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Copper may interact with selenite extracellularly in cultured HT-29 cells

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Abstract

Previous studies have demonstrated that copper (15.7 μmol/L) can inhibit selenite (12.6 μmol/L)–induced cytotoxicity and apoptosis in HT-29 cells. However, the exact nature of the interactions between selenium and copper is not fully understood. In this study, the effect of copper on the cell cycle arrest induced by selenite or selenocystine was examined. Both selenite and selenocystine were effective in inhibition of cell growth and cell cycle progression. Cell cycle analysis revealed that selenite (3–5 μmol/L) caused a decrease in G1 phase cells that corresponded with an increase in S and G2 phase cells, and that 0.625 or 1.25 μmol/L copper sufficiently inhibited selenite-induced cell cycle arrest. In contrast, selenocystine caused an increase in G1 phase cells that corresponded with a decrease in S and G2 phase cells. Interestingly, 0.625 or 1.25 μmol/L copper did not inhibit selenocystine-induced cell cycle arrest. In addition, cell free gel shift assay demonstrated that selenite suppressed the inhibitory effect of copper on SP-1 DNA binding. Furthermore, although 5 μmol/L selenite in culture media significantly increased the intracellular selenium content, 1.25 μmol/L copper sulfate blocked this increase of the intracellular selenium content. Collectively, these data demonstrate that selenite and selenocystine cause cell cycle arrest via distinct mechanisms, and suggest that copper may interact with selenite extracellularly, which represents the basis of antagonism between copper sulfate and selenite.

Keywords: Copper; Selenium; Cell cycle; Colon cell

1. Introduction

Selenium (Se) is an essential trace element for humans and many other forms of life [1]. Se is known to have anticancer effects that have been demonstrated in cultured cells [2,3], laboratory animals, and human studies [4,5]. Se can either stimulate or inhibit cell growth, depending on the concentration and chemical form of Se [6]. At concentrations higher than nutritional requirements, Se has anticancer effects. These effects may be mediated through changes in the proliferation of certain cells (such as promotion of immune cells), cell cycle, apoptosis, and/or toxic effects on cancer cells [6,7]. Over the years, numerous elements such as As, Cu, Zn, Cd, Hg, Sn, Pb, Ni, Co, Sb, Bi, Ag, Au, and Mo have been found to inhibit anticancer effects of selenium [8].

Copper (Cu), an essential trace element for humans, is also known to have anticancer effects in laboratory animals [9]. It is well known that copper sulfate is distributed widely in drinking water, soil, food, or the environment [10]. Antagonism between Cu and Se has been documented in animal studies [11], and an understanding of which is directly related to nutrition and cancer prevention. Recently, it has been reported that Cu as $CuSO_4$ protects human colonic cancer cells (HT-29) against selenite induced cytotoxicity and apoptosis and acts as an antioxidant [12]. Several hypotheses were proposed to explain the above observations [12], but further studies are needed to determine the mechanisms. The present study was conducted to test the hypothesis that Cu blocks selenite-induced cell cycle arrest through an extracellular mechanism.

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The control of cell cycle progression plays a key role in terminal differentiation, growth, development, and tumorigenesis [13,14]. In general, cell cycle arrest occurs before or coexists in the event of cytotoxicity and apoptosis, and therefore the detection of cell cycle progression is a very sensitive measurement [13,14]. Se and Cu in human blood have been reported to be up to 2–5 μmol/L and 11~22 μmol/L, respectively [15,16]. In the present study, the effect of interactions between Se compounds (3–5 μmol/L) and Cu (0.625–1.25 μmol/L) on cell cycle progression was examined. These concentrations of Se compounds and Cu are much lower than that of a previous report [12] in HT-29 cells. Our data provide a mechanistic basis for considering specific interaction between Cu and Se at concentrations that may be influenced by human nutrition and dietary supplementation.

2. Methods and materials

2.1. Chemicals

Na_2SeO_3, dithiothreitol, selenocystine, and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO). Adenosine 5′-triphosphate (γ-^{32}P) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Oligonucleotides were synthesized by Gibco BRL (Rockville, MD).

2.2. Cell culture and cell growth

HT-29 cells were maintained in DMEM media (GIBCO-BRL, Grand Island, NY) with 10% FBS in a humidified atmosphere 95% air/5% CO_2 at 37°C, and cell passage was between 133 to 150. Cells were seeded at 400,000 to 600,000 cells/mL into the well of a 6-well plate. After 24 hours or 48 hours, $CuSO_4$, selenite, selenocystine, or an equal volume of phosphate-buffered saline (PBS) was added to culture media to examine the effect on cell cycle/growth. Cells were counted by a hemocytometer. Cells that excluded trypan blue after incubation with an equal volume of PBS containing 0.4% trypan blue dye were considered viable.

2.3. Cell cycle analysis

Cell cycle was analyzed using flow cytometry with propidium iodide (PI) staining. HT-29 cells were tryspinsized and washed once with PBS and incubated in 70% (v/v) ethanol at −20°C. After the incubation, cells were washed with PBS, and stained with 50 mg PI/L with 6000 U RNase A/L. The DNA contents of cells were determined by flow cytometry. Data were stored as list mode files of at least 10,000 single cell events and analyzed by EPICS profile II and ModFit LT software (Coulter Corp., Miami, FL, and Topsham, ME).

2.4. Nuclear protein extraction

HT-29 cells were tryspinsized and washed twice with ice-cold PBS and subjected to centrifugation. Briefly, the cells were lysed for 15 minutes on ice in extraction buffer A (100 μL/10^6 cells) (20 mmol/L HEPES, pH 7.6, 20% glycerol, 10 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L dithiothreitol [DTT], and 0.1% Nonidet P-40-containing protease inhibitor cocktail [1:100 dilution] [Sigma, St. Louis, MO]). The nuclei were collected by centrifugation at 300 × g for 4 minutes, lysed for 30 minutes on ice in extraction buffer B (the same as buffer A but with 0.5 mol/L NaCl instead of 10 mmol/L NaCl), and then centrifuged for 15 minutes at 15,000 × g and 4°C. The supernatant was designated the nuclear fraction and kept at −80°C. Protein concentrations were determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

2.5. Electrophoretic gel mobility shift assay

The double-stranded SP-1 consensus binding sequence was annealed into double-strand oligonucleotides, respectively, and ^{32}P end-labeled. Gel shift experiments and nuclear protein preparation were performed as described previously [7]. Briefly, nuclear protein was incubated with 25,000 cpm of the above ^{32}P-end-labeled doubled strand oligonucleotides, individually (1–5 fmol) in a total volume of 40 μL for 30 minutes on ice and then 10 minutes at room temperature with sheared, nondenatured salmon sperm DNA (0.5 μg) as a nonspecific competitor. After electrophoresis in a 5% nondenaturing polyacrylamide gel, the dried gels were exposed to a phosphorimaging screen (Molecular Dynamics Image-Quant System, Sunnyvale, CA) for 16 hours at room temperature and gel images were visualized by the Molecular Dynamics Software (Sunnyvale, CA).

2.6. Selenium and copper analyses

First, HT-29 cells were washed with Se-free PBS. Second, these cells were then dissolved in 2.0 mL of 0.2 mol/L NaOH and 0.2% SDS, and protein was measured using the bicinchoninic acid [17]. Cellular Se and serum Se were determined by hydride-generation atomic absorption spectrometry [18]. Samples were prepared for analysis by predigestion in nitric acid and hydrogen peroxide, followed by high-temperature ashing in the presence of $Mg(NO_3)_2$ as an aid to prevent Se volatilization. Serum Cu was determined by inductively coupled argon plasma atomic emission spectrometry after digesting samples with nitric acid and hydrogen peroxide.

2.7. Statistical analysis

Results are given as means ± SEM. The effects of Cu and selenite on SP-1 DNA binding were tested by using a

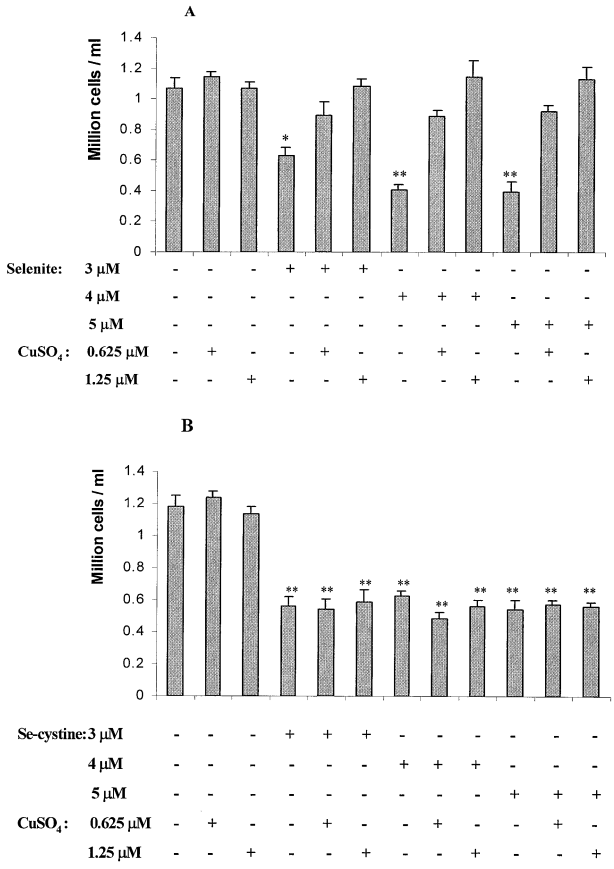

Fig. 1. Effect of interaction between copper and selenium on HT-29 cell growth. (*A*) Effect of Cu on HT-29 cell growth in the presence of selenite at concentrations indicated for 48 hours. (*B*) Effect of Cu on HT-29 cell growth in the presence of selenocystine at concentrations indicated for 48 hours. Viable cells were determined as described in the material and methods section. $n = 5$. *$P < 0.05$ and **$P < 0.0001$ as compared with the control group.

one-way analyses of variance (ANOVA) followed by Dunnett's multiple comparisons to the control group. Statistical analyses for all other variables were performed by two-way ANOVA followed by Dunnett's multiple comparisons to the control group. Differences with a P value < 0.05 were considered significant.

3. Results

3.1. Effect of Cu and selenite/selenocystine on cell growth and cell cycle progression in HT-29 cells

To determine the effect of interaction between Cu and selenite/selenocystine on cell growth, HT-29 cells were treated with different concentrations of Cu (0.625, 1.25 μmol/L) and/or selenite/selenocystine (3, 4, and 5 μmol/L) for 48 hours. The presence of Cu (0.625, 1.25 μmol/L) did not affect cell growth. However, there was a decrease in cell growth of HT-29 cells treated with sodium selenite or selenocystine in the absence of Cu (Figs. 1A, 1B). More importantly, Cu suppressed the inhibitory effect of selenite on cell growth when co-incubated with Cu (0.625 or 1.25 μmol/L) and selenite (3, 4, and 5 μmol/L) (Fig. 1A). In contrast, Cu did not suppresses the inhibitory effect of selenocystine on cell growth when co-incubated with Cu (0.625 or 1.25 μmol/L) plus selenocystine (3, 4, and 5 μmol/L) (Fig. 1B).

The effect of interaction between Cu and selenite on cell growth was further demonstrated by cell cycle analysis. The G1, S, and G2 phase-distribution of HT-29 cells after 24 hours of treatment with different concentrations of Cu, selenite, selenocystine, or the combination of Cu and selenite/selenocystine were compared with that of control cells. G1 phase cell distribution decreased but S + G2 phase cell distribution increased in selenite-treated cells (Table 1). In contrast, G1 phase cell distribution increased but S + G2 phase cell distribution decreased in selenocystine-treated cells (Table 2). Cu (0.625 or 1.25 μmol/L) did not affect on cell cycle progression. Interestingly, copper treatment suppressed selenite-induced cell cycle arrest but not selenocystine-induced cell cycle arrest (Tables 1 and 2).

3.2. Effect of selenite, Cu, and their interaction on a gel shift assay

To determine the interaction of selenite and Cu in a cell-free system, the effects of selenite, Cu, and a combination of the two were determined on SP-1 DNA binding

Table 1
Comparison of cell cycle phase-distributions of HT-29 cells treated with Cu and/or sodium selenite (Na_2SeO_3) for 24 h

	Phase-specific cells/total detected cells, %											
Na_2SeO_3, μmmol/L	0	0	0	3	3	3	4	4	4	5	5	5
$CuSO_4$, μmmol/L	0	0.625	1.25	0	0.625	1.25	0	0.625	1.25	0	0.625	1.25
	Mean ± SEM											
G1 phase	49 ± 1.6	48 ± 0.9	48 ± 0.6	40 ± 4.7*	46 ± 1.7	47 ± 0.9	29 ± 2.7*	46 ± 2.6	47 ± 1.7	27 ± 1.7*	42 ± 3.2	47 ± 1.8
S phase	35 ± 2.4	32 ± 2.9	36 ± 2.1	35 ± 2.3	35 ± 1.5	35 ± 1.9	41 ± 2.9	35 ± 2.4	36 ± 2.1	43 ± 3.6	34 ± 3.8	36 ± 1.2
G2 phase	16 ± 1.3	20 ± 2.9	16 ± 2.6	25 ± 2.8	19 ± 0.8	19 ± 1.5	30 ± 3.2*	19 ± 0.9	18 ± 1.6	30 ± 3.2*	24 ± 2.4	17 ± 1.7
S + G2 phase	51 ± 1.6	52 ± 0.9	52 ± 0.6	60 ± 4.7*	54 ± 1.7	53 ± 0.9	71 ± 2.7*	54 ± 2.6	53 ± 1.7	73 ± 1.7*	58 ± 3.2	53 ± 1.8

Values are means ± SEM, $n = 5$, unless otherwise indicated.
* $P < 0.01$ indicates significant differences between control and cells treated with sodium selenite.

Table 2
Comparison of cell cycle phase distributions of HT-29 cells treated with Cu and/or selenocystine (SeCys) for 24 h

	Phase-specific cells/total detected cells, %											
SeCys, μmmol/L	0	0	0	3	3	3	4	4	4	5	5	5
$CuSO_4$, μmmol/L	0	0.625	1.25	0	0.625	1.25	0	0.625	1.25	0	0.625	1.25
	Mean ± SEM											
G1 phase	48 ± 1.6	48 ± 1.2	48 ± 1.1	65 ± 1.7*	62 ± 1.5*	61 ± 1.4*	64 ± 0.7*	65 ± 1.5*	65 ± 0.8*	66 ± 1.3*	63 ± 0.5*	65 ± 1.1*
S phase	34 ± 1.1	35 ± 2.5	35 ± 2.8	20 ± 3.0*	19 ± 1.9*	22 ± 2.4*	16 ± 1.5*	18 ± 2.2*	19 ± 1.9*	15 ± 1.5*	14 ± 1.8*	15 ± 2.8*
G2 phase	19 ± 1.3	17 ± 1.8	18 ± 2.2	15 ± 1.5	19 ± 0.4	18 ± 1.4	20 ± 2.0	17 ± 1.9	16 ± 1.2	19 ± 2.2	22 ± 2.1	19 ± 3.9
S + G2 phase	52 ± 1.6	52 ± 1.2	52 ± 1.1	35 ± 1.7*	38 ± 1.4*	39 ± 1.7*	36 ± 0.7*	35 ± 1.5*	35 ± 0.8*	34 ± 1.2*	37 ± 0.5*	35 ± 1.1*

Values are means ± SEM, $n = 4$, unless otherwise indicated.
* $P < 0.01$ indicates significant differences between control and cells treated with Se-cystine.

activity. Although selenite (2.5 or 25 μmol/L) or Cu (1.25 or 12.5 μmol/L) decreased SP-1 DNA binding, the combination (2.5 μmol/L selenite + 1.25 μmol/L Cu or 25 μmol/L selenite + 12.5 μmol/L Cu) had much lesser inhibitory effects on SP-1 DNA binding activity when compared with that of Cu alone (Fig. 2).

3.3. Intracellular Se content analysis

Se content was significantly increased when cells were exposed to 5 μmol/L selenite for 24 hours. Interestingly, the increase of Se content was much less when cells were exposed to 5 μmol/L selenite plus 1.25 μmol/L Cu simultaneously in culture media (Fig. 3).

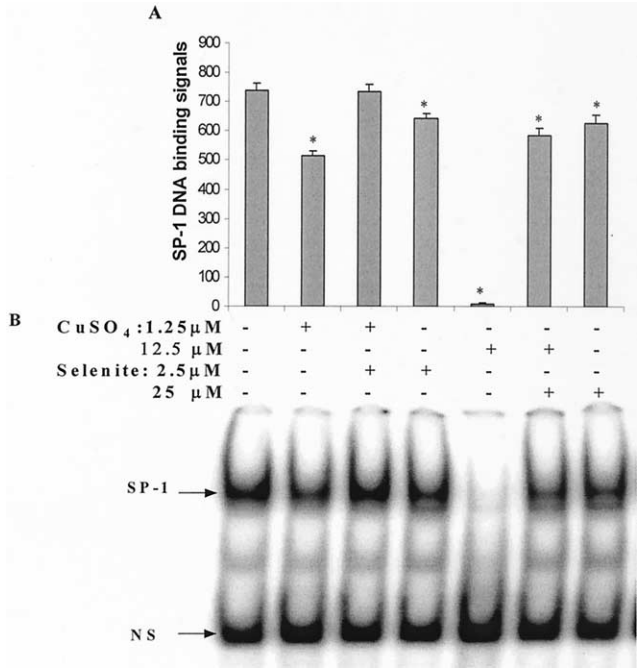

Fig. 2. Effect of interaction between Cu and Selenite at concentrations indicated on SP-DNA binding. (*A*) SP-1 DNA binding signals were quantified by the Molecular Dynamics Image-Quant system. NS = nonspecific DNA binding. $n = 6$. *$P < 0.01$ as compared with the control group. (*B*) Representative SP-1 gel shift assay showed specific SP-1 DNA binding signals.

4. Discussion

It is well documented that the biological activity of Se is dependent on its chemical form and not on the element per se [19]. Although selenocystine is an important chemical form of Se in nutritional and pharmacological studies, little is known about its role in cell growth [20,21]. In agreement with previous reports [19,22], the present study demonstrated that selenite or selenocystine (3, 4, and 5 μM) inhibited HT-29 cell growth and that selenite caused S-G2/M cell cycle arrest. In contrast, our data demonstrated for the first time that selenocystine caused G1 cell cycle arrest in HT-29 cells. These findings further strengthen the previous observation that inorganic and organic Se compounds inhibit cell growth through distinctly different modes [19,22].

In view of the observations that both Se and Cu have anticancer activities and that human diets routinely contain a combination of diverse mineral elements [6–12], the effects of Cu (0.625, 1.25 μmol/L) on selenite or selenocystine (3, 4, and 5 μmol/L) in cell cycle progression were examined. Results indicated that Cu strongly suppressed

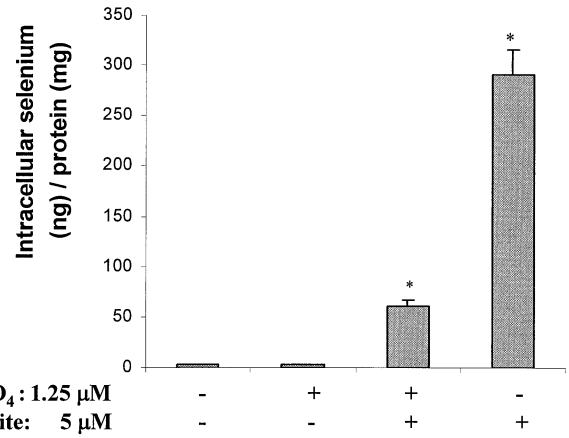

Fig. 3. Effect of 1.25 μmol/L Cu on the intracellular selenium (ng)/protein (mg) in the presence of 5 μmol/L selenite in the culture media for 24 hours. $n = 6$. *$P < 0.05$ as compared with the control group, and also indicated the difference between with and without 1.25 μmol/L Cu in the presence of 5 μmol/L selenite.

selenite-induced cell cycle arrest. However, under the same exact conditions, Cu did not affect selenocystine-induced cell cycle arrest. Similarly, Cu strongly suppressed selenite-induced cell growth arrest but not selenocystine-induced cell growth arrest. The above observation is not likely dependent on copper-salt forms because DMEM media contain endogenous SO_4^{2-} (800 μmol/L), which is about 800-fold higher than the additional SO_4^{2-} from copper sulfate (0.625, 1.25 μmol/L) in these experiments.

Se and Cu concentrations in fetal bovine serum (FBS) were considerately lower than that was reported in human blood [15,16]. According to our analysis of Se and Cu contents in FBS, Se and Cu in DMEM media with 10% FBS were 0.018–0.025 μmol/L and 0.22–0.24 μmol/L, respectively. It is well known that most of blood Se and Cu are covalently coupled with proteins such as Se and Cu dependent enzymes, and only a small fraction of Se and Cu is in ionic form. Because Se and Cu salts are in ionic forms in culture media, it is necessary to use the lowest concentrations of Se and Cu to study their interaction. The data may help to explain Se and Cu interaction when high doses are consumed in gastrointestinal tract because HT-29 cells are colon cancer cells. Previous studies have demonstrated that selenite can be more active than selenocystine in the generation of superoxide and in the reduction of cytochrome c by glutathione, and Cu (II) has been shown specifically to inhibit the selenite-catalyzed generation of superoxide [23,24]. The present finding is therefore consistent with the previous observation, suggesting that Cu may interact with inorganic but not organic form of Se.

The complexing of Se with Hg, Cu and Cd has been suggested as a mechanism for reversal of Se toxicity in animals [11,25]. The earlier report demonstrated that the pretreatment with copper did not have protective effect on selenite-induced cytotoxity [12]. An attractive hypothesis regarding the interaction between Cu and selenite is that they interact extracellularly [12,24]. However, such an interaction at the cellular level remains to be demonstrated.

The regulation of gene expression is an essential feature in maintenance of normal cellular function. The binding of transcription factors to their *cis*-DNA elements is a critical step in the gene transcription process. SP-1 transcription factor bound to GC-rich elements is ubiquitously expressed in mammalian tissue and is essential for the constitutive expression of many housekeeping genes [26]. The observation that SP-1 DNA binding activity is inhibited much less by the combination of selenite plus Cu than by Cu or selenite alone suggests that the direct interaction could take place in the extracellular biological buffer environment. Furthermore, Cu (1.25 μmol/L) in culture media significantly reduced intracellular Se content when cells were exposed to 5 μmol/L selenite. These findings provide direct evidence that Cu (II) can interact with selenite extracellularly and block selenite from getting through the cell membrane. It is likely that one copper ion may interact with multiple selenite molecules. However, the stoichiometry of the Cu-selenite complexing and whether that complexing occurs at even lower concentrations remain to be determined, and such information is needed for human nutrition and mineral supplementation design.

Taken together, our results demonstrate that selenite and selenocystine can cause cell cycle arrest via different mechanisms, and show that Cu may interact directly with selenite extracellularly with high specific affinity.

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