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Differential Induction of Growth Arrest Inducible Genes by Selenium Compounds

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ABSTRACT. The effects of two types of selenium compounds on the expression levels of growth arrest and DNA damage-inducible (*gadd*) genes and on selected cell death genes were examined in mouse mammary MOD cells to test the hypothesis that the diversity of selenium-induced cellular responses to these compounds could be distinguished by unique gene expression patterns. Whereas the expression patterns of known cell death-related genes (*bcl-2* and *bax*) were not informative with respect to the cellular response patterns upon exposure to selenium compounds, time-dependent and selenium species-specific induction patterns were observed for *gadd34*, *gadd45* and *gadd153* genes. It was also observed that the MOD cells expressed a truncated *p53* transcript but no detectable immunoreactive P53 protein, indicating a null *p53* phenotype. The fact that selenium compounds induced growth arrest and death of these cells and that these compounds induced specific patterns of expression of *gadd* genes indicates that these genes may mediate some selenium-induced cellular responses. The findings further imply that selenium compounds may be effective chemopreventive agents for human breast carcinogenesis, in which *p53* mutations are frequent. BIOCHEM PHARMACOL 53;7:921–926, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. selenium; *gadd* gene expression; *p53*; mammary cells

It is well established that the biological activity of selenium is dependent on the chemical form of selenium and not the element *per se* [1, 2]. The specificity of the cellular response to a particular form of selenium can be assessed in cell culture models without the complications of systemic metabolism. We have described, in several recent reports, the effects of different inorganic and organic selenium compounds on cell membrane integrity, cell proliferation, cell death, and DNA fragmentation in cell culture models [3–6]. In designing the experiments in this study, it was hypothesized that the diversity of selenium-induced cellular responses could be classified into two broad categories that might be distinguished by unique patterns of gene expression. Based on our previous work, the two response categories were: (1) growth inhibition due to DNA single-strand breaks, accompanied by a decrease in cell proliferation and an increase in cell death that manifested as both necrosis and apoptosis; and (2) growth inhibition due to a decrease in cell proliferation and an increase in cell death in which apoptosis was the prominent mechanism and with no involvement of DNA single-strand breaks. Representative compounds that induced these two types of cellular responses were selenite and selenide (Category 1 response) and MSeCN‖ and Se-methylselenocysteine (Category 2 response), respectively. Because the panel of endpoints described above included growth arrest, DNA damage, and cell death induction, we selected four genes associated with growth arrest and DNA damage responses, *gadd34*, *gadd45*, *gadd153* [7–10] and *p53* [11–15], and two genes involved in the regulation of apoptotic cell death, *bcl-2* and *bax* [16–19] for investigation. The effects on cultured mouse mammary epithelial MOD cells of the concentration and duration of treatment with selenite and MSeCN on the expression of these genes were investigated at the steady-state mRNA level by Northern blot analysis. The effects on gene expression were studied at the selenium concentrations of each compound that gave a comparable extent of cell number reduction within 24 hr of treatment.

MATERIALS AND METHODS

Chemicals

Sodium selenite pentahydrate was purchased from J. T. Baker, Inc. (Phillipsburg, NJ). MSeCN was synthesized by Dr. S. Vadhanavikit as described elsewhere [20].

Cell Culture

A subline of the MOD mouse mammary epithelial tumor cell line was used. Cells were cultured as described else-

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‖ *Abbreviations:* MSeCN, methylselenocyanate.

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where [3–5]. Briefly, near confluent cells were trypsinized and plated in either 75- or 25-cm^2 tissue culture flasks. Twenty-four hours after plating when cells were in the log phase of growth, the culture medium was replaced with fresh medium, and a specific selenium compound was added. At 4 and 24 hr after selenium addition, RNA was extracted for gene expression detection by Northern blot analyses. Cell enumeration, membrane integrity, DNA synthesis, filter elution assay for DNA strand breaks, and flow cytometry analysis were carried out as previously described [3, 4].

RNA Extraction and Northern Blot

Cells were washed three times in ice-cold PBS and gently lysed in 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 8.6, 0.5% Triton X-100, 1 mM dithiothreitol, 20 mM vanadylate ribonucleotide complex. Cell debris and nuclei were removed by centrifugation (1000 *g* for 5 min at 4°). The supernatant was digested in an equal volume of 2x protease K digestion buffer (50 μg/mL in 0.2 M Tris, pH 8, 25 mM EDTA, 0.3 M NaCl, 2% SDS) for 30 min at 37°. Poly $(A)^+$ RNA was enriched by binding to 20 mg of oligo-dT-cellulose equilibrated with binding buffer (0.5 M LiCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 0.1% SDS) for 45 min. Poly $(A)^+$ RNA was electrophoresed in 1.2% agarose gels with formaldehyde, transferred onto Hybond C membranes (Amersham, Arlington Heights, IL) by capillary blotting in 20x SSPE (175.3 g/L sodium chloride, 27.6 g/L $NaH_2PO_4 \cdot H_2O$, 7.4 g/L EDTA, pH 7.4), and cross-linked to the membrane matrix by baking at 80° for 2 hr. Hybridization was carried out in 50% formamide at 42° according to the manufacturer's instructions. The stringency of washes varied with probe affinity. cDNA probes were labeled with α-[^{32}P]dCTP by random priming using an Amersham kit.

Gene Probes

A mouse cDNA probe for *p53* was provided by L. Bemis, AMC Cancer Research Center. The rat *bcl-2* and human *bax* cDNA probes were obtained from J. Reed, La Jolla Cancer Research Foundation, La Jolla, CA. The hamster *gadd34* and *gadd45* cDNA probes were obtained from A. Fornace, Jr., National Cancer Institute, NIH, Bethesda, MD. A hamster *gadd153* cDNA probe was obtained from N. Holbrook, National Institute of Aging, NIH, Baltimore, MD. A rat cyclophilin (clone p1B15) probe was used as an internal control for gel loading correction. The respective cDNA fragments were released from the vector plasmids by digestion with appropriate endonucleases and purified by agarose gel electrophoresis. The purified DNA fragments (25 ng) were used as template for each labeling reaction.

Immunodetection of P53 by Western Blot

Cells were rinsed with ice-cold PBS and lysed in the following buffer: 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 5 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride (added immediately before use). The lysates were scraped off the cell culture flasks and sonicated briefly. The supernatants after 14,000 *g* centrifugation were boiled for 10 min, electrophoresed in an SDS-polyacrylamide gel (10%), and transferred to a nitrocellulose membrane. After blocking non-specific binding with 1% non-fat dried milk–0.5% Tween-20 in PBS, the membrane was incubated with rabbit anti-mouse P53 antiserum (CM-5, Oncogene Science, Inc., Uniondale, NY) and washed. P53 protein was detected by ^{125}I-protein A binding and autoradiography.

Results

Category I Response

EFFECT OF SELENITE. As shown in Table 1, treatment of cells with selenite effectively blocked thymidine incorporation into DNA within 4 hr and reduced adherent cell number in a time-dependent manner. These effects were accompanied by an increase in cell lysis as indicated by a greater leakage of an intracellular enzyme, and by increases in both DNA single- and double-strand breaks. Figure 1B shows that cells exposed to selenite were arrested in the S/G_2-M phases of the cell cycle by 24 hr. Figure 2A shows representative gene expression data by Northern blot analysis. Figure 2B shows densitometric quantitation of the expression level of each gene. The expression level of *gadd34* and *gadd45* increased in a selenium concentration-dependent manner in response to treatment with selenite at both time points, whereas the expression of *gadd153* was absent at 4 hr and was strongly induced at 24 hr. Both *bcl-2* and *bax* were observed to be highly expressed, but the level of expression was unaffected by treatment with selenite.

The steady-state level of a gene transcript that hybridized with the *p53* probe was unaffected. However, as shown in Fig. 3, this cell line appeared to possess a mutant form of *p53*: the mRNA size was truncated in comparison to the wild-type p53 mRNA found in mouse kidney (Fig. 3A) and no P53 protein was detected by the polyclonal P53 antiserum (CM-5) (Fig. 3B). The relevance of this p53 alteration to the cancer inhibitory activity of selenium is discussed later.

Category II Response

EFFECT OF **MSeCN.** As shown in Table 1, MSeCN induced a time-dependent reduction in adherent cell number and in the rate of cell proliferation as measured by thymidine incorporation into DNA. These effects were observed in the absence of DNA single-strand breaks or membrane leakage at 4 hr, but apoptotic cell death was observed beyond this duration of treatment and was accompanied by increased membrane leakage and DNA double-strand breaks in the absence of an increase in DNA single-strand breaks. Figure 1C shows that MSeCN retarded cell transit

TABLE 1. Representative data illustrating the distinct response patterns of MOD cells to selenite and MSeCN

	Selenite (5 μM)		MSeCN (5 μM)	
	4 hr	24 hr	4 hr	24 hr
Cellular selenium*†, ng/10^6 cells	24	36	4	4
Thymidine incorporation* (untreated control = 100)	16	7	59	45
Adherent cell number* (untreated control = 100)	93	44	98	41
DNA single-strand breaks* (untreated control = 1)	12.5	5.3	1	0.7
DNA double-strand breaks* (untreated control = 1)	6.4	3.5	1	2.4
Membrane leakage (untreated control = 1)	3.8	7.4	1.3	3.3
Morphological cell death	Acute lysis	Apoptosis	Null	Apoptosis

* These parameters were assessed on cells that remained adherent to the cell culture flasks after the designated period of selenium treatment and therefore reflected the status of cells that had survived to that point of time. In this model system, cells retract and detach from the culture flask as they undergo apoptotic cell death.

† The selenium content of untreated cells was approximately 0.1 ng/million cells.

into the S-phase of the cell cycle, indicating that the point of its action is likely at the G_1 phase of the cell cycle. It is particularly noteworthy that the observed effects of the two selenium compounds were achieved with differences in cellular selenium contents of approximately an order of magnitude (Table 1).

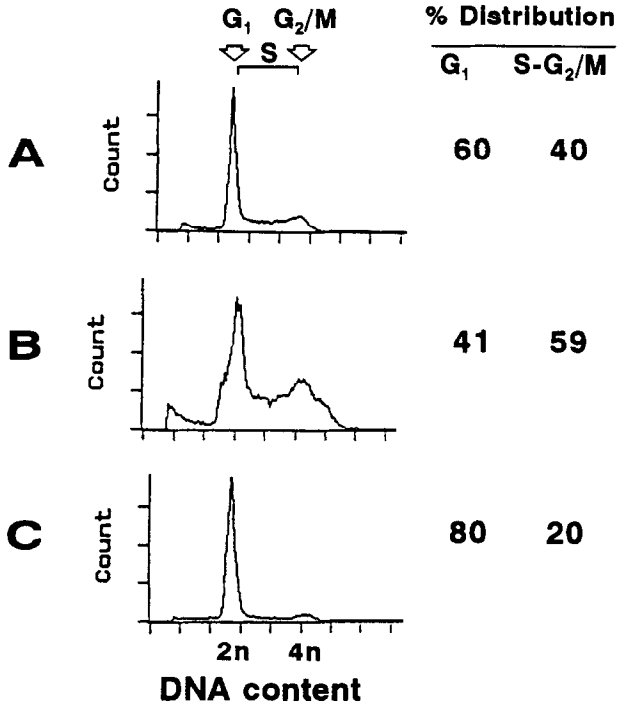


FIG. 1. Flow cytometry analyses of cell cycle distribution of mouse mammary MOD cells after a 24-hr exposure to selenium compounds. Shown here are representative histograms of (A) untreated control cells, (B) cells treated with 5 μM sodium selenite, and (C) cells treated with 5 μM MSeCN. The percent distribution of cells in G_1/G_0(DNA = 2n) and in the other phases (DNA > 2n) is shown to the right of each histogram.

As shown in Fig. 2, MSeCN induced expression of *gadd34* and *gadd45* within 4 hr of treatment, an effect that was diminished at 24 hr. The *gadd153* expression level was elevated at 4 hr of exposure to MSeCN, but was not different from the control at 24 hr. The levels of expression of the truncated *p53* message and of *bcl-2* and *bax* were not affected by MSeCN despite the observation of apoptosis at 24 hr, as evidenced by morphology and oligonucleosomal DNA fragmentation [3].

DISCUSSION

The experiments presented in this study were designed to examine the hypothesis that specific responses to various forms of selenium are associated with distinct patterns of gene expression. The data presented in Table 1 and Fig. 1 provided the basis for the categorization of response as described. It is apparent from the results presented in Fig. 2 that the expression patterns of the *gadd* genes were critically dependent on the chemical forms of selenium, while those of the known cell death-related genes (*bax* and *bcl-2*) were less informative. The finding implies that changes in the expression of the *gadd* genes may reflect the differential effects of the action of the chemical species of selenium in cell culture.

The *gadd* genes were first identified in experiments in which changes in gene expression in response to genotoxic agents were investigated in Chinese hamster ovary cells [7]. The initial rationale for studying the effects of selenium compounds on the expression of *gadd* genes was based on our observation that treatment of cells with selenite induced DNA single-strand breaks [3, 4, 6]. It is paradoxical that at 4 hr of exposure to selenium, a time point prior to

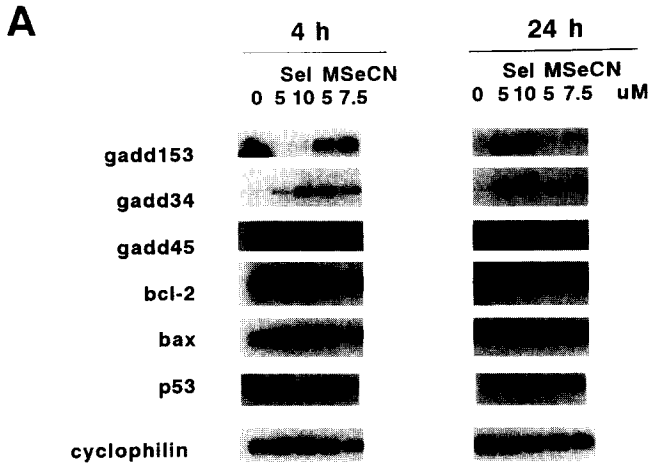
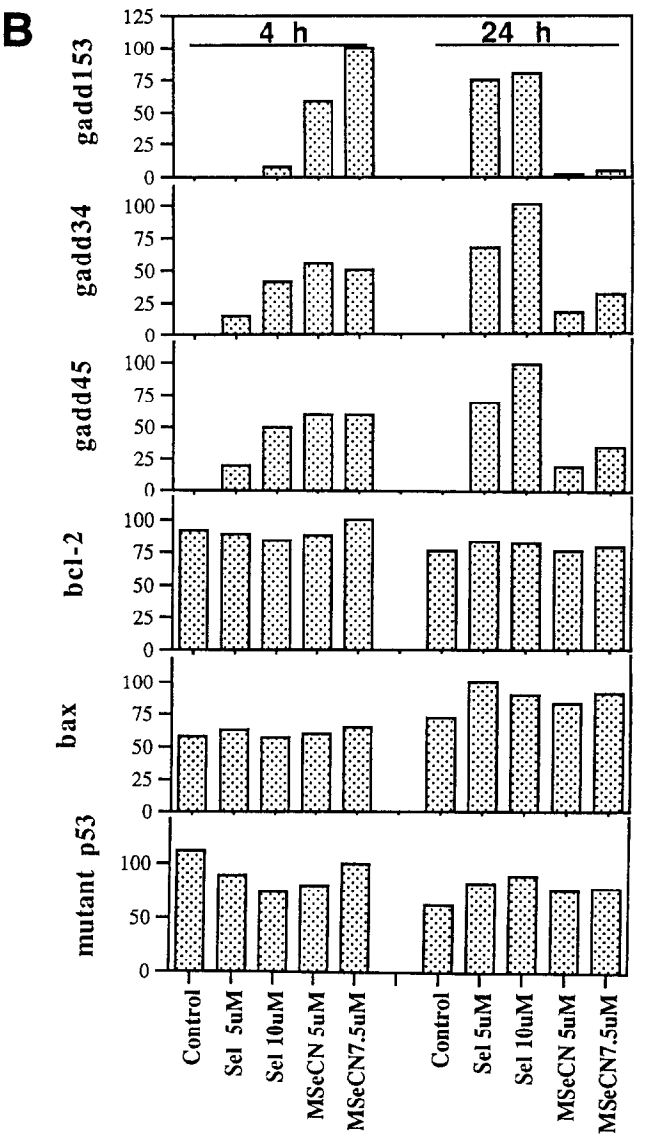


FIG. 2. Gene expression changes induced by selenium compounds in mouse mammary MOD cells. (A) Representative autoradiographs of gene expression by Northern blot analyses of mRNA. Abbreviations: Sel, selenite; and MSeCN, methylselenocyanate. Cyclophilin (clone p1B15) was used to serve as an internal control for correcting gel loading difference. The dark signal in the untreated cells at 4 hr for *gadd153* was due to a spurious ^{32}P spot. (B) Densitometric quantitation. The expression level of each gene was corrected with that of the cyclophilin loading control. The expression level of each gene was normalized to the maximal data point set as 100%. The results presented here were representative of two independent experiments conducted 6 months apart.



the occurrence of observable cellular effects, all three *gadd* genes were induced by MSeCN which did not induce DNA single-strand breaks (Table 1 and [3]). Conversely, selenite failed to induce *gadd153*, even though by this time extensive DNA single-strand breaks were induced by this form of selenium (Table 1 and [3]). These observations are in agreement with reports of induction of *gadd* genes by conditions that do not involve DNA damage [21]. It has been established recently that the *gadd34* gene is the homologue of the murine myeloid differentiation primary response gene *MyD116* and that *gadd45* is homologous to the murine *MyD118* gene [10]. The *gadd153* gene is a homologue of the murine *CHOP-10* gene, encoding a factor that negatively regulates C/EPB-controlled gene transcription [22]. These facts and the report that *gadd* genes cooperate in synergy to cause growth arrest when overexpressed [10] lend credence to the hypothesis that the *gadd* genes may play fundamental roles in cell cycle regulation, cell proliferation, and cell differentiation in addition to the response to DNA damage.

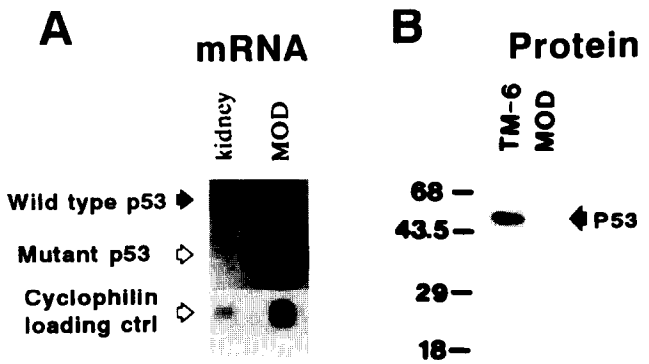


FIG. 3. The *p53* status of the mouse mammary MOD cells. (A) Northern blot detection of *p53* gene transcripts. The size of the *p53* transcript in MOD cells is shorter than that of the wild-type *p53* from a mouse kidney. Cyclophilin was used as an internal control for correcting gel loading differences. (B) Western blot detection of P53 protein. MOD cells lack any immunologically reactive P53, whereas the mouse TM-6 mammary cells contain a readily detectable amount. Molecular weight standards are indicated by the numbers on the left.

Future work will seek to determine the role of *gadd* induction in mediating the cancer inhibitory activity of selenium through strategies that block *gadd* gene functions.

A noteworthy comment is that the MOD cell line appears to lack wild-type *p53*. The present work and our previous work [3–5] as well as unpublished data from our group (Lu *et al.*, 1996) suggest that selenium is effective against cells lacking functional P53. Despite the lack of P53 function in the MOD cells, treatment with either selenium compound investigated here led to growth inhibition and cell death. As both selenium compounds are effective in preventing mammary carcinogenesis *in vivo* [20] and given that mutations in *p53* are among the most common pathogenetic alterations in human breast cancer [15], these data indicate that selenium is a good candidate for a chemopreventive agent for human breast cancer.

Another point requiring additional discussion is that two genes known to be involved in cell death regulation, *bax* and especially *bcl-2*, were observed to be constitutively expressed at high levels in the MOD mammary epithelial tumor cell line. To our knowledge there is little information in the literature concerning the expression of these genes in mammary epithelial cells *in vitro*. Expression of *bcl-2* is associated with protection against the induction of apoptosis, whereas bax expression has been reported to oppose the action of bcl-2, and the bcl-2/bax ratio is thought to be a rheostat for induction of apoptotic cell death [16]. Failure to observe differences in the expression of these genes in response to selenium is difficult to interpret. First, the situation is complicated by the lack of a competent *p53* gene in the MOD cell line used in this study. Given that the *bcl-2* and *bax* genes possess P53-response elements [17–19], it is conceivable that the high constitutive level of *bcl-2* expression was related to the lack of a functional P53, which represses *bcl-2* expression. Unpublished results (Lu *et al.*, 1996) with a p53-competent mammary epithelial cell line (TM12 obtained from D. Medina, Baylor College of Medicine) appear to corroborate this possibility: bcl-2 mRNA was not detectable in TM12 cells by Northern blot analysis even after a 7-day exposure of the X-ray film, whereas it was readily detectable in MOD cells after an overnight exposure under similar detection conditions. Furthermore, treatment of TM12 cells with various selenium compounds resulted in the same patterns of cellular responses as described in this study, yet no change in *bax* expression level was detected. These results indicate that alternative signaling pathways independent of the bax/bcl-2 rheostat, such as the other bcl-2 or bax family members, may mediate the effects of selenium on cell death induction. It is also possible that the regulation of cell bcl-2 and bax function is at the level of protein, rather than that of mRNA, which was measured in the present study. These questions will require further investigation.

In summary, the data presented here indicate that studying the effects of various selenium compounds on the expression of *gadd* genes in appropriate *in vitro* and *in vivo* models may provide new insights about the mechanisms that account for the cancer inhibitory activity of selenium. Differential modulation by selenium compounds of the expression of these genes may set the stage for studying gene- and/or pathway-specific cancer prevention by selenium and other chemopreventive agents.

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