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Cancer Letters 117 (1997) 35–40

CANCER LETTERS

Induction of differentiation and apoptosis by sodium selenite in human colonic carcinoma cells (HT29)

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Received 6 February 1997; received in revised form 20 March 1997; accepted 20 March 1997

Abstract

To explore the mechanism(s) by which selenium (Se) exerts its cancer chemopreventive activity, we studied the effect of selenite (0–100 μM) on cell growth, viability, differentiation, detachment, DNA fragmentation and apoptosis in human colonic carcinoma cells (HT29). Selenite ($\geq$5 μM) decreased cell growth, increased cell detachment and decreased intracellular levels of reduced glutathione (GSH), whereas $\geq$10 μM selenite induced cell differentiation and apoptosis. The chemopreventive effects of selenite may be related in part to the generation of reactive oxygen species (ROS) resulting from the reaction between selenite and GSH. © 1997 Elsevier Science Ireland Ltd.

Keywords: Selenite; Glutathione; Colon cancer; Differentiation; Apoptosis

1. Introduction

The trace element selenium (Se) has been shown to be cytotoxic in vitro in multiple cell models including Ehrlich ascites [1], mouse leukemic L1210 cells [2] and human mammary tumor cells [3]. Selenium supplementation in laboratory animals decreases tumorigenesis in several tumor models including skin, liver, colon and pancreas [4]. Epidemiological studies suggest an inverse relationship between Se intake (and tissue levels of Se) and cancer incidence and mortality rate for several forms of cancer [5]. Clark et al. [6] recently found that the incidence of lung, prostate and colon cancer was lower in Se-supplemented patients.

The mechanism(s) by which Se exerts it chemopreventive activity is unknown, however, several plausible explanations have been postulated including the role of Se in inducing DNA strand breaks [2,7,8], apoptosis [2,9] and the catalytic properties of Se with glutathione which result in the generation of reactive oxygen species (ROS) [10,11]. The latter is of particular importance because ROS also induce DNA strand breaks [12] and apoptosis [13]. The chemopreventive activity related to the antioxidant role of Se as a constituent of glutathione peroxidase (GSHPx) is also well established [14].

The chemopreventive effects of Se have been studied using various Se compounds, however, selenite has been investigated most extensively and is one of the most effective Se compounds studied. In the present study, we examined the effect of selenite on growth, viability, differentiation, detachment, DNA fragmentation and apoptosis in human colonic carcinoma cells (HT29). While the effects of selenite on these variables have been reported by several authors

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PII S0304-3835(97)00212-7

using other cell lines, to our knowledge this is the first report that comprehensively shows the multitude of effects of selenite on a single cancer cell line.

2. Materials and methods

2.1. Cells

HT29 cells were obtained from American Type Culture Collection (ATCC; Rockville MD) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum. Cells were seeded at 0.3×10^6 cells/ml, and incubated (37°C, 5% CO_2) for 4 days (except for DNA fragmentation assay; 24 h) in selenite supplemented (0–100 μM) DMEM. Cell counts were obtained for both detached and attached cells and viability was assessed by trypan blue exclusion.

2.2. Alkaline phosphatase

Following selenite (0, 0.1, 1.0, 2.0, 5.0, 10.0 μM; $n = 3$) treatment, both detached and attached cells were put into 1 ml 10 mM Tris–HCl buffer (pH 8.0), sonicated at 0°C for 15 s and then centrifuged at 9000 rev./min for 15 min at room temperature. Undiluted supernatant was used for the alkaline phosphatase assay, using Sigma's procedure 245. Sigma units/ml of alkaline phosphatase was calculated from the difference in absorption. Specific activity was then calculated per mg protein. Sodium butyrate treatment (4 mM) was used as the positive control since this compound is a known differentiation agent in HT29 cells [16].

2.3. DNA fragmentation ladders

The DNA sample (20 μL) mixed with 5 μl of 5 × blue juice (20% Ficoll, 0.01 M Tris–HCl (pH 8.0), 0.01 M disodium EDTA (pH 8.0), 0.1% xylene cyanol FF, 0.1% bromphenol blue) and DNA standard digests (1 μl of PhiX174 RF DNA Hae III Digest (New England Biolabs, Beverly, MA), 9 μl TE buffer, 10mM Tris–HCl (pH 8.0), 1 mM disodium EDTA (pH 8.0), 2.5 μl of 5 × blue juice) were loaded onto a 1.7% agarose gel (1.7 g agarose in 100 ml 1 × TAE buffer with 3.5 μg/ml ethidium bromide for staining). The gels were run for 2 h at 60 V. DNA ladders were visualized with UV light and photographed with a Fotodyne adapted Polaroid camera.

2.4. Reduced glutathione

Following selenite (0–10 μM) treatment of HT29 cells, the enzymatic determination of total reduced glutathione (GSH) was performed using the spectrofluorometric method of Hissin and Hilf [15]. Samples were prepared by scraping 100 mm culture dishes of selenite treated cells into homogenization buffer (4:1 mixture of phosphate/EDTA buffer and 25% metaphosphoric acid). Cells were homogenized on ice using a polytron homogenizer at $100\,000 \times g$ for 30 min at 4°C, and the supernatant was collected for analysis. Using 0.1% o-phthalaldehyde (in methanol) as a fluorescent agent, GSH levels were determined using a spectrofluorometer (RF-1501; Shimadzu) at an excitation wavelength of 350 nm and emission wavelength of 420 nm. GSH standards contained 0.25–2.5 μg GSH/ml.

2.5. Apoptag™ assay

Following selenite (0–20 μM) treatment, HT29 cells were stained using the Apoptag™ in situ peroxidase apoptosis detection kit (Oncor, Gaithersburg, MD) based on the TdT-mediated dUTP-digoxigenin (TUNEL) method. Since some Se compounds have been shown to induce strand breaks in DNA [22] and since TdT requires free 3′OH ends of DNA, we were concerned about the possibility of artifactual end-labeling. Gorczyco et al. [31] observed that strand breaks induced by ionizing radiation caused no immediate increase in the TdT assay, but increases were seen after 3 h. However, Chapman et al. [32] noted that drug induced DNA damage is not identified by the TdT assay unless it is accompanied by the apoptotic response.

3. Results

3.1. Cell detachment

Selenite treatment of 10 μM or greater caused greater than 50% of the cells to detach from the cul-

Table 1

Cell quantitation data for HT29 cells grown in the treatments indicated for 4 days.

Treatment	Total cell count ($\times 10^6$)	% Detached cells
Control (no Se)	23.4 ± 1.9	1.1 ± 0.1
0.1 μM selenite	20.1 ± 0.1	1.0 ± 0.1
1 μM selenite	21.7 ± 1.4	0.9 ± 0.1
5 μM selenite	6.3 ± 0.5	12.0 ± 0.8
10 μM selenite	1.7 ± 0.1	56.0 ± 1.2
25 μM selenite	1.7 ± 0.1	72.0 ± 2.6
50 μM selenite	1.8 ± 0.2	70.0 ± 3.1
100 μM selenite	1.8 ± 0.1	76.0 ± 2.7

HT29 cells were seeded at 4.3×10^6/100 mm culture dish.

ture dishes. At this level of selenite there was also evidence of inhibition of cell proliferation (Table 1). Cell viability assayed by trypan blue dye exclusion was between 95% and 99% for attached cells and 94% and 99% for detached cells.

3.2. Cell differentiation

Cell treatments with 10–50 μM selenite induced alkaline phosphatase. As expected, treatment with 4 mM sodium butyrate induced alkaline phosphatase (Table 2).

3.3. Apoptosis

Gel electrophoresis of DNA from detached cells revealed nucleosomal fragmentation ladders suggestive of apoptosis. No DNA ladders were associated

Table 2

Units of alkaline phosphatase activity per mg total protein

Treatment	Alkaline phosphatase activity
Control (no Se)	0.23 ± 0.00
0.1 μM selenite	0.10 ± 0.00
1 μM selenite	ND[a]
2 μM selenite	ND
5 μM selenite	0.10 ± 0.00
10 μM selenite	1.08 ± 0.07
25 μM selenite	12.72 ± 1.13
50 μM selenite	20.28 ± 0.79
4 mM Na butyrate	23.03 ± 3.00

Values represent mean ± SEM based on triplicate samples.
[a]None detected.

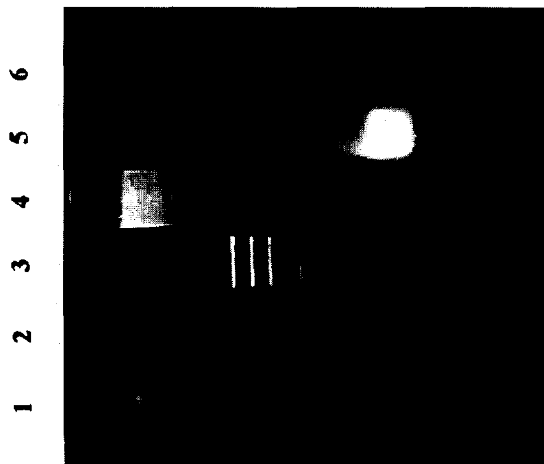

Fig. 1. Photograph of UV-illuminated DNA from detached cells resolved on 1.7% agarose gels by electrophoresis, stained with ethidium bromide. Fragmented DNA from detached cells of the 5.0 μM selenite treatment demonstrated a laddering effect consistent with nucleosomal fragmentation of DNA associated with apoptosis. 20 μl of sample DNA and 5 μl of tracking dye were loaded on lane 1 (4 mM butyrate, intact DNA), lane 2 (4 mM butyrate, fragmented DNA), lane 4 (5.0 μM selenite, intact DNA), lane 5 (5.0 μM selenite, fragmented DNA) and lane 6 (control, fragmented DNA). 1 μl of Phi × 174 RF DNA Hae III Digest diluted in 9 μl of TE buffer, with 2.5 μl of tracking dye was loaded in lane 3.

with the DNA isolated from the attached cells (Fig. 1). Selenite-induced apoptosis was detected in the attached cells using the TUNEL assay. The percent of apoptotic cells increased with selenite concentration in a dose dependent manner over a range of 0–20 μM selenite (Fig. 2).

3.4. Reduced glutathione

The concentration of GSH varied inversely with selenite concentration. GSH levels in attached cells were 43% and 68% lower in cells treated with 5 μM and 10 μM selenite, respectively, than in control cells. Treatment with 10 μM selenite resulted in a 2% decrease in GSH levels in detached cells.

4. Discussion

We have demonstrated that selenite induces differentiation, cell detachment, decreased cell proliferation, DNA fragmentation and apoptosis and these

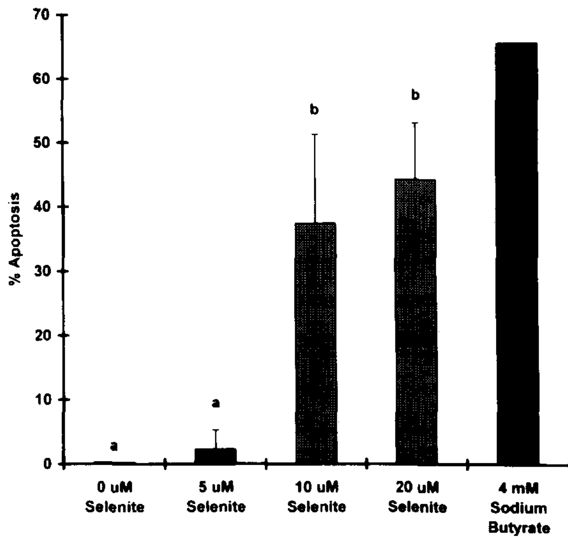

Fig. 2. Selenite-induced apoptosis in HT29 cells. HT29 cells were seeded at 0.3×10^6 cells/ml of selenite-supplemented medium and incubated for 4 days at 37°C in 5% CO_2. Data are presented as percentage of cells positive for apoptosis by Apoptag® staining ($n = 2$–5). Values with different letters are significantly ($P < 0.05$) different.

endpoints have all been measured in a single cell line, HT29 human colonic carcinoma cells. Cell differentiation has been demonstrated by using alkaline phosphatase as a marker for a more differentiated phenotype in intestinal epithelial cells [16]. Our studies confirm the ability of sodium butyrate to induce alkaline phosphatase in HT29 cells. Furthermore, we show that selenite (25–50 μM) also induces alkaline phosphatase activity, and that alkaline phosphatase is the standard marker of differentiation in HT29 cells [16,17]. To our knowledge, this is the first time that selenite has been shown to induce differentiation in any cell type. As reported for butyrate [16,17], the cell differentiation induced by selenite was greater in the detached cells than in the attached cells.

Our experiments confirm the findings of Yan and Frenkel [18] who first reported the inhibition of cell attachment of HeLa cells by 5–15 μM selenite. They did not observe a comparable effect with selenate, selenomethionine, selenocystine or sulfite. The question arises as to which process occurs first: detachment of the cells from the matrix or the DNA-related events described below.

Selenite treatment resulted in a decrease in HT29 cell proliferation as indicated by a decreased cell number. Selenite is well-documented as an inhibitor of cell growth in vitro in many cell lines [19–25] and appears to be a universal phenomenon when selenite exceeds 5 μM. Induction of DNA fragmentation and apoptosis selenite at similar doses has also been reported in multiple cell lines [9,22,23]. However, this report is the first demonstration that selenite can induce both cell differentiation and apoptosis in a single cell line, HT29 human colonic carcinoma cells.

GSH depletion in the HT29 cells by selenite appears to support the hypothesis that oxidant stress is the underlying cause of many of the cellular phenomena attributed to selenite. Selenite is a redox oxidizing agent that contributes to the loss of cellular GSH [26]. Lanfear et al. [9] has postulated that following absorption, selenite is reduced to selenodiglutathione (SDG), which has been reported to induce p53 in cells containing wild-type p53, as well as p53-independent apoptosis in cell lines with mutated p53; HT29 cells contain mutated p53. These authors [9] have further hypothesized that the variability in response to selenite in vitro by cell lines may be due to differences in the availability or regulation of GSH levels.

We demonstrate that selenite (5 and 10 μM) depletes cellular reduced GSH in HT29 cells. Caffrey and Frenkel [27] found that when HeLa cells were depleted of GSH by BSO, the ability of selenite to inhibit cell colony formation was reduced, suggesting that selenite exerts its cytotoxic activity by oxidation of GSH. In 1988, Seko et al. [28] reported that selenite reacted with GSH to produce the superoxide anion which has recently been shown to induce DNA fragmentation [29] and apoptosis [30]. Lu et al. [2] recently demonstrated that selenite induced DNA double strand breaks and cellular apoptosis in L1210 mouse leukemia cells and that both could be inhibited by zinc and aurintricarboxylic acid (ATA). One of us (R.L.D.) has recently demonstrated, in a cell-free system, that the interaction of selenite with GSH induces the generation of the superoxide anion ($O_2^{\cdot -}$), as indicated by lucigenin-dependent chemiluminescence, and that this process can be inhibited by zinc and ATA [10] and this observation was repeated using physiological levels of reduced glutathione and at physiological pH (Stewart, unpublished data). These findings, along with the results presented herein, suggest that selenite-induced differentiation and apopto-

sis may be mediated by the generation of $O_2^{\cdot-}$. Since it is well known that the form of selenium is important to its biological activity, and others [30] have shown that other selenium compounds, such as Se-methylselenocysteine, have some superior chemopreventive qualities, it is prudent to consider the relative efficacy of selenium compounds with regard to the endpoints discussed here and their application in chemoprevention.

Acknowledgements

This work was funded in part by the Texas Tech University Institute for Nutritional Sciences and The E.A. Franklin Endowment.

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