Original Article: Laboratory Investigation

Epigallocatechin-3-gallate and bicalutamide cause growth arrest and apoptosis in NRP-152 and NRP-154 prostate epithelial cells

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Aim: A number of epidemiological studies have suggested that consumption of green tea reduces the risk of prostate cancer. The aim of this study was to elucidate the effects of epigallocatechin-3-gallate (EGCG), one of the major constituents of green tea, on growth inhibition and apoptosis in prostate epithelial cell lines with and without bicalutamide.

Methods: The effects of EGCG and bicalutamide alone and in combination were examined on NRP-152 and NRP-154 cells derived from the dorso-lateral prostate of the Lobund-Wistar rat. Following treatments, cell number and levels of apoptosis were assessed.

Results: After treatment with EGCG, both cell lines displayed a dose-dependent decrease in cell number; this effect was more pronounced in NRP-154 cells. This decrease in cell number was caused by growth arrest in NRP-152 cells and apoptosis in NRP-154 cells. The apoptotic events in the NRP-154 cells were concurrent with a loss of manganese superoxide dismutase expression. Androgen ablation was achieved by androgen withdrawal using charcoal stripped serum or treatment with bicalutamide. Bicalutamide decreased cell number and induced apoptosis in a dose-dependent manner in both cell lines; however, androgen withdrawal did not. There was a loss of androgen receptor expression in NRP-152 cells with bicalutamide treatment. However, as the NRP-154 cells are androgen receptor negative, the loss in cell number and increased apoptotic events in these cells cannot be attributed to the anti-androgenic activity of bicalutamide. Cells treated with a combination of bicalutamide and EGCG also demonstrated a dose-dependent decrease in cell number that was significantly greater than bicalutamide alone.

Conclusions: This study demonstrates the potential use of EGCG and other antioxidants as therapeutic candidates for prostate cancer.

Key words: bicalutamide, epigallocatechin-3-gallate, NRP-152, NRP-154, prostate.

Introduction

The incidence of prostate cancer increases with age. Although there is clearly a genetic component to prostate cancer risk, the molecular mechanisms underlying the initiation and progression of spontaneous prostate cancer remain unknown. A number of epidemiological studies have suggested that consumption of green tea reduces the risk of prostate cancer.[1] Epigallocatechin-3-gallate (EGCG) is one of the major constituents of green tea. There is considerable evidence that EGCG and other catechins found in green tea act as potent antioxidants presumably by decreasing oxidative damage that may play a role in the mutator phenotype leading to tumor initiation and/or progression.[2,3]

EGCG causes alterations in cell cycle kinetics and apoptosis in prostate cancer cells both *in vitro* [4–6] and *in vivo.* [7] Animal studies include the use of EGCG on athymic mice inoculated with PC-3 and LNCaP 104-R cells. More recently studies on TRAMP mice treated with a polyphenolic fraction isolated from green tea show an inhibition of tumor growth after treatment[7,8] and suggest that EGCG may not be only chemopreventative, but may also have chemotherapeutic properties.

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Bicalutamide (ICI 176 334) is a non-steroidal anti-androgen typically used in combination with a luteinizing hormone-releasing hormone analog in the treatment of prostate cancer.[9] This treatment regime results in a complete androgen blockade and cell death in androgen dependent cells.

Normal rat prostate (NRP)-152 and NRP-154 are epithelial cell lines derived from the prostate of Lobund-Wistar rats treated with N-methyl-N-nitrosourea (MNU). The NRP-152 cell line has been described as a non-tumor forming basal epithelial cell and is androgen receptor positive, while NRP-154 cells are tumor forming secretory epithelial cells *in vivo* and are androgen receptor negative.[10]

We hypothesized that EGCG could be used as a therapy for prostate cancer in combination with the anti-androgen bicalutamide (ICI 176 334). The aim of this study was to elucidate the effects of EGCG on growth inhibition and apoptosis in the NRP-152 and NRP-154 cells with and without bicalutamide.

Methods

Cell culture and reagents

NRP-152 and NRP-154 cell lines were maintained in GM2 (HEPES-free, and antibiotic-free DMEM/F12 supplemented with 5% FBS, 20 ng/mL EGF, 10 ng/mL cholera toxin, 0.1 μmol/L dexamethasone, 5 μg/mL bovine insulin) supplemented with 50 U/mL penicillin, 50 mg/mL streptomycin and 10 nmol/L dihydrotestosterone and passaged every 3–4 days at subconfluence.[10]

Cells were treated in a growth defined Gc media (DMEM-F12 Phenol Red Free, 2 mg/mL bovine serum albumin [BSA], 0.5 mg/mL fetuin, 5 μg/mL insulin, 10 μg/mL transferrin, 50 nmol/L hydrocortisone, 0.1 nmol/L 3,3′,5-triiodo-L-thyronine, 25 nmol/L sodium selenite, 1 ng/mL EGF, Na-pyruvate 0.5 mmol/L) supplemented with 50 U/mL penicillin, 50 mg/mL streptomycin and 10 nmol/L dihydrotestosterone.[11] Bicalutamide was obtained from E. Von Angerer (Pharmacy Institute, Regensburg University, Germany) and EGCG from Sigma (St Louis, MO, USA).

Immunocytochemistry

Manganese superoxide dismutase

Cells were grown on chamber slides and treated with EGCG (0–40 μmol/L) for 72 h in Gc medium. Cells were fixed in 4% paraformaldehyde at 4°C for 20 min and permeablized in 100% methanol at −20°C for 5 min. Cells were then incubated with rabbit antirat manganese superoxide dismutase (MnSOD) 1/50 (Stressgen Biotechnologies, San Diego, CA, USA) in antibody buffer (3% w/v of BSA, 0.3% Triton X-100, in 10 mmol/L phosphate-buffered saline [PBS] at pH 7.2) for 1 h at room temperature, followed by incubation with a donkey antirabbit Cy2 (Jackson Immunochemical Laboratories, Bar Harbor, ME, USA) for 1 h at room temperature.

Androgen receptor

NRP-152 cells were treated with bicalutamide (40 μmol/L) for 48 h in Gc medium and were fixed and permeablized as above. Cells were incubated with 1/40 rabbit antiandrogen receptor (UBI, Lake Placid, NY, USA) in antibody buffer (3% w/v of BSA, 0.3% Triton X-100, in 10 mmol/L PBS pH 7.2) for 1 h at room temperature, followed by incubation with donkey antirabbit Cy2 (Jackson Immunochemical Laboratories) for 1 h at room temperature. The cells were incubated with Hoechst dye (1 ng/mL), coverslipped with mounting medium (Vector Laboratories, Burlingame, CA, USA) and photographed under indirect fluorescence documented using an Olympus AX70 microscope with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). Negative controls omitting primary antibody were performed in parallel.

Western blot analysis

Protein lysates were isolated in a RIPA buffer. Protein concentrations were determined using the bicinchoninic acid assay (Pierce, Rockford, IL, USA), electrophoresed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing gel, and transferred to 0.2 μm supported nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Non-specific binding was eliminated by preincubation in 1% heat-denatured casein for 1 h. Membranes were incubated in the appropriate dilution of primary antibody (rabbit antiandrogen receptor [UBI], rabbit anti-GAPDH [Biogenesis, Cambridge, UK], rabbit antirat MnSOD [Stressgen Biotechnologies], mouse antihuman catalase [Sigma] or mouse antihuman β-actin [Sigma]) for 1 h, followed by horseradish peroxidase-conjugated goat antimouse (Caltag Laboratories, Burlingame, CA, USA) or goat antirabbit (Bio-Rad Laboratories) and developed using SuperSignal (Pierce) and exposed to X-Omat film (Kodak, Rochester, NY, USA) at room temperature.

RT-PCR

Total RNA was harvested from NRP-152 and NRP-154 cells using Ultra Spec II RNA (Biotecx, Houston, TX, USA). Reverse transcription was performed in the presence or absence of SuperScript RT (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations, except for the addition of 10% glycerol. The androgen receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified using androgen receptor forward primer (CTTGTGCGCCTCTGGCCGAATGCAA) and reverse primer (GGCGATCCAGTGCTGGGTCCGGCTA) or GAPDH forward primer (TCAATGGCACAGTCAAGGCTGA) and reverse primer (GGCTCAGATCCACAACGGATA).

The PCR conditions for the androgen receptor were as follows: 3 min at 94°C and 35 cycles of 30 s at 94°C, 30 s at 65.8°C and 30 s at 72°C. PCR conditions for GAPDH were as follows: 1 min at 95°C and 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The primers were designed using Primers3 Software (http://www.genome.wi.mit.edu/cgibin/primer/primer3.cgi), based on the available sequences for rat androgen receptor and rat GAPDH. The gels were photographed using UV transillumination (306 nm), and photographed using Kodak ds1D software.

Apoptosis and viability assays

Apoptotic events were described as a percentage of total events with hypodiploid DNA assessed by propidium iodide (PI) incorporation. Cells were harvested by trypsinization, permeabilized with a hypotonic fluorochrome solution (50 μg/mL PI, 3.4 mmol/L sodium citrate, 1 mmol/L Tris, 0.1 mmol/L EDTA and 0.1% Triton X-100) and incubated on ice for 10 min prior to analysis. Viability assays were performed on the same samples. Cells were harvested by trypsinization, permeabilized with a hypotonic fluorochrome solution (50 μg/mL PI, 3.4 mmol/L sodium citrate, 1 mmol/L Tris, 0.1 mmol/L EDTA) and incubated on ice for 5 min prior to analysis. Samples were run on an Epics XL-MCL Coulter Elite Cytometer (Coulter Cytometry, Miami, FL, USA). 5000 events were gated on PI intensity and analyzed using Mplus software (Coulter Cytometry).

Assessment of DNA fragmentation by ApoBrdU staining

Assessment of apoptosis by flow cytometry-based TUNEL was performed according to standard methodology. Briefly, NRP-152 and NRP-154 cells were harvested by trypsinization, washed once with PBS and fixed in 1% paraformaldehyde in PBS at 4°C while shaking for 30 min. Cells were washed in PBS, permeabilized in 70% ice-cold ethanol and left overnight at −20°C. Then, 5×10^5 cells were washed twice with PBS/0.2% BSA, resuspended in 50 μL of DNA Labeling solution (25 U Terminal Transferase [TdT], 1 × reaction buffer, cobalt chloride, and Br-dUTP; Roche, Basel, Switzerland) and incubated for 60 min at 37°C. The cell suspension was washed with PBS/0.2% BSA and then rinse buffer 0.1% Triton-X100/1% BSA in PBS. Cell pellets were resuspended in 100 μL of antibody solution (5 μL of FITC-conjugated anti-BrdU [BD Pharmingen, San Diego, USA] in 95 μL of rinse buffer) and incubated for 30 min at RT. Then, 900 μL of PI staining solution (5 μg/mL PI, 10 μg/mL RNAse) (Sigma) was added to the antibody solution and the samples were run on a Coulter Epics XL Cytometer (Coulter Cytometry). Ten thousand events were gated using classical doublet exclusion based on PI staining (diagonal of the

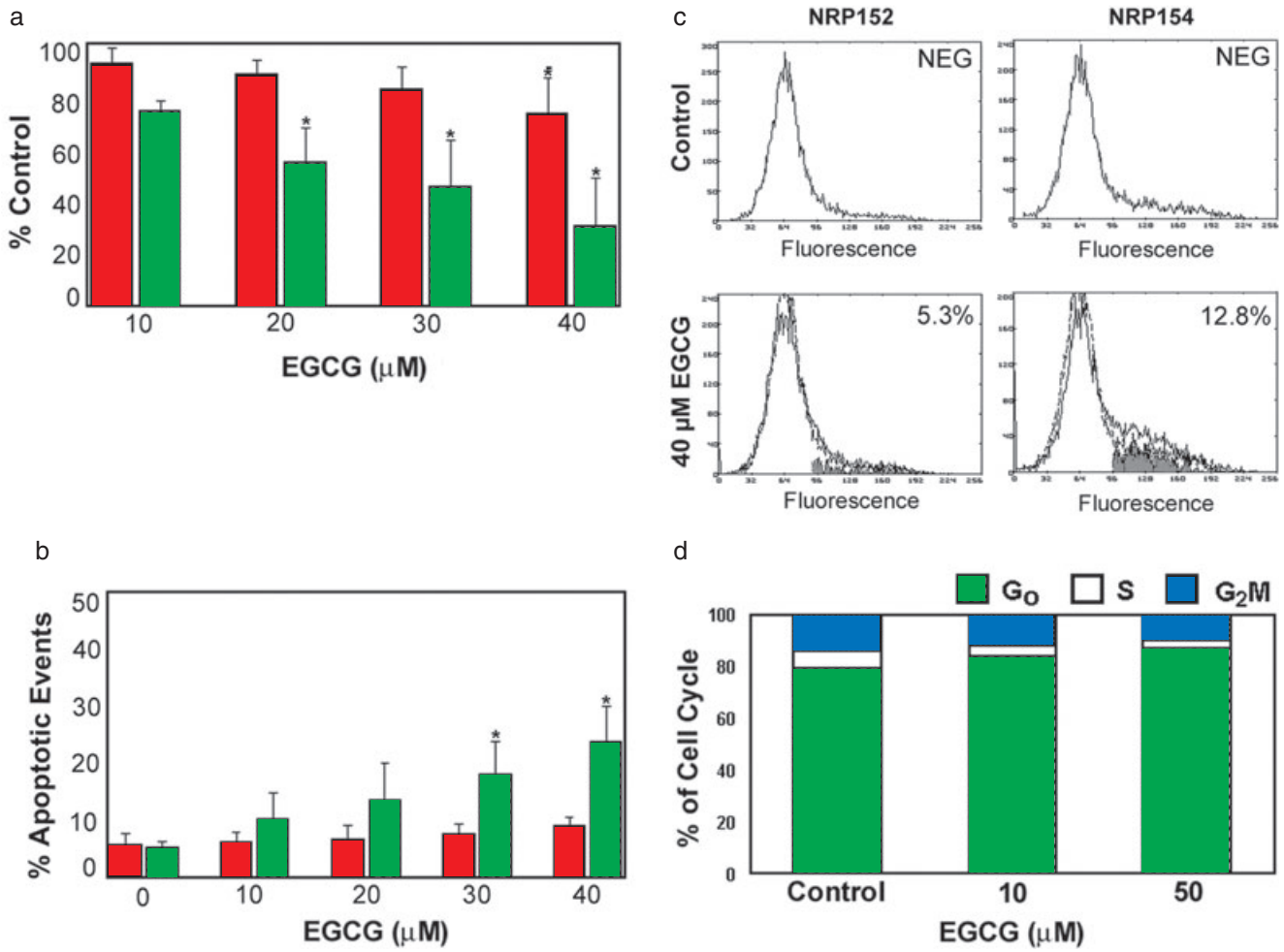

Fig. 1 Effect of EGCG on cell number and apoptosis in NRP-152 and NRP-154 cells. (a) NRP-152 (red) and NRP-154 (green) cells were brought to 40% confluence and cultured in Gc medium with increasing doses of EGCG for 72 h. Cell number was assessed by crystal violet assay ($n = 3$). Results are expressed as the mean ± SD. *Significantly different from control at $P < 0.05$. (b) Apoptotic events were assessed in NRP-152 (red) and NRP-154 (green) cells by percentage propidium iodide incorporation ($n = 6$). Results are presented as the mean ± SD. *Significantly different from control at $P < 0.05$; membrane integrity (viability) did not alter between doses (data not shown). (c) NRP-152 and NRP-154 cells were cultured in Gc medium with and without EGCG (40 μmol/L) for 72 h, dual stained with propidium iodide and APOBrDU-fluorescein and analyzed on a flow cytometer. The percentages and gray shading indicate APOBrDU positive cells. (d) Effect of increasing doses of EGCG on proliferation in NRP-152 cells. NRP-152 cells were brought to 40% confluence and cultured in Gc medium with 0, 10 or 50 μmol/L EGCG for 48 h. Cell cycle analysis was performed to ascertain the percentage of cells in G_2M, S and G0 phase of the cell cycle. Percentage of cells in S phase is 5.6% in Control, 3.9% in 10 μmol/L EGCG and 2.7% in 50 μmol/L EGCG. This is one example of three independent experiments.

peak *vs* integral signal). The events were subsequently assessed for apoptosis by a logarithmic increase in FITC intensity over untreated samples using Mplus software.

Cell number

NRP-152 and NRP-154 cells were grown in 24-well plates and treated for 72 h in Gc media supplemented with 50 U/mL penicillin, 50 mg/mL streptomycin and 10 nmol/L dihydrotestosterone. Cells were fixed in 1% gluteraldehyde solution for 15 min at room temperature, rinsed with PBS, and incubated with 1% crystal violet solution (Sigma) for 30 min at room temperature. Plates were washed with water and left to air dry. The dye was solubilized with 1% Triton X-100 for 20 min at room temperature and absorbance read at 590 nm using a spectrophotometer (Tecan, Zurich, Switzerland).

Statistical analysis

Significance was determined by one way ANOVA using Minitab software (Minitab, State College, PA, USA). $P < 0.05$ was considered significant.

Results

Effect of EGCG on cell number and apoptosis

The NRP-152 and NRP-154 cells displayed a dose-dependent decrease in cell number following treatment with EGCG. This decrease in cell number was more evident in the NRP-154 cells, which displayed at least a twofold reduction compared to the NRP-152 cells using 40 μmol/L EGCG (Fig. 1a). NRP-152 cells had no statistically

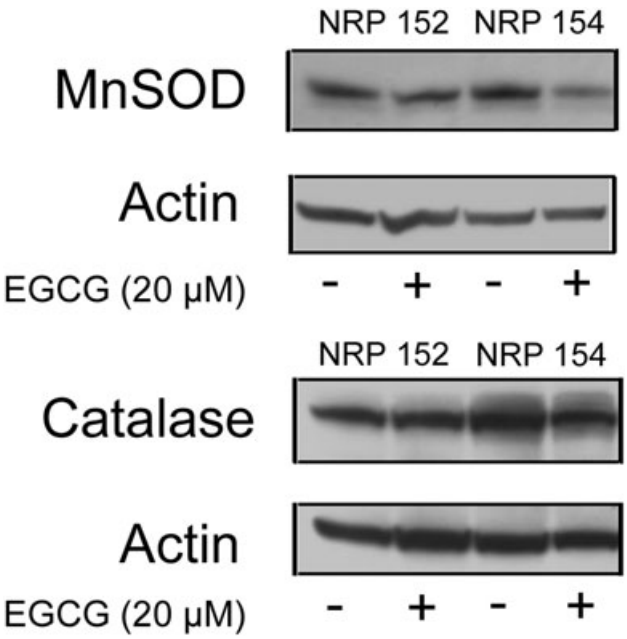

Fig. 2 Effect of EGCG on MnSOD and catalase expression in NRP-152 and NRP-154 cells. NRP-152 and NRP-154 cells were brought to 40% confluence and cultured in Gc media for 72 h with 0 or 20 μmol/L EGCG, cell lysates were isolated and western blotting analysis performed using antibodies to MnSOD and catalase, with β-actin as control ($n = 3$).

significant increase in apoptotic events with EGCG treatment; however, the NRP-154 cells had a dose-dependent increase in apoptotic events in response to EGCG (Fig. 1b). Membrane integrity (viability) did not alter between doses (data not shown).

Apoptosis in NRP-152 and NRP-154 cells was confirmed using the TUNEL assay. Untreated NRP-154 cells were mostly TUNEL negative (Fig. 1c). Following treatment with 40 μmol/L EGCG, NRP-154 cells had a significantly greater number of TUNEL positive results compared to NRP-152 cells (Fig. 1c).

As the loss in cell number in the NRP-152 cell line could not be attributed to apoptosis, we examined the effect of EGCG on cell cycle kinetics. With increasing doses of EGCG the percentage of cells in G_2M and S phase decreased with an increase in cells in the G0 phase of the cell cycle (Fig. 1d).

Effect of EGCG on MnSOD and catalase expression

The NRP 152 and NRP 154 cells were cultured in Gc media for 72 h with or without EGCG (20 μmol/L). Cells were treated with a lesser concentration of 20 μmol/L EGCG to discount the possibility that the decrease in MnSOD or catalase protein expression was due to cleavage during the apoptotic process, rather than an alteration in protein expression. There was no change in catalase steady state protein levels in either cell line after EGCG treatment (Fig. 2). MnSOD steady state protein levels did not alter in the NRP-152 cells; however, there was a decrease in MnSOD steady state protein levels in NRP-154 cells after EGCG treatment (Fig. 2).

These results were consistent with immunocytochemical staining of NRP-152 and NRP-154 cells for MnSOD cultured for 72 h with EGCG (0, 10 and 40 μmol/L). The cells displayed a punctate staining pattern consistent with the mitochondrial localization of MnSOD in the cell (Fig. 3).

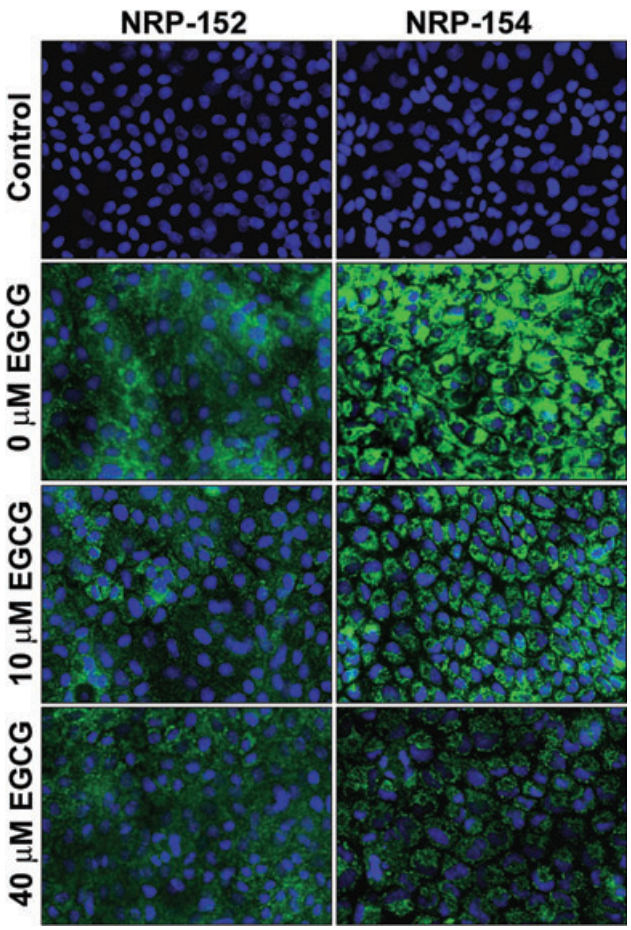

Fig. 3 Immunocytochemical analysis of MnSOD in EGCG treated NRP-152 and NRP-154 cells. NRP-152 and NRP-154 cells were plated on chamber slides and cultured in Gc medium for 72 h with 0, 10 or 50 μmol/L EGCG, fixed and incubated with an antibody for MnSOD. Controls omitted primary antibody. This experiment was done once.

Cytokeratin and androgen receptor expression

The androgen receptor status of the NRP-152 and NRP-154 cells was confirmed using RT-PCR, immunocytochemical and western blotting analysis. When cultured on glass chamber slides the NRP-152 cells formed a regular and enlarged cell type that stained positive for cytokeratin 14. NRP-154 cells displayed positive staining for cytokeratin 18 that was polarized within the cells. Cytokeratin expression was confirmed by western blotting analysis (data not shown).

Using RT-PCR, NRP-152 cells were positive for androgen receptor mRNA and NRP-154 cells were negative (Fig. 4a). The expression of the androgen receptor protein correlated with the mRNA expression by western blotting analysis (Fig. 4b) and immunocytochemical analysis (data not shown). These data clearly show that the NRP-152 cells express the androgen receptor and NRP-154 cells do not.

Immunocytochemical analysis of the androgen receptor status of bicalutamide-treated NRP-152 cells

To confirm the anti-androgenic effect of bicalutamide on the NRP-152 cells, we examined androgen receptor localization. The androgen

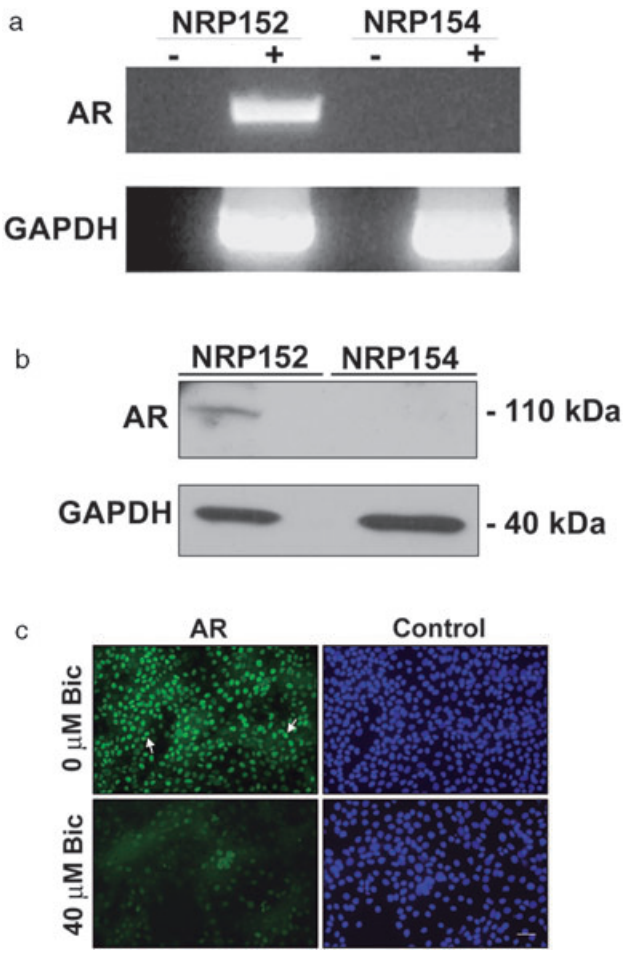

Fig. 4 Androgen receptor status of NRP-152 and NRP-154 cells and immunocytochemical analysis of the androgen receptor in bicalutamide-treated NRP-152 cells. (a) NRP-152 and NRP-154 cells were cultured in Gc medium for 72 h, total RNA was isolated, reverse transcribed and RT-PCR was performed with (+) or without (–) reverse transcriptase for the androgen receptor and GAPDH. (b) NRP-152 and NRP-154 cells were cultured in Gc medium for 72 h, cell lysates were isolated and western blotting analysis performed using an antibody specific to the androgen receptor, with GAPDH as a control. (c) NRP-152 cells were plated on chamber slides and cultured in Gc medium for 48 h, fixed and incubated with an antibody specific for the androgen receptor. Untreated, or bicalutamide (40 μmol/L) treated cells were incubated with an antibody specific for the androgen receptor. Hoechst dye staining indicates presence of cells. Arrows indicate positive nuclear staining. Bar = 50 μm. This experiment was done once.

receptor localized to the nucleus of the NRP-152 cells on immunocytochemical analysis (Fig. 4c). However, when treated with bicalutamide (40 μmol/L) there was a loss of androgen receptor reactivity in the nucleus of the NRP-152 cells (Fig. 4c).

Effect of bicalutamide on cell number and apoptosis

The NRP-152 and NRP-154 cells displayed a dose-dependent decrease in cell number when treated with bicalutamide (0–40 μmol/L) (Fig. 5a). The decrease in cell number may be attributed to apoptosis as the NRP-152 and NRP-154 cells displayed a dose-dependent increase

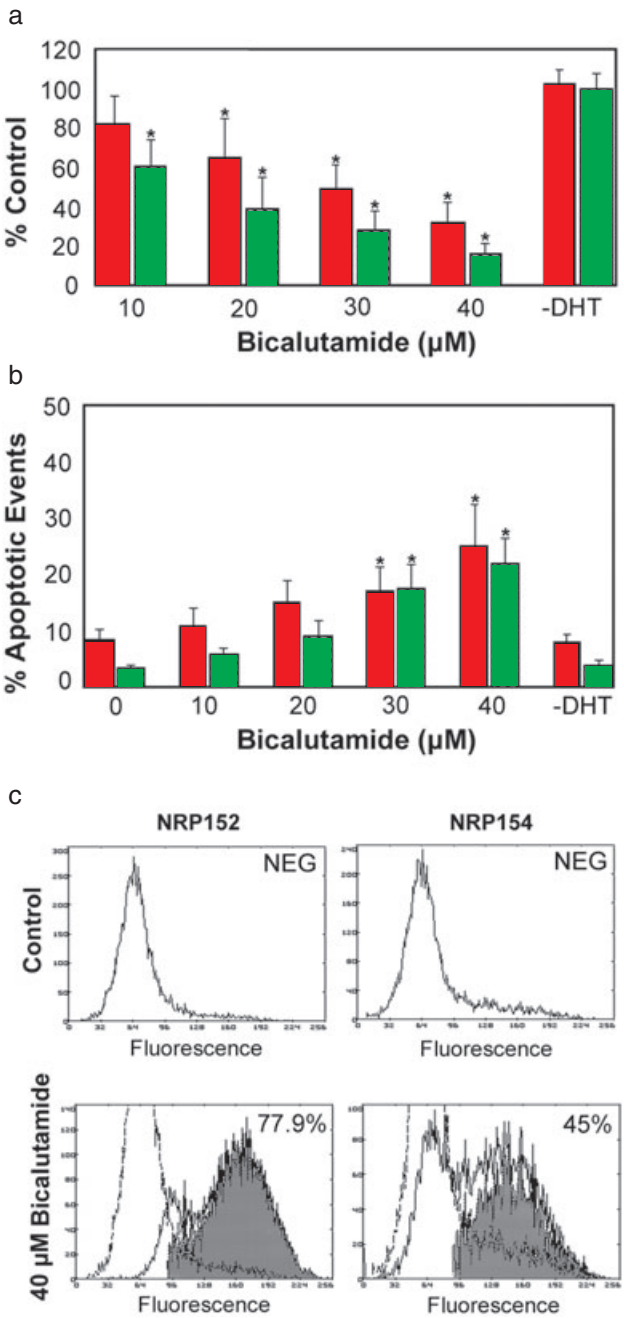

Fig. 5 Effect of bicalutamide on cell number and apoptosis in NRP-152 and NRP-154 cells. (a) NRP-152 (red) and NRP-154 (green) cells were brought to 40% confluence and cultured in Gc medium with increasing doses of bicalutamide for 72 h. Cell number was assessed by crystal violet assay ($n = 3$). Results are expressed as the mean ± SD. *Significantly different from control at $P < 0.05$. (b) Apoptotic events in NRP-152 (black) and NRP-154 (hatched) cells were assessed by percent propidium iodide incorporation ($n = 4$). Results are expressed as the mean ± SD. *Significantly different from control at $P < 0.05$. Membrane integrity (viability) did not alter between doses (data not shown). (c) NRP-152 and NRP-154 cells were cultured in Gc medium with and without bicalutamide (40 μmol/L) for 72 h, dual stained with propidium iodide and APOBrDU-fluorescein and analyzed on a flow cytometer. The percentages and gray shading indicate APOBrDU positive cells.

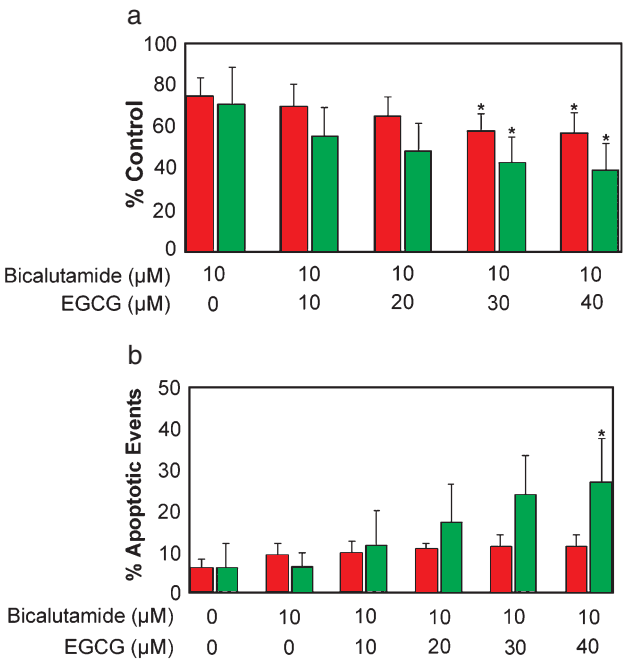

Fig. 6 Effect of EGCG in combination with bicalutamide on cell number and apoptosis in NRP-152 and NRP-154 cells. (a) NRP-152 (red) and NRP-154 (green) cells were brought to 40% confluence and cultured in Gc medium with DMSO (control), bicalutamide (10 μmol/L) alone or in combination with increasing doses of EGCG for 72 h. Cell number was assessed by crystal violet assay ($n = 5$). Results are expressed as the mean ± SD. *Significantly different from control at $P < 0.05$; (b) Apoptotic events in NRP-152 (black) and NRP-154 (hatched) cells were assessed by percent propidium iodide incorporation ($n = 3$). Membrane integrity (viability) did not alter between doses (data not shown). Results are expressed as the mean ± SD. *Significantly different from bicalutamide 10 μmol/L at $P < 0.05$.

in apoptotic events when treated with bicalutamide (0–40 μmol/L) (Fig. 5b). Membrane integrity (viability) was not altered between doses (data not shown). When dihydrotestosterone (DHT) was removed from the medium it had no effect on cell number or apoptosis in either cell line (Fig. 5a,b).

Apoptosis was confirmed by TUNEL assay. Untreated NRP-152 and NRP-154 cells were mostly TUNEL negative (Fig. 5c). However, following treatment of NRP-152 and NRP-154 with 40 μmol/L EGCG, the number of TUNEL positive events increased (Fig. 5c).

Effect of EGCG alone and in combination with bicalutamide on cell number and apoptosis

The NRP-152 and NRP-154 cells displayed a dose-dependent decrease in cell number when treated with bicalutamide (10 μmol/L) in combination with increasing doses of EGCG. This decrease in cell number was significantly different from bicalutamide (10 μmol/L) treatment alone. Even though these cells expressed no androgen receptor, the NRP-154 cells were more sensitive to this treatment (Fig. 6a). The NRP-152 cells had no statistically significant increase in apoptotic events with bicalutamide (10 μmol/L) in combination with increasing doses of EGCG. The NRP-154 cells, however, underwent apoptosis in response to EGCG treatment, displaying a dose-dependent increase in apoptotic events (Fig. 6b). This increase in apoptosis was significantly different from bicalutamide (10 μmol/L) treatment alone. There was no alteration in membrane integrity (viability) between any of the doses (data not shown).

Discussion

The cytokeratin and androgen receptor status of the NRP cell lines have previously been described.[10,12] The NRP-152 cells are non-tumoigenic, cytokeratin 14 and androgen receptor positive cells representing the basal epithelial phenotype, while the NRP-154 cells are tumorigenic, cytokeratin 18 positive and androgen receptor negative and appear to be derived from the secretory epithelial portion of the gland. However, a conflicting report by Richter *et al.* showed that NRP-154 cells express low levels of the androgen receptor by northern blotting analysis.[13] In our initial studies, we confirmed the androgen receptor status of the NRP-152 and NRP-154 cells as their androgen receptor status was central to our hypothesis.

The NRP-152 and NRP-154 cells display dose-dependent decreases in cell number when treated with EGCG. This decrease in cell number was more evident in the NRP-154 cells. EGCG causes growth arrest in the non-tumorigenic NRP-152 cells, whereas the tumorigenic NRP-154 cells are more susceptible to cell death using the same concentrations of EGCG. This mechanism of action for EGCG is different from that of a protective antioxidant reducing the cumulative genetic damage to the cells. Instead, EGCG appears to have a direct effect on the NRP-154 cells *in vitro* inducing cell death and slowing cell cycle kinetics.

We would suggest that the secretory epithelial component of the prostate is more susceptible to cell death than the basal component by potent antioxidants such as EGCG. Previous studies suggest that EGCG can cause an increase in reactive oxygen species and mitochondrial depolarization. The role of the Bcl-2 proteins in this process was discounted by Chung *et al.* in DU-145 cells, but another group suggested that EGCG blocks the expression of hyperphosphorylated Bcl-xL causing cytochrome C release and cell death in growth arrested prostate cancer cells.[14] If the Bcl-2 family of proteins play a role in this process, this might explain the resistance of the NRP-152 cells to apoptosis as the basal component of the prostate express higher levels of Bcl-2 than the secretory portion protecting the cells from a mitochondrial mediated cell death pathway.[15–17]

Cell cycle kinetics and mitochondrial activity are regulated by the oxidative environment. EGCG treatment may produce a differential oxidative environment in the tumorigenic NRP-154 cells *vs* the non-tumorigenic NRP-152 cells. This would explain the apoptotic effect that occurs in the NRP-154 cells while the NRP-152 cells display slowing cell cycle kinetics.[18]

EGCG treatment had no effect on MnSOD expression or apoptosis in the NRP-152 cells. We observed a decrease in MnSOD expression and an increase in apoptosis in EGCG treated NRP-154 cells. Zhang *et al.* also observed a loss of MnSOD activity and an increase in apoptotic events in EGCG treated HL-60 cells.[19] The loss of MnSOD expression in the NRP-154 cells may be the result of mitochondrial dysfunction at the early stages of apoptosis in response to changes in the oxidative environment.

Bicalutamide is a potent anti-androgen.[9] We used EGCG in combination with bicalutamide to determine if we could exaggerate the effects of bicalutamide on cell number *in vitro*. If EGCG could be used in combination with lower doses of bicalutamide this may reduce side-effects *in vivo* (hot flashes, body pain and constipation). Also, both compounds used in combination with different properties may be more effective as a combination therapy for prostate cancer compared to monotherapy.

As in previous studies, we observed the loss of the androgen receptor in the NRP-152 cell line after bicalutamide treatment.[20] The induction

of apoptosis in both cell lines after treatment with bicalutamide was unexpected. If the NRP cell lines were androgen dependent (requiring androgens for survival) withdrawal of DHT from the medium should result in spontaneous apoptosis; however, it does not. When treated with bicalutamide both cell lines show a dose-dependent increase in apoptotic events and loss of cell number. If this increase in apoptotic events is due to androgen blockade, complete androgen withdrawal should elicit the same response; however, this effect was not observed.

Moreover, we have confirmed that under our growth conditions the androgen receptor is not expressed by the NRP-154 cells, indicating that the induction of apoptosis in the NRP-154 cells by bicalutamide is via an androgen receptor independent pathway. In addition, the doses of bicalutamide used in these assays *in vitro* are not out of the range of those prescribed *in vivo*, for example, 50 mg/day results in a serum bicalutamide mean steady-state concentration of approximately 20 μmol/L.[21]

Lee *et al.* have suggested that bicalutamide induces cell death by a pathway that is independent of changes in mitochondrial membrane potential and Bcl-2 actions.[22] Baron *et al.* have suggested a non-genomic link between androgens and the PI3-K/AKT signaling pathway in governing cell survival.[23] However, the mechanism of action of bicalutamide in androgen receptor negative cells is currently unknown.

Finally, when EGCG was used in combination with bicalutamide, NRP-152 and NRP-154 cells displayed a significant dose-dependent decrease in cell number when compared to bicalutamide treatment alone. However, there was no synergistic effect of the combined treatment on apoptosis. The combination treatment of bicalutamide and EGCG had an additive effect on decreasing NRP-152 cell numbers, but had no additive effect on NRP-154 cell numbers at higher concentrations of EGCG. This suggests that therapeutic doses of EGCG may be useful as a part of a combination therapy with bicalutamide inducing apoptosis and slowing cell cycle kinetics independently of the androgen receptor mediated cell death pathway.

More recently, synthetic analogs of the green tea polyphenols have been analyzed that can inhibit the proteasome *in vitro* and *in vivo.* [24] The development of more analogs of EGCG and other potent antioxidants may result in a more potent compound with greater activity *in vivo*. Finally, understanding the molecular mechanisms of action of EGCG and other potent antioxidants could lead to the development of more effective therapies for prostate cancer.

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