Effects of Selenium on Cell Proliferation in Rat Liver and Mammalian Cells as Indicated by Cytokinetic and Biochemical Analysis

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ABSTRACT

Studies were conducted in vivo with regenerating liver and in vitro with mammalian cells to determine the effects of selenium on cell proliferation and the stages of the cell cycle affected by selenium. Six ppm selenium as Na₂SeO₃ fed to weaning male F344 rats for 6 wk significantly reduced the percentage of ³H-labeled hepatocyte nuclei by one-half compared to 0.1 ppm selenium when [methyl-³H]thymidine was injected at 23 h post-two-thirds hepatectomy. Sampling was done at 30 h post-hepatectomy. A trend towards decreased ³H per DNA per labeled cell was also observed, suggesting that selenium decreased the rate of DNA synthesis as well as delaying the entry of cells into S phase (i.e., increasing the duration of G₂-G₁). Studies in vitro with H-4 "minimal deviation" hepatomas and 3T3 mouse fibroblasts demonstrated that selenium decreased the growth of these cells in a dose-dependent manner, and this inhibition was reversible upon removal of selenium from the growth medium. Cytokinetic analysis using fluorescence flow cytometry and microscopic techniques indicated that selenium treatment increased the duration of G₁, S, and G₂ phases of the cell cycle, while having no effect on mitosis under the conditions of our experiments.

Biochemical analyses of H-4 cells demonstrated that selenium treatment caused a significant dose-dependent increase in oxidized and reduced glutathione (GSSG and GSH) as well as in the GSSG/GSH ratio as was previously observed in liver in vivo. In addition, glutathione reductase activity as well as the oxidized nicotinamide adenine dinucleotide phosphate:reduced nicotinamide adenine dinucleotide phosphate ratio was significantly increased with selenium treatment. These results indicate that selenium affects all "synthetic" stages of the cell cycle, and elevated GSSG or the GSSG/GSH ratio may explain the antiproliferative effects of selenium on cells.

INTRODUCTION

Recent studies from our laboratory have demonstrated that, compared to 0.1 ppm selenium, 3.0 and 6.0 ppm dietary selenium as Na₂SeO₃ fed to rats after diethylnitrosamine administration (28) can decrease the growth of hepatic enzyme-altered foci induced by a carcinogenic dose of diethylnitrosamine without affecting the number of "preneoplastic" lesions. A similar effect was observed when high selenium was fed concurrently with N-acetylaminofluorene, and foci development was monitored over time using quantitative stereological techniques. These results suggest that high dietary selenium can decrease cell proliferation of preneoplastic lesions which may represent a fundamental mechanism by which selenium inhibits or delays carcinogenesis as shown previously. Reports by others have demonstrated decreased cell proliferation by high selenium as indicated by decreased [³H]thymidine incorporation into colon DNA (21), decreased small intestine weights (18), and decreased cell proliferation in vitro (30, 36, 37).

The first purpose of the experiments reported herein was to determine if selenium, at concentrations comparable to those shown to have antitumor effects, can decrease cell proliferation, and if so, what stages of the mammalian cell cycle are affected. Effects of selenium on cell proliferation were studied in vivo using regenerating liver and in vitro using a "minimal deviation" hepatoma cell line and fibroblasts. Fluorescence flow cytometry and microscopic techniques were used for cell cycle analysis. The second purpose of these experiments was to establish a relevant in vitro cell model to study the biochemical effects of selenium which may be related to decreased cell proliferation. Following high dietary selenium feeding as Na₂SeO₃ to rats, the hepatic glutathione status shifted towards a more oxidized state which was subsequently followed by adaptive changes in glutathione metabolism in an attempt to maintain a nearly normal GSSG³/GSH ratio (27). As will be discussed, these alterations in glutathione metabolism may be associated with changes in synthetic processes in the cell necessary for cell reproduction (23). Therefore, the establishment of an in vitro cell system which behaves biochemically similar to the liver in response to selenium could provide a useful approach in the understanding of the effects of selenium on cell reproduction and the role of selenium in carcinogenesis. The cell line chosen for study in vitro was the H-4-II-EC₃ minimal deviation hepatoma, which demonstrates many biochemical characteristics of normal adult liver (45).

MATERIALS AND METHODS

Effects of Selenium on Regenerating Liver. Weanling male F344 rats (Harran-Sprague Dawley, Madison, WI) were received and housed in hanging wire stainless steel cages at 3 rats per cage. A 20% casein diet supplemented with 0.2% methionine and 0.4% threonine as described previously was fed ad libitum. Selenium was supplemented to the diet at 0.1 or 6.0 ppm selenium as Na₂SeO₃ as a selenium-glucose monohydrate premix. The 0.1 ppm selenium-supplemented diet was analyzed fluorometrically (42) to contain 0.13 ppm selenium. Eight rats per experimental group were fed the indicated diets for 6 wk, at which time PH were performed (22). Rats received injections at 23 h post-PH with [methyl-³H]thymidine (specific activity, 20 mCi/mmol in sterile aqueous solution; New England Nuclear) at 1 μCi/g body weight. At

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2 To whom requests for reprints should be addressed.
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30 h post-PH, rats were killed, and their livers were prepared for autoradiography and biochemical analysis. For autoradiography, acetic acid:chloroform:methanol (1:3:8)-fixed standard liver sections were prepared from the liver lobes, and autoradiography was performed using NTB-2 nuclear emulsion (Eastman Kodak, Rochester, NY) with an exposure time of 2 wk at 4°C. The percentages of [3H]thymidine-labeled hepatocyte nuclei were quantitated on hematoxylin:cosin-stained slides, and a nucleus was considered labeled if it contained 5 or more grains. Two samples from each rat were evaluated for labeled nuclei, and the values were averaged. A total of 1500 hepatocyte nuclei was evaluated for each rat. For the determination of the amount of [3H]thymidine incorporated into liver DNA, DNA was isolated according to Schmidt and Thanhauser as modified by Munro (40, 49) and quantitated using diphenylamine (16), and the number of [3H] counts per sample was determined using scintillation techniques. The amount of [3H] in the perchloric acid-soluble fraction of the liver homogenate was also determined as an approximation of the [3H]thymidine specific activity of the liver.

**Cells and Culture Conditions.** H-4-ll-EC3 (H-4 cells) minimal deviation hepatomas (45) were obtained from Dr. V. R. Potter, and NIH-3T3 mouse fibroblasts were obtained from Jon Horwitz of McAllrde Laboratory, Madison, WI. Both cell lines were maintained at 37°C in an atmosphere of 5% CO2:95% air-saturated air. H-4 cells were grown in Swin’s Medium 77 (GIBCO, Grand Island, NY) modified with CaCl2, phenol red, NaHCO3, L-cystine, and L-glutamine (4 mx) according to Morse and Potter (39). The medium was supplemented with 20% horse serum (GIBCO), 5% fetal bovine serum (KC Biologicals, Lenexa, KS), and Penicillin:G:sodium (100 units/ml of medium) (Sigma). Stock cultures of cells were subcultured weekly by trypsin detachment, and cells were fed fresh medium on Days 0, 3, and 5. 3T3 cells were subcultured every 6 days with fresh medium supplied on Days 0 and 3.

**Growth Curves.** Growth curves were determined for cells plated at 1 × 10³ cells (low density) or 3 × 10³ cells (high density) per 50-× 15-mm culture dish (Falcon Plastics, Inc., Los Angeles, CA) with 4 ml of growth medium. Twenty-four h after plating, the growth medium was replaced (defined as T = 0) with medium containing 50 μM Na2SeO3 (control), 50 μM Na2SeO4, or 100 μM Na2SeO3. Aqueous solutions of Na2SeO2 or Na2SeO4 were added to the growth medium immediately before use at a Na2SeO4/Na2SeO2 ratio of 1:20 (vol:vol) to obtain the desired final Na2SeO3 or Na2SeO4 concentrations. Concentrations of 50 and 100 μM selenium were chosen for these studies following preliminary dose-response studies. For those experiments with a 48-h time point, cells were fed fresh medium containing Na2SeO3 or Na2SeO4 at 24 h. For cell counting, cells were detached from the plates with cell dispersion solution (g/liter: sodium phosphate (pH 7.8) containing 1 mM EDTA and 100 μg/ml sodium pyruvate, 100 μg/ml glucose, 1.1; NaCl, 8.0; KCl, 0.4; NaHPO4, 12H2O, 0.39; KH2PO4, 0.15) containing trypsin (0.5 mg/ml) (Sigma) and 0.5 mM EDTA (8) and suspended in trypsin blue. Cell counts were determined with a hemocytometer, and the percentage of viability was quantitated. Population doubling times (TP) were calculated from average cell densities determined for a given treatment group. With the exception of a single 12-h experiment, growth curves of H-4 cells at high and low cell densities were repeated 2–4 times with a minimum of 4 samples per treatment group at each time point per experiment. Growth curves of 3T3 cells were constructed from 1 experiment at both high and low densities with 5 samples per treatment group at each time point.

**Fluorescent Flow Cytometry and Cell Cycle Analysis.** For cell cycle analysis, growth conditions and Na2SeO4 (Na2SeO3) additions were identical to those used for growth curve determinations. Cells were prepared for fluorescent flow cytometry and stained with ethidium bromide (Sigma) at 100 μg/ml in 0.15 M NaCl containing 15 mM MgCl2 as previously described (8, 9). Each ml of staining solution contained approximately 3 × 10⁶ cells. Flow cytometry was performed on a Becton-Dickinson FACS IV (Sunnyvale, CA) with a laser excitation line of 457 nm, and the distributions of cells in G₁, S, and G₂–M were determined from the DNA profiles using computer fit analysis (10). The percentage of mitotic cells was determined directly on cell monolayers washed once with 1% sodium citrate and then incubated in 1% citrate for 10 min followed by cell fixation with ice-cold methanol:acetic acid (3:1). Six hundred cells per plate were scored for mitotic figures. The length of mitosis for a given cell treatment was calculated from the population doubling time and the average mitotic index (50). An approximation of the length of the G₁, S, and G₂–M stages of the cell cycle was calculated from the average percentage of cells in G₁, S, and G₂–M as determined by flow cytometry and population doubling times for a given treatment according to the graphical method of Okada (43).

**Biochemical Analysis.** For biochemical determinations, performed only on H-4 cells, 1.86 × 10⁶ cells were plated per 150-× 25-mm dish with 25 ml of growth medium, which is an identical density to that used for the high cell density and cell cycle kinetic experiments. Na2SeO3 or Na2SeO4 treatment of cells was identical to that described for the growth studies. All biochemical determinations were made after 24 h of cell treatment. For GSH and GSSG determinations, cells were rinsed twice with 0.02 μl EDTA at 4°C and rapidly detached with a rubber policeman into 1.5 ml of 0.02 μl EDTA. Cell suspension (0.9 ml) was then rapidly added to 0.1 ml of cold 50% trichloroacetic acid containing 0.1 nmol HCl and homogenized by hand with a Teflon pestle which tightly fit the centrifuge tube. Samples were centrifuged at 10,000 × g for 10 min (Serva, type SM rotor; Ivan Sorval, Inc., Norwalk, CT), and GSH and GSSG analysis was performed on undiluted supernatants as previously described (27) according to Tietze (52) as modified by Griffith (19) for GSSG. A portion of the original EDTA cell suspension was kept frozen for DNA analysis.

For the determination of GSSG-Rd activity, cells were rinsed twice with 5 mm sodium phosphate (pH 7.6) containing 1 mm EDTA and detached with a rubber policeman into 0.7 ml of the same. Cell suspensions were frozen prior to GSSG-Rd analysis (34) and protein determination.

For pyridine nucleotide analysis, cells were rinsed twice with cold phosphate-buffered saline (g/liter: NaCl, 8; KCl, 0.2; Na2HPO4, 1.14; KH2PO4, 0.2) and rapidly detached with a rubber policeman at 4°C. Extraction and analysis of the nucleotides were performed according to Giblin and Reddy (15). A portion of the unextracted cell suspension was kept for DNA analysis.

To confirm the validity of expressing GSH, GSSG, and pyridine nucleotide concentrations as per μg DNA, the amount of DNA and protein per cell was determined on cells detached with growth medium plus trypsin plus EDTA. One portion of the suspension was prepared for cell counting, while the other was centrifuged (1200 rpm; Model PR-6 International centrifuge for 5 min at 4°C) and washed with phosphate-buffered saline 3 times at 4°C. The washed cell pellet was finally resuspended in distilled, deionized water. DNA was determined fluorometrically (26), and protein was determined according to the method of Lowry et al. (33).

**Statistics.** The data were analyzed for significant effects of selenium using ANOVA, Dunnett’s new multiple range test, and Student’s t test (51).

**RESULTS**

**Effect of Dietary Selenium Concentration on Liver Regeneration.** Six ppm dietary selenium as Na2SeO3 fed to weanling male F344 rats for 6 wk prior to PH caused a significant reduction (P < 0.001) of 50% in the percentage of [3H]thymidine-labeled hepatocyte nuclei compared to 0.1 ppm selenium-fed controls when [3H]thymidine was injected at 23 h post-PH, which represents the time at which maximum DNA synthesis occurs following PH as shown by others (4) (Table 1). The specific activity of [3H]DNA was also significantly decreased (P < 0.01) by 60%
with high compared to low selenium feeding. When the average specific activity per \(^3\)H-labeled cell was calculated, a significant decrease (\(P < 0.05\)) of 22\% with 6.0 ppm selenium was observed compared to 0.1 ppm selenium. Selenium treatment had no significant effect on the amount of DNA per g of liver or the number of counts per minute per g of liver in the perchloric acid-soluble fraction representing the free \(^3\)H pool (data not shown). Six ppm selenium caused a significant reduction (\(P < 0.01\)) in the final rat body weights from 194 ± 5 g in controls to 167 ± 4 g in the high selenium-fed rats.

**Effect of Medium Selenium Concentration on Exponential Cell Growth.** The effect of medium selenium concentration on H-4 cell growth at low and high cell densities is shown in Chart 1. For low density cells, the values shown for each treatment at the 24-h time point represent the means for 10-18 individual samples collected from 2-4 separate experiments. Selenium treatment caused a significant decrease in cell number (\(P < 0.005\)) in a dose-dependent manner at 50 and 100 \(\mu\)M selenium compared to controls and, within the limits of this determination, established a new steady-state growth rate through 48 h of selenium treatment. When selenium-containing medium was replaced with control medium after 24 h of selenium treatment, cells previously exposed to selenium grew at a rate comparable to or slightly greater than controls as determined at 48 h. There was no evidence of cell loss into the medium, and viability was greater than 95\% with all treatments. H-4 cells grown at high density also showed a significant decrease of cell number due to selenium (\(P < 0.005\)) without cell loss or decreased viability at 24 h of treatment. Selenium treatment of high density cells caused a greater growth inhibition by 48 h compared to low density cells. Cell detachment and shedding into the medium preceded by cell “rounding up,” indicative of toxicity, were observed with continuous 100 \(\mu\)M selenium treatment of high density cells by 48 h. The effect of selenium on growth of high density cells observed at 24 h was also reversible when control medium was fed to the cells previously fed selenium. Population doubling times, determined after 24 h of selenium treatment and used in the calculations for cell cycle stage durations, are shown in Tables 2 and 3 for the H-4, low and high cell density experiments, respectively. A single experiment conducted at 12 h after selenium feeding indicated that the relative rates of cell growth with selenium treatment compared to controls were similar to those observed at 24 h of selenium treatment (data not shown).

**Table 1**

<table>
<thead>
<tr>
<th>Treatment (ppm selenium)</th>
<th>% of (^3)H-labeled nuclei</th>
<th>(^3)H/mg DNA (10(^{-8}) x cpm/mg)</th>
<th>(^3)H/mg DNA/labeled nucleus (10(^{-8}) x cpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>26.7 ± 2.7(^a)</td>
<td>6.37 ± 0.63</td>
<td>24.8 ± 2.2</td>
</tr>
<tr>
<td>6.0</td>
<td>13.8 ± 2.1(^b)</td>
<td>2.64 ± 0.55</td>
<td>18.9 ± 1.9(^c)</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± SE for 8 animals per treatment group.
\(^b\) Significantly different from 0.1 ppm selenium at \(P < 0.001\) according to the Student\'s t test.
\(^c\) Significantly different from 0.1 ppm selenium at \(P < 0.05\).

The growth of 3T3 fibroblasts was also significantly decreased (\(P < 0.005\)) by selenium in a dose-dependent manner at low and high cell densities analogous to what was observed with H-4 cells (Chart 2). The effect of selenium on growth was also reversible at low density, but cell growth did not recover to a control rate when control medium was added after selenium treatment at the high density. Cell detachment and shedding into the medium were observed with 100 \(\mu\)M selenium treatment by 48 h at both low and high cell densities. Population doubling times calculated at 24 h for low density, 3T3 cells are listed in Table 4. **Fluorescence Flow Cytometry and Cell Cycle Kinetics.** Typ-
The values shown in Table 2 for low density represent the mean of 11 individual samples obtained from 3 separate experiments. Each sample was run in duplicate on the FACS IV. Selenium treatment caused a significant decrease \((P < 0.005)\) in the percentage of cells in G1 compared to controls (Table 3). In addition, a significant decrease \((P < 0.005)\) in the percentage of cells in S phase was observed, while selenium had no effect on the percentage of cells in G2-M. In terms of cycle stage durations, G1 was again increased by selenium in a dose-dependent manner (Table 3). S and G2-M were unchanged with 50 \(\mu M\) selenium but were increased with 100 \(\mu M\) selenium. The length of M was not determined at the high density. Therefore, with the H-4 cells, at 12 and 24 h as well as at low and high cell densities, selenium treatment caused an increase in the duration of all stages of the cell cycle except M; G1 was the stage initially affected at the lower selenium concentration.

In order to compare the results obtained with the H-4 cells to a non-tumor-derived cell line, cell cycle kinetics was determined on 3T3 cells in the presence and absence of selenium. Cell cycle kinetics was determined only at low cell densities. Representative DNA profiles from 3T3 cells are shown in Chart 3. The values for the percentages of distribution of cells in G1, S, G2, and M are shown in Table 4. The values shown represent the mean of 7 samples for each treatment from 2 separate experiments. Each sample was run in duplicate on the FACS IV. Selenium treatment caused a significant decrease \((P < 0.005)\) in the percentage of cells in G2-M. Stage durations for 3T3 cells are shown in Table 4. Similar to what was observed with the H-4 cells, selenium caused a significant dose-dependent decrease \((P < 0.005)\) in the mitotic index compared to controls but had no effect on the length of mitosis. Selenium treatment caused the largest percentage of increase in the duration of G2 compared to controls; however, the increase in G2 was not enough to account for the entire increase in the population doubling time; thus increases in the duration of G1 and S were also observed. Therefore, while some differences were present, both H-4 and 3T3 cell lines showed an increase in the duration of all stages of the cell cycle except mitosis with selenium treatment.

**Biochemical Analysis.** As a possible explanation for the decreased rate of cell proliferation observed with selenium, glutathione status of H-4 cells was determined. These studies were conducted at high cell density. Selenium at 50 and 100 \(\mu M\) caused a significant dose-dependent increase \((P < 0.005)\) in cellular GSSG, GSH, and the GSSG:GSH ratio (Table 5), indicating a shift in the glutathione status of the cells towards a more...
Selenium, Cell Proliferation, and Glutathione Metabolism

Chart 3. DNA profiles for H-4 and 3T3 cells grown at low density and treated with selenium for 24 h. Experimental conditions were identical to those described in the legend to Chart 1. Cells were harvested after 24 h of selenium treatment and prepared for flow cytometric analysis of DNA as described in "Materials and Methods." The profiles shown are traces of 3 individual samples which are representative of the mean distributions for a particular treatment group control (---), 50 μM selenium (- - - -), and 100 μM selenium (-----).

Relative Cell Number

Relative Amount of DNA

Table 2

Effect of selenium concentration on cell cycle kinetics of low density H-4 cells determined after 24 h of selenium (NaSeO₃) treatment

The experimental protocol used for cell cycle analysis was identical to that used for the growth curves experiments (see legend to Chart 1). Cell preparation for FACS IV analysis is described in "Materials and Methods." The percentages of cells in G₁, S, and G₂-M were obtained from DNA distributions as shown in Chart 3 by computer fit analysis. The duration of mitosis was determined from the mean mitotic index and the mean population doubling time (T₀) for a given treatment group. The durations of the other stages were determined from the mean percentage of distribution cells in G₁, S, and G₂-M and the mean T₀ for a given treatment using the graphical method of Okuda.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>50 μM selenium</th>
<th>100 μM selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₁ (%)</td>
<td>43.1 ± 2.1b</td>
<td>46.0 ± 1.3</td>
<td>49.4 ± 1.5c</td>
</tr>
<tr>
<td>S</td>
<td>48.3 ± 1.6</td>
<td>44.9 ± 1.5</td>
<td>43.8 ± 1.6</td>
</tr>
<tr>
<td>G₂-M</td>
<td>8.6 ± 0.9</td>
<td>9.1 ± 0.5</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>T₀ (h)c, e</td>
<td>19.7 ± 0.6</td>
<td>25.2 ± 2.2</td>
<td>28.7 ± 1.9 (146)c</td>
</tr>
<tr>
<td>Mitotic index (%)p, h</td>
<td>4.2 ± 0.4</td>
<td>3.3 ± 0.2</td>
<td>2.5 ± 0.1f</td>
</tr>
<tr>
<td>Duration of stages (h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G₁</td>
<td>6.6</td>
<td>9.6 (145)</td>
<td>12.0 (182)</td>
</tr>
<tr>
<td>S</td>
<td>10.6</td>
<td>12.6 (118)</td>
<td>14.1 (133)</td>
</tr>
<tr>
<td>G₂-M</td>
<td>1.4</td>
<td>1.8 (129)</td>
<td>1.6 (114)</td>
</tr>
<tr>
<td>M</td>
<td>1.1</td>
<td>1.2 (109)</td>
<td>1.0 (91)</td>
</tr>
</tbody>
</table>

- Selenium treatment significantly increased the percentage of cells in G₁ (P < 0.05) compared to controls as indicated by ANOVA.
- Mean ± SE for 11 samples per treatment group collected from 3 experiments.
- Significantly different from control at P < 0.05 according to Duncan's new multiple range test.
- Values for population doubling times (T₀) represent the mean ± SE for average T₀s obtained from 4 experiments. Average values for T₀s from a given experiment were obtained from 4-5 pooled samples for a given treatment group.
- Selenium treatment significantly increased T₀ (P < 0.025) compared to controls as indicated by ANOVA.
- Numbers in parentheses, percentage of controls.

Table 3

Effect of selenium concentration on cell cycle kinetics of high density H-4 cells determined after 24 h of selenium (NaSeO₃) treatment

See Table 2 for legend.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>50 μM selenium</th>
<th>100 μM selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td>G₁ (%)</td>
<td>41.9 ± 0.6b</td>
<td>48.7 ± 1.3c</td>
<td>47.6 ± 1.4c</td>
</tr>
<tr>
<td>S</td>
<td>50.6 ± 1.2</td>
<td>45.3 ± 1.7c</td>
<td>42.6 ± 1.6c</td>
</tr>
<tr>
<td>G₂-M</td>
<td>7.8 ± 0.9</td>
<td>6.1 ± 1.0</td>
<td>9.8 ± 0.8</td>
</tr>
<tr>
<td>T₀ (h)c, e</td>
<td>17.3 ± 1.7</td>
<td>19.6 ± 2.6 (113)c</td>
<td>28.0 ± 0.1 (162)c</td>
</tr>
</tbody>
</table>

- Selenium treatment significantly increased the percentage of cells in G₁ (P < 0.01) compared to controls as indicated by ANOVA.
- Mean ± SE for 7 samples per treatment group collected from 2 experiments.
- Significantly different from controls (P < 0.05) according to Duncan's new multiple range test.
- Selenium treatment significantly decreased the percentage of cells in S (P < 0.005) compared to controls as indicated by ANOVA.
- Values for T₀ represent the mean ± SE for average T₀s obtained from 2 experiments. Average values for T₀s from a given experiment were obtained from 4-5 pooled samples for a given treatment.
- Selenium treatment significantly increased T₀ (P < 0.05) compared to controls as indicated by ANOVA.
- Numbers in parentheses, percentage of control.

- Selenium treatment significantly increased TD (P < 0.025) compared to controls as indicated by ANOVA.
- Numbers in parentheses, percentage of control.

Oxidation of GSH by selenium during the preparation of the cells at 4°C in 0.02 M EDTA, pH 4.7, is also unlikely (54). The increase in GSH with selenium treatment may represent an "adaptive" increase in an attempt to maintain a normal GSSG:GSH ratio. GSSG-Rd activity was also increased significantly (P < 0.005) by approximately 43% at both selenium concentrations com-
SELENIUM, CELL PROLIFERATION, AND GLUTATHIONE METABOLISM

Table 4

Effect of selenium concentration on cell cycle kinetics of low density 3T3 cells determined after 24 h of selenium treatment

See Table 2 legend.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>50 μM</th>
<th>100 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell distribution (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>33.0 ± 2.24</td>
<td>35.5 ± 0.9</td>
<td>30.5 ± 1.8</td>
</tr>
<tr>
<td>S</td>
<td>52.4 ± 1.86</td>
<td>48.0 ± 1.16</td>
<td>45.4 ± 2.00</td>
</tr>
<tr>
<td>G2-M</td>
<td>14.6 ± 1.0</td>
<td>16.5 ± 1.2</td>
<td>24.1 ± 1.2</td>
</tr>
<tr>
<td>Td (h)</td>
<td>16.0</td>
<td>18.1 (113)</td>
<td>32.6 (204)</td>
</tr>
<tr>
<td>Mitotic index (%)</td>
<td>3.98 ± 0.28</td>
<td>3.24 ± 0.19</td>
<td>1.87 ± 0.12</td>
</tr>
<tr>
<td>Duration of cycle stages (h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>4.1</td>
<td>5.3 (129)</td>
<td>8.0 (195)</td>
</tr>
<tr>
<td>S</td>
<td>9.1</td>
<td>9.0 (99)</td>
<td>14.6 (160)</td>
</tr>
<tr>
<td>G2</td>
<td>1.9</td>
<td>3.0 (158)</td>
<td>9.1 (480)</td>
</tr>
<tr>
<td>M</td>
<td>0.9</td>
<td>0.8 (92)</td>
<td>0.9 (100)</td>
</tr>
</tbody>
</table>

* Mean ± SE for 7 samples collected over 2 experiments.
* Selenium treatment significantly decreased the percentage of cells in S (P < 0.05) compared to controls as indicated by ANOVA.
* Significantly different from controls (P < 0.05) according to Duncan’s new multiple range test.
* Selenium treatment significantly increased the percentage of cells in G2-M (P < 0.005) compared to controls as indicated by ANOVA.
* Numbers in parentheses, percentages of controls.
* Mean ± SE from 3 samples.
* Selenium treatment significantly decreased the mitotic index (P < 0.005) compared to controls as indicated by ANOVA.

Table 5

Effect of selenium concentration on GSH and GSSG concentrations, GSSG-Rd activity, and pyridine nucleotide phosphate concentrations of H-4 cells

H-4 cells were plated at 1.86 x 10^5 cells per 150- x 25-mm dish. Twenty-four h after cell plating, 50 μM Na_2SeO_3 (control), 50 μM, or 100 μM selenium as Na_2SeO_3 was added to the cells in fresh medium. Cells were harvested after 24 h of selenium treatment, and biochemical analysis was performed as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>50 μM selenium</th>
<th>100 μM selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (nmol/mg DNA) (10)</td>
<td>185 ± 12</td>
<td>229 ± 27</td>
<td>310 ± 24</td>
</tr>
<tr>
<td>GSSG (nmol/mg DNA) (10)</td>
<td>1.70 ± 0.13</td>
<td>3.37 ± 0.38</td>
<td>10.80 ± 1.30</td>
</tr>
<tr>
<td>GSG-GSH (%) (10)</td>
<td>0.94 ± 0.11</td>
<td>1.40 ± 0.14</td>
<td>3.34 ± 0.34</td>
</tr>
<tr>
<td>GSSG-Rd activity (10)^a</td>
<td>10.83 ± 0.79</td>
<td>15.30 ± 0.52</td>
<td>15.80 ± 0.63</td>
</tr>
<tr>
<td>x enzyme unit (mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADP (nmol/mg DNA) (4)</td>
<td>6.0 ± 1.4</td>
<td>8.3 ± 1.6</td>
<td>12.0 ± 1.7</td>
</tr>
<tr>
<td>NADPH (nmol/mg DNA) (4)</td>
<td>7.3 ± 1.5</td>
<td>6.0 ± 1.0</td>
<td>7.2 ± 0.6</td>
</tr>
<tr>
<td>NADP: NADPH (4)</td>
<td>0.78 ± 0.11</td>
<td>1.35 ± 0.11</td>
<td>1.70 ± 0.10</td>
</tr>
</tbody>
</table>

* Selenium treatment caused a significant increase in GSH, GSSG, the GSSG:GSH ratio, GSSG-Rd activity, and the NADP:NADPH ratio (P < 0.005) as indicated by ANOVA.
* Numbers in parentheses, percentages of controls.
* Mean ± SE for the number of samples collected from 2-3 separate experiments.
* Significantly different from controls (P < 0.01) according to Duncan’s new multiple range test.
* One enzyme unit of GSSG-Rd activity is that amount of enzyme which catalyzes the oxidation of 1 μmol NADPH per min at 25 °C as described in “Materials and Methods.”

Discussion

Our results indicate that 6.0 ppm dietary selenium inhibited or more likely delayed the regenerative response following PH. To determine whether this represents an inhibition or delay would require data at more than a single time point. A preliminary experiment indicated that liver weights at 14 days after PH were comparable in 6.0 and 0.1 ppm selenium-fed rats, thus suggesting that selenium merely delayed the regenerative response. Numerous other agents including toxicants and carcinogens have been shown to inhibit or delay liver regeneration (6). Our data indicate that selenium decreases the rate at which hepatocytes traverse from G2-G1 into S phase of the cell cycle as indicated by the decreased labeling index at 23 h post-PH. A trend toward decreased ^3H per mg of DNA per labeled cell also suggests that selenium can decrease the rate of DNA synthesis. Alterations in diurnal rhythm and feeding patterns have been shown to alter the pattern of ^3[H]thymidine incorporation into DNA following PH (1). Preliminary studies have indicated, however, that 6.0 ppm selenium feeding as Na_2SeO_3 does not affect the diurnal rhythm of rats. An additional experiment could be designed to determine the effect of selenium on DNA synthesis by using a radioactive label in the presence and absence of selenium.

In agreement with the inhibitory effect of selenium on cell proliferation in vitro, selenium in the growth medium of H-4 and 3T3 cells, at concentrations comparable to those shown to alter carcinogenesis and affect liver regeneration, decreased cell proliferation. Cells grown at high density were more sensitive to inhibitory effects of selenium on proliferation and to selenium-induced cytotoxicity compared to low density cells by 48 h of selenium treatment. This may indicate that a density-dependent process, such as amino acid transport for example (3), is necessary to protect cells from the cytokinetic and cytotoxic effects of selenium.
of selenium. It is unlikely that decreased cell proliferation caused by selenium was due to depletion of serum or medium components. If this were the case, a relative increase in the inhibition of proliferation by selenium within a given medium change would be expected from 0-24 h. This was not observed at 12 versus 24 h of selenium treatment.

Cytokinetic analysis with H-4 cells indicated that G_1, S, and G_2 stages of the mammalian cell cycle showed a dose-dependent increase with selenium at both cell densities, while the length of mitosis was unaffected. Population doubling times and the durations of the stages of the cell cycle in our control cultures were similar to those reported previously (25). In addition, it was demonstrated previously (25) that the growth fraction of H-4 cells was 100%, therefore making population doubling time used for stage duration calculations a good approximation for generation time. A possible source of error in the calculation of stage durations with selenium treatment could arise if, unlike the controls, population doubling times were not comparable to generation times. Cell viability was greater than 95% with selenium treatment in the cell cycle analysis experiments; therefore a correction of the population doubling time for dead cells (57) was not necessary. Selenium treatment could also cause an "out of cycle" state for some cells while allowing others to progress normally through the cycle. If this were the case with selenium treatment, out of cycle cells would have to occur in all stages of the cell cycle except M to account for the observed DNA distributions. Future studies should address the possibility that selenium treatment induces heterogeneity in cell cycling (17).

As extensively reviewed (2, 44, 46), the synthesis of RNA and protein is essential for normal passage of cells through the cell cycle. In addition, the cell cycle demonstrates stage- and dose-dependent responses to inhibitors of RNA and protein synthesis. Mitosis is unique in that dramatically reduced rates of protein and RNA synthesis are observed compared to G_1, S, and G_2 stages of the cell cycle. Therefore, RNA and protein synthetic activities in the cell cycle correlate well with the stages of the cell cycle affected by selenium in our experiments.

A moderate accumulation of H-4 cells in G_1 occurred with selenium treatment as indicated by DNA distributions. It has previously been demonstrated that inhibition of protein synthesis by cycloheximide causes an accumulation of cells in G_1 (47). The accumulation of cells in G_1 by selenium was not enough, however, to account for the observed increase in the population doubling times; thus the duration of S and G_2 of H-4 cells was also increased by selenium in our studies. Protein synthesis inhibitors in addition to cycloheximide can affect S and G_2 stages of the cell cycle (11, 38, 46).

It has also been demonstrated that transformed cells are partially or completely deficient in the G_1 cell cycle arrest observed with cycloheximide treatment (38). Because the growth of transformed cells can be slowed with little or no alterations in the cell cycle distribution (38, 47) similar to what was observed with selenium treatment of H-4 cells, we also studied the effect of selenium on nontransformed 3T3 fibroblasts. In contrast to what was observed with the H-4 cells, the percentage of cells in G_2-M was increased with selenium treatment, particularly with 100 μM selenium. The increase in the percentage of cells in G_2-M was again not sufficient to account for the observed increase in the population doubling time with selenium treatment. Therefore, similar to the H-4 cells, the lengths of G_1, S, and G_2 were increased with selenium while M was unaffected. A number of drugs and X-irradiation, which cause DNA damage, cause arrest of cells in G_2 (53). Selenium has also been shown to induce DNA damage at concentrations comparable to those used in our studies (31, 41, 58); thus the increased duration of G_2 observed with selenium treatment in 3T3 cells may represent a partial G_2 arrest due to DNA damage. It is likely that selenium can have multiple effects on cells such as inhibition of macromolecular synthesis and DNA damage which could affect different stages of the cell cycle. Depending on the cells under study, which may have different capacities to metabolize selenium, culture conditions, which can alter the effective concentration of selenium by selenium binding to serum proteins (35), and cell densities, different patterns of cell cycle perturbations by selenium may be observed. In addition, the binding of selenium to serum components in the growth medium (35) may explain why relatively high concentrations of selenium were required to cause effects on cell proliferation in our studies. While this paper was in preparation, it was reported that selenium as Na_2SeO_3 at concentrations of 5 × 10^{-6} and 5 × 10^{-5} M in the growth medium caused an increase in the percentage of mouse mammary cells in S and G_2-M as indicated by cytofluorometric analysis compared to cells with no selenium added (36). Differences in cell cycle response to selenium in the recent study (36) compared to ours may be due to a number of factors including differences in cell type and density as evidenced in our study, as well as nutritional considerations.

As a possible biochemical explanation for decreased cell proliferation observed with selenium treatment, we examined cellular glutathione metabolism. Selenium as Na_2SeO_3 in the growth medium of H-4 cells caused a dose-dependent shift in the glutathione and pyridine nucleotide status towards a more oxidized state compared to controls. "Adaptive" increases in GSH, which may occur in response to increased GSSG or the GSSG:GSH ratio (27), and increased GSSG-Rd activity also occurred with selenium treatment similar to what was previously observed in rat liver in vivo with 6.0 ppm selenium feeding as Na_2SeO_3. This finding substantiates the relevancy of H-4 cells as an in vitro cell model to study selenium-induced biochemical changes associated with decreased proliferation. Increased GSSG may also be responsible for the decreased cell proliferation caused by selenium.

Moderate increases in GSSG comparable to those observed by us in vivo and in vitro with selenium treatment have been shown to inhibit protein synthesis in reticulocyte lysates (24). This inhibition is mediated through a protein kinase, the activity of which is stimulated by GSSG and phosphorylates eukaryotic initiation factor 2 (eIF-2), thus inactivating it (12). It has also been demonstrated that selenite can inactivate eIF-2, and the effect of selenium is similarly mediated through a protein kinase (48). It therefore seems plausible that inhibitory effects of selenium on protein synthesis and cell proliferation are mediated through increased cellular GSSG. Several investigators have demonstrated inhibition of protein synthesis by selenium in cells in culture (20, 30, 55).

An alternate mechanism of inhibition of protein synthesis by selenium has been demonstrated in vitro in which selenodiglutathione (GSSeSeG), a reductive intermediate of selenite, can inhibit elongation of peptide synthesis by inactivating eukaryotic elongation factor 2 (eEF-2) (55). It has also been demonstrated...
that, when the GSH-GSSSeG ratio exceeds 4:1, the unstable GSSSeG is rapidly reduced to glutathione-ditosulfide (GSSeH) and H2Se (14). Even with high selenium feeding or exposure in the medium, the ratio of GSH to selenium in the cell approaches 100 to 1, thus favoring the formation of GSSeH or H2Se. Therefore, attaining sufficient GSSeG in the cell to inhibit protein synthesis seems unlikely. The seemingly contradictory result of an increased amount of protein per 10^6 H-4 cells with 100 μM selenium in light of the proposed decreased protein synthesis by selenium may be explained by an even greater decrease in the rate of protein degradation with selenium treatment. In addition, alterations in the rates of synthesis and degradation of specific proteins involved in the regulation of cell reproduction may be involved (32).

Numerous other possibilities, in addition to altered protein synthesis, exist as an explanation for the observed inhibition of cell proliferation by selenium. As reviewed (23), alterations in cellular glutathione status can affect a number of biochemical events involved in cell proliferation. While it has been demonstrated that increased cellular GSSG can activate eIF-2 protein kinase (12), increased GSSG caused by selenium may enhance the phosphorylation of other proteins by GSSG-activated kinases. Changes in the phosphorylation state of the cell can alter cellular activities which are associated with cell proliferation (7, 44). In addition, it has been recently reported that daily injections of Na2SeO3 into hepatoma-bearing mice caused an increase in hepatoma cyclic AMP while not affecting tumor host liver (59). The reported increase in cyclic AMP by selenium, which could affect cell proliferation, was due to a decrease in tumor cyclic AMP phosphodiesterase activity.

In summary, selenium as Na2SeO3 has been shown to decrease cell proliferation both in response to PH in vivo and in cultured mammalian cells in vitro. The duration of the "synthetic" stages of the cell cycle, i.e., G1, S, and G2, was increased with selenium treatment, while mitosis was unaffected, and under given conditions, these effects are reversible with no loss of cell viability. Increased cellular GSSG observed with selenium treatment may explain the antiproliferative effects of selenium. The similarities in biochemical effects of selenium in H-4 cells compared to liver in vivo substantiate the use of H-4 cells as a relevant in vitro model for further study to determine the biochemical effects of high selenium on cells and the role of selenium in cell proliferation.

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Selenium, Cell Proliferation, and Glutathione Metabolism


Effects of Selenium on Cell Proliferation in Rat Liver and Mammalian Cells as Indicated by Cytokinetic and Biochemical Analysis

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