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EFFECT OF METHYLATED FORMS OF SELENIUM ON CELL VIABILITY AND THE INDUCTION OF DNA STRAND BREAKAGE

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Abstract—Selenobetaine (SB) and selenobetaine methyl ester (SBME) are methylated selenonium derivatives that undergo metabolism to release methyl selenide and dimethylselenide, respectively, as primary metabolites. Since methylation of selenium is considered to be detoxifying, the toxicologic activity of SB or SBME may differ from that of inorganic forms of selenium, such as selenite, that undergo reduction and can induce cell damage. In this study, the effects of SB, SBME and selenite on the viability and long-term growth potential of a mouse leukemia cell line (L1210) were compared. Treatment with 20 μM selenite reduced the rate of cell doubling and the long-term growth potential of cells as measured by colony-forming ability. These effects of selenite were accompanied by a reduction in DNA integrity, assessed by alkaline elution analysis for single-strand breaks. Exposure to 500 μM SB or SBME for 24 hr reduced the colony-forming ability of cells in the absence of any effect on dye exclusion or induction of single-strand breaks in DNA. Exposure of cells to 500 μM SB or SBME resulted in levels of intracellular selenium similar to those after exposure to 20 μM selenite. These observations indicate that it is possible to maintain high intracellular levels of selenium, by exposure to methylated selenocompounds, without affecting DNA integrity. These findings also suggest that DNA fragmentation resulting from exposure to selenite occurs during its reductive metabolism and not from the accumulation of a methylated metabolite of selenium. The fact that SB or SBME reduced the ability of L1210 cells to form colonies in agar in the absence of either DNA fragmentation or any effect on the ability of treated cells to exclude a vital dye suggests that both methylated compounds alter the long-term proliferative potential of cells via a mechanism(s) distinct from that associated with cell injury and death by necrosis. Efforts are underway to determine the origin of these effects.

Selenium is an essential nutrient [1, 2], although its toxicity at levels higher than the dietary requirement is widely recognized [3]. A relatively narrow range of exposures separates the level of selenium essential for health from that which is toxic [4]. Because selenium is present in only trace amounts in most foods, toxicity is rare. However, there is increasing evidence that selenium has potent anticarcinogenic activities at levels of intake above the physiologic requirement in the diet (0.1 to 0.3 ppm), i.e. at levels of 2–5 ppm dietary selenium [5–7]. Because little is known about the adverse effects of chronic exposure to these levels of selenium, there is caution concerning the use of this element as an anticarcinogenic agent. Consequently, a need exists to define the mechanisms of both the toxicity and anticarcinogenicity of selenium and to determine if they are dissociable. It is important to bear in mind that the biological activity of selenium is a consequence of its particular chemical form and not of the element *per se* [3]. As shown in Fig. 1, reduction and methylation reactions are prominent features of selenium metabolism in animals and thereby metabolism can confer or eliminate biological activity. There is evidence that an inorganic form of selenium, such as hydrogen selenide, is the precursor for its incorporation into glutathione peroxidase [8]. Alternatively, hydrogen selenide undergoes methylation, and recent studies suggest that methylated selenides may be the forms of selenium active in cancer chemoprevention [9–11]. This is relevant to the question of anticarcinogenicity and toxicity because the methylation of selenium is considered to be detoxifying [3].

Our laboratory recently found that a 24-hr exposure of L1210 cells to 5 μM selenite, a widely investigated and highly effective anticarcinogenic form of selenium, induced single-strand breaks in DNA that were detectable by alkaline elution analysis.¶ The cause of this DNA damage has yet to be identified, and its relationship to the anticancer activity of selenium is unknown. We report here studies with selenobetaines (SBs**), methylated selenocompounds that generate the distal methylated metabolites independent of hydrogen selenide (Fig.

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** Abbreviations: SB, selenobetaine; SBME, selenobetaine methyl ester; and FBS, fetal bovine serum.

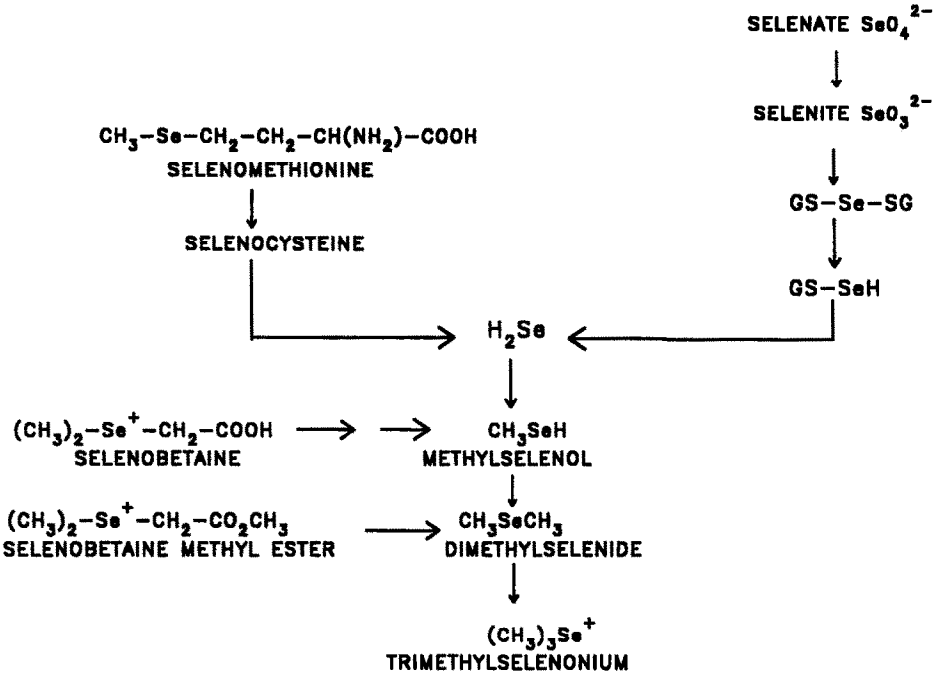

Fig. 1. Intermediary metabolism of selenium.

1). SB tends to lose a methyl group before conversion to methylselenol whereas selenobetaine methyl ester (SBME) undergoes facile breakage and release of dimethylselenide [10]. The effects of these betaines on cell viability, colony-forming ability and the induction of DNA strand breakage in L1210 cells were investigated.

MATERIALS AND METHODS

Selenium compounds. Sodium selenite was purchased from J. T. Baker as $Na_2SeO_3 \cdot 5H_2O$. SB and SBME were synthesized as described elsewhere [11].

Cell culture. Murine lymphocytic leukemic cells (L1210) obtained from the American Type Culture Collection were maintained in suspension culture in RPMI 1640 with phenol red and bicarbonate (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. Cells were grown in 75 cm^2 tissue culture flasks (Corning, NY) at 37° in a 5% CO_2 atmosphere. Cells were subcultured prior to confluence ($<1.5 \times 10^6$ cells/mL) and seeded at 125,000 cells/mL. For selenite treatment, stock selenite (1 mg selenium equivalent/mL) was diluted to 800 μM with RPMI 1640 medium. The stock solutions of SB and SBME were diluted similarly with RPMI 1640 medium and adjusted to a neutral pH with HEPES buffer. The working solutions of each compound were added to the cell suspension to give the required final concentration.

Cell viability. Cell viability was defined by trypan blue exclusion (0.5%, w/v) after a 5-min incubation. Long-term ability to propagate was assessed by colony formation. Following a 24-hr exposure to selenium in its various forms, L1210 cells were centrifuged (500 *g* for 10 min) and washed twice with medium. Cells were resuspended at 100,000/mL and serial dilutions (×10) were made down to 100 cells/mL; 1.5 mL of cell suspension was taken in triplicate and added to 4.5 mL agar [final agar concentration 0.1% (w/v) in RPMI/10% FBS/2 mM glutamine]. Initial cell numbers ranged from 150 to 15,000 cells/tube and cells were incubated at 37° for 7–10 days until colonies grew visible for counting.

Alkaline elution analysis. The basic principles and methodology involved in the detection of DNA damage by the pumping method of alkaline elution have been reviewed in detail [12]. A detailed description of this procedure will be outlined elsewhere.*

Selenium analysis. Cells were harvested via filtration onto glass fiber discs (0.2 μM pore size, Whatman, Maidstone, U.K.) and washed with phosphate-buffered saline. Filters were transferred to digestion flasks and wet ashed in a concentrated nitric/perchloric acid mixture (3:1, v/v). Selenium content of the digest, measured as diaminonaphthalene reactive fluorescence, was determined as previously described [13].

Statistical analysis. Data were evaluated for their compliance with assumptions of distributional normality and then subjected to analysis of variance and/or regression analyses [14].

RESULTS

Selenium accumulation

When exponentially growing L1210 cells were

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Table 1. Effect of form and concentration of selenium on the ability of L1210 cells to exclude dye and form colonies in soft agar

Selenium compound	Concentration (μM)	Cell number ($\times 10^5$/mL)	Percent excluding dye (%)	Colony-forming ability (% of controls)
None	—	7.5 ± 0.2[a]	98.6 ± 0.8[a]	100.0 ± 5.9[a]
Selenite	5	6.7 ± 0.2[b]	98.5 ± 0.4[a]	106.0 ± 11.9[a]
	10	4.9 ± 0.2[c]	98.6 ± 0.3[a]	44.3 ± 10.3[b]
	20	5.1 ± 0.2[c]	98.1 ± 1.1[a]	32.3 ± 6.4[b]
Selenobetaine	20	8.3 ± 0.5[a]	97.8 ± 0.1[a]	118.4 ± 24.7[a]
	100	7.0 ± 0.1[a]	98.7 ± 0.2[a]	70.0 ± 15.9[a]
	500	6.3 ± 0.6[b]	99.1 ± 0.3[a]	48.6 ± 14.7[b]
Selenobetaine methyl ester	20	8.3 ± 0.9[a]	99.4 ± 0.3[a]	104.8 ± 8.8[a]
	100	7.6 ± 0.5[a]	98.7 ± 0.1[a]	93.0 ± 7.6[a]
	500	6.0 ± 0.3[b]	97.6 ± 0.4[a]	34.01 ± 8.1[b]

L1210 cells were seeded at 1.25×10^5 cells/mL and incubated for 24 hr in a medium containing the form and concentration of selenium as indicated. Each value is the mean ± SEM of two experiments, in which each treatment was evaluated in triplicate flasks. Values in a column with different superscripts are statistically different, $P < 0.05$.

treated with graded concentrations of selenite (0–20 μM), SB (0–500 μM) or SBME (0–500 μM) for 24 hr, total cellular selenium increased in a concentration-dependent manner for each compound (Fig. 2). Regression analyses of these data indicate that the increase in response to selenite was linear ($r^1 = 0.97$, $P < 0.01$), whereas the increase in response to SB or SBME was curvilinear ($P < 0.01$). We observed that exposure to 500 μM SB was required to give an intracellular selenium level similar to that found following 20 μM selenite. The intracellular selenium level following exposure for 24 hr to 500 μM SBME was lower than that observed with 20 μM selenite or 500 μM SB.

Cell number and viability. Table 1 shows the effect of form and concentration of selenium on the number and the viability of L1210 cells. As the concentration of selenium increased, the number of cells observed following a 24-hr incubation period was lower, indicating a reduction in cell doubling. The effect of form of selenium was selenite ≫ SBME > SB. The ability of cells to exclude trypan blue was greater than 90% following a 24-hr exposure to all forms and doses of selenium. However, the ability of L1210 cells to form colonies in agar was reduced significantly following exposure to ≥10 μM selenite and to ≥100 μM SB or 500 μM SBME (Table 1). Collectively these data indicate that in the absence of membrane damage associated with loss of ion homeostasis and necrosis, cells treated with SB or SBME and judged viable by dye exclusion did not retain long-term proliferative capacity measured by colony formation.

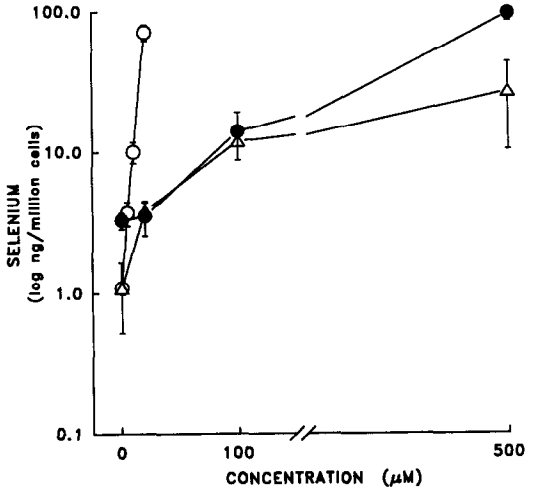

Fig. 2. Effect of form and concentration of selenium in medium on the intracellular concentration of selenium. L1210 cells were exposed for 24 hr to selenite (○—○), SB (●—●), or SBME (△—△).

Alkaline elution analysis. Figure 3 shows the alkaline elution profile for single-strand breaks in DNA of selenium-exposed cells. Cells were exposed to graded concentrations of selenite, SB or SBME. Treatment with up to 500 μM SB or SBME for 24 hr failed to induce DNA strand breaks. In contrast, 20 μM selenite induced extensive single-strand breakage of DNA. Furthermore, the alkaline elution profile observed in selenite-exposed cells was biphasic. This profile indicates that some cells may have contained random breaks and that other cells had no breaks in their DNA or that the DNA within each cell was broken in a non-random manner.

DISCUSSION

Our laboratories have been investigating the mechanism(s) that accounts for selenium toxicity and recently observed that exposure of L1210 cells to sodium selenite, at concentrations greater than 5 μM, induced single-strand breaks in DNA detected by alkaline elution analysis.* This effect of selenite, which is dependent on concentration, duration of exposure, and passage number of exposed cells is in agreement with findings from other laboratories that have reported DNA fragmentation in cells exposed to selenite [15, 16]. Work by others has indicated

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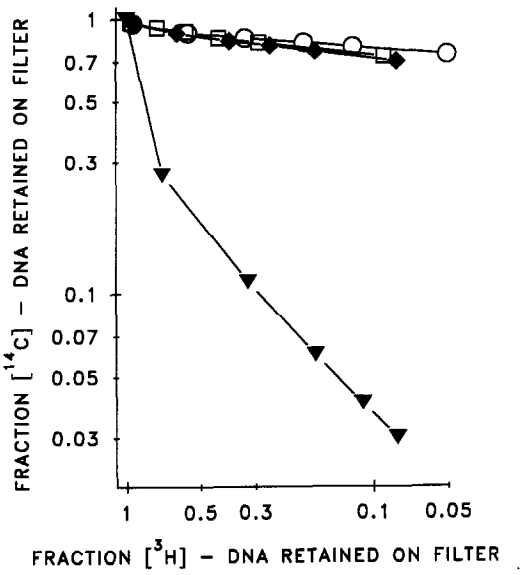

Fig. 3. Effect of form of selenium on the induction of alkaline labile damage to DNA measured as single-strand breaks. L1210 cells were exposed for 24 hr to medium containing no supplemental selenium (○), 20 μM selenite (▼), 500 μM SB (□), or 500 μM SBME (◆).

that a radical species may be formed during the reaction of selenium with glutathione [17] while other data indicate that a DNA damaging species is formed during the oxidative metabolism of hydrogen selenide (H_2Se) [16]. A different line of investigation indicates that the toxicity of selenite is mediated by a reduction in cellular NAD(P)H levels resulting from glutathione-dependent redox cycling [18]. Further studies have shown that DNA damage plays a modulating role in selenite toxicity by activation of poly(ADP)ribose polymerase that leads to a reduction in NAD levels and impaired energy production [15]. The evidence to date, therefore, indicates that selenite does not induce DNA fragmentation directly but rather must first be activated to a genotoxic species. The role of oxygen-centered radicals in this process has yet to be clarified [15, 16].

In this study, we exposed cells to concentrations of SB or SBME that resulted in intracellular levels of selenium similar to that achieved by a concentration of selenite that induces a significant amount of DNA damage. SB and SBME enter the intermediary pathway of selenium metabolism beyond the initial reductive steps of metabolism (Fig. 1). Under these conditions of concentration and duration of exposure, neither SB nor SBME induced DNA strand breaks detectable by alkaline elution analysis. This finding implies that the reductive metabolism of selenium is the origin of selenite's DNA damaging activity. The fact that a 24-hr exposure to SB or SBME did not alter the ability of cells to exclude a vital dye indicates that it is possible to maintain high cellular levels of selenium without causing cell membrane damage and loss of ion homeostasis. This observation is particularly noteworthy since both SB and SBME inhibit the development of experimentally induced breast cancer [11]. While these observations point to reductive metabolism as an origin of the genotoxic and cell-damaging effects of selenite, they do not address the issue of whether a selenometabolite is directly responsible for DNA fragmentation or if genotoxicity is an indirect consequence of metabolism. This question merits further investigation.

It is of particular interest that exposure to SB or SBME decreased the colony-forming ability of L1210 cells without inducing DNA strand breaks. This is in contrast to the effects observed with selenite and is indicative of a specific mechanism of action distinct from genotoxicity. Caffrey and Frenkel [19] recently reported that selenite decreased the colony-forming ability of HeLa cells. In that study it was suggested that the selenium metabolite responsible for reduced colony-forming ability is selenodiglutathione. Our findings show that colony-forming ability of cells is altered by either SB or SBME. The fact that both compounds enter metabolism below the hydrogen selenide pool suggests that a methylated form of selenium could be responsible for the effect on colony-forming ability. This observation is consistent with other reports that methylated forms of selenium are not inert products of selenium detoxification, but rather are biologically active species [3, 9, 11]. This does not, however, exclude the possibility that intermediates other than methylated derivatives of selenium may be responsible for our observations. Since SB and SBME have been reported to have anticarcinogenic activity, it is critical to determine if this anticancer activity is related to our observations that these compounds reduce the long-term ability of cells to propagate. We have shown recently that selenite induced cell death in L1210 cells not only by necrosis but also by apoptosis, an intrinsic, controlled type of cell elimination.* At present it is not known what relationship, if any, exists between selenium-induced apoptosis and necrosis, nor the relationship of these types of cell death to the anticancer or toxic activities of selenium observed *in vivo*. However, the observed effects of SB and SBME are consistent with effects expected in cell populations undergoing apoptosis. We are currently investigating whether SB and SBME induce cell death by apoptosis and how agents capable of inducing apoptosis affect cell proliferation and differentiation.

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