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# Induction of apoptosis in prostate cancer cell lines by the green tea component, (–)-epigallocatechin-3-gallate

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**Abstract**

Green tea components exert many biological effects, including antitumor and cancer preventive activities. In the search for anticancer agents for prostate cancer the inhibitory effects of green tea components were tested on the prostate cancer cell lines LNCaP, PC-3 and DU145. (–)-Epigallocatechin-3-gallate (EGCG) proved to be the most potent catechin at inhibiting cell growth. The inhibition induced by EGCG was found to occur via apoptotic cell death as shown by changes in nuclear morphology and DNA fragmentation. Thus, we report the first evidence that EGCG is the active component in green tea and induces apoptosis in human prostate cancer cells. 

*Keywords:* Green tea; Apoptosis; Prostate cancer

## 1. Introduction

Prostate cancer is the most common cancer in American men and the second leading cause of male cancer death after lung cancer [1–3]. Epidemiological studies show that in Asian countries the incidence of prostate cancer is low compared to that in the West [4,5]. In addition, there is evidence that long term consumption of tea extracts or catechins has antitumor effects in animals. Liao et al. [6] showed that injections of EGCG rapidly reduced the size of human breast and prostate tumors in nude mice. Therefore, the low occurrence of prostate cancer in Asian countries may be due, in part, to the large consumption of green tea by these populations. Since green tea and its individual components have been suggested to have anticarcinogenic properties, this has created a rising interest in their application as a possible chemopreventive therapy.

Green tea is an aqueous infusion of dried unfermented leaves of *Camellia sinensis* (family Theaceae) for which numerous biological activities have been reported, including antimutagenic, antibacterial, hypocholesterolemic, antioxidant, antitumor and cancer preventive properties (see Ref. [7] and references therein). The tea leaves are distinguished by their content of methylxanthines and polyphenols, especially flavanols of the catechin type. Although its chemical composition varies with growing conditions, season, age of the leaves and variety cultivated, the major

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green tea phenols (GTPs) are (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin, (+)-gallocatechin (GC) and (+)-catechin [8]. Tea polyphenols and the major catechins are considered to be the main effectors of the anticarcinogenic actions of green tea (for a comprehensive review see Ref. [9]). However, the composition of green tea is very complex and although green tea polyphenols account for the majority of all components (about 36% by fresh weight), there are many components that might also contribute to its anticarcinogenic properties [10,11].

In this study we wanted to investigate whether the components of green tea have general growth effects on several human prostate cancer cell lines. Throughout these experiments not only was cell growth inhibited but the cells also appeared to undergo apoptosis. To confirm this, the cells were studied after treatment with EGCG for signs of apoptosis, such as nuclear morphological characterization and DNA laddering, neither of which have previously been described for this specific green tea polyphenol.

## 2. Materials and methods

### 2.1. Chemicals

The green tea components (EGCG, EGC, ECG and EC) were purchased from Sigma (St. Louis, MO) and the green tea polyphenols were purchased from LKT Laboratories (St. Paul, MN).

### 2.2. Cell culture

LNCaP, PC-3 and DU145 cells were obtained from the ATCC. All cells were maintained in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C. Cells ($2 \times 10^4$ per well (24-well plate) or $6.4 \times 10^3$ per well (96-well plate)) were seeded in RPMI media supplemented with 5% serum and incubated for 3 days at 37°C. Fresh medium was added and the cells were incubated for an additional 24 h prior to treatment. After treatment with the green tea compounds at varying concentrations (described in the text) the cells were incubated for a further 6 days at which time the cell number was determined using MTS assay (Promega, Madison, WI). Each experiment was carried out in quadruplicate and repeated at least three times.

### 2.3. Assay of growth inhibition

Six days after treatment the number of viable cells was calculated using the MTS colorimetric assay. The medium was removed and fresh Phenol-Red- and serum-free RPMI 1640 medium containing the MTS assay reagents at a 1:6 dilution was added (350 $\mu$l per well for 24-well plates and 100 $\mu$l per well for 96-well plates). The cells were incubated for 90 min at 37°C and the optical density was measured at 490 nm using a plate reader.

### 2.4. Characterization of nuclear morphology

Cells ($3 \times 10^4$/ml) were seeded onto 10 cm plates and incubated at 37°C for 3 days in RPMI 1640 medium containing 5% serum. The medium was changed and the cells were incubated for 24 h prior to treatment with 200 $\mu$M EGCG. After treatment the cells were incubated for a further 6 days and then collected using a Beckman J-6B centrifuge (JS 3.0 rotor) at 1000 rev./min for 5 min at 4°C. The cells were washed twice with phosphate-buffered saline (PBS) and then fixed for 5 min at 4°C with 300 $\mu$l 3% paraformaldehyde in PBS. After fixation the cells were collected at 8000 rev./min in a microfuge and then resuspended in water for 1 min and centrifuged as before. The cells were then stained with 300 $\mu$l bis-benzimide (Hoechst 33258) at a concentration of 0.3 $\mu$g/ml for 5 min at room temperature. The stained cells were centrifuged at 8000 rev./min for 5 min in a microfuge, washed once with PBS (500 $\mu$l) and then resuspended in an appropriate volume of PBS (usually 200 $\mu$l) [12]. The stained nuclei were viewed by fluorescent microscopy using an Axiophot microscope (Zeiss). Appropriate excitation filters were used for detection of bis-benzimide (365 nm excitation and 420 nm emission).

### 2.5. Gel electrophoresis and DNA fragmentation studies

Cells ($3 \times 10^4$/ml) were seeded onto 10 cm plates and incubated as described previously prior to the incubation with 200 $\mu$M EGCG. The cells were incu-

bated for 4 days and then collected using a Beckman J-6B centrifuge (JS 3.0 rotor) at 3000 rev./min for 5 min at 4°C. The DNA from treated cells was prepared for agarose gel analysis using essentially the method of Gunji et al. [13]. The cells were resuspended in 20 $\mu$l of 50 mM Tris–HCl (pH 8.0), 10 mM EDTA and 0.5 mg/ml proteinase K and incubated for 1 h at 50°C. Then 10 $\mu$l of a 0.5 mg/ml RNase A solution was added and the samples were incubated for an additional 1 h at 50°C. The sample was mixed with 10 $\mu$l of loading solution (10 mM EDTA (pH 8.0), 1% (w/v) low melting-point agarose, 0.25% (w/v) Bromophenol Blue and 40% (w/v) sucrose) preheated to 70°C. The DNA samples were loaded onto a 1.8% (w/v) agarose gel and sealed with 0.8% (w/v) low melting-point agarose. The DNA fragments were separated by electrophoresis at 25 V for 18 h at 4°C in TBE buffer. The DNA was visualized using ethidium bromide and photographed using an Alphaimager 2000 digital camera.

## 3. Results

### *3.1. Green tea components inhibit the growth of LNCaP, PC-3 and DU145 cells in culture*

In order to assess the inhibitory effects of green tea, three prostate cancer cell lines were treated with the individual purified green tea components and also a mixture of the green tea polyphenols (GTPs). A range of concentrations was used, i.e. from 1 to 400 $\mu$M for the single catechins and from 1 to 400 $\mu$g/ml for the GTPs. After treatment for 6 days the cell number was quantified by a colorimetric MTS assay measuring the number of viable cells remaining. The results are expressed as a percentage of the control cell number to show the extent of an inhibitory or proliferative effect of these compounds on cell growth (Fig. 1). In the hormone refractory prostate cancer cell lines, DU145 and PC-3, all of the green tea compounds, with the exception of EC, showed levels of cell growth inhibition as low as 80% at concentrations of 50 $\mu$M and above (Fig. 1a,b, respectively). At concentrations lower than this the catechin gallates, EGCG and ECG, and the GTPs still showed inhibitory effects on DU145 cell growth as great as 50% of control values. In the PC-3 cells, while all of the compounds showed an inhibitory effect even at concentrations as low as 1 $\mu$M, EGCG appeared to be the most potent because it inhibited cell proliferation by 45% when compared to control, at least 25% greater than any of the other compounds. As shown in Fig. 1c, the effect of EGCG was particularly potent in the androgen-responsive LNCaP cell line. At concentrations as low as 1 $\mu$M, EGCG was still able to inhibit cell growth by 90% compared to control cells. For this cell line the only other treatment to have a marked effect on cell proliferation was the GTP mixture which is probably due to the combined interaction of the polyphenols. EC only showed significant inhibitory effects on PC-3 and DU145 cells at the two highest concentrations and had no effect on the growth of LNCaP cells.

### *3.2. Induction of apoptotic cell death by EGCG*

We focused on EGCG because it was found to be the most potent inhibitor of cell growth in all three cell lines. LNCaP and DU145 cells were used for the following study because they represent androgen-responsive and -unresponsive cells, respectively. After a 24 h treatment with EGCG the majority of cells had become shrunken, clumped together and detached from the culture dishes. At this time the nuclei were stained with bis-benzimide (Hoechst 33258) in order to study any nuclear morphology changes. Cells undergoing apoptosis displayed profound structural changes, including a rapid blebbing of the plasma membrane and nuclear disintegration. The nuclei of cells treated with EGCG (Fig. 2) were clearly found to be fragmented with condensed chromatin, both of which are signs of apoptosis, as previously mentioned. Another marker of apoptosis, the DNA fragmentation laddering effect, was seen in both cell lines after EGCG treatment by separating DNA extracted from apoptotic cells on agarose gels (Fig. 3).

## 4. Discussion

The growth inhibitory effects of green tea have been shown in many tumor cell lines [7,14,15], however, this is the first study to investigate the effects of individual green tea components in prostate cancer cells. The results show that (–)-epigallocatechin-3-

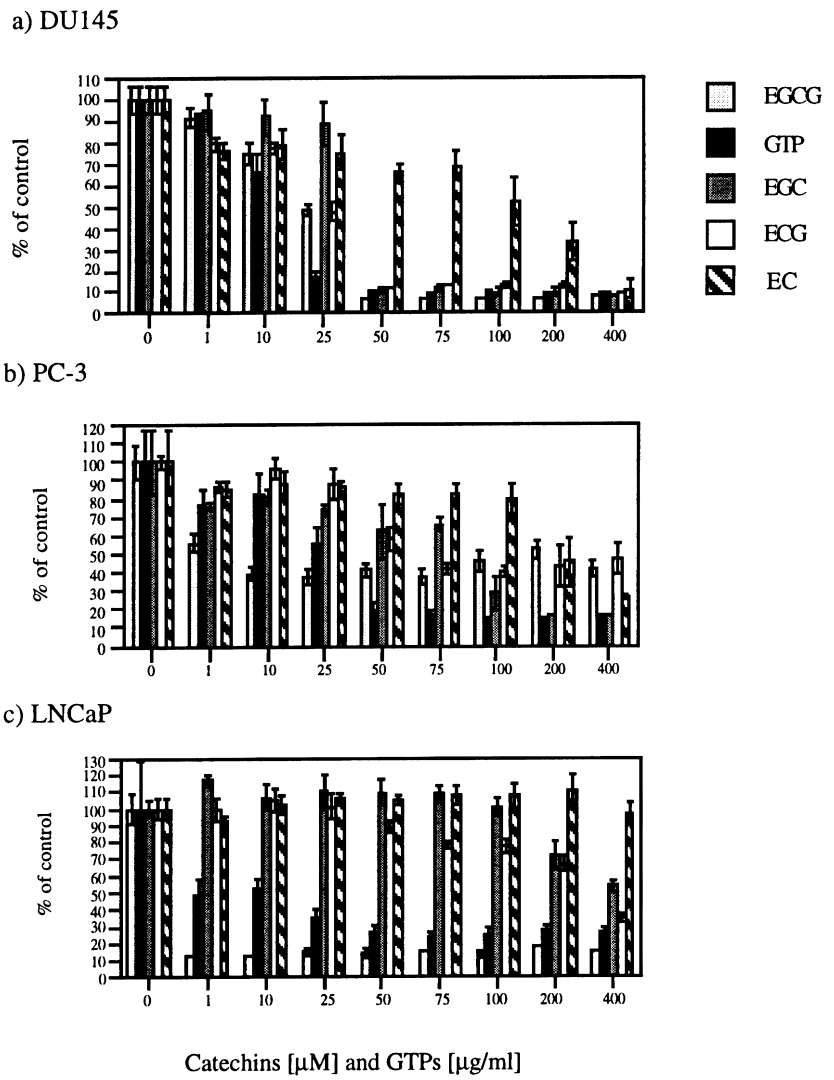


Fig. 1. The effects of green tea components on the growth of human prostatic adenocarcinoma cell lines. (a) DU145, (b) PC-3 and (c) LNCaP cells were treated for 6 days with increasing concentrations of green tea components. Viable cells were measured using an MTS assay and expressed as a percentage of the controls ($n = 3$). Standard errors are shown as a thin vertical line.

gallate was the most effective component at inhibiting the cell growth of all three prostate cancer cell lines studied. The reasons why EGCG should be so potent in this cell type are complex, although a number of enzymes, including the androgen-converting enzyme, 5$\alpha$-reductase [16], and urokinase [17], an enzyme known to be crucial for cancer spread [18], have been shown to be inhibited by EGCG. The development of prostate cancer is known to be androgen-dependent so the fact that EGCG inhibits a major enzyme involved in androgen action may be of importance. However, this is unlikely to be the only mechanism involved because the concentration of EGCG required to inhibit these enzymes is far greater than that needed to arrest cell growth. In addition, the mechanism of action for green tea is not necessarily an androgen-dependent one because two androgen refractory cell lines are still inhibited by EGCG. In fact, at higher concentrations GTPs and EGC can be seen to have more of an effect than EGCG in the

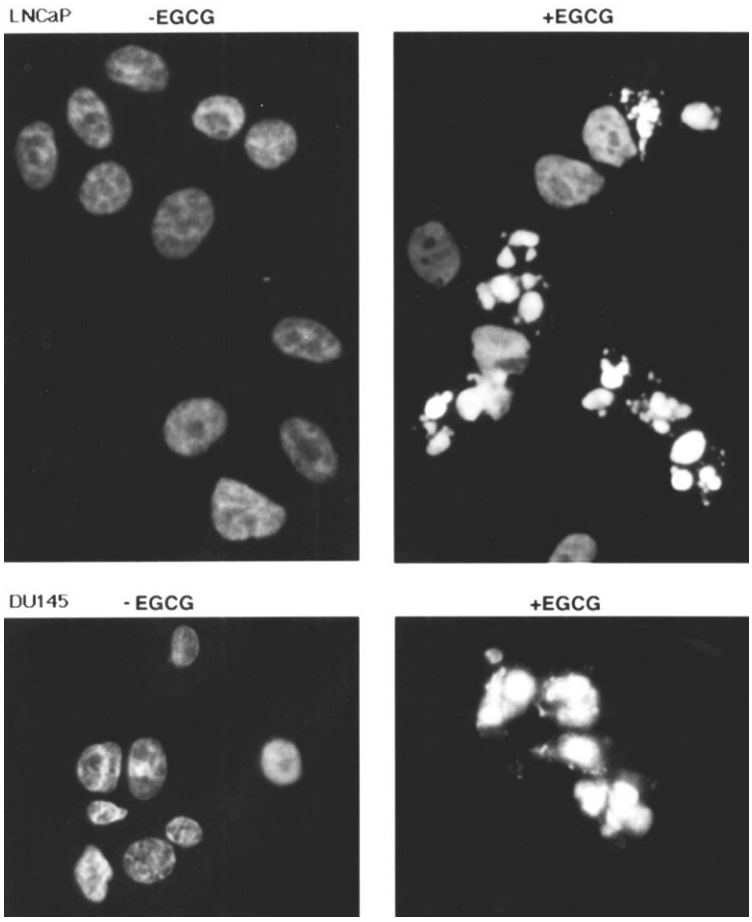


Fig. 2. Chromatin condensation and nuclear fragmentation in LNCaP and DU145 cells induced by EGCG. Cells were treated with 200 $\mu$M EGCG for 6 days and fixed in paraformaldehyde and the DNA was stained using bis-benzimide (Hoechst 33258) to show apoptosis.

hormone refractory cell lines. However, the ability of EGCG to exert its effects at such low concentrations may be important if green tea or EGCG alone are to be considered as chemopreventative agents for prostate cancer. Studies by Yang [19] indicated that the concentration of EGCG in the blood after more than two to three cups of green tea could only be a maximum of 0.6 $\mu$M. At a concentration of 1 $\mu$M the effect of EGCG in LNCaP cells was a 90% inhibition of cell growth. Therefore, it is possible to presume a similar response at even lower concentrations, such as those achieved by drinking three or more cups of green tea.

Apoptosis or programmed cell death has become of interest as an intervening target in cancer chemoprevention. There is much evidence that administration of naturally occurring compounds with antitumor activities triggers the apoptotic death of cancer cells [20–25]. Apoptosis is the most common form of eukaryotic cell death, acting as a physiological suicide mechanism to preserve homeostasis, and occurs naturally during tissue turnover [26]. Apoptosis has been shown to be the mechanism of action of tea polyphenols for inhibiting cell growth in HL-60 cells, a promyelocytic leukemic cell line [14], human epidermoid (A431) and keratinocyte (HaCaT) carcinoma cell lines and in DU145 [15], one of the prostate carcinoma cell lines studied here. Therefore, we wanted to further investigate whether the effect of these compounds was mediated via an apoptotic mechanism in prostate cancer cells. Changes in nuclear morphology and DNA fragmentation associated with EGCG-induced apoptosis were clearly seen in both LNCaP

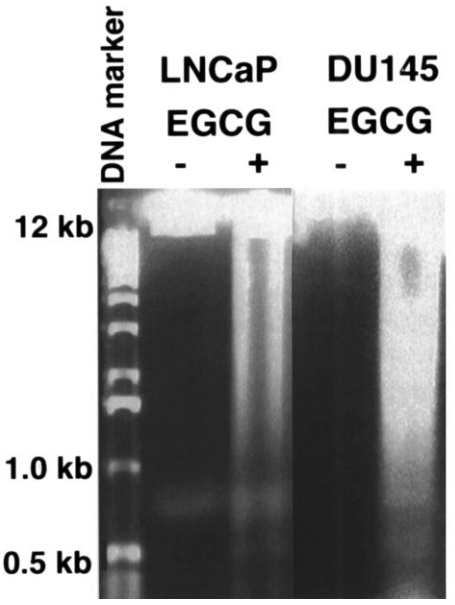


Fig. 3. Electrophoretic analysis of DNA from LNCaP and DU145 cells treated with EGCG. LNCaP and DU145 cells were incubated with and without 200 $\mu$M EGCG for 4 days. DNA was extracted and analyzed by electrophoresis on 1.8% agarose gels.

and DU145 cells by nuclear staining and DNA studies. The nuclear collapse is associated with extensive damage to chromatin and DNA cleavage into oligonucleosomal length DNA fragments after activation of a calcium-dependent endogenous endonuclease [27]. Therefore, EGCG is the active inhibitor of cell proliferation in green tea and one possible mechanism for its action in prostate cancer cells may be via the induction of apoptosis.

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