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Growth Inhibition, Cell-Cycle Dysregulation, and Induction of Apoptosis by Green Tea Constituent (-)-Epigallocatechin-3-gallate in Androgen-Sensitive and Androgen-Insensitive Human Prostate Carcinoma Cells

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Prostate cancer (PCA) is the most prevalent cancer diagnosed and the second leading cause of cancer-related deaths among men in the United States. Descriptive epidemiological data suggest that androgens and environmental exposures play a key role in prostatic carcinogenesis. Since androgen action is intimately associated with proliferation and differentiation, at the time of clinical diagnosis in humans most PCA represent themselves as a mixture of androgen-sensitive and androgen-insensitive cells. Androgen-sensitive cells undergo rapid apoptosis upon androgen withdrawal. On the other hand, the androgen-insensitive cells do not undergo apoptosis upon androgen blocking, but maintain the molecular machinery of apoptosis. Thus, agents capable of inhibiting growth and/or inducing apoptosis in both androgen-sensitive and androgen-insensitive cells will be useful for the management of PCA. In the present study, we show that (-)-epigallocatechin-3-gallate (EGCG), the major polyphenolic constituent present in green tea, imparts antiproliferative effects against both androgen-sensitive and androgen-insensitive human PCA cells, and this effect is mediated by deregulation in cell cycle and induction of apoptosis. EGCG treatment was found to result in a dose-dependent inhibition of cell growth in both androgen-insensitive DU145 and androgen-sensitive LNCaP cells. In both the cell types, EGCG treatment also resulted in a dose-dependent G_0/G_1-phase arrest of the cell cycle as observed by DNA cell-cycle analysis. As evident by DNA ladder assay, confocal microscopy, and flow cytometry, the treatment of both DU145 and LNCaP cells with EGCG resulted in a dose-dependent apoptosis. Western blot analysis revealed that EGCG treatment resulted in (i) a dose-dependent increase of p53 in LNCaP cells (carrying wild-type p53), but not in DU145 cells (carrying mutant p53), and (ii) induction of cyclin kinase inhibitor WAF1/p21 in both cell types. These results suggest that EGCG negatively modulates PCA cell growth, by affecting mitogenesis as well as inducing apoptosis, in cell-type-specific manner which may be mediated by WAF1/p21-caused G_0/G_1-phase cell-cycle arrest, irrespective of the androgen association or p53 status of the cells. © 2000 Academic Press

Key Words: **epigallocatechin-3-gallate; apoptosis; cell cycle arrest; chemoprevention; prostate cancer.**

Prostate cancer (PCA) is the most prevalent cancer diagnosed and the second leading cause of cancer-related deaths among men in the United States (Landis *et al.,* 1998; Whittemore, 1994). Descriptive epidemiologic data suggest that androgens and environmental exposures play a key role in prostatic carcinogenesis (Fleshner and Klotz, 1998). Because androgens are capable of both stimulating proliferation as well as inhibiting the rate of the glandular epithelial cell death within the prostate, palliation with radiation or androgen ablation therapy are commonly suggested for men with this non-organ-confined disease (Gleave *et al.,* 1998; Thompson *et al.,* 1995; Wilding, 1995). The initial response to androgen ablation generally results in tumor shrinkage due to the apoptotic death of androgen-sensitive PCA cells. However, the apoptotic pathway is not activated by androgen ablation or by cytotoxic drugs in androgen-insensitive cells. However, these cells are known to maintain the molecular machinery of apoptosis (Tang and Porter, 1997). Generally, patient's death relates directly to the proliferation of this subpopulation of cells (Tang and Porter, 1997; Denmeade *et al.,* 1996). It is also important to note that at the time of clinical diagnosis most PCAs represent a mixture of androgen-sensitive and androgen-insensitive cells. Therefore, the key to the control of PCA appears to lie in the elimination of both types of cells through mechanism-based preventive/therapeutic approaches.

Green tea, a popular beverage consumed worldwide, has been shown to possess cancer chemopreventive effects in a wide range of target organs in rodent carcinogenesis models (Kuroda and Hara, 1999; Katiyar and Mukhtar, 1996, 1997; Yang and Wang, 1993; Yang *et al.,* 1997; Stoner and Mukhtar, 1995). Much of the effects of green tea are believed to be mediated by the polyphenolic constituents, most notably (-)-epigallocatechin-3-gallate (EGCG), present therein (Katiyar and Mukhtar, 1996). The oral consumption or topical application of a polyphenolic mixture obtained from green tea or its

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major constituent EGCG has been shown to afford protection against all stages of carcinogenesis in a wide range of animal tumor bioassay systems (Conney *et al.,* 1997; Katiyar and Mukhtar, 1996; Landau *et al.,* 1998; Hiroshe *et al.,* 1997; Katiyar *et al.,* 1992, 1993a,b; Huang *et al.,* 1992; Xu *et al.,* 1992; Khan *et al.,* 1992). Many human epidemiological studies conducted around the world, though inconclusive, have shown encouraging results that green tea consumption affords cancer preventive effects against some cancer types (Bushman, 1998; Dreosti *et al.,* 1997; Hara, 1997; Imai *et al.,* 1997; Ji *et al.,* 1997; Kohlmeier *et al.,* 1997; Blot *et al.,* 1996). The chemopreventive effects of tea on human oral leukoplakia have also been demonstrated (Li *et al.,* 1999). More recent studies are showing that EGCG may also have cancer chemotherapeutic effects (Cao and Cao, 1999; Barthelman *et al.,* 1998; Ahmad *et al.,* 1997; Liao *et al.,* 1995; Mukhtar *et al.,* 1994). Based on these extensive data, clinical trials in human cancer patients with green tea are being conducted or planned (Yang *et al.,* 1999; Nakachi *et al.,* 1998; Yu *et al.,* 1995; Imai and Nakachi, 1995). At least two epidemiological studies have shown that people who regularly consume green tea have lower PCA incidence (Kinlen, 1988; Heilburn *et al.,* 1986). Further, in Chinese, Japanese, and some other Asian populations that consume tea, especially green tea, on a regular basis, the incidence of PCA is lower compared to that in the West (Wynder *et al.,* 1994). These associations remain inconclusive because the population in China and Japan also consumes soy, low-fat diets, and high-fiber diets among other variables.

Studies in culture system have shown that green tea extract and EGCG is capable of inhibiting the growth of a variety of mouse and human cancer cell types (Ahmad *et al.,* 1997; Valcic *et al.,* 1996; Yang *et al.,* 1998; Chen *et al.,* 1998). It has also shown that green tea and its polyphenolic constituents impart inhibitory effects on the activities of many enzymatic and metabolic pathways that has relevance in cancer development (Jankun *et al.,* 1997; Lea *et al.,* 1993; Austin *et al.,* 1992; Liao *et al.,* 1995). In addition, EGCG has been shown to cause growth inhibition and regression of human prostate and breast tumors in athymic nude mice (Liao *et al.,* 1995). Our earlier studies have shown that EGCG treatment results in an induction of apoptosis in a variety of human cancer cells including the human PCA cells DU145 (Ahmad *et al.,* 1997). Subsequent studies from other laboratories in other cell types supported these observations (Yang *et al.,* 1998; Hibasami *et al.,* 1998; Otsuka *et al.,* 1998). In a recent study, we provided evidence that both under *in vitro* as well as *in vivo* situations, a polyphenolic mixture obtained from green tea inhibits the androgen-mediated (i) growth of PCA cells, and (ii) ODC enzyme activity and mRNA expression in PCA cells (Gupta *et al.,* 1999). Furthermore, we have also shown that ODC is elevated in human PCA and could be developed as a target for the prevention/therapy of PCA (Mohan *et al.,* 1999). In the present study, we show that EGCG imparts negative growth effects against both androgen-sensitive and androgen-insensitive human PCA cells, and these antiproliferative effects are mediated by deregulation in cell cycle and induction of apoptosis. Based on data presented, we suggest that EGCG-induced G_0/G_1-phase cell-cycle arrest and apoptosis may be mediated by WAF1/p21 in both the cell types irrespective of their androgen or p53 status.

MATERIALS AND METHODS

Materials. Purified preparation of EGCG (>97% purity) was obtained from Mitsui Norin Co. Ltd., Shizuoka, Japan. The human PCA cells namely, DU145 and LNCaP, were obtained from American Type Culture Collection (Rockville, MD). Both cell lines were cultured in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. The cells were maintained at 37°C and 5% CO_2 in a humid environment. EGCG was dissolved in double-distilled water for treatment.

Cell viability. The LNCaP and DU145 cells were grown to 70% confluence and treated with EGCG (10, 20, 40, and 80 μg/ml doses) for 24, 48, and 72 h, respectively. The viable cells were determined by trypan blue exclusion assay.

Detection of apoptosis by DNA ladder assay. The LNCaP and DU145 cells were grown to about 70% confluency and treated with EGCG (10, 20, 40, and 80 μg/ml) for 48 h. Following this treatment, the cells were washed twice with phosphate-buffered saline (10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, and 0.5% Triton X-100), left on ice for 15 min, and pelleted by centrifugation (14,000*g*) at 4°C. The pellet was incubated with DNA lysis buffer (10 mM Tris, pH 7.5, 400 mM NaCl, 1 mM EDTA, and 1% Triton X-100) for 30 min on ice and then centrifuged at 14,000*g* at 4°C. The supernatant obtained was incubated overnight with RNAse (0.2 mg/ml) at room temperature and then with Proteinase K (0.1 mg/ml) for 2 h at 37°C. DNA was extracted using phenol:chloroform (1:1) and precipitated with 95% ethanol for 2 h at −80°C. The DNA precipitate was centrifuged at 14,000*g* at 4°C for 15 min and the pellet was air-dried and dissolved in 20 ml of Tris–EDTA buffer (10 mM Tris–HCl, pH 8.0, and 1 mM EDTA). Total amount of DNA was resolved over 1.5% agarose gel, containing 0.3 μg/ml ethidium bromide in 1× TBE buffer (pH 8.3, 89 mM Tris, 89 mM Boric acid, and 2 mM EDTA) (Bio Wittaker, Inc., Walkersville, MD). The bands were visualized under UV transilluminator (Model No. TM-36, UVP Inc., San Gabriel, CA) followed by polaroid photography (MP-4 Photographic System, Fotodyne Inc., Hartland, WI).

Detection of apoptosis by confocal microscopy. The cells were cultured over round glass coverslip in a 60-mm culture dish to about 70% confluency and then treated with EGCG (20, 40, and 80 μg/ml doses) for 48 h followed by incubation with 10 μM Hoechst 33258 dye for 20 min in complete medium at 37°C for labeling the nuclei. The coverslips were washed with PBS and mounted on the microscope stage. The Hoechst 33258 fluorescence was excited from an UV argon laser with a 351-nm line and imaged through a 460-nm dichroic mirror and 417–482-nm emission filter using a Zeiss 410 laser-scanning confocal microscope. Apoptosis was characterized by the morphological changes, viz., chromatin condensation, nuclear condensation, and formation of apoptotic bodies.

Quantification of apoptosis by flow cytometry. The cells were grown at a density of 1×10^6 cells in 100-mm culture dishes and were treated with EGCG (10, 20, 40, and 80 μg/ml doses) for 48 h. The cells were trypsinized, washed with PBS, and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by use of an Apo-direct apoptosis kit obtained from Phoenix Flow Systems (San Diego, CA) as per manufacturer's protocol. The labeled cells were then analyzed by flow cytometry.

DNA cell cycle analysis. The cells (70% confluent) were starved for 36 h to arrest them in G_0 phase of the cell cycle, after which they were treated with

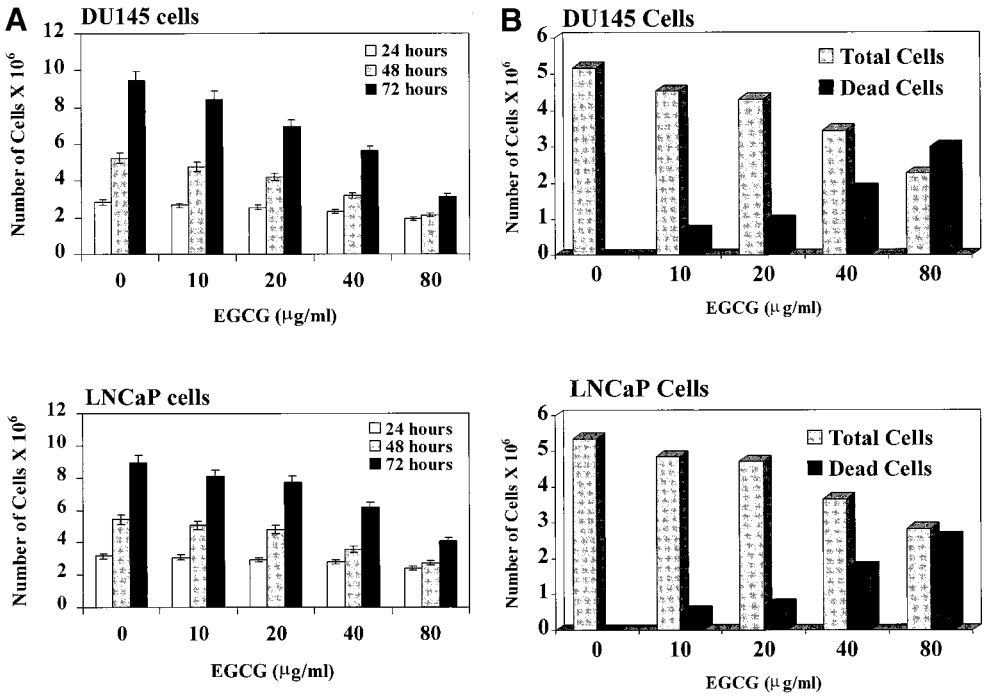

FIG. 1. (A) Effect of EGCG on DU145 and LNCaP cell growth at various time intervals and (B) viability of DU145 and LNCaP cells following EGCG treatment for 48 h. The cells were incubated with EGCG (10, 20, 40, and 80 μg/ml doses) and the total number of cells and the viable cells were measured by trypan blue exclusion assay. The data is represented as number of cells $\times 10^6$. Data shown are mean values $\pm$ SE determined from three independent experiments.

EGCG (10, 20, 40, and 80 μg/ml doses) in RPMI-1640 complete media for 24 h. The cells were trypsinized thereafter, washed twice with cold PBS, and centrifuged. The pellet was resuspended in 50 μl cold PBS and 450 μl cold methanol for 1 h at 4°C. The cells were centrifuged at 1100 rpm for 5 min, pellet washed twice with cold PBS, suspended in 500 μl PBS, and incubated with 5 μl RNAse (20 μg/ml final concentration) for 30 min. The cells were chilled over ice for 10 min and stained with propidium iodide (50 μg/ml final concentration) for 1 h and analyzed by flow cytometry.

Western blot analysis. The cells were treated with EGCG (10, 20, 40, and 80 μg/ml doses) in RPMI-1640 complete media for 48 h. For time-dependent assay, the cells were treated for 24, 48, and 72 h with 40 μg/ml of EGCG. After this the media was aspirated, the cells were washed with cold PBS (10 mM, pH 7.4), and ice cold RIPA buffer (0.15 mM NaCl/0.05 mM Tris–HCl, pH 7.3/1% Triton X-100/1% sodium deoxycholate) was added to the plates that were then placed over ice for 30 min. The cells were scraped; the lysate was collected in a microfuge tube and passed through a 21-gauge 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 −80°C. The protein concentration was determined by DC Bio-Rad assay using the manufacturer's protocol. For Western blot analysis, 25–50 μg protein was resolved over 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (5% nonfat dry milk/1% Tween 20; in 20 mM TBS, pH 7.6) for 1 h at room temperature, incubated with appropriate monoclonal or polyclonal primary antibody (human reactive anti-WAF1, anti-p53; Santa Cruz Biotechnology) in blocking buffer for 1 h to overnight at 4°C followed by incubation with anti-mouse or anti-rabbit secondary antibody horseradish peroxidase conjugate (Amersham) and detected by chemiluminescence and autoradiography using XAR-5 film (Eastman Kodak). Densitometric measurements of the bands in Western blot analysis were performed using Scion Image computer software program (Scion Corporation, Frederick, MD).

RESULTS

The trypan blue exclusion assay demonstrated that EGCG (10 to 80 μg/ml) treatment resulted in a dose-dependent inhibition of cell growth, as compared to untreated controls, in both androgen-insensitive DU145 and androgen-sensitive LNCaP PCA cells (Fig. 1A). EGCG treatment of cells also resulted in time-dependent inhibition of cell growth in both cell types and the effect was more pronounced at 48-h posttreatment (Fig. 1A). Similarly, EGCG treatment to DU145 and LNCaP PCA cells resulted in a maximum loss of cell viability at 48 h (Fig. 1B).

In the next series of experiments we assessed the effect of EGCG on apoptosis in these cell types. Both androgen-insensitive DU145 and androgen-sensitive LNCaP PCA cells were treated with different doses of EGCG (10, 20, 40, and 80 μg/ml). As shown in Fig. 2, compared with vehicle-treated control, EGCG (40 and 80 μg/ml for 48 h) treatment of the cells resulted in the formation of DNA fragments in both cell types. The induction of apoptosis by EGCG was also evident from the morphologic alterations as shown by confocal microscopy after labeling the cells with Hoechst 33258 dye. Both androgen-insensitive DU145 and androgen-sensitive LNCaP cells were treated with 20, 40, and 80 μg/ml doses of EGCG. The vehicle-treated control as well as 20 μg/ml treatment with EGCG did not cause any alterations in the nuclear morphology of DU145 cells (Fig. 3A, panels a and b). However, at 40 and

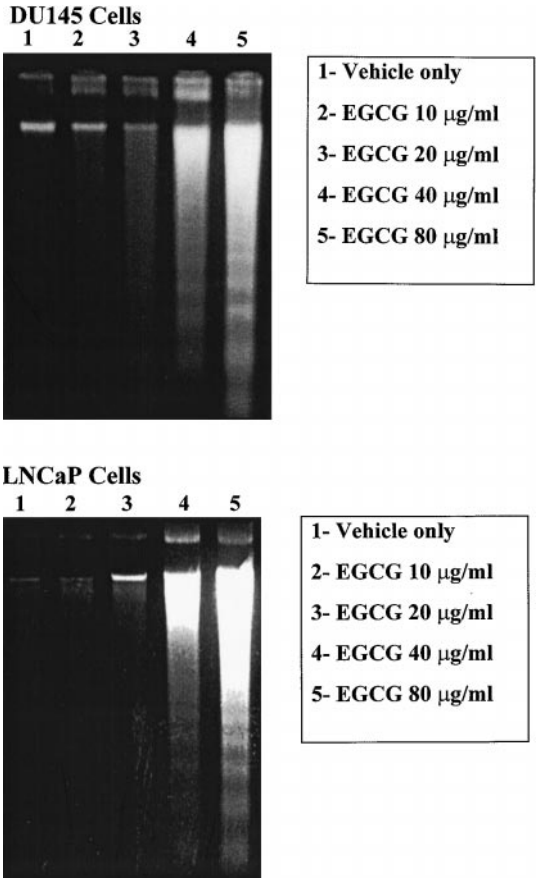

FIG. 2. DNA fragmentation by EGCG in DU145 and LNCaP cells. The cells were grown to 70% confluency and were treated with various doses of EGCG for 48 h. The DNA was isolated and resolved over 1.5% agarose gel electrophoresis followed by visualization of bands as described in the Materials and Methods section. Data shown are from a representative experiment repeated three times with similar results.

80 μg/ml dose of EGCG the cells showed significant apoptosis as evident from advanced chromatin condensation, nuclear condensation, and formation of apoptotic bodies (Fig. 3A, panels c and d). Essentially similar results were obtained with androgen-sensitive LNCaP cells following treatment with EGCG (Fig. 3B, panels a–d). In addition, the number of cells that could be observed on the coverslip was markedly diminished at 40 and 80 μg/ml doses of EGCG, indicating that the treatment resulted in a detachment of the apoptotic cells.

We next quantified the extent of apoptosis by flow cytometric analysis of the cells labeled with deoxyuridine triphosphate and propidium iodide. The androgen-insensitive DU145 and androgen-sensitive LNCaP cells were treated with 10, 20, 40, and 80 μg/ml of EGCG for 48 h. As shown by data in Fig. 4A, EGCG treatment resulted in 13.9, 19.1, 42.2, and 58.1% of apoptotic cells in androgen-insensitive DU145 cells and 9.2, 13.4, 36.9, and 52.5% of apoptotic cells in androgen-sensitive LNCaP cells, respectively (Fig. 4B). While the induction of apoptosis was almost negligible at the lowest dose (10 μg/ml), the highest dose (80 μg/ml) resulted in a massive apoptosis and a drastic decline in the number of cells as determined by flow cytometry.

Since the induction of apoptosis may be mediated through the regulation of cell cycle, we also examined the effect of EGCG on the cell-cycle perturbations. Compared with the vehicle-treated controls, the EGCG treatment resulted in an appreciable arrest of DU145 and LNCaP cells in G_0/G_1 phase of cell cycle after 24 h of the treatment. The treatment caused an arrest of 53.7% cells in G_0/G_1 phase of the cell cycle at 20 μg/ml that further increased to 63.42% at 40 μg/ml and 76.33% at the highest dose of 80 μg/ml in DU145 cells (Fig. 5A). Essentially similar observations were recorded with LNCaP cells where following EGCG treatment of cells at 20, 40, and 80 μg/ml doses resulted in 54.99, 61.79, and 68.17% arrest, respectively (Fig. 5B). This increase in G_0/G_1 cell population was accompanied with a concomitant decrease of cell number in S-phase and G2-M phase in both PCA cell lines. However, the extent of arrest in G_0/G_1 phase was more in DU145 cells than in LNCaP PCA cells. Taken together, these data suggest that essentially similar mechanism of EGCG-mediated cell growth inhibition, cell cycle arrest, and apoptosis occurs in both androgen-insensitive DU145 cells and androgen-sensitive LNCaP cells.

Employing Western blot analysis, we next assessed the protein expression of p53 that is also known to play an important role in cell-cycle regulation. The two cell lines employed in this study differ in their p53 status as the DU145 cells carry mutant p53, whereas LNCaP cells harbor wild-type p53; we also studied the effect of EGCG on the protein expression of p53 in these cell types. As shown in Fig. 6, EGCG treatment resulted in an appreciable increase in the levels of p53 protein at 40 μg/ml (1.6-fold) and 80 μg/ml (2.2-fold) in LNCaP cells (carrying wild-type p53) but not in DU145 cells (carrying mutant p53). In a time-dependent study with 40 μg/ml dose of EGCG, a modest increase in the level of p53 protein was observed at 48-h posttreatment in LNCaP cells and remained stable at 72-h posttreatment. However, the treatment of DU145 cells with EGCG (40 μg/ml) did not cause any appreciable change in the level of p53 protein. We also assessed the effect of EGCG on the induction of WAF1/p21 that, either in response to p53 or independent of p53, is known to regulate the cell-cycle progression in G_1 phase of the cell cycle and induce apoptosis. EGCG (40 and 80 μg/ml) treatment results in a significant induction of WAF1/p21 in both androgen-insensitive DU145 and androgen-sensitive LNCaP PCA cells, when compared with the basal levels (Fig. 6). The time-dependent treatment of DU145 and LNCaP PCA cells with 40 μg/ml dose of EGCG resulted in a significant increase in WAF1/p21 at 24, 48, and 72 h respectively in both the cell lines. Taken together, these results suggest that EGCG-caused growth inhibitory effects in human PCA may be mediated by WAF1/p21, irrespec-

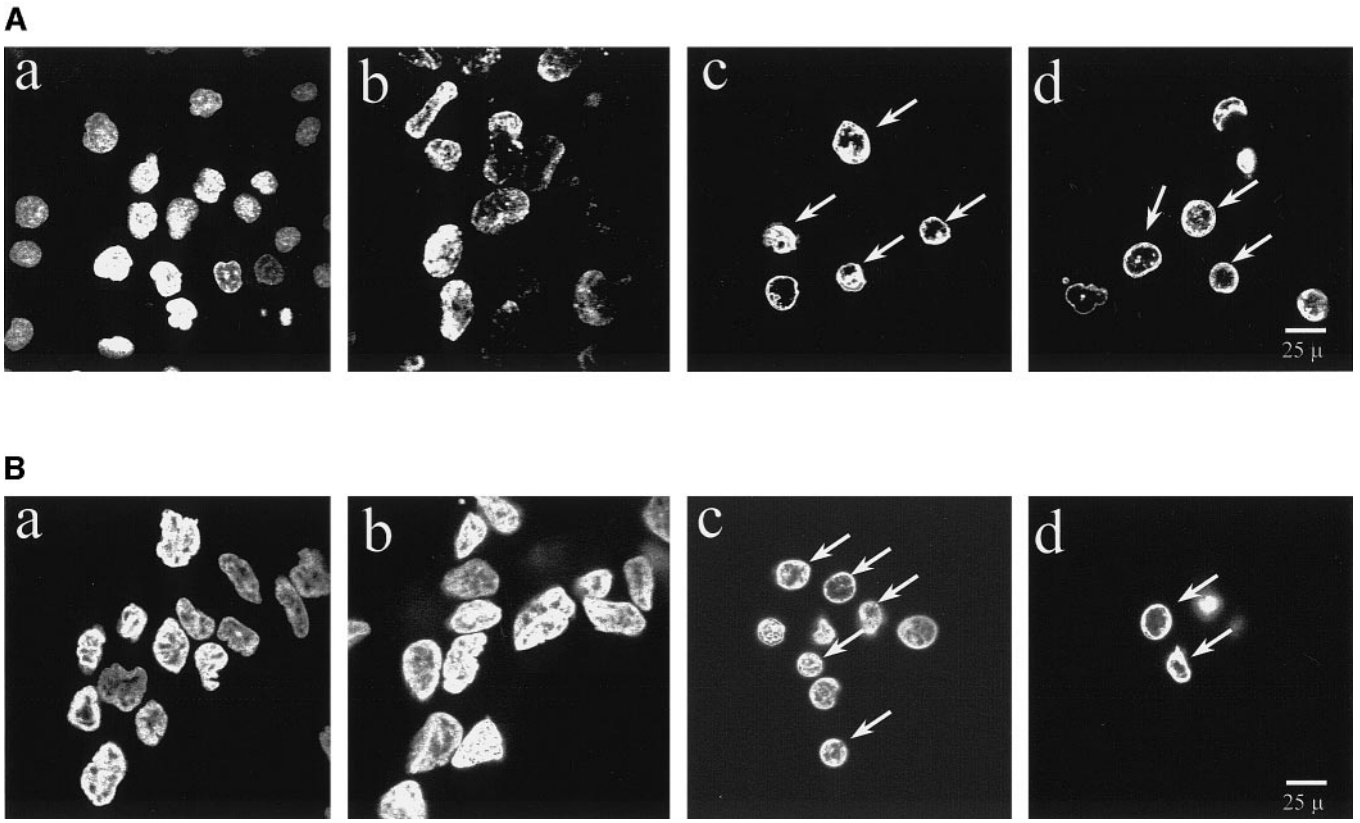

FIG. 3. (A) Morphological changes in DU145 cells and (B) LNCaP cells following EGCG treatment as evident by confocal microscopy. (a) Vehicle only; (b–d) 20, 40, and 80 μg/ml doses of EGCG, respectively, for 48 h. Morphologic changes denoting apoptotic cells are shown by arrows. Bar represents 25 μm. Data shown are from a representative experiment repeated four times with similar results.

tive of the androgen association of the cells and their p53 status.

DISCUSSION

PCA is one of the leading causes of cancer-related deaths in men in the United States. Unfortunately, research efforts against PCA have lagged far behind compared to the cancer of other body sites due to their heterogeneous entity of androgen-sensitive and androgen-insensitive cells, since most PCA respond initially to androgen ablation and the population of androgen-sensitive cells undergo rapid apoptosis upon androgen withdrawal. However, androgen-insensitive PCA cells do not undergo apoptosis upon androgen blocking. Therefore, it is warranted to intensify the efforts for a better understanding of this disease, and for the development of novel mechanism-based approaches for its prevention and treatment (Weisburger, 1998). Studies conducted in cell culture system and animal models as well as human epidemiological studies have shown that the polyphenols present in green tea afford protection against a variety of cancer types (reviewed by Katiyar and Mukhtar, 1996; Ahmad and Mukhtar, 1999; Bushman, 1998; Dreosti *et al.*, 1997; Hara, 1997; Imai *et al.*, 1997; Ji *et al.*, 1997; Kohlmeier *et al.*, 1997; Blot *et al.*, 1996). Recent studies also suggest that these polyphenols present in green tea, especially EGCG, could be developed as therapeutic agents against cancer including PCA (Cao and Cao, 1999; Barthelman *et al.*, 1998; Ahmad *et al.*, 1997; Liao *et al.*, 1995; Paschka *et al.*, 1998). It is important to emphasize here that the incidence of PCA is very low in Chinese and Japanese population, which consume green tea on a regular basis. It must also be emphasized that in these studies a definite correlation could not be established because the diet in this population differs from that of the western world in many other ways.

Studies from this and other laboratories have shown that EGCG, the major polyphenolic antioxidant present in green tea, results in an induction of apoptosis in several human carcinoma cells (Ahmad *et al.*, 1997; Yang *et al.*, 1998; Hibasami *et al.*, 1998; Otsuka *et al.*, 1998; Paschka *et al.*, 1998). This study was designed to determine the antiproliferative potential of EGCG on androgen-sensitive and androgen-insensitive human PCA cells and to understand the mechanistic basis of EGCG-mediated inhibition. To achieve our goal, in

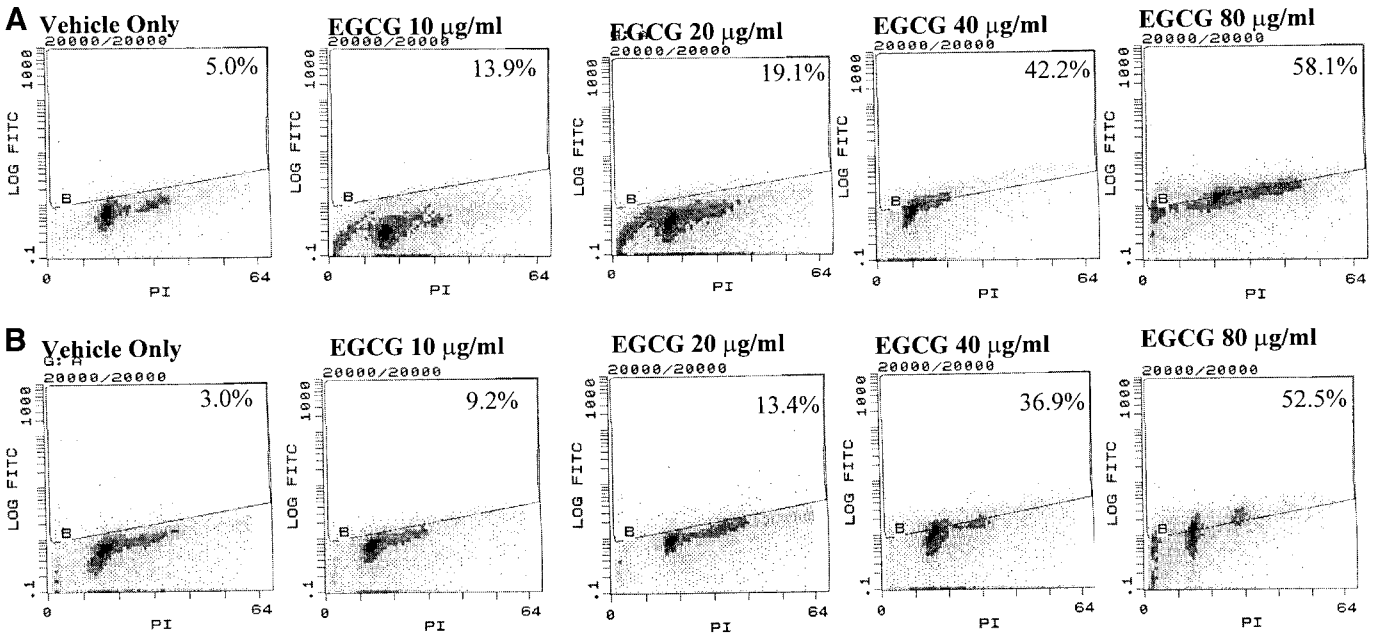

FIG. 4. (A) Quantitation of apoptosis in DU145 cells and (B) LNCaP cells by flow cytometry. The cells were treated with various doses of EGCG for 48 h, labeled with deoxyuridine triphosphate using terminal deoxynucleotide transferase and propidium iodide. Cells showing deoxyuridine triphosphate fluorescence above that of control population, as indicated by the line in each histogram, are considered as apoptotic cells and their percentage population is shown in each box. Data shown are from a representative experiment repeated three times with similar results.

this study, we employed two types of human PCA cell lines that differ in their responsiveness toward androgen, viz. androgen-insensitive DU145 and androgen-sensitive LNCaP cells. This choice was based on the fact that the regulation of normal growth, development, and function of the prostate is intimately associated with androgen action. PCA is known to undergo a transition from an early androgen-sensitive form of cancer to a late (metastatic) androgen-insensitive cancer (Tang and Porter, 1997; Denmeade *et al.,* 1996). Because of this reason, at the time of clinical diagnosis most PCAs represent a mixture of androgen-sensitive and androgen-insensitive cells. Therefore, for effective PCA treatment there is a need to develop approaches aimed toward the elimination of both androgen-sensitive and androgen-insensitive PCA cells. In this study, we found that EGCG is capable of inhibiting the PCA cell growth irrespective of their androgen association, in a dose- and time-dependent fashion.

Because there has been an appreciation that regulation of apoptosis and cell cycle could be important targets for cancer chemoprevention (Ahmad *et al.,* 1997; Hartwell and Kastan, 1994) and also because the antiproliferative effects of EGCG have been shown to be mediated by apoptosis and/or cell-cycle dysregulation (Ahmad *et al.,* 1997; Yang *et al.,* 1998), we investigated whether or not EGCG causes apoptosis in PCA cells with different androgen responsiveness. Our results demonstrated that EGCG results in the apoptosis of both androgen-insensitive DU145 as well as androgen-sensitive LNCaP cells. This observation was verified by DNA fragmentation, flow cytometry, and confocal microscopy where the cells were stained with Hoechst 33258 dye that is specific for apoptosis. This could be an important observation that may be useful for devising strategies for the management of human PCA because apoptosis is a physiological and discrete way of cell death different from necrotic cell death and is regarded to be an ideal way of cell elimination (Ahmad *et al.,* 1997; Hartwell and Kastan, 1994; Schafer, 1998). Growth inhibitory effects of EGCG toward cancer cells, but not normal cells, have been reported (Chen *et al.,* 1998; Ahmad *et al.,* 1997). Since the two different PCA cell types employed in our study differ in androgen response and their origin, it appears that EGCG could be useful in inhibiting growth of PCA at any stage.

Because a controlled cell-cycle progression is also an important physiological event that is regarded to be essential for normal tissue homeostasis (Schafer, 1998) and also because in recent years the concept of cell-cycle-mediated apoptosis is being increasingly appreciated, we studied the effect of EGCG on cell-cycle distribution in both PCA cell types. Our studies demonstrated a dose-dependent arrest of cells in G_0/G_1 phase of the cell cycle in androgen-sensitive as well as androgen-insensitive PCA cells. This observation further substantiates our suggestion that EGCG may be developed as a useful agent for the management of PCA in humans because most of the cancer types including PCA possess defects in one or more cell-cycle checkpoints (Hartwell and Kastan, 1994; Schafer, 1998; Mercer, 1998). The loss of cell-cycle checkpoints results in the selection of cells that have a growth advantage that may result in drug resistance, invasion, and metastasis. Thus, the G_0/G_1-phase cell-cycle arrest of PCA cells by EGCG suggests

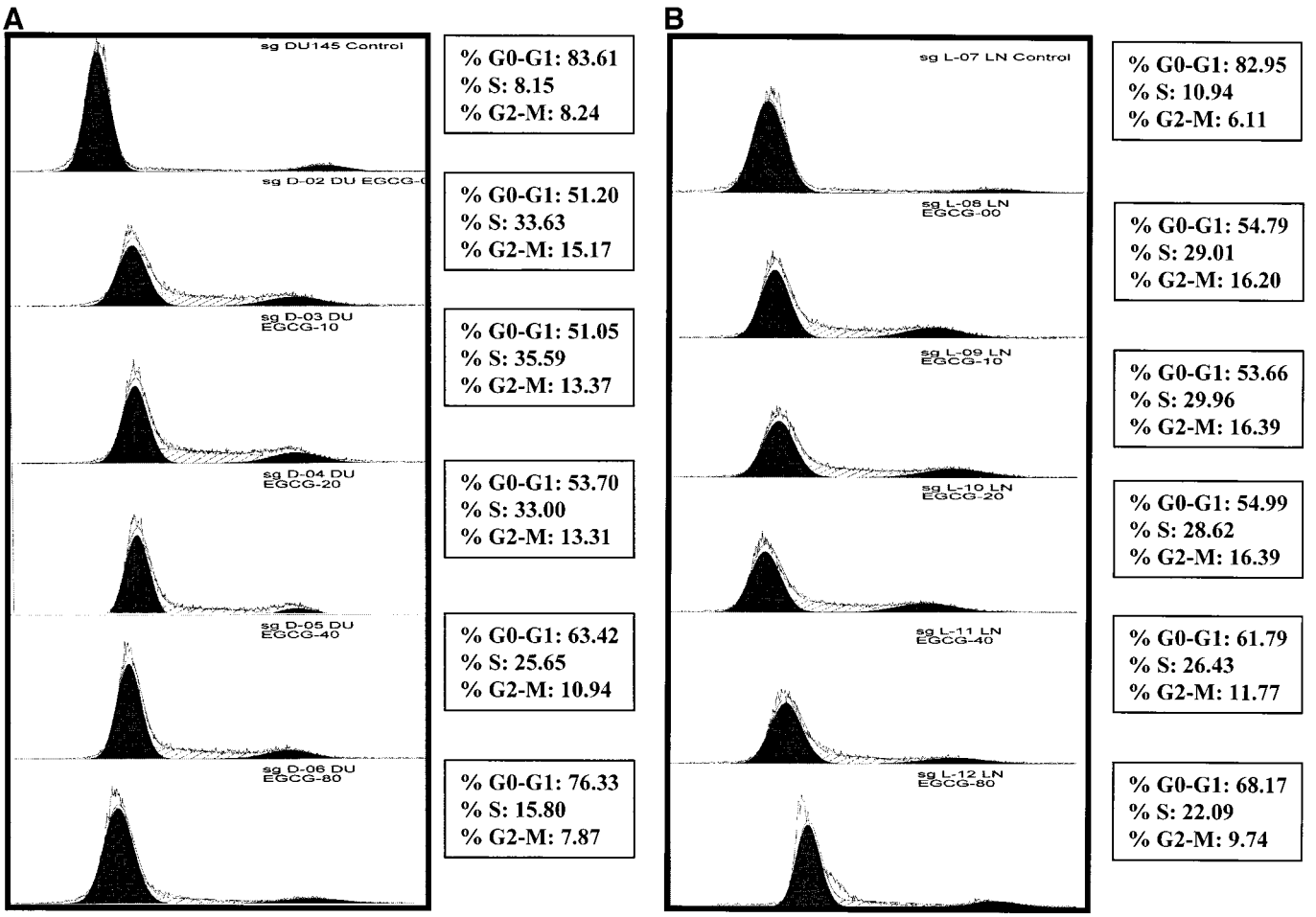

FIG. 5. (A) DNA cell-cycle analysis of DU145 cells and (B) LNCaP cells. The cells were synchronized by serum starvation for 36 h after which they were treated with vehicle or EGCG (10, 20, 40, and 80 μg/ml doses) for 24 h and analyzed by flow cytometry. Percentage of cells in G_0/G_1, S, and G_2-M phase were calculated using cell-fit computer software and are represented in the right side in histograms. Data shown here are from a representative experiment repeated three times with similar results.

that this agent may slow down the growth of cancer cells by artificially imposing the cell-cycle checkpoint.

Since many studies have shown the involvement of p53-mediated induction of the cyclin kinase inhibitor WAF1/p21 during G_0/G_1-phase cell-cycle arrest and apoptosis (Agarwal *et al.*, 1998; King and Cidlowski, 1998; Morgan and Kastan, 1997; Yonish-Rouach, 1996), we studied the effect of EGCG on these parameters. The p53 tumor suppressor gene is the most frequently mutated gene found in human malignancies including PCA. Generally, no correlation between p53 mutations and early stage PCA has been noticed but p53 mutations are shown to be associated with a small group (10–20%) of advanced PCA patients with high Gleason score and distant metastasis (Meyers *et al.*, 1998; Navone *et al.*, 1993). It is important to emphasize here that the two human PCA cell lines employed in this study also differ in their p53 status as the DU145 cells carry mutant p53, whereas the LNCaP cells carry wild-type p53. In this study, EGCG was found to result in an upregulation of p53 in LNCaP cells (with wild-type p53) but not in DU145 cells (with mutant p53). Our data also showed that EGCG treatment results in a significant induction of WAF1/p21 in both the cell types employed. This induction of WAF1/p21, therefore, appears to be p53-dependent in LNCaP cells (with wild-type p53) and p53-independent in DU145 cells (with mutant p53).

Therefore, it appears that the G_0/G_1 arrest produced by EGCG is dependent on p53 status in LNCaP cells, but not in DU145 cells, but is mediated by WAF1/p21 in both the cell types. This is an interesting observation because EGCG appears to be effective in imparting its apoptotic and cell-cycle deregulatory responses independent of p53 status. Therefore, EGCG seems to be capable of imposing its responses in the PCA with or without mutations in p53. However, more detailed studies are needed to investigate the association of p53, p53 mutation, and the other related events such as cyclins, cyclin-dependent kinase network, and involvement of bcl-2 and bax during the biological responses of EGCG in PCA.

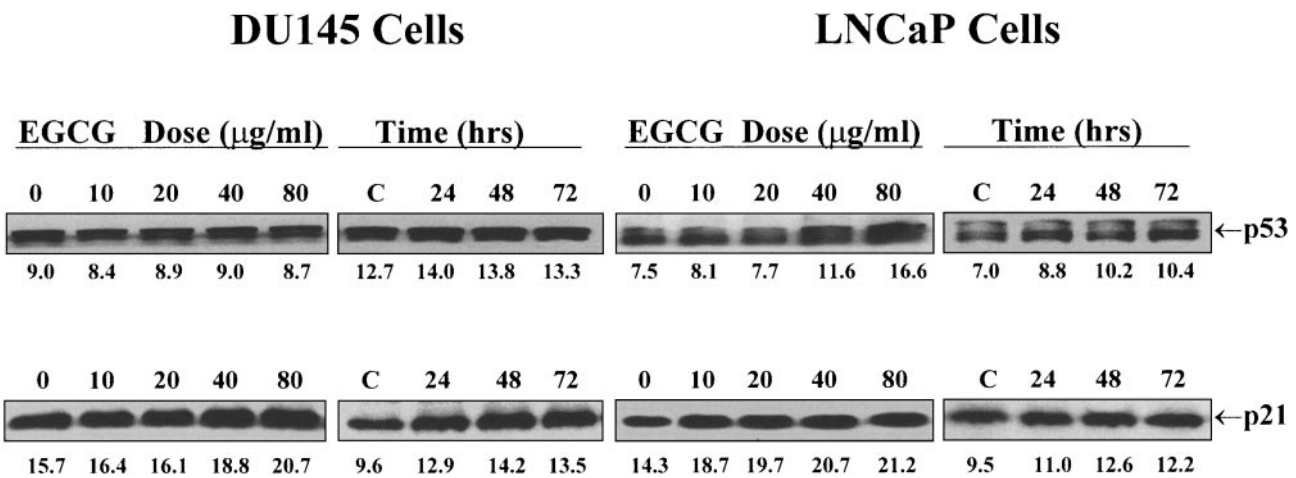

FIG. 6. Dose-dependent and time-dependent effect of EGCG on protein expression of p53 and WAF1/p21 in DU145 and LNCaP cells. The cells were treated with various doses of EGCG for 48 h. For time-dependent studies cells were treated with 40-μg/ml dose of EGCG for 24, 48, and 72 h, respectively. Total cell lysates were prepared and 25–50 μg protein was subjected to SDS–PAGE, followed by immunoblot analysis and the detection of protein. The lower value represents densitometric measurements of the bands normalized to β-actin. For normalization of protein loading, the membrane was stripped and reprobed with β-actin primary antibody and appropriate secondary HRP conjugate. The details are described under Materials and Methods.

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