Redox modulation of human prostate carcinoma cells by selenite increases radiation-induced cell killing

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Abstract

Although selenium compounds have been extensively studied as chemopreventative agents for prostate cancer, little is known about the potential use of selenium compounds for chemotherapy. We have shown that selenite inhibits cell growth and induces apoptosis in androgen-dependent LAPC-4 prostate cancer cells. LAPC-4 cells were more sensitive to selenite-induced apoptosis than primary cultures of normal prostate cells. Selenite-induced apoptosis in LAPC-4 cells correlated with a decrease in the Bcl-2:Bax expression ratio. Selenite-induced oxidative stress and apoptosis are dependent upon its reaction with reduced GSH. LAPC-4 cells treated with selenite showed decreased levels of total GSH and increased concentrations of GSSG. Thus, selenite altered the intracellular redox status toward an oxidative state by decreasing the ratio of GSH:GSSG. Because increased levels of Bcl-2 and GSH are associated with radioresistance, we examined the ability of selenite to sensitize prostate cancer cells to \( \gamma \)-irradiation. Both LAPC-4 and androgen-independent DU 145 cells pretreated with selenite showed increased sensitivity to \( \gamma \)-irradiation as measured by clonogenic survival assays. Importantly, selenite-induced radiosensitization was observed in combination with a clinically relevant dose of 2 Gy. These data suggest that altering the redox environment of prostate cancer cells with selenite increases the apoptotic potential and sensitizes them to radiation-induced cell killing.

Keywords: Selenite; Prostate cancer; Radiation therapy; GSH; Bcl-2; Free radicals

Introduction

Prostate cancer is the most common malignancy among American men [1]. Radiation therapy is frequently used to treat localized prostate cancer, but unfortunately this treatment is not curative in many of these patients. For patients who present with T1–T2 disease and are treated with external beam radiation, the 5-year estimates of overall survival, disease-specific survival, and freedom from biochemical failure (PSA recurrence) are 85.0, 95.1, and 65.8, respectively [2]. The inherent resistance of prostate cancer cells to apoptosis is a major factor responsible for the failure of radiation therapy. The development of an effective radiosensitizer that increases the apoptotic potential of prostate cancer cells could significantly improve local control and cure rates in prostate cancer.

Glutathione (GSH), a cysteine-containing tripeptide, is the most abundant intracellular thiol-based antioxidant [3]. The intracellular redox status of a cell is often measured by the ratio of GSH (reduced):GSSG (disulfide) [4]. Antioxidants such as GSH play an important role in maintaining concentrations of reactive oxygen species (ROS) compatible with cellular function. The generation of ROS is well known to be an important component of ionizing radiation-induced cytotoxicity. GSH can reduce ROS and repair DNA radicals produced by ionizing radiation [5]. Increased levels of intracellular GSH correlate with radioresistance in vitro [6], and GSH levels are frequently elevated in cancer cells compared to normal cells [7,8]. Conversely, when GSH levels are depleted, tumor cells are rendered more susceptible to radiation-induced cell killing [6]. Therefore, GSH...
seems to play an important role in the adaptive response of tumor cells to oxidative stress generated by γ-irradiation and is an attractive target for radiosensitization.

Bcl-2 family members are important regulators of apoptosis. Antiapoptotic Bcl-2 associates with the outer mitochondrial membrane to regulate the mitochondrial membrane potential, counteract the effect of the proapoptotic Bax, and block the release of cytochrome c [9]. Increased Bcl-2 expression has been associated with the progression of prostate cancer from androgen-responsive to androgen-independent or refractory disease that is poorly responsive to radiation therapy [10–13]. Furthermore, patients whose prostate tumors show elevated Bcl-2:Bax ratios are at increased risk of radiation therapy failure [14]. Therapeutic measures to decrease the level of functional Bcl-2 have delayed the progression of prostate cancer in xenograft models to a state of androgen independence and enhanced sensitivity to radiation-induced apoptosis [15,16]. Therefore, novel therapies that inhibit the antiapoptotic Bcl-2 pathway are necessary for the improved management of prostate cancer.

The anti-tumor activities of selenium compounds are dependent upon the dose and chemical form. The inorganic form of selenium, selenite (SeO$_3^{2-}$), undergoes thiol-dependent reduction to selenide (H$_2$Se), which supplies selenium for the synthesis of selenoproteins [17]. Selenium is incorporated into antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase as the amino acid selenocysteine [17]. At lower concentrations, the major effects of selenite are related to its role as a micronutrient. However, at higher concentrations, selenite may become toxic. The GSH-dependent reduction of selenite and the further oxidative metabolism of the resulting selenide can produce superoxide anions and inhibit the antiapoptotic Bcl-2 pathway necessary for the improved management of prostate cancer.

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The present studies describe the effects of selenite on LAPC-4 prostate cancer cells in vitro. Our results show that selenite-induced apoptosis in LAPC-4 cells correlates with a decrease in Bcl-2:Bax expression. Selenite was also shown to alter the redox status toward oxidation by decreasing the ratio of GSH:GSSG. Furthermore, selenite sensitized LAPC-4 cells, as well as DU 145 cells, to clinically relevant doses of γ-irradiation. These observations suggest that selenite may be a novel radiosensitizer for the treatment of prostate cancer.

Material and methods

Cell culture and treatments

LAPC-4 prostate cancer cells (provided by Dr. Charles Sawyers, UCLA, Los Angeles, CA, USA) were cultured in phenol red-free RPMI 1640 (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA, USA) at 37°C in a humidified atmosphere with 5% CO$_2$. DU 145 prostate cancer cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 (plus phenol red) supplemented with 10% fetal bovine serum. Primary cultures of prostatic epithelial cells from normal prostate tissue were established by methods previously described [27]. When the cells reached 50% confluence the medium was replaced and selenite was added at the noted concentrations. All chemicals and reagents were supplied by Sigma (St. Louis, MO, USA) unless otherwise noted. A $^{137}$Cs irradiator was used to deliver γ-irradiation.

Cell proliferation assay

Cells were seeded in 96 well plates (10,000 cells/well) and treated with selenite for 48 h. Cell survival was assayed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). The MTS tetrazolium solution was added directly to the wells and incubated at 37°C for 3 h, and then the absorbance was read at 490 nm with a 96 well plate reader (Vmax Kinetic Microplate Reader; Molecular Devices, Sunnyvale, CA, USA).

Detection of apoptosis

Flow cytometry analysis of propidium iodide (PI)-stained cells was used to quantify the percentage of apoptotic cells as the fraction of cells with a hypodiploid amount of DNA (sub-$G_0$). Cells were fixed and permeabilized with 100% ice-cold ethanol at 4°C overnight. The cells were resuspended in 500 µl of buffer [phosphate-buffered saline, 5 mM EDTA] and incubated with 200 µg/ml RNase A for 30 min at room temperature and then 50 µg/ml PI for 10 min at room temperature. The cell cycle distribution was analyzed using FACStar flow cytometry (Becton-Dickinson, San Jose, CA, USA). Cleavage of caspase-3 as a marker of caspase-mediated apoptosis was also detected by immunoblot analysis (see below).
Western blot analysis

Cell pellets were resuspended in lysis buffer (50 mM Hepes, pH 7.5, 0.5% NP-40, 0.5% sodium deoxycholate, 50 mM sodium chloride, 1 mM EDTA, and 0.1 mM sodium orthovanadate), incubated on ice for 20 min, and spun at 14,000 × g to collect whole cell lysates. Total cell lysates were run on NuPAGE 10% Bis–Tris gels (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to PVDF membranes and blocked with 5% milk/Tris-buffered saline (100 mM Tris–HCl, pH 7.5, 150 mM NaCl/0.1% Tween 20). Primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and included rabbit polyclonal anti-caspase-3 (H-277), goat polyclonal anti-actin (C-11), mouse monoclonal anti-Bcl-2 (100), mouse monoclonal anti-Bcl-xL (H-5), and mouse monoclonal anti-Bax (B-9). The secondary antibodies were conjugated to horseradish peroxidase and detected with ECL Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

Determination of intracellular GSH and GSSG content

The determination of intracellular GSH and GSSG content after exposure to selenite was performed using the GSH reductase recycling assay [28]. This assay measures the reaction of GSH with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Briefly, cells were rinsed in cold stock buffer (143 mM sodium phosphate, 6.3 mM EDTA, pH 7.4) and lysed by repeated freeze-thawing in 10 mM HCl. Proteins were precipitated on ice for 15 min with 6.5% sulfosalicylic acid and supernatants were collected. The 96 well plate assay was prepared by mixing 20 μl of sample or known GSH standard, 20 μl of stock buffer, 200 μl of reaction buffer (1 mM DTNB and 0.34 mM nicotinamide adenine dinucleotide phosphate in stock buffer), and 40 μl of GSH reductase (8.5 U/ml). The reaction was recorded kinetically at 30 s intervals for 6 min at a wavelength of 405 nm. GSSG was measured after GSH derivatization with 2-vinylpyridine and triethanolamine. GSSG was measured as described above using known GSSG standards. The concentrations of GSH and GSSG were expressed as nmol/mg of protein.

Clonogenic survival assays

LAPC-4 and DU 145 cells were treated with 10 μM selenite for 6 or 12 h before treatment with 2 or 5 Gy of γ-irradiation. After irradiation cells were trypsinized, counted, and seeded in triplicate into 60 mm dishes. At least two dilutions of cells were used for each treatment group. Plated cells were allowed to grow for 17 days before being stained with 0.25% crystal violet in 75% ethanol. Resulting colonies with ≥50 cells were scored. The surviving fraction was calculated as the plating efficiency of treated cells divided by the plating efficiency of untreated cells. SF2 is the survival fraction of exponentially growing cells that were irradiated at the clinically relevant dose of 2 Gy. The SF2 enhancement ratio (SF2 ER) is defined here as the SF2 without treatment divided by the SF2 for cells treated with selenite.

Results

Selenite-induced apoptosis in LAPC-4 prostate cancer cells

Selenite inhibited cell growth and induced apoptosis in a dose-dependent fashion in androgen-dependent LAPC-4 human prostate cancer cells in vitro. The proliferation of LAPC-4 cells was measured using the MTS assay after incubation with selenite for 48 h. Cell proliferation was 53.3% of control after treatment with 10 μM selenite and 33.4% of control after treatment with 25 μM selenite (Fig. 1A). Apoptosis was measured as the percentage of cells in the sub-G1 fraction of the cell cycle. The percentage of sub-G1 cells after treatment with 10 or 25 μM selenite for 48 h was 14.5 and 26.1%, respectively (Fig. 1B). Cleavage of caspase-3, a marker of apoptosis, was also detected in selenite-treated LAPC-4 cells by Western blotting (Fig. 1C). In contrast, primary cultures of normal prostate epithelial cells were more resistant to selenite-induced apoptosis than LAPC-4 cells (Fig. 1D). These results are consistent with other reports showing that selenite has differential effects in prostate cancer versus normal cells [22,25].

Effects of selenite on Bcl-2, Bcl-xL, and Bax expression in LAPC4 cells

Bcl-2, Bcl-xL, and Bax are members of the Bcl-2 family that play key roles in the regulation of apoptosis [9]. Therefore, we measured the expression levels of Bcl-2, Bcl-xL, and Bax after exposure to selenite for 48 h by Western blotting (Fig. 2). The expression levels of antiapoptotic Bcl-2 and Bcl-xL were decreased after treatment with selenite, and this reduction was coupled to an increased expression of proapoptotic Bax. The decreased Bcl-2:Bax expression ratio suggests that selenite-induced apoptosis in LAPC-4 cells correlates with a shift in the balance of Bcl-2 family member expression from a prosurvival to an apoptotic state.

Effects of selenite on intracellular GSH and GSSG in LAPC-4 cells

Because oxidative stress is induced by selenite, we measured changes in the intracellular redox state by measuring total GSH and GSSG concentrations at 6, 24, and 48 h after treatment with 10 μM selenite. Selenite decreased total GSH levels in a time-dependent fashion from a basal level of 52.1 ± 5.6 to 11.8 ± 2.1 nmol/mg at 48 h (Fig. 3A). The concentration of GSSG increased after selenite treatment and was maximal after 24 h (Fig. 3B). As
a result, the ratio of GSH:GSSG in LAPC-4 cells was decreased by selenite (Fig. 3C). As early as 6 h after treatment with 10 μM selenite, the GSH:GSSG ratio was decreased from 129.4 ± 13.6 to 15.1 ± 2.3. These changes in intracellular GSH content in response to selenite indicate a dramatic shift in the cellular redox balance toward an oxidative state.

Selenite sensitizes LAPC-4 cells to radiation-induced cell killing

Because selenite-induced apoptosis in LAPC-4 cells correlated with decreased Bcl-2:Bax and GSH:GSSG ratios,
we next tested the ability of selenite to sensitize LAPC-4
cells to γ-irradiation. LAPC-4 cells were treated with 10 μM
selenite for 6 h before receiving γ-irradiation, and survival
was measured using a clonogenic assay. This treatment
regimen was based upon our data showing that treatment of
LAPC-4 cells with 10 μM selenite for 6 h decreased the
GSH:GSSG ratio 88.3%. The surviving fraction of LAPC-4
cells after treatment with selenite alone was 0.431 ± 0.021
(data not shown). In experiments in which selenite was
combined with radiation, the results were normalized for the
killing from selenite alone. We found that selenite enhanced
radiation-induced inhibition of colony formation (SF2 =
0.056) compared to cells treated with radiation alone (SF2 =
0.244) (Fig. 4). These results indicate that selenite inhibits
the clonal growth of LAPC-4 cells and enhances the effect
of radiation on these cells.

Radiosensitization of androgen-independent DU 145 cells
by selenite

We expanded our studies to include an androgen-
independent prostate cancer cell line to determine if selenite
would similarly sensitize these cells to γ-irradiation. The
androgen-independent DU 145 prostate cancer cell line was
chosen because previous studies have shown that selenite can
inhibit growth and induce apoptosis in these cells [20].
Again, changes in intracellular GSH and GSSG were
measured 6 and 12 h after treatment with 10 μM selenite.
The ratio of GSH:GSSG decreased from a basal level of
146.4 ± 14.0 in control cells to 57.5 ± 13.8 and 5.7 ± 1.8 at
6 and 12 h, respectively (Fig. 5A). Next, the effects of selenite
on the response of DU 145 cells to γ-irradiation were studied
using clonogenic survival assays. The surviving fractions of
DU 145 cells treated with 10 μM selenite for 6 or 12 h alone
were 0.941 and 0.409, respectively (data not shown). After
normalization for the killing from selenite alone, we found
that pretreatment with selenite enhanced radiation-induced
cell death (SF2 = 0.343 and 0.199 at 6 and 12 h, respectively)
compared to cells treated with radiation alone (SF2 = 0.554)
(Fig. 5B). These results are summarized in Table 1.

Discussion

Increasing information suggests that selenium may be an
effective chemopreventative agent for prostate cancer;
however, little is known about the potential use of selenium
for the treatment of prostate cancer. We characterized the
response of androgen-dependent LAPC-4 prostate cancer

Table 1
Radiosensitization of LAPC-4 and DU 145 cells by selenite

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>SF2</th>
<th>SF2_E.R.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAPC-4</td>
<td>Radiation alone</td>
<td>0.244</td>
<td>–</td>
</tr>
<tr>
<td>LAPC-4</td>
<td>10 μM selenite 6 h before radiation</td>
<td>0.056</td>
<td>4.36</td>
</tr>
<tr>
<td>DU 145</td>
<td>Radiation alone</td>
<td>0.554</td>
<td>–</td>
</tr>
<tr>
<td>DU 145</td>
<td>10 μM selenite 6 h before radiation</td>
<td>0.343</td>
<td>1.62</td>
</tr>
<tr>
<td>DU 145</td>
<td>10 μM selenite 12 h before radiation</td>
<td>0.199</td>
<td>2.78</td>
</tr>
</tbody>
</table>
cells to selenite in vitro. Selenite-induced growth inhibition and apoptosis were found to correlate with changes in Bcl-2 family member expression and altered intracellular GSH status. Selenite was also found to enhance the response of LAPC-4 and androgen-independent DU 145 prostate cancer cells to γ-irradiation. The finding that selenite can sensitize prostate cancer cells to radiation therapy is significant because the inherent radioresistance of prostate tumors is a major reason for failure to control local disease with radiation therapy.

The ability to override the antiapoptotic mechanisms in prostate cancer is essential for effective therapy. Overexpression of antiapoptotic Bcl-2 is associated with the progression of prostate cancer to an androgen-independent state that is resistant to radiation therapy [10–13]. Furthermore, Rosser et al. found that patients undergoing radical prostatectomy after radiotherapy failure had a significantly higher rate of Bcl-2 overexpression than did patients who underwent surgery as the initial treatment [29]. We have shown that selenite-induced apoptosis correlates with decreased expression of Bcl-2 and Bcl-xL in LAPC-4 cells. In addition, exposure to selenite increased the expression of proapoptotic Bax, which targets the mitochondria to antagonize the function of Bcl-2 and promote the release of cytochrome c [9]. The mechanism(s) responsible for the changes in Bcl-2, Bcl-xL, and Bax expression may be related to the induction of oxidative stress by selenite. The alteration of the cellular redox state can affect the DNA binding and transactivating activity of redox-sensitive proteins such as nuclear factor κB, which has been implicated in the regulation of bcl-2 and bcl-xL gene expression [30,31], via the oxidation of critical cysteine residues. Furthermore, ROS produced as a by-product of selenite metabolism may potentially induce DNA strand breaks, resulting in p53 activation and upregulation of Bax [32,33].

GSH is a major component of the overall antioxidant defense in cells. However, GSH can also act as a pro-oxidant by facilitating selenite metabolism [34]. Selenite undergoes thiol-dependent reduction via reduced GSH, which generates ROS and alters the intracellular redox state [17–19]. The GSH:GSSG ratio is an important measure of the intracellular redox status [4]. A dynamic balance is maintained between GSH synthesis, its recycling from GSSG, and its utilization. The GSH:GSSG balance plays a crucial role in the regulation of pathways essential for homeostasis, normal cell function, and survival. Selenite-induced apoptosis in LAPC-4 cells correlated with a decrease in the ratio of GSH:GSSG. In response to selenite, GSH was consumed and GSSG was produced. These results demonstrate that selenite induced a dramatic shift in the intracellular redox state toward oxidation. A prolonged oxidative state could have profound effects on cellular function, because irreversible cell damage occurs when a cell is no longer able to maintain normal levels of GSH [35]. In addition, GSH-depleted cells are more vulnerable to other toxic insults such as ionizing radiation [6].

The sensitivity of human prostate cancer cells to ionizing radiation correlates with their ability to undergo apoptosis. The rationale for testing the effects of selenite with γ-irradiation was based upon our results showing that selenite can modulate the apoptotic potential of prostate cancer cells by decreasing the GSH:GSSG and Bcl-2:Bax ratios, two factors which have been previously shown to correlate with radioresistance. The clonogenic assay results demonstrate that selenite administered before irradiation has a synergistic effect on cell killing in LAPC-4 cells. Selenite was also found to sensitize androgen-independent DU 145 cells to γ-irradiation. Selenite-induced radiosensitization was observed in combination with a clinically relevant dose of 2 Gy, which is commonly used as a daily treatment for patients receiving radiation therapy for treatment of prostate cancer. In DU 145 cells, the degree of radiosensitization seemed to correlate with the time of exposure to selenite before irradiation and the magnitude of change in GSH:GSSG. Thus, the enhanced radiation response may be related to the inability of GSH-depleted cells to inactivate ROS and repair DNA radicals induced by γ-irradiation.

The increased sensitivity of androgen-dependent LAPC-4 cells to selenite and radiation compared to androgen-independent DU 145 cells may be related to their p53 and Rb status. Whereas LAPC-4 cells have wild-type p53 and Rb tumor suppressor genes, DU 145 cells contain mutant p53 and Rb [36–39]. Selenite is known to induce p53 and we have shown that selenite-induced apoptosis in LAPC-4 cells is associated with the upregulation of Bax [32]. In DU 145 cells, a mutant p53 and the absence of Bax expression may explain, at least in part, their relative resistance to selenite. The presence of mutated p53 is also a marker of resistance to radiation, because a dysfunctional p53 will increase tolerance to DNA damage and may allow tumor growth despite treatment with radiation [40,41]. Furthermore, the combination of ionizing radiation and adovaliral p53 gene therapy has been shown to have synergistic effects in DU 145 cells [42]. Rb is another key tumor suppressor gene that interacts with E2F transcription factors to prevent the transcription of genes required for progression through the cell cycle [43]. The reintroduction of Rb into DU 145 cells has also been shown to increase their sensitivity to radiation-induced apoptosis [44,45].

Although these results show that high levels of selenite (10 μM) can induce apoptosis and sensitize prostate cancer cells to radiation, it is not clear whether these findings can be extrapolated to animal models or humans. The majority of selenium literature over the past 20 years has focused on animal and human research showing that dietary selenium can prevent or reduce the incidence of naturally occurring or chemically induced cancer. There have been few reports on the treatment of established tumors in animals with selenite. However, these limited studies have shown that high levels of selenite can retard tumor growth in vivo without apparent ill effects on the host and suggest that the use of selenite for the treatment of tumors is worthy of further study [46–50].
In addition, our results showing that normal prostate cells are more resistant to selenite-induced apoptosis than LNCaP-4 cells and the previous work of Menter et al., which showed that prostate carcinoma cell lines (LNCaP, PC-3, and DU 145) are more sensitive to growth inhibition and the induction of apoptosis by selenite than primary cultures of normal human prostate cells, suggest that this difference has the potential to be exploited therapeutically [22]. Still many questions remain regarding the types of tumors that may be sensitive to selenite and the dosage regimens that are safe and effective.

To our knowledge, these data demonstrate for the first time that selenite can sensitize prostate cancer cells to radiation therapy. Other thiol-depleting agents such as diethyl maleate have been previously shown to sensitize radiation therapy. Other thiol-depleting agents such as time that selenite can sensitize prostate cancer cells to and effective.

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References


