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DISSOCIATION OF THE GENOTOXIC AND GROWTH INHIBITORY EFFECTS OF SELENIUM

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Abstract—The effects of forms of selenium compounds that enter the cellular selenium metabolic pathway at different points were investigated in a mouse mammary carcinoma cell line. The goal of these experiments was to determine if the genotoxicity of selenium, defined as its ability to induce DNA single-strand breaks, could be dissociated from activities proposed to account for its cancer inhibitory activity. The results demonstrated that growth inhibition, measured as inhibition of cell proliferation and induction of cell death, was induced by all the forms of selenium evaluated. However, sodium selenite and sodium selenide, which are metabolized predominantly to hydrogen selenide, caused the rapid induction of DNA single-strand breaks as an early event that preceded growth inhibition. Interestingly methylselenocyanate and Se-methylselenocysteine, which are initially metabolized predominantly to methylselenol, induced growth inhibition in the absence of DNA single-strand breakage. Differences in the time course of selenium retention, in the occurrence of membrane damage, and in the induction of morphological changes by selenite versus methylselenocyanate were noted. Collectively, these data indicate that different pathways affecting cell proliferation and cell death are induced depending on whether selenium undergoes metabolism predominantly to hydrogen selenide or to methylselenol.

Key words: selenium; apoptosis; DNA strand breaks; methylselenol

Inorganic forms of selenium undergo reductive metabolism through a number of intermediate steps leading to the generation of hydrogen selenide, or they enter directly into this metabolic pool [1]. Examples are illustrated in Fig. 1. Depending on cellular requirements for selenium, hydrogen selenide is either used for selenoprotein synthesis [2] or is further metabolized via methylation in the process of its elimination from the cell [1]. The complete methylation of selenium occurs in three steps in which methylselenol, dimethylselenide and trimethylselenonium are formed. This methylation pathway is considered detoxifying [1].

While the above-referenced metabolic scheme identifies the origins of the nutritional effects of selenium, i.e. the synthesis of selenoproteins using selenium derived from hydrogen selenide [2], efforts to determine the origin of the cancer inhibitory activity of selenium have resulted in divergent viewpoints. Recently, it has been argued that the cancer inhibitory activity of selenium is inseparable from its ability to generate oxy-radicals [3], which are known for their DNA damaging activity [4]. It was hypothesized that oxy-radical species could be generated from hydrogen selenide [5] and/or by reaction between "prooxidant catalytic" selenium species and glutathione and other simple thiol compounds [3, 6]. The idea that cancer inhibitory activity is a consequence of the metabolism of various selenium species to hydrogen selenide and the generation of oxy-radicals is plausible, since evidence has been published indicating that hydrogen selenide can be formed in amounts sufficient to replete selenium-dependent glutathione peroxidase activity from essentially every form of selenium that has cancer inhibitory activity, including methylated selenium compounds as well as those that undergo reductive metabolism [7–12]. An alternative to the oxy-radical hypothesis also has been proposed [7–9]. The cancer inhibitory activity of selenium is hypothesized to be derived from a selenium species generated during its methylation. A likely candidate is methylselenol [7, 8]. Cancer inhibition by this mechanism is postulated to be independent of the biological activity(ies) associated with selenium in the hydrogen selenide pool [7, 8, 13]. This possibility also is plausible since essentially every metabolized form of selenium that has cancer inhibitory activity is ultimately methylated prior to its elimination from the cell [1].

The experiments reported in this study were designed to determine in a mammary tumor cell line if the DNA single-strand breakage activity of selenium could be dissociated from the biological effects that are associated with cancer inhibition. Since the effects of agents that induce free radicals that damage DNA can be detected by methods that measure DNA single strand breaks, we used the sensitive alkaline elution technique as the indicator for this type of genotoxicity. As indicators of cancer inhibitory activity, we evaluated the effects of selenium compounds on both cell proliferation and

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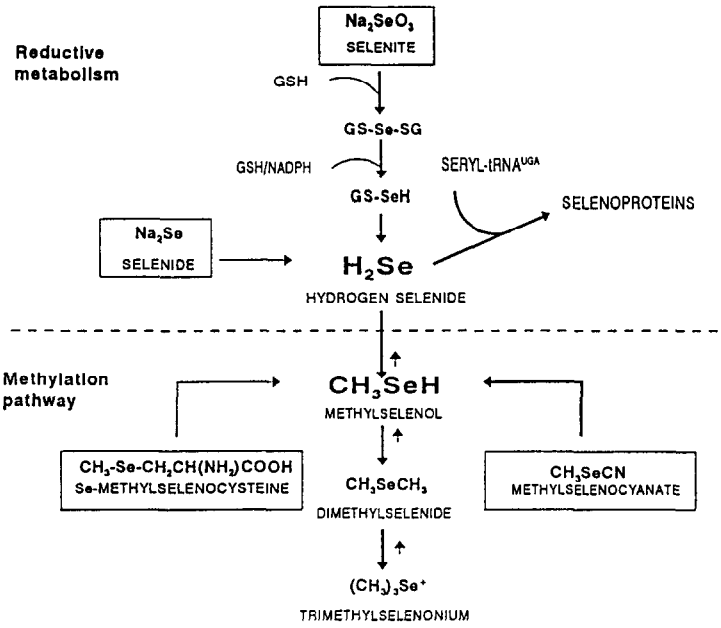

Fig. 1. Schematic representation of the selenium metabolic pathway. Most of the conversion steps were established by Ganther's group for the rat liver [1]. Selenocompounds in boxes were employed in the present study.

cell death, the two parameters that determine tumor growth kinetics. The following two groups of selenium compounds were studied: (i) sodium selenite and sodium selenide, forms of selenium that metabolically generate hydrogen selenide; and (ii) methylselenocyanate and Se-methylselenocysteine, which metabolically convert to methylselenol.

MATERIALS AND METHODS

Chemicals. Sodium selenite pentahydrate was purchased from J. T. Baker, Inc. (Phillipsburg, NJ), and sodium selenide from Alfa Products (Thiokol/Ventron Division, Danvers, MA). Methylselenocyanate was synthesized by reacting KSeCN with an equimolar amount of iodomethane in ethanol at room temperature as described elsewhere [12]. Se-methylselenocysteine was synthesized from DL-selenocysteine (Sigma Chemical Co., St. Louis, MO) as described previously [8].

Cell culture. Cells were cultured as described elsewhere [14]. A subline of the MOD mouse mammary epithelial tumor cell line was used for the experiments reported. It was derived by infection of MOD cells with a retroviral vector carrying the bacterial *lacZ* gene, which encodes β-gal. β-Gal activity is constitutively expressed at high levels in this subline of MOD cells. Much like the assay of lactate dehydrogenase leakage as an indicator for membrane damage, the assay of culture medium from these cells for β-gal activity by the method of Lederberg [15], using *o*-nitrophenylgalactopyranoside (ONPG) as substrate, provides a more sensitive measure of the loss of membrane integrity and is an indirect indicator of cell death. Cell number estimation was accomplished by DNA measurement using a Hoechst dye binding method [16].

Filter elution analyses for DNA strand breaks. Adherent cells were scraped off the culture flasks in ice-cold PBS containing 5 mM EDTA and were evaluated by a gravity-flow filter elution method as described previously [17]. The alkaline elutable fraction estimates the relative extent of DNA single-strand breaks and/or alkaline labile sites, whereas the neutral elutable fraction estimates the relative extent of DNA double-strand breaks. Using this assay, our laboratory has shown that DNA that elutes in the neutral fraction has predominantly the ladder-type pattern of DNA fragmentation characteristic of apoptosis [18]. The elution activities were defined as:

$$\text{Double-strand breaks as neutral elution} = [N/(N + A + F)] \times 100\%$$

$$\text{Single-strand breaks as alkaline elution} = [A/(A + F)] \times 100\%$$

where N is ^3H collected in lysis and proteinase K digestion steps, A is ^3H collected in alkaline elution, and F is ^3H retained on the filter.

DNA nucleosomal fragmentation detection. DNA was isolated and electrophoresed as described previously [14].

Selenium assay. Cells were washed with PBS three times and harvested by trypsinization. Cell pellets after centrifugation at 200 *g* for 5 min were wet ashed, and the selenium content was determined by the fluorometric method as described in Ref. 14.

Rate of DNA synthesis. Cells were pulse-labeled with [*methyl*-^3H]thymidine (20 Ci/mmol, DuPont-

Table 1. Effects of sodium selenite or methylselenocyanate on mouse mammary tumor MOD cell-associated selenium content, adherent cell number, [^{3}H]thymidine incorporation into DNA, and membrane integrity*

Selenium compound	Cellular Se (ng/10^6 cells)	Adherent Cell#/flask ($\times 10^{-6}$)	[^{3}H]Thymidine incorporation relative to control	β-Gal leakage†
		4-hr treatment		
Untreated	BDL‡	5.4 ± 0.3	100 ± 10	1.0 ± 0.1
Selenite				
5 μM	23.5 ± 0.5	5.0 ± 0.3	16 ± 1	3.8 ± 0.5
10 μM	35.8 ± 2.0	4.4 ± 0.2	13 ± 2	4.8 ± 0.4
Methylselenocyanate				
2 μM	3.0 ± 0.2	5.3 ± 0.3	77 ± 1	1.3 ± 0.1
5 μM	4.2 ± 0.3	5.4 ± 0.1	59 ± 5	1.3 ± 0.1
10 μM	5.0 ± 0.3	5.2 ± 0.1	40 ± 2	1.3 ± 0.1
ANOVA P (regression)				
Selenite effect	<0.001	<0.05	<0.01	<0.01
Methylselenocyanate effect	<0.01	NS§	<0.001	NS
		24-hr treatment		
Untreated	0.15	13.3 ± 0.6	100 ± 3	1.0 ± 0.1
Selenite				
5 μM	32.9 ± 0.2	5.8 ± 0.3	7 ± 1	7.4 ± 0.6
10 μM	71.8 ± 9.2	4.0 ± 0.3	8 ± 1	9.9 ± 0.5
Methylselenocyanate				
2 μM	3.2 ± 0.3	8.8 ± 0.2	85 ± 5	1.2 ± 0.1
5 μM	3.6 ± 0.2	5.5 ± 0.2	45 ± 5	3.3 ± 0.5
10 μM	6.2 ± 2.3	2.3 ± 0.2	4 ± 1	18.1 ± 1.5
ANOVA P (regression)				
Selenite effect	<0.001	<0.001	<0.001	<0.01
Methylselenocyanate effect	<0.01	<0.001	<0.01	<0.01

* Values are means ± SD, N = 3 independent flasks.
† The β-gal activity in the medium of untreated cells at the respective time points was taken as unity for normalization.
‡ Below detection limit.
§ NS = not significant.

New England Nuclear, Wilmington, DE) for 1 hr prior to harvesting. The pulse-labeled cells were washed three times with PBS and trypsinized off the flasks. Aliquots were taken for trichloroacetic acid (TCA) precipitation and subsequent scintillation counting to measure the TCA-precipitable ^{3}H and also for DNA measurement. The radioactive counts were normalized against the respective DNA values to estimate the rates of DNA synthesis.

Statistical analyses. The effects of selenium compounds on selected parameters of cellular responses were evaluated by analysis of variance and regression analyses [19].

RESULTS

Cell-associated selenium content. Representative data on the selenium content of adherent cells after treatment with selenite or methylselenocyanate of 4 or 24 hr are shown in Table 1. Selenite treatment increased the cell-associated selenium in a concentration- and time-dependent manner. In contrast, methylselenocyanate treatment at various concentrations resulted in only a small increase in cell-associated selenium content above the level in the untreated cells, and selenium retention did not show any time dependence.

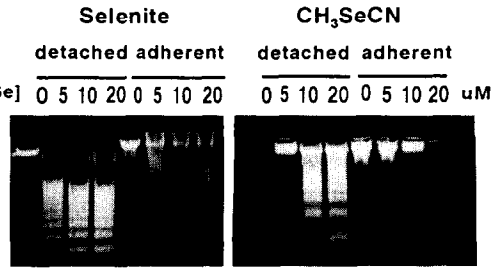

Fig. 2. Gel electrophoretic analysis of DNA isolated from detached cells (dead) and adherent cells after selenium treatment for 24 hr. DNA nucleosomal fragmentation was observable as early as 8–10 hr of selenium treatment. CH_3SeCN = methylselenocyanate.

Effects on cell growth and cell death. To assess the effects of selenite and methylselenocyanate on cell growth, the number of adherent cells after 4 or 24 hr of treatment was estimated by DNA measurement (Table 1). After 4 hr of selenium treatment within the concentration range of 10 μM, methylselenocyanate did not decrease the cell number nor did it affect

cell membrane integrity as measured by leakage of β-gal into the culture medium, whereas selenite induced a selenium concentration-dependent reduction of cell number. The latter coincided with a selenite concentration-dependent induction of membrane damage, i.e. a 3- to 4-fold increase in β-gal activity in the medium (Table 1). By 24 hr of selenium treatment, both selenite and methylselenocyanate reduced the adherent cell number in a selenium concentration-dependent manner. Note that the same magnitude of cell number reduction was achieved with approximately a 10-fold difference of cellular selenium content. The reduction in cell number caused by both forms of selenium was associated with the inhibition of DNA synthesis and the induction of cell death. However, the manner by which each selenium compound influenced these processes was quite different. While selenite treatment at both concentrations dramatically reduced DNA synthesis as measured by [^{3}H]thymidine incorporation into the TCA-precipitable fraction (>84% by 4 hr and >90% by 24 hr, respectively), methylselenocyanate moderately inhibited this parameter in a concentration-dependent manner that was also time dependent (Table 1). Significant differences in cell morphology were induced by these compounds. Selenite treatment induced cytoplasmic vacuolation in patches of cells and often caused sheets of cells to detach and float in the medium (details reported in Ref. 14). Methylselenocyanate treatment caused cells on the periphery of cell patches to retract and detach as small clusters and those cells in the center to become tightly compacted without cytoplasmic vacuolation. The time course of the effect of selenite on DNA synthesis, membrane damage (increase in β-gal activity in the medium) and cell morphological changes was more rapid than the effect of methylselenocyanate (compare 4 hr data in Table 1). Despite the differences observed, cells treated with either selenite or methylselenocyanate detached from the culture flask and displayed DNA nucleosomal fragmentation that is characteristic of apoptosis [20, 21] (Fig. 2).

DNA integrity. Figure 3 presents data from cells treated for 4 hr with selenite or methylselenocyanate on membrane damage, as indicated by leakage of β-gal into the cell culture medium (panel A), and the occurrence of DNA single-strand breaks (panel B) and double-strand breaks (panel C). Selenite treatment caused a concentration-dependent increase in DNA single-strand breaks measured by alkaline elution (Fig. 3B) and double-strand breaks measured by neutral elution (Fig. 3C). Time–course experiments showed that selenite treatment induced significant single-strand breaks as early as 1 hr, which was followed by the appearance of double-strand breaks (Fig. 4). In contrast, methylselenocyanate treatment for 4 hr was nearly devoid of DNA single-strand breaking activity. This was true at a concentration level of methylselenocyanate sufficient to induce a comparable or greater extent of membrane damage than did selenite (Fig. 3A). While the filter elution profile for selenite-treated cells for 24 hr was essentially the same as that at 4 hr with the exception of the reduced magnitude of single-strand breaks, the elution profile for

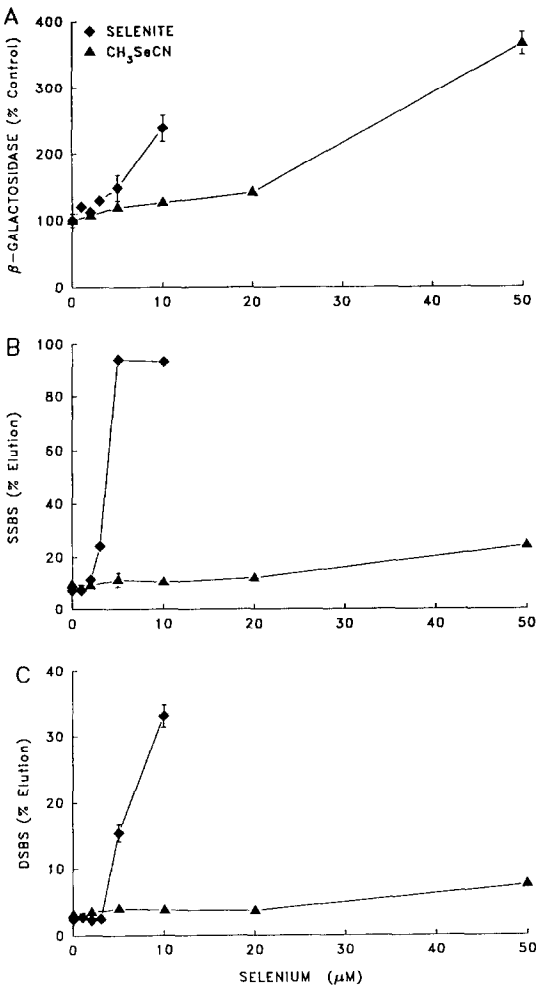

Fig. 3. Effects of treatment with selenite and methylselenocyanate (CH_3SeCN) for 4 hr on cell membrane integrity and DNA strand break induction. (A) Cell membrane damage as measured by leakage of β-galactosidase into the cell culture medium. The β-gal activity of the untreated cells in the culture medium was taken as unity (100%). (B) Single-strand breaks (SSBS) as alkaline elution fraction. (C) Double-strand breaks as (DSBS) as neutral elution fraction. Elution parameters were as defined in Materials and Methods. Each point represents the mean ± 1 SD of triplicate measurements.

methylselenocyanate-treated cells at 24 hr showed a greater selenium concentration-dependent increase in double-strand breaks in the absence of an increase in single-strand breaks (Table 2).

To further evaluate our hypothesis that forms of selenium that are metabolized predominantly to hydrogen selenide induce DNA single-strand breaks whereas those that are metabolized predominantly to methylselenol do not, the effects of two other selenium compounds were examined. These compounds were sodium selenide (Na_2Se) and Se-methylselenocysteine (see Fig. 1). Sodium selenide treatment induced DNA single-strand breaks and double-strand breaks in a pattern similar to that

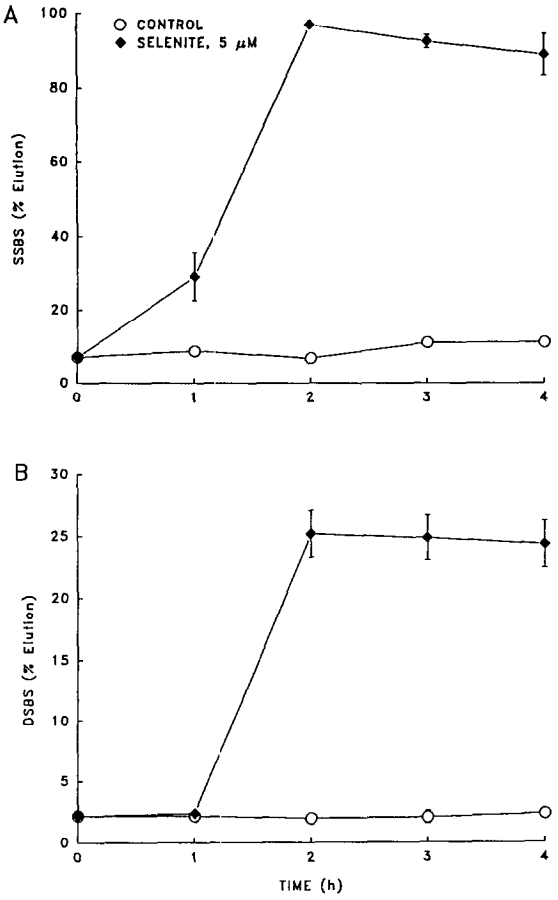

Fig. 4. Time course of selenite treatment-induced DNA single-strand breaks (A) and double-strand breaks (B). Each point represents the mean ± 1 SD of triplicate measurements.

induced by selenite treatment (Fig. 5). Se-methylselenocysteine did not induce DNA single-strand breaks or double-strand breaks at 4 hr (Fig. 5). After Se-methylselenocysteine treatment for 24 hr, significant induction of double-strand breaks and cell death was observed in the absence of single-strand breaks (Table 2).

DISCUSSION

In the experiments reported here, we compared the effects of methylselenocyanate or Se-methyl-selenocysteine with those of sodium selenite or sodium selenide. The former pair of compounds represent forms of selenium that are metabolized predominantly via the methylation pathway without undergoing reductive metabolism, whereas selenite and selenide undergo reductive metabolism prior to entering the methylation pathway (see Fig. 1). While all the compounds inhibited cell proliferation and induced cell death, which are *in vitro* markers of cancer inhibitory activity, only selenite and selenide led to a rapid induction of DNA single-strand breaks, a measure of genotoxicity that can be induced by oxy-radical species. These observations indicate that genotoxicity, as reported in this paper, can be dissociated from growth inhibitory activity, based on the point at which selenium enters the intracellular pathway for its metabolism. The ability of selenite and its proximal metabolites to induce DNA single-strand breaks has been demonstrated in several other cell types [4, 13, 17, 22], and recently our laboratory has reported data linking DNA single-strand breaks to cell death induction in L1210 cells [17]. Because superoxide and other oxy-radicals have been reported to be produced during selenium reductive metabolism [4–6], the induction of DNA single-strand breaks by selenite and its proximal metabolites is likely to be mediated by an oxy-radical-mediated mechanism. In fact, free radicals and membrane lipid peroxidation have been implicated as an underlying mechanism of cell death induction by diverse agents [23]. As recently reviewed by Eastman [24], DNA damaging agents can induce unprogrammed cell death by apoptosis. Selenite and selenide and those selenium compounds that are metabolized predominantly to hydrogen selenide may induce growth arrest and cell death at pharmacological levels of exposure by this mechanism. A recent report [25] indicates that

Table 2. Filter elution profiles of mouse mammary tumor MOD cells treated with selenium compounds for 24 hr

Selenium compound	μM	Single-strand breaks (% elution)	Double-strand breaks (% elution)
Untreated	0	11.2 ± 1.3[c]	3.8 ± 0.2[d]
Sodium selenite	1	51.9 ± 3.8[b]	4.8 ± 0.1[d]
	2	58.6 ± 0.8[a]	5.5 ± 0.3[d]
	5	59.2 ± 4.4[a]	14.6 ± 1.2[b]
Methylselenocyanate	2	8.4 ± 0.2[c]	3.3 ± 0.2[d]
	5	8.0 ± 0.8[c]	9.1 ± 0.1[c]
	7	9.0 ± 2.5[c]	21.1 ± 0.7[a]
Se-Methylselenocysteine	20	10.5 ± 3.3[c]	3.2 ± 0.2[d]
	50	8.3 ± 0.4[c]	4.1 ± 0.5[d]
	100	7.5 ± 1.0[c]	11.7 ± 0.2[b,c]

Values are means ± SD, N = 3 measurements.
[a–d] Different superscripts indicate significant differences among means ($P < 0.05$).

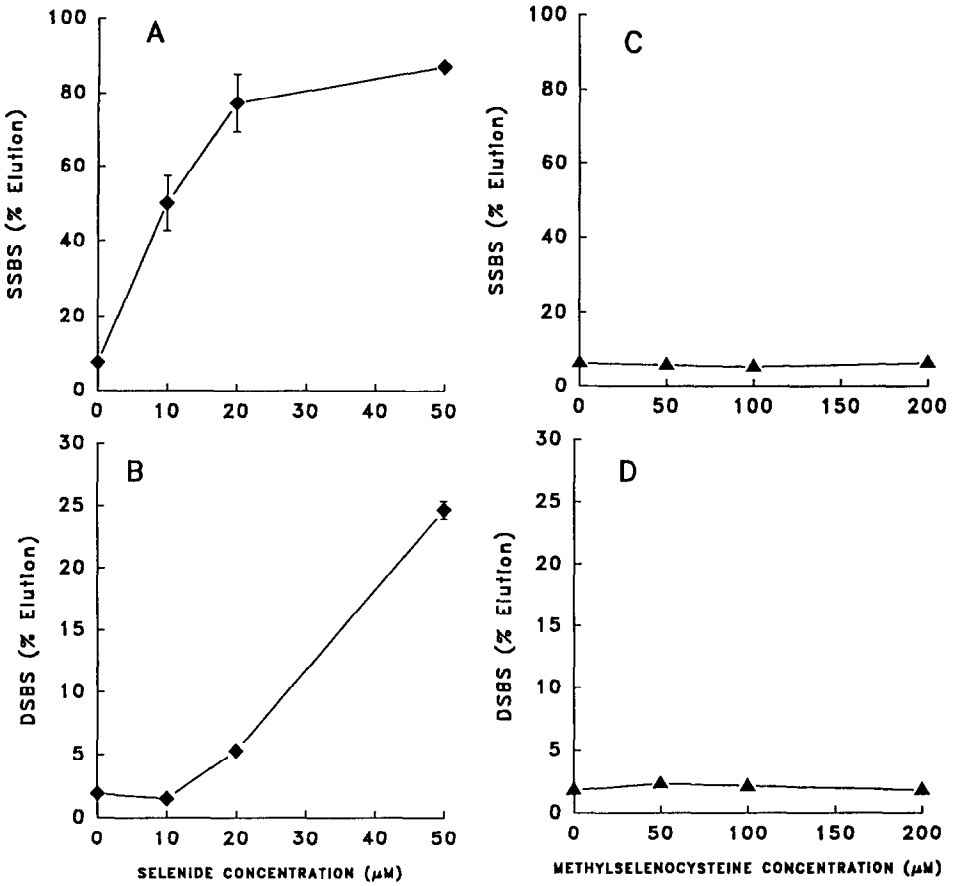

Fig. 5. Effects of treatments for 4 hr with sodium selenide (A, B) and Se-methylselenocysteine (C, D) on DNA strand break induction. The concentration ranges chosen produced a comparable degree of membrane damage as assessed by the β-gal leakage assay. Each point represents the mean ± 1 SD of triplicate measurements. SSBS = single-strand breaks; and DSBS = double-strand breaks.

selenodiglutathione, an intermediate in the reductive pathway between selenite and hydrogen selenide, induces p53 expression at the protein level. This observation is consistent with the role of wild-type p53 protein as a cell cycle check point to ensure repair of DNA damage [26, 27]. Therefore, genotoxic selenium compounds are likely to induce cell death by a p53-dependent pathway. The fact that methylselenocyanate and Se-methylselenocysteine did not induce DNA single-strand breaks yet inhibited cell proliferation and induced cell death indicates that different molecular mechanisms are induced by these different selenium compounds. Because of their lack of DNA single-strand breaking activity, it is speculated that methylselenocyanate and Se-methylselenocysteine induce growth inhibition by a mechanism that is independent of the p53-mediated pathway. Identification of this pathway may provide insights into both the specific mechanism of selenium chemopreventive activity and the specific gene products and genetic lesions that identify the subpopulations of tumors, the development of which selenium can be anticipated to inhibit.

In summary, the present data indicate that in cell culture, the pathways to cancer cell growth inhibition induced by methylselenol precursors are different from those of selenium compounds that induce DNA single-strand breaks as an early biochemical event. Future work will focus on identifying the genetic and cellular sequence of events in methylselenol-induced growth inhibition. In the development of new selenium compounds for cancer prevention, the design of chemical forms of selenium that bypass reductive metabolism and directly enter the intracellular methylselenol pool appears warranted to avoid the genotoxicity (i.e. DNA single-strand breakage) associated with selenium compounds that undergo reductive metabolism.

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