Effects of Phytoestrogens on Growth and DNA Integrity in Human Prostate Tumor Cell Lines: PC-3 and LNCaP

Julie H. Mitchell, Susan J. Duthie, and Andrew R. Collins

Abstract: Prostate cancer is one of the most common male cancers in Western countries, yet the incidence of this fatal disease remains low in Asian populations. Environmental factors such as diet play an important role in hormone-dependent cancer etiology, and a high phytoestrogen intake may be one factor contributing to the low prostate cancer mortality in Eastern populations. In this study, we investigated the effects of the phytoestrogens genistein, daidzein, coumestrol, and equol on cell growth and DNA damage (strand breakage) in two human prostate tumor cell lines: androgen receptor-positive LNCaP and androgen receptor-negative PC-3. Each compound caused growth inhibition at physiologically relevant concentrations (<10 μM). Genistein induced DNA damage in both cell lines at <10 μM. Daidzein inhibited cell growth at 10–100 μM yet had no effect on DNA damage at up to 500 μM. Thus, despite their structural similarities, different phytoestrogens inhibit prostate tumor cell growth by independent mechanisms.

Introduction

Prostate cancer is one of the most common cancers to affect men in Western countries, whereas in Africa, Eastern Europe, and Japan the risk of this disease remains low. However, the prevalence of latent cancers, discovered postmortem, is similar between high- and low-risk populations, with genetic and lifestyle factors implicated in the progression to the malignant form of the disease (1). The marked increase in prostate (and breast) cancer incidence in migrant populations relocating from low- to high-risk geographical areas suggests that environmental factors, such as diet, play a major role in hormone-dependent cancer etiology (2). Many epidemiological studies correlating dietary factors, such as fat and animal protein intake, with cancer incidence are inconclusive. However, nonnutrient components of plant foods are being investigated as chemopreventive agents, particularly in association with hormone-related cancers (3,4).

Phytoestrogens are naturally occurring chemicals in plants that induce weak estrogenic and antiestrogenic responses in mammalian tissues by binding to estrogen receptors (5). Isoflavones and lignans, which are particularly abundant in soy and flaxseed products, represent the main classes of phytoestrogens. They are present in appreciable quantities in Asian diets, with an average isoflavone intake of 50 mg/day in Japanese populations (6). The isoflavone genistein is a well-recognized inhibitor of protein tyrosine kinases (7), whereas a range of phytoestrogens increase synthesis of sex hormone-binding globulin \textit{in vivo}, thus reducing the plasma concentrations of free, unbound sex hormones (8,9).

Cell culture studies indicate that phytoestrogens may also protect against hormone-responsive cancers by inhibition of sex hormone-metabolizing enzymes, such as 17β-hydroxysteroid dehydrogenase (10), 5α-reductase (11), and aromatase (12). These studies provide considerable insight into the possible actions of phytoestrogens, but high, nonphysiological doses are often required to show significant effects. Phytoestrogens may also act as cellular antioxidants, protecting against oxidative damage (13). Again, high concentrations (0.1–1 mM) were required to demonstrate measurable antioxidant activity in model systems (14). Consequently, in the present study we examined the effects of the phytoestrogens genistein, daidzein, equol, and coumestrol over a wide concentration range (0–500 μM) on cell growth and toxicity to DNA (strand breakage) in two human prostate tumor cell lines: the androgen receptor (AR)-positive LNCaP and the AR-negative PC-3 cell lines.

Materials and Methods

Materials

Genistein, daidzein, equol, and coumestrol were purchased from Apin Chemical (Oxon, UK). RPMI 1640 medium was purchased from ICN Flow (Irvine, UK). Fetal calf serum (FCS) was obtained from Globepharm (Surrey, UK). Dr. R. Cunningham (Dept. of Biological Sciences, State University of New York, Albany, NY) generously supplied the \textit{Escherichia coli} strain overproducing the endonuclease III. 4′,6-Diamidine-2-phenylindole dihydrochloride was purchased from Boehringer Mannheim (East Sussex, UK). All other chemicals were obtained from BDH (Leicestershire, UK) or Sigma Chemical (Dorset, UK).

The authors are affiliated with the Division of Cellular Integrity, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK.
Cells and Culture Conditions

Human prostate carcinoma PC-3 cells were a kind gift from Dr. John Kavanagh (Dept. of Urology, South Manchester University Trust, Manchester, UK) and were maintained in RPMI 1640 medium supplemented with 10% FCS. Androgen-sensitive human prostate carcinoma LNCaP cells were purchased from American Type Culture Collection (CRL-10995, ATCC, Manassas, VA) and maintained in RPMI 1640 medium adjusted to contain 4.5 g/l glucose, supplemented with 10% FCS. Cell stocks were grown in 80-cm² tissue culture flasks at 37°C in a humidified atmosphere of 5% CO₂-95% air and passaged using 0.25% trypsin in 0.02% EDTA.

Inhibition of Growth Studies

PC-3 cells were seeded in 25-cm² flasks at a density of 2 × 10⁵/flask and allowed to attach overnight (18 h). Fresh medium containing phytoestrogen at 0, 0.01, 0.1, 1, 10, or 100 µM in a maximum of 2% dimethylsulfoxide was added. Cells were allowed to grow undisturbed for three or six days before being harvested using trypsin-EDTA and counted using a Neubauer improved hemocytometer. LNCaP cells grow in clusters and only adhere lightly to flasks, so the cells were seeded in six-well plates at a density of 2 × 10⁵/well and allowed to grow undisturbed for 48 hours before exposure to phytoestrogen as described above. Cells were harvested for counting by gentle scraping with a soft cell scraper (Starstedt, Leicestershire, UK) and counted as described above. Cell viability was determined using trypan blue exclusion at the end of each experiment and was typically 93%.

Toxicity to DNA

Cells were seeded in six-well plates at a density of 2 × 10⁵/well and allowed to attach for 18 hours (PC-3) or 48 hours (LNCaP). Cells were then exposed to fresh medium containing 0, 10, 50, 100, 250, or 500 µM phytoestrogen in 1% dimethylsulfoxide (maximum 100 µM coumestrol owing to its limited solubility) for 24 hours. DNA damage was determined using single-cell gel electrophoresis (the comet assay) (15,16).

Comet Assay

The comet assay is a rapid and sensitive method for the determination of DNA damage at the level of individual cells (15,16). Briefly, cells were washed with cold phosphate-buffered saline (4°C) and harvested by gentle scraping. The cells were then suspended in 1% low-melting-point agarose in phosphate-buffered saline on frosted microscope slides and lyzed to remove cellular proteins. Supercoiled nucleoid DNA was then allowed to “unwind” at alkaline pH before electrophoresis, upon which strand breakage allows DNA to move toward the anode, forming a comet tail. Slides were stained with 4’6-diamidine-2-phenylindole dihydrochloride and scored visually using a fluorescence microscope (Zeiss Axioskop). One hundred comets per slide are classified as 0, 1, 2, 3, or 4 according to the relative intensity of the fluorescence in the tail, with 0 being undamaged and 4 being maximally damaged. Thus the total score for a slide could range from 0 (all undamaged) to 400 (all maximally damaged). Figure 1 shows examples of Classes 0, 2, and 4.

Results

Inhibition of Cell Growth

Each of the four phytoestrogens inhibited the growth of AR-negative PC-3 and AR-positive LNCaP cells when pres-

Figure 1. Comet images of PC-3 cells: Classes 0 (A), 2 (B), and 4 (C).
ent in the culture medium at 100 \( \mu M \) for three or six days (Table 1, 3 days exposure; Figure 2, 6 days exposure). Genistein inhibited LNCaP cell growth at \( \geq 10 \) \( \mu M \) after three days (Table 1) and \( \geq 1 \) \( \mu M \) after six days (Figure 2). Coumestrol showed a pattern of inhibition similar to that of genistein in LNCaP cells, resulting in a reduced cell number at \( \geq 10 \) or \( \geq 1 \) \( \mu M \) after three or six days of exposure, respec-

tively (Table 1, Figure 2). Equol inhibited LNCaP cell growth at \( \geq 10 \) \( \mu M \) after three and six days of exposure, whereas in PC-3 cells, equol at \( \geq 0.1 \) \( \mu M \) significantly inhibited cell growth after six days (Table 1, Figure 2). Cell growth tended to be inhibited at lower phytoestrogen concentrations and/or to a greater extent in LNCaP than in PC-3 cells.

### Table 1. Effect of Genistein, Daidzein, Coumestrol, and Equol on Cell Growth in PC-3 and LNCaP Human Prostate Tumor Cells After Three Days of Exposure$^a,b$

<table>
<thead>
<tr>
<th>Cell number $\times 10^5$</th>
<th>Control</th>
<th>0.01 ( \mu M )</th>
<th>0.1 ( \mu M )</th>
<th>1 ( \mu M )</th>
<th>10 ( \mu M )</th>
<th>100 ( \mu M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>3.42 ± 0.44</td>
<td>3.27 ± 0.30</td>
<td>3.31 ± 0.37</td>
<td>3.75 ± 0.36</td>
<td>2.94 ± 0.27</td>
<td>1.95 ± 0.17 $^\dagger$</td>
</tr>
<tr>
<td>LNCaP</td>
<td>3.60 ± 0.18</td>
<td>3.41 ± 0.29</td>
<td>2.98 ± 0.12</td>
<td>2.95 ± 0.30</td>
<td>2.32 ± 0.31 $^\dagger$</td>
<td>0.71 ± 0.10 $^\dagger$</td>
</tr>
<tr>
<td>Daidzein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>9.22 ± 0.75</td>
<td>8.44 ± 0.77</td>
<td>8.28 ± 0.21</td>
<td>8.81 ± 0.64</td>
<td>7.64 ± 0.32*</td>
<td>5.23 ± 0.32 $^\dagger$</td>
</tr>
<tr>
<td>LNCaP</td>
<td>3.33 ± 0.18</td>
<td>3.08 ± 0.27</td>
<td>3.49 ± 0.06</td>
<td>2.95 ± 0.24</td>
<td>3.02 ± 0.11</td>
<td>1.31 ± 0.09 $^\dagger$</td>
</tr>
<tr>
<td>Coumestrol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>7.34 ± 0.56</td>
<td>7.20 ± 0.75</td>
<td>7.32 ± 0.43</td>
<td>7.37 ± 0.66</td>
<td>7.54 ± 0.22</td>
<td>1.61 ± 0.11 $^\dagger$</td>
</tr>
<tr>
<td>LNCaP</td>
<td>3.38 ± 0.24</td>
<td>3.33 ± 0.15</td>
<td>3.00 ± 0.09</td>
<td>2.91 ± 0.42</td>
<td>2.24 ± 0.24 $^\dagger$</td>
<td>1.67 ± 0.12 $^\dagger$</td>
</tr>
<tr>
<td>Equol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC-3</td>
<td>4.29 ± 0.26</td>
<td>4.38 ± 0.23</td>
<td>3.16 ± 0.16*</td>
<td>4.34 ± 0.32</td>
<td>4.02 ± 0.43</td>
<td>1.98 ± 0.27 $^\dagger$</td>
</tr>
<tr>
<td>LNCaP</td>
<td>2.35 ± 0.14</td>
<td>2.14 ± 0.19</td>
<td>2.29 ± 0.20</td>
<td>2.73 ± 0.22</td>
<td>1.53 ± 0.23 $^\dagger$</td>
<td>1.31 ± 0.21 $^\dagger$</td>
</tr>
</tbody>
</table>

$^a$: Values (means ± SEM) represent number of cells ($\times 10^5$) per flask; $n > 4$.

$^b$: Statistical significance is as follows: *, $p < 0.5$; †, $p < 0.01$; ‡, $p < 0.001$.

![Figure 2](image-url)  

**Figure 2.** Effect of genistein, daidzein, coumestrol, and equol on cell growth in PC-3 and LNCaP human prostate tumor cells after 6 days of exposure. Values (means ± SEM) represent number of cells $\times 10^6$/flask; $n > 4$. *, $p < 0.5$; **, $p < 0.01$; ***, $p < 0.001$. 

Vol. 38, No. 2 225
Toxicity to DNA

Genistein was the most genotoxic of the four phytoestrogens, inducing DNA strand breakage at <10 μM in PC-3 and LNCaP cell lines within 24 hours of exposure (Figure 3). Coumestrol, at up to 100 μM, had no effect in LNCaP cells but induced DNA breakage in PC-3 cells at >50 μM. Equol increased DNA strand breakage at >250 μM in both cell lines. Daidzein did not induce DNA damage at any of the concentrations used (Figure 3).

Discussion

Isoflavones, when present in high concentrations (>10 μM), inhibit the growth of many human cell lines in culture (17,18). In this study we have shown that each of the phytoestrogens, genistein, daidzein, coumestrol, and equol, inhibits the growth of human prostate tumor cells in vitro at physiologically relevant concentrations (1–10 μM) (6). In addition, we suggest that different mechanisms may be involved in the inhibition of growth by these different compounds. Plasma levels of phytoestrogens in soya-consuming populations can reach concentrations up to 1 μM, and higher levels can be achieved by dietary supplementation (6,19). Furthermore, levels of phytoestrogens may be higher in prostatic fluid than in plasma, and there are no reports of maximum concentrations occurring in cells (20).

Growth inhibition was achieved at lower phytoestrogen concentrations in LNCaP than in PC-3 cells. The AR in

Figure 3. Effect of genistein, daidzein, coumestrol, and equol on DNA strand breakage in LNCaP and PC-3 human prostate tumor cell lines. Cells were exposed to phytoestrogens for 24 h before DNA damage was assessed using comet assay. DNA damage is represented as arbitrary units. Values are mean ± SEM; n > 4.
LNCaP cells is mutated, which relaxes the specificity and allows other ligands, such as phytoestrogens, to bind (21). However, many other genetic differences exist between the two cell lines, besides the presence of the AR. In addition, a number of non-receptor-related mechanisms are likely; genistein was originally thought to inhibit the autophosphorylation of the epidermal growth factor receptor (7). However, subsequent studies indicated that genistein acts through tyrosine kinase inhibition at other points in signal transduction or through different mechanisms related to cell proliferation and differentiation (18). Genistein has also been shown to inhibit cell growth and induce apoptosis by modulating transforming growth factor-β signaling pathways (22,23). Growth inhibition at high phytoestrogen concentrations (100 µM) was similar in LNCaP and PC-3 cells, indicating a non-receptor-related action.

Genistein induced DNA strand breakage at <10 µM in PC-3 and LNCaP cell lines. This action could possibly be related to inhibition of DNA topoisomerase enzymes, which function in DNA cleavage and religation during DNA replication and repair (24). Genistein inhibits topoisomerase II function and stabilizes the intermediate “cleavable complex” between DNA and the enzyme in a fashion similar to the chemotherapeutic drug etoposide (25,24). The comet assay provides a sensitive indicator of DNA strand breakage that may precede apoptosis. Apoptotic cells are easily distinguishable in the comet assay by the movement of most of the DNA from the head into the tail of the comet, which cannot be accounted for by tail length alone (26). Thus, even Class 4 comets may be considered preapoptotic. In agreement with this, 10 µM genistein resulted in <1% of cells being maximally damaged (Class 4 comets) and did not affect cell growth in PC-3 cells. Daidzein, present in the culture medium at 10–100 µM, significantly inhibited cell growth in both cell lines, yet it had no effect on DNA damage at up to 500 µM. This indicates that daidzein, despite its structural similarity to genistein, is nongenotoxic and may inhibit cell growth by mechanisms unrelated to DNA. Similarly, equol was not genotoxic at up to 250 µM. Thus phytoestrogens exert their effects on cell growth and DNA by independent mechanisms. However, soybeans contain genistein and daidzein (or conjugated forms thereof), with the latter being metabolized to equol, giving soy foods chemopreventive and chemotherapeutic potential.

A number of chemopreventive properties have been attributed to phytoestrogens (7–12). A recent study using modern analytic technology to analyze dietary intakes of individual phytoestrogens in patients with prostate cancer or controls indicated a significant protective effect of genistein, daidzein, and coumestrol (27). Furthermore, in an illustrative case study, a 66-year-old prostate cancer patient took a phytoestrogen supplement (160 mg/day) for one week before radical prostatectomy. On histological examination of the prostatectomy specimen, significant apoptosis in tumor cells suggestive of tumor regression was evident compared with the preoperative needle biopsy (28). Genistein, the most widely investigated of the phytoestrogens, has also been shown to inhibit the growth of benign prostate hypertrophy and prostate cancer tissue in histoculture (29) and to suppress the growth of tumor cell implants in rats (30). Nevertheless, the antiimitogenic action of phytoestrogens against tumor cells remains inconclusive.

Acknowledgments and Notes

The authors thank David Simpson for the photographs of comet classes in PC-3 cells (Figure 1). This work was performed at the Rowett Research Institute and was supported by the Ministry of Agriculture, Fisheries, and Food/Food Standards Agency, the World Cancer Research Fund, and the Scottish Executive Rural Affairs Department. Address correspondence to Dr. Susan J. Duthie, Div. of Cellular Integrity, Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK. Phone: +44(0) 1224 712 751 (ext. 2324). FAX: +44(0) 1224 716629. E-mail: sd@rri.sari.ac.uk.

Submitted 29 March 2000; accepted in final form 16 August 2000.

References


