to undergo G1 arrest, while Bax is a proapoptotic member of the Bcl-2 family of proteins, whose upregulation favors apoptosis.

The stability of p53 is determined by its transcriptional target MDM2 which acts as an E3 ligase and targets p53 to proteasomal degradation (Kubbutat *et al*., 1997). Therefore, increased p53 activity causes increased expression of its own negative regulator MDM2, thereby forming an autoregulatory feedback loop. The interaction of MDM2 with p53 in turn is governed by the tumor suppressor $p14^{ARF}$ (called as $p19^{ARF}$ in mice). $p19^{ARF}$ is an alternative reading frame protein from the INK4a locus, the other being $p16^{INK4a}$ (Sherr, 2001). $p14^{ARF}$ acts upstream of p53 and activates p53 function by restraining the p53-antagonist, MDM2 (Tao and Levine, 1999; Weber *et al*., 1999).

In contrast to the role of wild-type p53 as a negative regulator of growth signals, the NF-κB family of transcription factors initiate cell survival pathways. NF-κB is a family of ubiquitously expressed, dimeric, sequence-specific transcription factors. The importance of NF-κB family members in modulating cellular growth, apoptosis and development is well documented. In most unstimulated cells, the NF-κB dimers are bound in the cytoplasm by inhibitory IκB molecules (Verma *et al*., 1995). Phosphorylation of IκB by IκB kinases (IKK), IKKα and IKKβ, triggers its ubiquitin-mediated degradation. This allows nuclear translocation and transcriptional activation of NF-κB.

Since both NF-κB and p53 are activated in response to a variety of stimuli, it is conceivable that these transcription factors, which have largely opposite effects, modulate each other's activities. Competition for limiting pools of transcriptional coactivators p300 and CBP has been reported to mediate a bidirectional repression between NF-κB and p53 (Webster and Perkins, 1999). p53 is inactivated in over 50% of all human cancers, but in a significant fraction of cancers that express wild-type p53, this protein is inactivated by other mechanisms, such as increased MDM2 expression, increased Raf or Akt signaling. Another potential mechanism may involve the activation of antiapoptotic genes that compete with the proapoptotic pathways activated by p53. In this context, the upregulation of the antiapoptotic protein Bcl-2 by NF-κB might play a unique role in compromising the apoptotic abilities of p53. On the other hand, p53 can upregulate the levels of the proapoptotic protein Bax. Since cells maintain a fine balance between the concentrations of these two family members, shift in the levels of one of these proteins, that is, decreased expression of Bcl-2 or increased expression of Bax, can commit a cell to undergo apoptosis.

In the present study, we have used human prostate carcinoma LNCaP cells with wild-type p53 as a model to study the role of p53 and NF-κB in EGCG-mediated growth arrest and apoptosis. We show that EGCG treatment causes the stabilization of p53 via phosphorylation of critical serine residues on p53 and modulation of MDM2-$P14^{ARF}$ pathway. Simultaneously, EGCG inhibits the transcriptional activity of NF-κB. We further demonstrate that activation of p53-dependent downstream targets p21/WAF1 and Bax and downregulation of NF-κB-dependent Bcl-2 result in growth arrest and apoptosis.

Results

EGCG induces cell growth inhibition and apoptosis in LNCaP cells

To study the effect of EGCG on prostate carcinoma LNCaP cells, these cells were treated with 20–80 μM of EGCG for 24, 48 and 72 h and their viability was determined post-treatment by MTT assay. We observed that at 60 and 80 μM, EGCG inhibited cell growth at 24 and 48 h of treatment, while inhibition of cell growth was seen at all doses after 72 h of treatment with EGCG (Figure 1a). To determine if the loss of viability induced by EGCG was due to apoptosis, we measured apoptosis in LNCaP cells by ELISA and TUNEL assay. We observed significant apoptosis in LNCaP cells after 24 and 48 h of treatment with 60 and 80 μM of EGCG (Figure 1b,c). After 72 h of treatment, EGCG was found to induce apoptosis at all the doses tested. These results indicate that EGCG inhibits cell growth by inducing apoptosis in LNCaP cells as a function of dose and time.

EGCG increases the expression and transcriptional activation of p53 in LNCaP cells

To investigate if EGCG had an effect on p53 protein expression, we determined the p53 levels in LNCaP cells treated with 40 or 80 μM of EGCG for 24, 48 or 72 h. Immunoblot analysis showed that EGCG treatment increased protein expression of p53 (Figure 2a). The increased p53 protein expression correlated with increase in the levels of its transcriptional target p21/WAF1 (Figure 2a). To examine if increased p53 expression resulted in a concurrent upregulation of its transcriptional activity, LNCaP cells were transfected with PG-13 luciferase plasmid that contains p53 consensus binding sites. For normalizing transfection efficiency, LNCaP cells were cotransfected with β-galactosidase plasmid. At 24 h after transfection, these cells were treated with 20, 40 or 80 μM of EGCG and luciferase activity was determined after 24 h. We observed that there was a linear increase in the transcriptional activity of p53 as a function of EGCG dose (Figure 2b).

EGCG induces stabilization of p53 in LNCaP cells

Wild-type p53 is a short lived protein and its induction in response to cellular stress is followed by post-translational modifications such as phosphorylation and acetylation of critical serine residues. To examine the mechanism by which EGCG mediated increase in p53 levels, we determined the phosphorylation status of

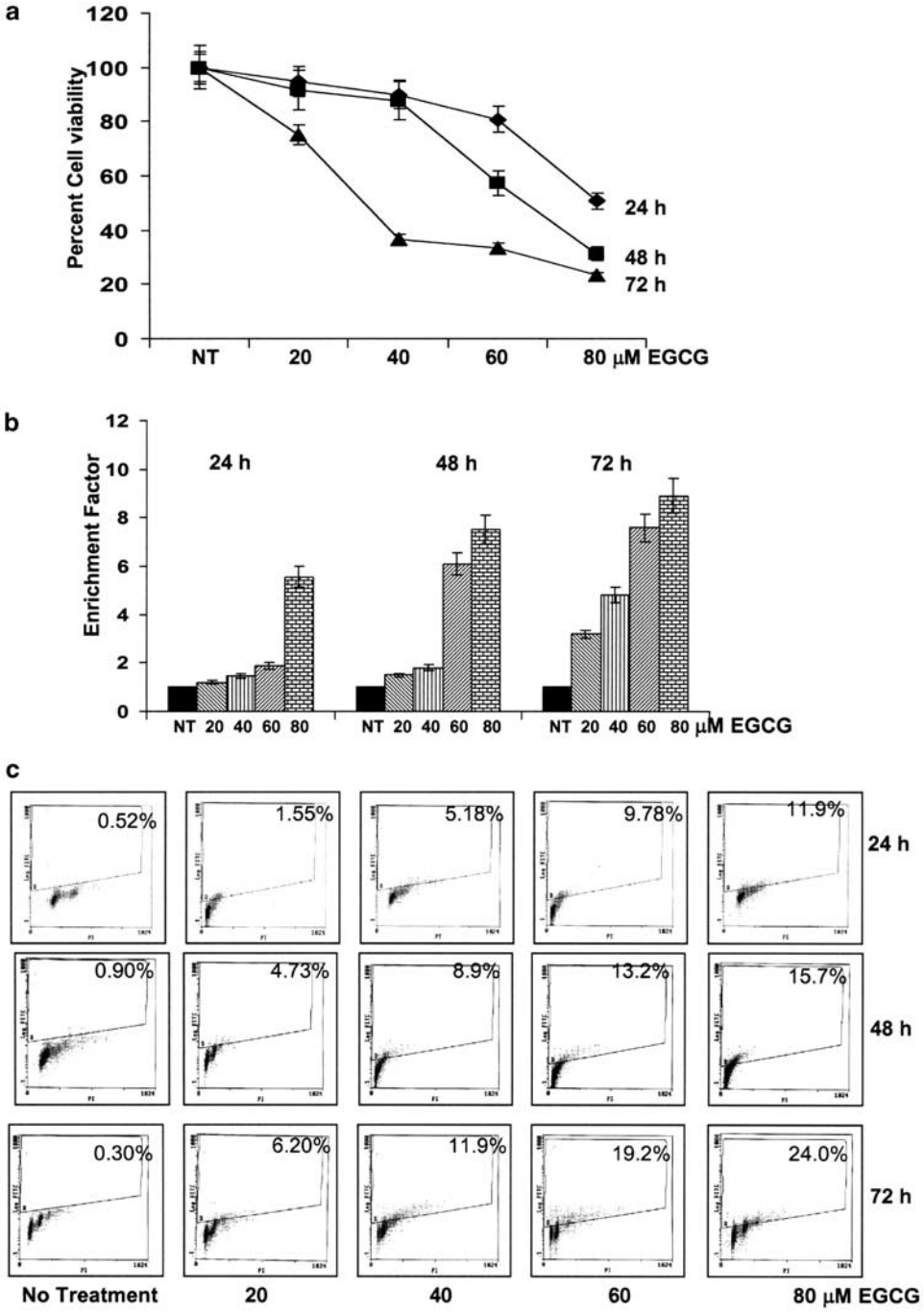

Figure 1 Growth inhibition and apoptosis in EGCG-treated LNCaP cells. The 60–70% confluent LNCaP cells were grown in 10% serum-containing medium in the presence of 20–80 μM EGCG for 24, 48 or 72 h. (**a**) Cell viability for each time point was determined by MTT assay. Mean $\pm$ s.e. for three independent experiments is shown. (**b**) Determination of apoptosis by ELISA. Data are expressed as enrichment factor (see Materials and methods). (**c**) Determination of apoptotic (TUNEL-positive) cells by flow cytometry. The abscissa of each histogram indicates fluorescence of PI, and the ordinate indicates fluorescence of FITC-labeled DNA. The number at each histogram is the percentage of cells with FITC fluorescence above the line (TUNEL-positive). Representative data from one experiment ($n = 3$) are shown

p53 at different serine residues that have been documented to increase the half-life of this protein. As shown in Figure 3a, 24 h of EGCG treatment resulted in phosphorylation of p53 at serine 6, 15, 20, 37 and 392, while there was no change at serine 9. Adriamycin which induces p53 and phosphorylates it at various serine residues was used as a positive control. Stabilization of p53 can occur via the MDM2–p14^{ARF} pathway. MDM2 is a ubiquitin ligase that binds and targets p53 to ubiquitin-dependent proteolysis. The interaction of

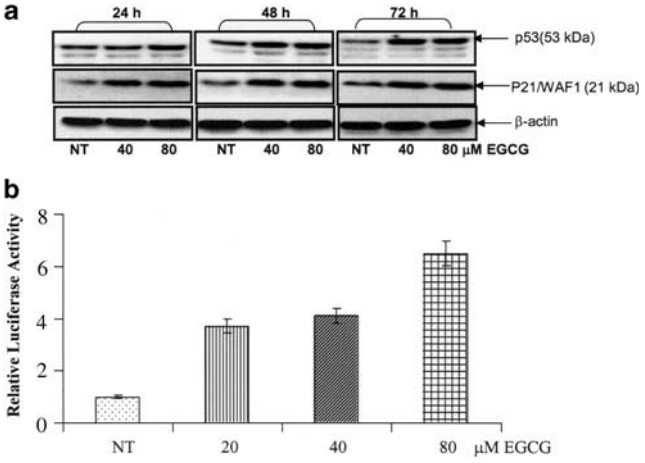

Figure 2 Effect of EGCG on p53 expression and transcriptional activity. (**a**) LNCaP cells were treated with the indicated doses of EGCG for 24, 48 or 72 h. Protein (50 μg) from total cell lysates from the treated and control cells was subjected to SDS–PAGE and Western blotting using p53 and p21 antibodies (**b**) Transcriptional activation of p53 was studied using p53-dependent PG-13 promoter. At 24 h post-transfection with the PG-13 luciferase plasmid, LNCaP cells were treated with the indicated concentration of EGCG. Luciferase assay was performed as described under Materials and methods

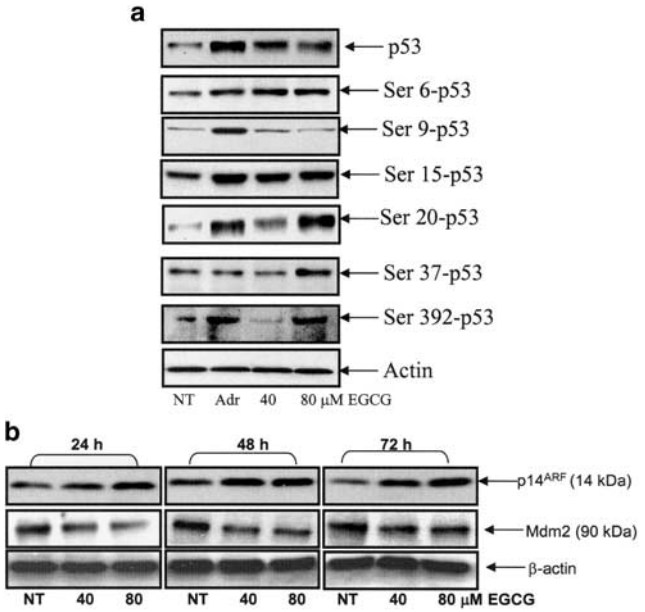

Figure 3 EGCG-induced stabilization of p53. (**a**) Total cell lysates prepared from LNCaP cells treated with the indicated doses of EGCG for 24 h were subjected to SDS–PAGE and Western blotting using antibodies against p53 phosphorylated at different serine residues. (**b**) LNCaP cells were treated with the indicated doses of EGCG for 24, 48 or 72 h. Protein (50 μg) from total cell lysates from the treated and control cells was subjected to SDS-PAGE and Western blotting using MDM2 and $p14^{ARF}$ antibodies

MDM2 with p53 is antagonized by the tumor suppressor $p14^{ARF}$, which averts the MDM2-mediated ubiquitination and degradation of p53. To investigate the role of MDM2–$p14^{ARF}$ in EGCG-mediated p53 stabilization, we immunoblotted lysates of LNCaP cells treated with EGCG for anti-MDM2 and anti-$p14^{ARF}$. As shown in Figure 3b, EGCG treatment resulted in an increase in $p14^{ARF}$ and a concomitant decrease in MDM2 expression. Taken together, these results suggest that EGCG induces stabilization of p53 by phosphorylation on critical serine residues and via $p14^{ARF}$ -mediated down-regulation of MDM2 protein.

EGCG decreases DNA binding and transcriptional activity of NF-κB

NF-κB family of transcriptional regulators is associated with increased malignancy and cellular transformation. Recent studies show that NF-κB transcriptional activity significantly reduced cell death and p53 accumulation in response to stress. Our experimental results indicated that EGCG-induced apoptosis was mediated by increased stability of p53 and subsequent upregulation of its downstream targets. In order to investigate if EGCG exerts a parallel effect on NF-κB, we examined the DNA binding activity of NF-κB in nuclear lysates of LNCaP cells by electrophoretic mobility shift assay (EMSA). The sequence used for DNA binding was 5′–AGT TGA GGG GAC TTT CCC AGG C–3′, 3′–TCA ACT CCC CTG AAA GGG TCC G–5′, which is a binding site for NF-κB/c-Rel homodimeric and heterodimeric complexes. As shown in Figure 4a, EGCG treatment decreased the DNA binding activity of NF-κB. Furthermore, EGCG also decreased TNFα (a known inducer of NF-κB, used as a positive control)-induced NF-κB activity. We also observed a decrease in the protein levels of the p65 subunit of NF-κB in nuclear lysates of LNCaP cells treated with EGCG. This indicated that reduced availability of NF-κB in the nucleus may be responsible for the decreased transcriptional activity (Figure 4b). To determine the specificity of this inhibition, NF-κB transcriptional activity was further assayed using the NF-κB-dependent E-selectin-luciferase reporter plasmid (pElam[-143]-luc) (Sizemore *et al.*,1999). TNFα, an inducer of NF-κB transcriptional activity, was used as a positive control in this experiment. We observed that EGCG treatment resulted in decrease of both endogenous and TNFα-mediated NF-κB dependent transcriptional activity (Figure 4c).

EGCG increases the ratio of Bax/Bcl-2

The ratio of the proapoptotic Bax and antiapoptotic Bcl-2 is critically balanced during cell proliferation such that an increase in the levels of Bax or a decrease in the level of Bcl-2 can shift the ratio and trigger a signal initiating apoptosis. The expression of the antiapoptotic protein Bcl-2 is positively regulated by the transcription factor NF-κB and negatively by p53. In contrast, the levels of the proapoptotic Bax can be transcriptionally upregulated by p53. Since EGCG induced p53 activity and decreased NF-κB activity, we hypothesized that EGCG treatment would result in altered levels of the

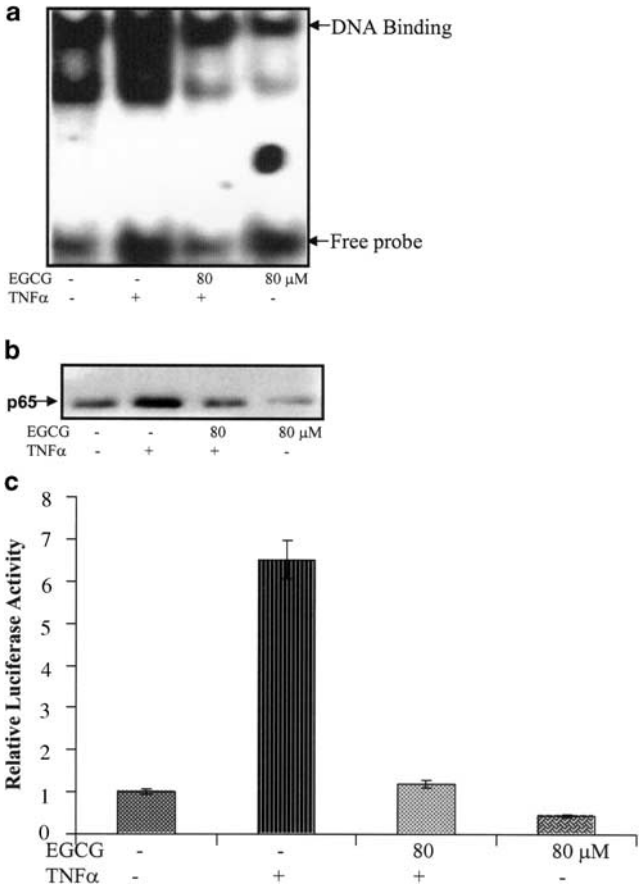

Figure 4 Decreased transcriptional activity of NF-κB by EGCG treatment. (**a**) LNCaP cells were treated with 80 μM of EGCG for 24 h followed by treatment with or without TNFα. Nuclear lysates (10 μg) from treated and control cells were subjected to EMSA using NF-κB binding consensus sequence as described under Materials and methods. (**b**) Protein (50 μg) from the above nuclear LNCaP lysates from was subjected to SDS–PAGE and Western blotting using NF-κB (p65 subunit) antibody. (**c**) Transcriptional activation of NF-κB was studied using NF-κB-dependent E-selectin promoter. At 24 h post-transfection with the E-selectin luciferase plasmid, LNCaP cells were treated with 80 μM of EGCG for 24 h followed by treatment with or without TNFα for 4 h. Luciferase assay was performed as described under Materials and methods

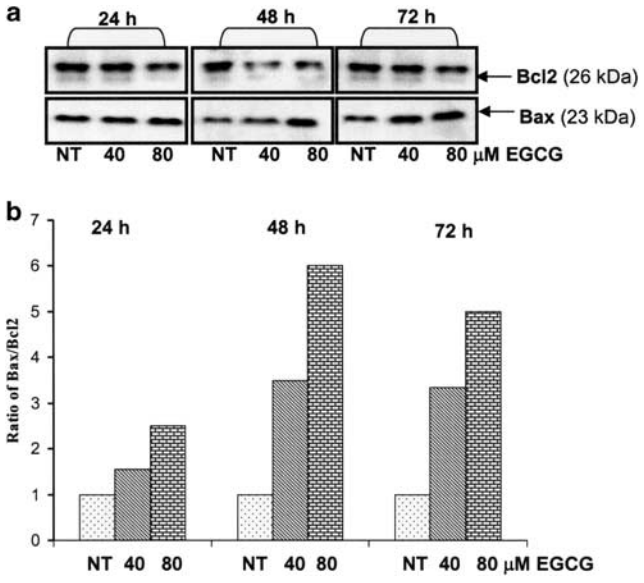

Figure 5 Modulation of Bax and Bcl-2 levels by EGCG treatment. (**a**) LNCaP cells were treated with the indicated doses of EGCG for 24, 48 or 72 h. Protein (50 μg) from total cell lysates was subjected to SDS–PAGE and Western blotting using Bax and Bcl-2 antibodies. (**b**) The graph represents densitometry of the blots in (**a**). The values were normalized to the untreated control and the ratio of Bax/Bcl-2 was calculated by dividing the relative density of Bax/relative density of Bcl-2

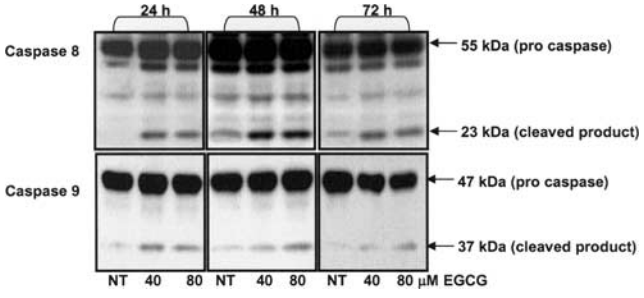

Figure 6 Activation of caspases 8, 9 by EGCG treatment. LNCaP cells were treated with the indicated doses of EGCG for 24, 48 or 72 h. Protein (50 μg) from total cell lysates was subjected to SDS–PAGE and Western blotting using caspase 8 and 9 antibodies

Bcl-2 family members. To test our hypothesis, we determined the protein levels of Bax and Bcl-2 in LNCaP cells treated with 40 and 80 μM of EGCG for 24, 48 or 72 h by Western blot analysis. We observed that EGCG induced a decrease in Bcl-2 levels with a concomitant increase in Bax levels (Figure 5a). Densitometry of the immunoblot indicated that the ratio of Bax/Bcl-2 was higher in EGCG-treated cells compared to the control (Figure 5b), thus supporting our contention that a shift in the Bax/Bcl-2 ratio activated the apoptotic pathway in EGCG-treated cells.

Activation of caspases and cleavage of poly (ADP-ribose) polymerase (PARP) during EGCG-mediated apoptosis

The downstream signals during apoptosis are transmitted via caspases, which upon conversion from pro to active forms mediate the cleavage of PARP and is followed by DNA fragmentation. To evaluate the nature of the apoptotic response mediated by EGCG, we studied the effect of EGCG on activation of initiator and activator caspases. Western blot analysis showed that 24 h of EGCG treatment led to activation and cleavage of initiator caspases 8 and 9 (Figure 6). The activation of initiator caspases resulted in activation of downstream effector caspase 3 (Figure 7a) as well as increase in caspase 3 activity as demonstrated by DEVDase assay (Figure 7b). The activation of the effector caspase 3 in response to EGCG treatment also resulted in cleavage of its *in vivo* substrate PARP (Figure 7c).

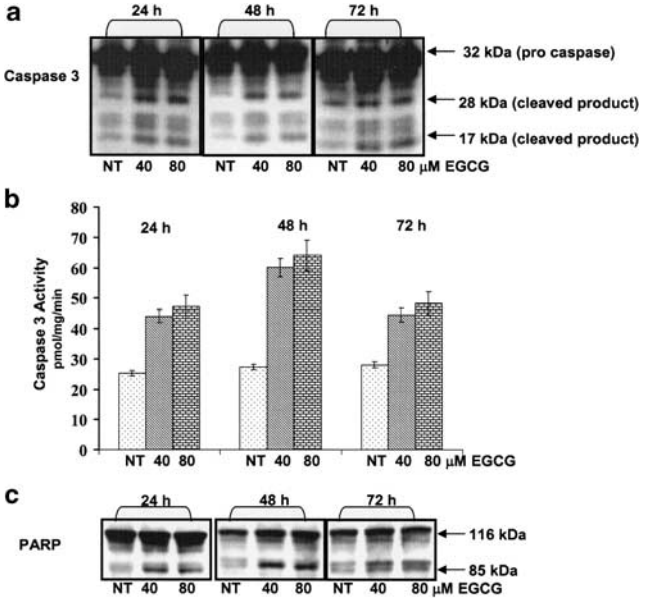

Figure 7 Activation of caspase 3 and PARP cleavage by EGCG treatment. (**a**) LNCaP cells were treated with the indicated doses of EGCG for 24, 48 or 72 h. Protein (50 μg) from total cell lysates was subjected to SDS–PAGE and Western blotting using caspase 3 antibody. (**b**) Caspase 3 activity was measured by AFC-DEVDase assay as described under Materials and methods. Representative data from one experiment ($n=3$) are shown. (**c**) PARP cleavage was determined by Western blotting of the above lysates using PARP antibody

Discussion

In this study, we have evaluated the role of two important transcription factors, p53 and NF-κB, and their downstream events in the antiproliferative effects of green tea polyphenol EGCG against prostate cancer LNCaP cells. p53, considered as the 'guardian of the genome', is the most frequently altered tumor suppressor in human malignancies with more than 50% of solid tumors having a loss of wild-type p53 expression due to deletion or point mutation (Agarwal *et al*., 1998). p53 is responsible for cell cycle arrest upon DNA damage (Burns *et al*., 2001) and is also a key regulator of apoptosis. The p53-dependent apoptotic response is a well-documented anticancer mechanism (Vousden and Lu, 2002).

In this study, we have demonstrated a role for p53-associated events during the antiproliferative effects of green tea polyphenol EGCG against prostate cancer cells. Accumulating evidence shows that dietary factors can activate apoptosis (Ahmad *et al*., 1997). Recent studies indicate that green tea and its polyphenolic constituents exert inhibitory effect on the activity of several enzymatic and metabolic pathways of relevance to the development and progression of cancer (Levites *et al*., 2002; Pianetti *et al*., 2002). EGCG, by virtue of its ability to selectively induce apoptosis in cancer cells and not in normal cells, is potentially an important cancer chemopreventive agent.

Our results demonstrate that EGCG treatment caused a significant decrease in the percentage of viable cell along with a concurrent induction of apoptosis; however, the percentage of apoptotic cells does not coincide with the percent of viable cells. This can be because, along with inducing apoptosis, EGCG is also responsible for G1 arrest in the LNCaP cells (Gupta *et al*., 2000). Secondly the pathway involving cell cycle deregulation has been studied in one of our recent studies (Gupta *et al*., 2003). Therefore, difference in cell viability and apoptosis can be explained by cell cycle arrest induced by EGCG.

Our results show that in response to EGCG treatment, there is an increase in the cellular levels of p53. This increase in p53 expression was accompanied by phosphorylation of p53 at serine 6, 15, 20, 37 and 392, but not serine 9. Phosphorylation of p53 at serine 15 and 20 has been shown to stimulate p53-dependent transcriptional activation (Dumaz and Meek, 1999) and also results in reduced interaction of p53 with its negative regulator MDM2 both *in vivo* and *in vitro* (Shieh *et al*., 1997). EGCG treatment also resulted in increased expression of $p14^{ARF}$, a tumor suppressor that negatively regulates MDM2 (Weber *et al*., 1999). Our results indicate that EGCG stabilizes p53 via both phosphorylation of p53 and modulation of the ARF-MDM2 pathway. Interestingly, we observed a decrease in the levels of MDM2 protein in response to EGCG treatment. We speculate that this decrease may be directly mediated by EGCG at the transcriptional level (Levites *et al*., 2002) or as a result of EGCG-mediated increase in $p14^{ARF}$ levels (Jackson *et al*., 2001).

The importance of p53 in EGCG-mediated apoptosis was shown in a recent study from our laboratory where we showed that PC-3 cells (null for p53) are not as sensitive to EGCG-mediated apoptosis as LNCaP cells (wild-type p53). Moreover, we have demonstrated that after introducing wild-type p53 in PC-3 cell, these cells become very sensitive to EGCG-mediated G1 arrest and apoptosis (Hastak *et al*., manuscript under preparation).

EGCG-induced stabilization of p53 caused an increase in its transcriptional activity, thereby resulting in an upregulation of its downstream target p21/WAF1. p21/WAF1 can inhibit cdk2, 4 and 6 causing cell cycle arrest (Bates and Vousden, 1999). Studies from our laboratory indicate that EGCG-mediated upregulation of p21/WAF1 is critical in inducing both cell cycle arrest and apoptosis (Gupta *et al*., 2003; Hastak *et al*., manuscript in preparation). This premise is supported by several independent studies, which show that targeted overexpression of p21/WAF1 increases apoptosis (Fotedar *et al*., 1999; Shibata *et al*., 2001). The mechanisms by which p21 may promote apoptosis are not currently understood, but could be related to its ability to interact and possibly regulate components of DNA repair machinery (Gartel and Tyner, 2002). Another transcriptional target of p53 is the proapoptotic protein Bax whose levels increased in response to EGCG treatment.

The NF-κB protein stimulates cell survival and promotes cell proliferation, and its increased activity is

positively associated with many cancer types including prostate cancer. We show that EGCG effectively decreased NF-κB transcriptional activity in human prostate carcinoma LNCaP cells, which may be due to decrease in the nuclear levels of the p65 subunit of NF-κB. Recent studies suggest that NF-κB activates the expression of antiapoptotic proteins Bcl-2 and Bcl-xL (Tamatani *et al*., 1999; Chen *et al*., 2000). Our data show that EGCG-induced decrease in NF-κB transcriptional activity is accompanied by a decrease in the levels of the antiapoptotic protein Bcl-2. However, a genetic approach using dominant-negative NF-κB along with Bcl-2 reporter study will effectively help study the possible mechanism. Moreover, a direct role of p53-dependent repression of Bcl-2 cannot be ruled out (Sheikh and Fornace, 2000). High level of Bcl-2 is reported to inhibit p53-mediated transactivation of target genes like p21/WAF1, Bax and gadd45 and potentially inhibit p53-mediated apoptosis (Miyashita *et al*., 1994). Decrease in Bcl-2 expression and a concurrent increase in p53's transcriptional activity shown in our study support this hypothesis.

The ratio of Bax/Bcl-2 is critical to cell survival such that an increase in Bax levels can shift the ratio in favor of apoptosis. Activation of Bax results in its translocation to the mitochondrial membrane causing membrane permeabilization and release of cytochrome *c* from the mitochondria. Cytochrome *c* complexes with apoptotic protease activating factor–1 (Apaf-1) and procaspase-9 to form 'apoptosome'. The apoptosome recruits procaspase 3, which is cleaved and activated by caspase 9 to induce apoptosis. The activation of caspase 8 may be due to direct activation via the death receptor pathway and, therefore, we do not rule out this possibility in our study. The role of EGCG in activation of death receptor has already been demonstrated by another group (Kuo and Lin, 2003). However, this study and the pathway that we have described herein is novel and has not been elucidated. Secondly, activation of caspase 8 may directly activate procaspase -3 or cleave Bid to t-bid, which then subsequently induces cytochrome *c* release, to form apoptosome and activation of caspase 9 (Zimmermann *et al*., 2001). Thus, these pathways are interrelated and one cannot rule out role of these molecules in activating each other.

Our data thus suggest that EGCG treatment may result in upregulation of Bax via p53 and a parallel downregulation of Bcl-2, which might ultimately initiate the activation of the casapase cascade leading to apoptosis. In Figure 8, we provide an overview of the possible mechanisms by which EGCG induces apoptosis in prostate carcinoma cells by modulating the transcriptional activities of p53 and NF-κB.

Materials and methods

Materials

A purified preparation of EGCG (>98% pure) was kindly provided by Dr Yukihiko Hara of Mitsui Norin Co., Ltd. (Schizoku, Japan).

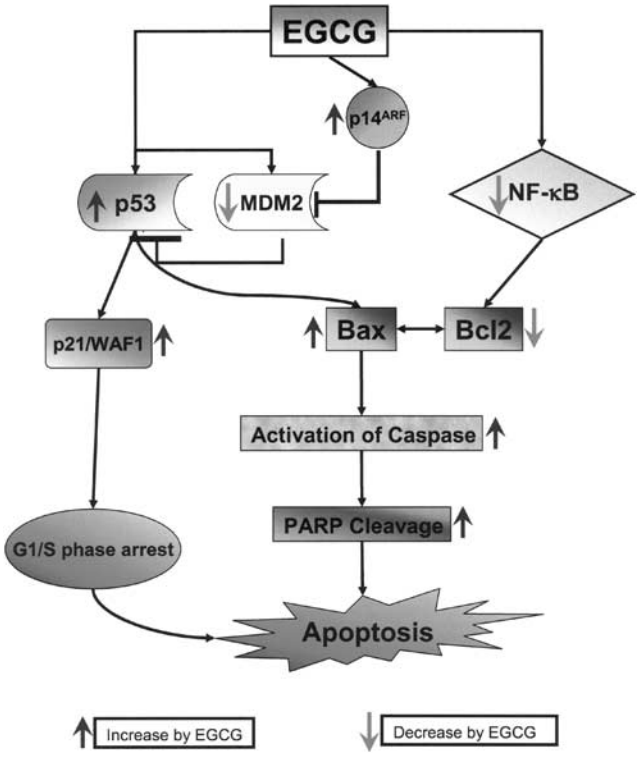

Figure 8 Proposed mechanism of EGCG-induced apoptosis by modulation of p53 and NF-κB activation

Cells culture

LNCaP cells were maintained in RPMI 1640 with 10% FBS and 1% penicillin and streptomycin.

Cell viability

In all, cells (60–70% confluent) were treated with EGCG (20–80 μM) for 24, 48 or 72 h and the cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2-5-diphenyl tetrazolium-bromide) assay as described previously (Ahmad *et al*., 1997).

Apoptosis by TUNEL assay

Cells were grown at a density of 1 x 10^6 cells in 100-mm culture dishes and were treated with desired concentrations of EGCG for 24, 48 or 72 h. Following this treatment, the cells were trypsinized, washed with PBS and labeled with fluorescein-tagged bromodeoxyuridine triphosphate (Br-dUTP) and propidium iodide according to the manufacturer's protocol using the APO-BRDU apoptosis kit (Phoenix Flow Systems).

Apoptosis by ELISA

Following EGCG treatment (20–80 μM of EGCG for 24, 48 or 72 h) of cells, the extent of apoptosis was determined by Cell Death Detection ELISA^{PLUS} (Roche Biochemical) assay. The assay is based on the enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cells due to degradation of DNA by endogenous endonuclease (that cleaves double-stranded DNA at the internucleosomal linker regions). ELISA was carried out according to the manufacturer's protocol. The enrichment factor was calculated by dividing the absorbance of the sample by the absorbance of the controls without treatment.

Caspase 3 activity by DEVDase assay

LNCaP cells were treated with 40 and 80 μM of EGCG for 24, 48 or 72 h, and caspase 3 activity was measured by Ac-DEVD-AFC (*N*-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl coumarin)), a fluorogenic substrate for caspase 3, according to the manufacturer's protocol (BIOMOL Research Laboratories, Inc.,. PA, USA). The sequence DEVD is based on PARP cleavage site Asp-216 for caspase 3. Upon activation, caspase 3 cleaves the substrate, releasing the fluorophore –AFC, which was measured quantitatively spectrofluorometrically (Ex-400 nm, Em-505 nm).

Preparation of cell lystates and immunoblot assay

Following treatment of the cells, the medium was aspirated and the cells were washed twice with cold PBS (10 mM, pH 7.4). 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) was added to the plates, which were then placed over ice for 30 min. The cells were scraped, and the lysate was collected in a microfuge tube and passed through a 21G needle to break up the cell aggregates. The lysate was cleared by centrifugation at 14 000 *g* for 15 min at 4°C and the supernatant (total cell lysate) was either used immediately or stored at −70°C. The protein concentration was determined by the DC Bio-Rad assay using the manufacturer's protocol (Bio-Rad Laboratories).

For immunoblot analysis, 25–50 μg of protein was resolved over 8–12% polyacrylamide–SDS gels and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer (5% nonfat dry milk, 1% Tween 20; in 20 mM TBS, pH 7.6) for 2 h at room temperature followed by incubation with an appropriate concentration of primary antibody (p21/WAF1 and phosphorylated Rb – Santa Cruz Biotechnology; caspases 3, 8 and p53 – Pharmingin Laboratories; Bcl-2, Bax and PARP – Upstate Biotechnology; MDM2, $p14^{ARF}$, $p16^{INK4A}$ – Neomarker Laboratories; caspase 9 and p53 phosphorylated at serine 6, 9, 15, 20, 37 and 392 – Cell Signaling Technologies). To study levels of phosphorylated p53, LNCaP cells were treated with adriamycin (ADR) (200 ng/ml) for an additional 24 h as positive control. For NF-κB p65 subunit (Santa Cruz Biotechnology), nuclear lysate was prepared as described in EMSA. This was followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate (Amersham Life Sciences, Inc.), and the protein expression was detected by chemiluminescence using an ECL detection kit (Amersham Life Sciences, Inc.) and autoradiography using XAR-5 film (Eastman Kodak Co., Rochester, NY, USA).

Reporter assays for p53 and NF-κB

p53 reporter plasmid PG13, NF-κB reporter plasmid E-selectin and β-galactosidase plasmid used for normalization were kind gifts from Dr Agarwal (Cleveland Clinic Foundation). LNCaP cells were cotransfected with 500 ng of PG13 or E-selectin plasmid and 1 μg for β-galactosidase plasmid using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 24 h after transfection, fresh medium was added to the cells followed by treatment with a desired concentration of EGCG for an additional 24 h. Luciferase and β-galactosidase (β-Gal) activities were determined with the luciferase assay system (Promega) and with chemiluminescent reagents (Promega), respectively. Luciferase activity was normalized to β-Gal activity to control for transfection efficiency.

EMSA

LNCaP cells were treated with 80 μM of EGCG for 24 h. For positive control, cells were stimulated with 25 ng/ml of TNFα (Roche, Indianapolis, IN, USA) for 15 min. To prepare the nuclear lysate, approximately 5×10^6 cells were washed with ice-cold PBS and resuspended in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1 mM leupeptin). After 15 min at 4°C, 25 μl of 10% Nonidet P-40 was added. Cells were vortexed briefly, nuclei were pelleted by microcentrifugation, and supernatants were removed (cytoplasmic extracts). Pellets were resuspended in 200 μl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 1 mM leupeptin). After 30 min at 4°C, lysates were centrifuged and supernatant was removed (nuclear extract). Protein concentration of extracts was measured. Nuclear extracts (10 μG) were mixed with 0.02 U of poly (DI-dC) (Pharmacia Biotech Inc.) and end-labeled NF-κB oligonucleotide probe 5′–AGT TGA GGG GAC TTT CCC AGG C-3′, 3′–TCA ACT CCC CTG AAA GGG TCC G–5′ (Upstate Biotechnology, NY, USA) (1×10^4–2.5×10^4 c.p.m. per reaction) in binding buffer (2.5 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 1.8 mM dithiothreitol, 10% glycerol) and were incubated for 20 min at room temperature. Samples were separated on native 6% polyacrylamide gels in low ionic strength buffer (0.25 × Tris-borate-EDTA). Dried gels were exposed to a storage phosphor screen (Kodak) overnight and autoradiography using XAR-5 film (Eastman Kodak Co., Rochester, NY, USA).

Abbreviations

EGCG, (−)-epigallocatechin-3-gallate; PARP, poly (ADP-ribose) polymerase; Ac-DEVD-AFC, *N*-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethylcoumarin); adriamycin, ADR; MDM2, murine double minute 2.

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