Selenium Modulation of Cell Proliferation and Cell Cycle Biomarkers in Human Prostate Carcinoma Cell Lines

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ABSTRACT

Prostate cancer (PCA) is the most common histological malignancy and the second leading cause of cancer deaths among North American men. There has been considerable interest in the chemopreventative properties of selenium. In this study, we assessed whether selenium inhibits cell growth and associated cell cycle regulatory proteins. Human PCA cells (LNCaP, PC3-AR2, and PC3-M) were incubated with and without selenium (Seleno-DL-methionine, 150 μM) for 24, 48, and 72 h. Cells were fixed and stained with propidium iodide for flow cytometry analysis. In parallel experiments, total protein was extracted, immunoprecipitated with cyclin E antibody, and analyzed by Western blot for the expression of cell cycle markers. Treatment with selenium caused G1 arrest and an 80% reduction in the S phase of LNCaP with no effect on PC3. However, PC3 cells transfected with the androgen receptor (PC3-AR2) exhibited a G2/M arrest and a marked reduction (57%) in the S phase during cell cycle progression. In the analysis of cell cycle regulatory molecules, selenium-treated cells demonstrated a significant induction of cyclin-dependent kinase inhibitors Cip1/p21 and Kip1/p27. These data suggest that selenium possesses strong antiproliferative properties in regard to human PCA. This effect appears to be dependent on the presence of a functioning androgen receptor. This provides a theoretical basis for Phase III studies of selenium in PCA prevention.

INTRODUCTION

PCA is the most common histological malignancy and the second leading cause of cancer deaths among North American men (1, 2). The induction of human PCA is a multistage process, involving progression from small latent carcinomas of low histological grade to high-grade metastatic cancers (3). Current accepted risk factors for PCA include: age, race, dietary habits, and androgen levels (4). Epidemiological and laboratory studies suggest that diet and androgens may alter PCA risk via a conjoint etiological pathway (5–7).

There is considerable interest in the role of selenium in cancer prevention. There is an indirect association between levels of bioavailable selenium in the environment and cancer mortality (8). Numerous studies have demonstrated that selenium can inhibit experimental carcinogenesis from a variety of inciting causes (9–14). In one randomized trial of selenium supplementation among skin cancer patients, a 63% reduction in PCA incidence was noted among men who were randomized to selenium (6).

A potential link between diet, androgens, and PCA is oxidative stress (15–18). Oxidative stress refers to the generation of reactive oxygen species, which may trigger carcinogenesis via genetic and epigenetic mechanisms (19–23). Ripple et al. (24) have shown recently that increase oxidative stress in human PCA cell lines occurs in response to androgen. Dietary habits can influence this process via known antioxidant properties of micronutrients such as selenium.

DNA replication and mitosis are controlled by the activation of S phase- and M phase-specific cyclins and CDKs. The catalytic subunits of these kinases are only active when complexed with their specific regulatory subunits, cyclins (25). Targets for the cyclins and CDKs include the retinoblastoma protein pRB (26) and the CKIs (such as p21 and p27; Ref. 27). PCA is associated with loss of cell cycle control, resulting in unregulated growth of cells. We and others have shown that cell cycle arrest is induced by high levels of DHT (a steroid hormone which stimulates prostate cell growth at lower levels; Ref. 28). This arrest is mediated by p27 and p21. It is within this context that we report the cell cycle effects of selenium in androgen-sensitive and androgen-resistant human PCA cell lines.

MATERIALS AND METHODS

Cell Culture. Four established human PCA cell lines were used. LNCaP (androgen responsive) and PC3 (androgen independent) were obtained from the American Type Culture Collection (Rockville, MD); PC3-AR2 (androgen receptor transfected) and PC3-M (mock transfectants) were from Ted Brown (Mt. Sinai Hospital, Toronto, Ontario, Canada). LNCaP cells was cultured in RPMI 1640 with l-glutamine (Life Technologies, Inc., Grand Island, NY), supplemented with 10% FBS, and 100 μM penicillin and 100 μg/ml streptomycin. PC3 cells were cultured in DMEM/F12 medium with 10% FBS and antibiotics. PC3-AR2 and PC3-M were cultured in RPMI 1640 with l-glutamine (Life Technologies, Inc.), supplemented with 5% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin, fungizone (250 μg/ml amphotericin B and 250 μg/ml sodium deoxycholate; Life Technologies, Inc.), and 100 μg/ml Hygromycin B (Calbiochem). All of the cell types were maintained at 37°C in a humidified atmosphere of 5% CO2 in air. Cells were grown to 80% confluency in 10-cm tissue culture plates and split 1:8. Cell populations growing asynchronously were treated with variable concentrations of the antioxidant. Controls received the vehicle alone. Treatment was started after 24 h of attachment. Cells were incubated for a maximum of 72 h after selenium treatment before recovery for flow cytometry and protein analysis.

Dietary Antioxidants/Micronutrients. Selenium (Seleno-DL-methionine) was reconstituted in 0.005 n HCl. This was added to the cultures at a concentration of 150 μM. Control cultures received the vehicle alone.

Cell Proliferation. To evaluate cell proliferation, cells were plated onto 24-well cell culture plates at 5000 cells/well in 1 ml of culture medium with FBS as described earlier. Before treatment cells were allowed to adhere to the bottom of the plate for 24 h, a concentrated stock solution of selenium (1 mM) was prepared, and then diluted so that the concentration delivered to the cells was 50–200 μM. Controls were treated with the vehicle alone. At 24, 48, and 72 h of treatment at 37°C, the cells were harvested by suspension in 0.025% trypsin in 0.02% EDTA solution. Cell counts were performed in triplicates using a hemocytometer, with trypan blue exclusion to identify viable cells. Growth curves were generated for each cell line.

Mitochondrial Activity (MTT Assay). To evaluate cellular mitochondrial as well as the antioxidantive activity, the MTT assay was used (29, 30). Five thousand cells per well of each cell type were placed in 96-well cell culture plates with 100 μl culture medium and incubated for 24 h to attach to the bottom of the well. Cells were treated with selenium (50–200 μM). Controls were treated with the vehicle alone. Plates were incubated for 24, 48, and 72 h after selenium addition, then 15 μl of MTT solution was added to each well and incubated for an additional period of 4 h at 37°C in a humidified incubator. The stop solution (100 μl) was added to solubilize the formazan product, and the absorbance at 570 nm was recorded using a 96-well plate reader. The

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2 The abbreviations used are: PCA, prostate cancer; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BrdUrd, bromodeoxyuridine; FACS, fluorescence-activated-cell sorter.
570-nm absorbance reading is directly proportional to the number of cells normally used in the proliferation assay.

**Flow Cytometric Analysis.** Asynchronously growing cells were pulse labeled with 10 mM BrdUrd for 2 h with or without previous treatment of the selenium. Cells were then harvested, fixed with 70% ethanol, treated with 0.1% HCl, and heated for 10 min at 90°C to expose the labeled DNA. Cells were stained with anti-BrdUrd-conjugated FITC (Becton Dickinson) and counterstained with propidium iodide. Cell cycle analysis was carried out on a Becton-Dickinson FACScan using Lysis II software.

**Immunoblotting.** Cells were lysed in ice-cold NP40 lysis buffer [0.1% NP40, 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 0.02 mg/ml each of aprotonin, leupeptin, and pepstatin]. Lysates were sonicated and clarified by centrifugation. Proteins were quantitated by Bradford analysis and 20–100 μg protein/lane resolved by SDS-PAGE. Transfer and blotting was carried out (31) and proteins detected by electrochemiluminescence. Densitometry was performed using the Molecular Dynamics Imaging system and Image Quant software to quantitate the relative amounts of p27 protein detected on Western blots. For the detection of cyclin E-associated proteins by immunoprecipitation-Western analysis, cyclin E was immunoprecipitated from 200 μg protein lysates, complexes resolved, blotted, and blots probed with cyclin E, cdk2, p21, and p27 antibodies.

**Antibodies.** The following antibodies were used in the immunoblotting experiments. β-Actin mouse monoclonal from Sigma Laboratories; cyclin E, mouse monoclonal E172 for immunoprecipitation and E12 for immunoblotting, from E. Harlow (Massachusetts General, Boston, MA); cdk2 mouse monoclonal PSTAIRE antibody was a gift from S. Reed (The Scripps Research Institute, La Jolla, CA); p27 mouse monoclonal antibody from transduction Laboratories (Lexington, KY); p21 rabbit polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA); and α-BrdUrd-FITC conjugate (Cedarlane, Hornby, Ontario, Canada).

**RESULTS**

**Dose- and Time-dependent Inhibitory Effect of Selenium on Cell Growth.** To assess the minimal required dose of selenium to cause maximal growth inhibition, we examined various doses of selenium (25–200 μM) with time. Treatment of cells grown on 10% FBS with selenium resulted in a highly dramatic to complete inhibition of their growth on both a dose- and time-dependent manner. An inhibitory effect in LNCaP was evident as early as 24 h with a dose of 150 μM of selenium. A profound effect was observed 48–72 h after treatment. (Fig. 1).

**Selenium Inhibits Cell Proliferation without Cell Death or Cytotoxicity.** The results are expressed as the mean of three experiments each carried out in triplicates. In control cultures without selenium, cells grew at a sustained rate, as determined by counting cells (Fig. 2a). Selenium slowed the rate of growth in LNCaP but not in PC3. The number of LNCaP cells were lower in the selenium-treated group as compared with the controls after 24 h of treatment. Suppression of cell numbers by selenium was time and dose dependent. Maximum reduction was seen between 150–200 μM concentrations of selenium.

The MTT assay was used in conjunction with trypan blue counting. Results from the analysis revealed maximum response to 150–200 μM of selenium in LNCaP with no effect on PC3. Maximum effect in LNCaP was seen 24 h after treatment (Fig. 2b).

**Selenium Causes G1 Arrest of LNCaP with No Effect on PC3.** Treatment of asynchronously growing LNCaP and PC3 cells with 150 μM of selenium for up to 72 h induced growth arrest of LNCaP as early as 24 h after treatment as demonstrated by DNA content histo-

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Fig. 1. Dose- and time-dependent inhibitory effect of selenium on cell growth. LNCaP cells were harvested at indicated time intervals for FACS analysis, after the addition of various concentrations (25–200 μM) of selenium to the culture medium.

Fig. 2. *a,* in vitro growth curves of LNCaP and PC3 cells after treatment with selenium. Cells treated with various concentrations (50–200 μM) of selenium were harvested at different time intervals and counted using trypan blue. Selenium treatment resulted in decreased cell numbers compared with controls in LNCaP and not in PC3. *b,* effect of selenium on LNCaP and PC3 cells measured by the MTT assay. Cells were treated with various concentrations of selenium (50–200 μM). The absorbance was read by microplate reader at 570 nm at different time intervals; bars, ±SD.
grams from flow cytometric studies (Fig. 3a). The percentage reduction of cells in S phase was 58.1% by 24 h of treatment. The cell arrest persisted for 72 h in medium supplemented with selenium reducing the cells in the S phase to a maximum of 80.3% (Table 1). No growth arrest was observed in controls treated with the vehicle alone. In contrast, treatment of PC3 cells with selenium demonstrated no alteration in the number of cells in S phase even after 72 h of treatment thereby indicating absence of growth arrest (Fig. 3b; Table 1). A typical histogram flow cytometric analysis of LNCaP and PC3 cells treated with and without selenium indicated no cytotoxicity or cell death as seen by the absence of the hypodiploid (sub-G1) peak (Fig. 4).

**Effect of Selenium on Cell Cycle Regulatory Molecules in LNCaP and PC3.** Defects in the regulation of cell cycle progression are thought to be one of the most common features of transformed cells (32). Eukaryotic cell cycle progression is regulated by a series of CDKs, their activity positively regulated by cyclins and negatively regulated by CKIs (33). The significance of growth factors and the signaling pathway(s) mediated by them to regulate the progression of cell cycle in eukaryotes has been identified as an important component of their function (34, 35). Therefore, we assessed whether inhibition of growth was associated with the alteration in cell cycle regulatory molecules in PCA cell lines. The CKI p21 is known to be involved in both G1 and G2/M phase arrest. Because there was a doubling of the number of cells in the G2/M phase, we investigated whether p21 was implicated in selenium-induced inhibition of PCA cell growth. Levels of p21 were examined in both LNCaP and PC3 cells after selenium treatment. As shown in Fig. 5a there was an up-regulation of CKI Cip1/p21 protein levels in LNCaP with no such alteration in PC3. Maximum up-regulation was observed at 72 h after treatment. Similar to Cip1/p21, treatment of LNCaP and PC3 with selenium resulted in a significant up-regulation of another CKI, Kip1/p27, which was also dependent on the time of treatment (Fig. 5, a and b).

**Selenium Causes an Increase in p27.** Inhibition of cyclin E/cdk2 activity was observed within 24 h after treatment with selenium in LNCaP. To evaluate the mechanism of inhibition of cyclin E/cdk2, these complexes were examined by immunoprecipitation of cyclin E followed by Western blotting to detect associated proteins (Figs. 6a and 7). The expression levels of the cell cycle regulatory proteins were compared with their respective time point controls (data not shown).

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**Table 1 Inhibitory effect of selenium**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% Reduction S phase</th>
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<tbody>
<tr>
<td>LNCaP</td>
<td>58.1 70.0 80.3</td>
</tr>
<tr>
<td>PC3</td>
<td>3.6 5.9 8.4</td>
</tr>
</tbody>
</table>

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**Fig. 3.** FACS analysis of LNCaP (a) and PC3 (b) cells treated with 150 μM of selenium. Cells were harvested and prepared for FACS analysis at various time intervals after the addition of selenium to culture medium. G1 arrest was seen in LNCaP.

**Fig. 4.** Histogram of flow cytometric analysis showing the effect of selenium on cell cycle in LNCaP (a) and PC3 (b) cells after 72 h of treatment. There was no cytotoxicity or cell death as seen by the absence of the hypodiploid (sub-G1) peak.

**Fig. 5.** Cell cycle regulators during arrest by selenium. Cells were harvested at the indicated time intervals after the addition of selenium to the culture medium. Lysates were prepared and resolved using SDS-PAGE: (a) LNCaP and (b) PC3. Proteins were detected by immunoblotting with the indicated antibodies.
The amount of E-associated p27 in LNCaP increased from 80 to 100% by densitometry (Fig. 6b) as the cells entered G_1 arrest. In contrast to LNCaP, there were no detectable levels of p27 until the end of the treatment period (Fig. 7) among the selenium-treated PC3 cells.

**Fig. 6.** a, cyclin E immune complexes (LNCaP). Cells were recovered at various time intervals after addition of selenium to the culture medium. Cyclin E was immunoprecipitated, complexes resolved by SDS-PAGE, and immunoblots were probed with antibodies to detect associated proteins. b, cyclin E-associated p27 was quantitated by densitometry from the blots in a above. LNCaP cells show an increase in cyclin E-bound p27.

**Fig. 7.** Cyclin E immune complexes (PC3). Cells were recovered at various time intervals after the addition of selenium to the culture medium. Cyclin E was immunoprecipitated, complexes resolved by SDS-PAGE, and immunoblots were probed with antibodies to detect associated proteins. PC3 cells show no detectable levels of cyclin E-bound p27 up to 72 h after treatment.

The amount of E-associated p27 in LNCaP increased from 80 to 100% by densitometry (Fig. 6b) as the cells entered G_1 arrest. In contrast to LNCaP, there were no detectable levels of p27 until the end of the treatment period (Fig. 7) among the selenium-treated PC3 cells.

**Androgen Receptor-transfected (PC3-AR2) Cells Demonstrated Inhibition of Cell Proliferation with No Cytotoxicity.** Selenium slowed the rate of growth in PC3-AR2 (Fig. 8a) but not in PC3-M (data not shown). The number of cells in PC3-AR2 were lower as compared with the controls after 24 h of treatment. Suppression of cell numbers by selenium was time and dose dependent. Maximum reduction was seen between 150 and 200 μM concentrations of selenium.

The MTT assay revealed maximum response to 150–200 μM of selenium in PC3-AR2, as early as 24 h after treatment (Fig. 8b). PC3-M cells demonstrated a behavior similar to the nontransfected PC3 (data not shown).

**Selenium Arrests the Androgen Receptor-transfected Cells.** Treatment of asynchronously growing androgen receptor-transfected PC3-AR2 cells with 150 μM of selenium induced a growth arrest by 72 h of treatment. There was a 57% reduction in the S phase (Fig. 9a), with no such alteration seen in the mock transfectants (PC3-M; Fig. 9b). A histogram flow cytometric analysis of PC3-AR2 cells treated with and without selenium indicated no cytotoxicity or cell death (no hypodiploid (sub-G_1) peak; Fig. 10).

**Fig. 8.** a, in vitro growth curves of PC3-AR2 cells after treatment with selenium. Cells treated with various concentrations (50–200 μM) of selenium were harvested at different time intervals and counted using trypan blue. Selenium treatment resulted in decreased cell numbers compared with the untreated control. b, effect of selenium on PC3-AR2 cells measured by the MTT assay. Cells were treated with various concentrations of selenium (50–200 μM). The absorbance was read by microplate reader at 570 nm at different time intervals; bars, ±SD.

**Fig. 9.** FACS analysis of PC3-AR2 (a) and PC3-M (b) cells treated with 150 μM of selenium. Cells were harvested and prepared for FACS analysis at various time intervals after the addition of selenium to culture medium. Cell cycle arrest was seen in PC3-AR2 and not in PC3-M.
human prostate cell line LNCaP. In addition to the trypan blue studies, and/or associated CDKs (32, 33).

Clinical outcome among men after radical prostatectomy (44). Cell p27 inhibits cell cycle progression (32, 33). Previous data from our laboratory reveal that men from countries with a low incidence of PCA tend to acquire an increased incidence rate (38, 39). Data also suggest that the origins of microfocal cancer occur in the 4th decade of life, as 30% of men in the age group of 30–39 possess microfocal cancer (40). The progression from microfocal to clinical PCA is a relatively slow process, because tumors are generally detected in the 6th or 7th decade of life. In this context, prevention refers to the delayed progression of microfocal disease, as true primary prevention requires intervention before age 30.

Selenium is a trace element that has been identified as a potential chemopreventive agent. Clark et al. (6) conducted a randomized study of selenium supplementation in 934 men with nonmelanomatous skin cancer. At 6.5 years of follow-up, a 63% reduction in PCA incidence was noted among men who were assigned the selenium arm of the trial. Critics of the study cite the fact that this was not a priori end point of the study. Nonetheless, because the majority of the men at study entry harbored subclinical PCA, the results suggest that selenium possesses antiproliferative properties. In light of the provocative findings of the aforementioned study, we examined the effects of selenium supplementation on human PCA cell lines with particular reference to cell cycle effects.

CDKs are important cell cycle regulatory proteins that are positively and negatively regulated by cyclins and CKIs, respectively (41–43). A relative abundance of CKIs such as Cip1/p21 and Kip1/p27 inhibits cell cycle progression (32, 33). Previous data from our laboratory have shown that expression of Kip1/p27 is associated with clinical outcome among men after radical prostatectomy (44). Cell cycle arrest can also be achieved by a decrease in the levels of cyclins and/or associated CDKs (32, 33).

The data generated from this series of investigations demonstrates that selenomethionine exerts an antiproliferative activity against the human prostate cell line LNCaP. In addition to the trypan blue studies, MTT assays demonstrate that this effect is not because of cytotoxicity (Fig. 2, a and b). The IC_{50} for selenomethionine ranged from 25 to 200 μM, which is consistent with data published previously in a host of human malignant cell lines (45, 46). We demonstrate that a single exposure of asynchronously growing LNCaP cells with 150 μM of selenium induced an arrest as early as 24 h after treatment as demonstrated by DNA content histograms from flow cytometric studies (Fig. 3a). In contrast, the hormone-insensitive PC3 cell line was not inhibited by selenium. This is consistent with a previous report demonstrating growth suppression with selenium in LNCaP but not in PC3 or DU145 (45). LNCaP cells treated with selenium exhibited no observed morphological change (data not shown).

Our choice of selenium dosage requires mention. Previous studies in prostate and nonprostate cell lines have demonstrated dose-dependent inhibition of growth using a range of selenium concentrations similar to those used in these experiments (47, 29, 30). Human blood concentration of selenium ranges between 82 and 620 μg/liter, the higher dose reflecting human supplementation at 600 μg/day (48–51). Therefore, the dosages used in this study were between 5 and 8 times higher than human plasma concentrations. Although these dosages are slightly supra-physiological, it is difficult to make comparisons between in vitro and in vivo studies. Tissues which contain stable selenoproteins are likely to contain higher levels of selenium than plasma, although the measurement of tissue levels of selenium has not been described.

Exposure to selenium was noted to up-regulate levels of CKIs, Cip1/p21, and Kip1/p27 in LNCaP. Previous data from our laboratory have noted a similar effect when human PCA cells were treated with vitamin E but not the antioxidant zinc. In addition, LNCaP but not PC3 demonstrated up-regulation of cyclin E-bound p27 when exposed to selenium. This observation likely accounts for the observed differential growth inhibition between the two cell lines.

To investigate the discordant response among hormone-sensitive and-insensitive cell lines, we exposed androgen receptor-transfected PC3 cells to selenium and observed growth inhibition (Fig. 9, a and b). This novel finding suggests that selenium-induced growth inhibition in human PCA cells requires a functioning androgen receptor. One possible reason for the differential observed response to selenium exposure might be because of the human selenium binding protein (Hsp56). Yang et al. have shown that this protein is present in LNCaP but not PC3 cells. The expression of the Hsp56 gene has also been shown to be regulated by androgens in PCA cells (52). Additional work is needed to better define the role of this gene in prostate carcinogenesis.

These experimental observations have shown that selenium can inhibit cell cycle progression in human PCA cell lines. This is consistent with the known descriptive epidemiology of prostate carcinogenesis. Additional work including completion of randomized trials of selenium supplementation will be needed to determine the clinical utility of this compound.

REFERENCES


V. Venkateswaran, N. E. Fleshner, and L. H. Klotz, Modulation of cell proliferation and cell cycle regulators by vitamin E in human prostate carcinoma cell lines, submitted for publication.

Fig. 10. Histogram of flow cytometric analysis showing the effect of selenium on cell cycle in PC3-AR2 cells after 72 h of treatment. There was no cytotoxicity or cell death as seen by the absence of the hypodiploid (sub-G1) peak.

DISCUSSION

Epidemiological evidence suggests that diet play an important role in the progression of PCA (36). Despite a large global variation in PCA death rates, small foci of PCA exist ubiquitously in 42–80% of males in their 8th decade (1, 37). Studies of migrating populations reveal that men from countries with a low incidence of PCA tend to acquire an increased incidence rate (38, 39). These experimental observations have shown that selenium can inhibit cell cycle progression in human PCA cell lines. This is consistent with the known descriptive epidemiology of prostate carcinogenesis. Additional work including completion of randomized trials of selenium supplementation will be needed to determine the clinical utility of this compound.


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