Effects of Dietary Selenium Concentration on the Development of Enzyme-altered Liver Foci and Hepatocellular Carcinoma Induced by Diethylnitrosamine or N-Acetylaminofluorene in Rats

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ABSTRACT

Three protocols were used to determine the effects of dietary selenium concentration on the development of \( \gamma \)-glutamyltranspeptidase (GGT)-positive foci and hepatocellular carcinoma induced by either diethylnitrosamine (DEN) or N-acetylaminofluorene in rats. In the first experiment, foci were induced by a carcinogenic dose of DEN (100 mg/kg body weight, p.o.) at 20–22 h after two-thirds partial hepatectomy. One wk after DEN administration, during which time 0.1 ppm (representing a control level), 3.0, or 6.0 ppm selenium as Na\( _{2} \)SeO\(_{3} \) was fed for 8 or 16 wk, at which time focal analysis was conducted using quantitative stereology. The results demonstrated that 3.0 and 6.0 ppm dietary selenium, initiated 1 wk following carcinogen administration, decreased focal growth rate without affecting the number of GGT foci compared to 0.1 ppm selenium. Decreased focal growth was temporary and reversible with 6.0 ppm selenium which may be related to chronic selenium observed after 16 wk of 6.0 ppm selenium feeding.

A second experiment involved a noncarcinogenic dose of DEN (25 mg/kg body weight, p.o.), then 0.1 or 6.0 ppm selenium feeding for 8 wk, followed by 0.05% phenobarbital (PB), a liver tumor promoter in a diet containing 0.1 ppm selenium. Analysis of GGT foci at 5 or 8 wk of PB feeding indicated that 6.0 ppm selenium caused a trend towards an increase in the number of foci/cm\(^2\) of liver and mean focal volume and a significant increase in GGT focal volume as a percentage of liver volume by 8 wk of PB feeding. Thus, high dietary selenium concentrations prior to PB enhance the tumor-promoting ability of PB.

In a third experiment, using male Fischer 344 rats (150 g), 0.1 or 6.0 ppm selenium was fed concurrently with 0.02% AAF which was fed in a cyclic regimen. After 4 cycles, where 1 cycle equalled 4 wk of AAF, followed by 1 wk of control diet (0.1 ppm selenium), 6.0 ppm selenium significantly decreased the mean focal volume and focal volume as a percentage of liver volume, while not affecting the number of foci/cm\(^2\) of liver, again indicating a selenium effect on focal growth while not affecting the number of "preneoplastic" lesions in the liver. Six ppm selenium feeding after AAF treatment had no effect on the percentage of incidence of hepatocellular carcinoma (100%) but did cause a significant decrease in the percentage of liver volume occupied by macroscopic subcapsular liver lesions compared to 0.1 ppm selenium. Three ppm selenium was without effect. The results from these studies are discussed in terms of an inhibitory effect of high selenium on cell proliferation and thus an inhibitory or delaying effect on carcinogenesis.

INTRODUCTION

Numerous studies have demonstrated anticarcinogenic effects of dietary selenium in experimental animals when administered in the food or drinking water at concentrations of 20 to 60 times the nutritional requirement. Protection by selenium has been demonstrated in chemically induced carcinogenesis in liver (6, 10, 13), colon (1, 2, 20–22), mammary gland (16, 17, 19, 31, 34, 52, 55), and skin (49). The genesis of spontaneous mammary tumors in mice (33, 47) as well as the growth of transplanted tumor cells (9, 18, 35, 53, 54) is also inhibited by high dietary and injected selenium. Several reports have suggested that the effect of selenium on chemically induced cancer may be mediated through alterations in carcinogen metabolism (4, 5, 11, 21, 23, 30). Other mechanisms of inhibition must also be present as indicated by those studies in which high dietary selenium fed after carcinogen administration caused a significant reduction in hepatic and mammary tumor incidence (10, 16, 52, 55). This interpretation is also supported by studies in which selenium exerted protective effects against tumor development and growth in nonchemically induced tumor systems (9, 18, 33, 35, 47, 53, 54).

The purpose of our experiments was to study the effect of dietary selenium concentration on early and late stages of hepatic carcinogenesis induced by DEN and AAF. Two of the experiments were designed such that any observed selenium effects would be independent of alterations in carcinogen metabolism. For our experimental end points, we quantitated GGT-positive foci to study early stages of hepatocellular carcinoma and macroscopic subcapsular protrusions to study late stages of hepatocarcinogenesis. Our results indicate that high dietary selenium can affect postinitiation stages of hepatocarcinogenesis as indicated by a decreased growth of "preneoplastic" foci. In addition, selenium can decrease or enhance the carcinogenic process, depending on the dietary concentration of selenium, the duration of selenium feeding, and the experimental system under study.

MATERIALS AND METHODS

Diet. The diet composition for the experiments involving DEN was previously described (25), with the exception that 20% casein was used instead of 15%, at the expense of glucose monohydrate, and an addi-
Selenium Effects on Liver Foci and Carcinoma Development

Dietary selenium treatment: 8-mg of zinc as ZnCl₂ per kg diet was supplemented due to the fact that the animals in the present studies were housed in stainless steel cages. This diet met all nutritional requirements of the laboratory rat according to the National Research Council (36). Selenium supplementation as Na₂SeO₃ at 0.1, 3.0, and 6.0 ppm selenium was accomplished by adding a selenium-glucose monohydrate premix. The 0.1 ppm selenium-supplemented diet which represents a control concentration of selenium was analyzed fluorometrically to contain 0.13 ppm selenium (37). The diet used for the experiments involving AAF was similar to that described above for the DEN experiments with the exception that 0.2% (37). The diet used for the experiments involving AAF was originally designed to last 50 wk post-AAF feeding, a length of time shown previously (15) to be necessary for carcinoma development using a cyclic feeding protocol. Amino acid supplementation to the diet was an attempt to minimize selenium toxicity over the 50-wk feeding period (28, 43, 56). AAF (Aldrich, Milwaukee, WI) was supplemented to give a final dietary concentration of 0.02% by adding a premix of 1% AAF in glucose monohydrate to the basal diet.

DEN:Selenium Experiment (Chart 1a). Female Sprague-Dawley rats (Harlan-Sprague Dawley, Madison, WI) weighing approximately 200 g were received and housed in hanging stainless steel wire cages at 3 rats/cage. They were fed a basal diet containing 0.1 ppm selenium for 1 wk. PH (14) was then performed on the rats followed by intragastric administration of DEN (100 mg/kg body weight; Eastman Kodak, Rochester, NY) in 0.9% saline at 20–22 h post-PH. This dose of DEN is capable of causing hepatocellular carcinoma without further treatment (44). This large dose of DEN was used in order to induce hepatic lesions which required no further exogenous modifiers for the subsequent development of hepatocellular carcinoma. In this way, the direct effect of selenium on the development of liver GGT foci could be evaluated.

RESULTS

DEN:Selenium Experiment (Chart 1a; DEN, 100 mg/kg Body Weight). Dietary selenium treatment had no effect on body or liver weight after 8 wk of selenium feeding, but 6.0 ppm selenium caused a reduction in body weight and an increase in liver weight by 16 wk which was significant compared to 0.1 ppm (Table 1).
Survival of animals was greater than 95% in all groups.

The effect of dietary selenium concentration on focal development was evaluated in 2 separate experiments after 8 wk of selenium feeding to test the reproducibility of this protocol. Individual values from each experiment are listed in Table 2, and statistics were performed on pooled replicates from both experiments. Dietary selenium concentration had no effect on the number of foci/cm² of liver, while both 3.0 and 6.0 ppm selenium caused a significant reduction of mean focal volume and focal volume as a percentage of liver volume by an average of 45% and 50%, respectively, compared to 0.1 ppm selenium. The reproducibility between individual experiments was excellent. At 16 wk, there was a significant increase in the number of foci/cm² of liver compared to 8 wk, and dietary selenium had no effect on this increase expressed as the number of foci/cm² of liver (Table 2) or total foci per liver (data not shown). Similar to the 8-wk time point, 3.0 ppm selenium maintained a significant decrease of 42% in mean focal volume and 41% in focal volume as a percentage of liver volume compared to 0.1 ppm selenium but did not increase the relative difference between 0.1 and 3.0 ppm selenium at 16 wk compared to that observed at 8 wk.

### Table 1

<table>
<thead>
<tr>
<th>Treatment (ppm selenium)</th>
<th>Body wt (g)</th>
<th>Liver wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 wk of selenium feeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>264 ± 5</td>
<td>8.3 ± 0.4</td>
</tr>
<tr>
<td>3.0</td>
<td>259 ± 5</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td>6.0</td>
<td>263 ± 5</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td>16 wk of selenium feeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>292 ± 5</td>
<td>9.8 ± 0.4</td>
</tr>
<tr>
<td>3.0</td>
<td>286 ± 6</td>
<td>10.6 ± 0.3</td>
</tr>
<tr>
<td>6.0</td>
<td>274 ± 7p</td>
<td>11.1 ± 0.4p</td>
</tr>
</tbody>
</table>

* Mean ± SE for 12 animals/treatment group.

** Significantly different (P < 0.05) from 0.1 but not 3.0 ppm selenium at 16 wk according to Duncan’s multiple range test.

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** Table 2 **

<table>
<thead>
<tr>
<th>Treatment (ppm selenium)</th>
<th>No. of animals/group</th>
<th>No. of foci/cm²</th>
<th>Mean focal volume (mm²)</th>
<th>Focal/liver volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 wk selenium feeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>7</td>
<td>95 ± 17a</td>
<td>0.089 ± 0.025</td>
<td>0.87 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>111 ± 12 (105 ± 10)b</td>
<td>0.072 ± 0.011 (0.078 ± 0.011)</td>
<td>0.86 ± 0.21 (0.86 ± 0.18)</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>110 ± 14</td>
<td>0.045 ± 0.007c</td>
<td>0.50 ± 0.11c</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>83 ± 11</td>
<td>0.041 ± 0.04</td>
<td>0.33 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>110 ± 9 (98 ± 7)</td>
<td>0.044 ± 0.005 (0.042 ± 0.004)c</td>
<td>0.48 ± 0.08 (0.42 ± 0.05)c</td>
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<tr>
<td>16 wk selenium feeding</td>
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<td></td>
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<tr>
<td>0.1</td>
<td>12</td>
<td>177 ± 15d</td>
<td>0.082 ± 0.014</td>
<td>1.53 ± 0.36d</td>
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<tr>
<td></td>
<td>3.0</td>
<td>179 ± 14d</td>
<td>0.046 ± 0.004d</td>
<td>0.90 ± 0.14d</td>
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<tr>
<td></td>
<td>6.0</td>
<td>180 ± 11d</td>
<td>0.086 ± 0.014d</td>
<td>1.49 ± 0.22d</td>
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</tbody>
</table>

* Mean ± SE.

** Numbers in parentheses, mean ± SE for pooled animals from 2 separate experiments, for which individual means ± SE are also shown. Only one experiment was conducted with the 3.0-ppm selenium treatment.

** Significantly different (P < 0.05) from means in a column not followed by Footnote c at 8 or 16 wk according to Duncan’s multiple range test.

** Significantly different (P < 0.001) from means of the same dietary treatment group at 8 versus 16 wk.

* Numbers in parentheses, mean ± SE for pooled animals from 2 separate experiments, for which individual means ± SE are also shown. Only one experiment was conducted with the 3.0-ppm selenium treatment.
SELENIUM EFFECTS ON LIVER FOCI AND CARCINOMA DEVELOPMENT

<table>
<thead>
<tr>
<th>Treatment (ppm selenium)</th>
<th>Number of animals/group</th>
<th>Number of foci/cm³</th>
<th>Mean focal volume (mm³)</th>
<th>Focal/liver volume (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 wk PB feeding</td>
<td></td>
<td>0.1</td>
<td>5</td>
<td>156 ± 61 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>4</td>
<td>199 ± 81</td>
</tr>
<tr>
<td>8 wk PB feeding</td>
<td></td>
<td>0.1</td>
<td>12</td>
<td>118 ± 29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.0</td>
<td>12</td>
<td>154 ± 21</td>
</tr>
</tbody>
</table>

* Mean ± SE.

Table 3

Effect of dietary selenium concentration on the development of liver GGT (+) foci when selenium is fed after DEN and to PB administration (DEN:selenium:PB experiment)

Female Sprague-Dawley rats (200 g) were given DEN (25 mg/kg body weight) (p.o.) at 20 h post-PH. Rats were fed a diet containing 0.1 ppm selenium for 1 wk post-DEN and then either 0.1 or 6.0 ppm selenium for 8 wk. Rats were subsequently fed a diet containing 0.1 ppm selenium for 1 wk, followed by a diet containing 0.05% PB with 0.1 ppm selenium for 5 or 8 wk (Chart 1b), at which time focal analysis was conducted.

The age of 5.4 with PB treatment following DEN. With no PB treatment, the focal volume as a percentage of liver volume was less than 0.03% regardless of selenium treatment. This low frequency of transsections prevented a meaningful statistical evaluation of results. Thus, the DEN:selenium experiment was conducted with the higher dose of DEN (100 mg/kg body weight) so as to yield sufficient transsections for accurate quantitative analysis.

AAF Experiment 1 (Chart 1, c1; Concurrent AAF:Selenium Feeding). Six ppm selenium fed concurrently with AAF had no effect on body weight (data not shown) after 4 cycles of AAF but prevented the increase in liver/body weight caused by AAF feeding with 0.1 ppm selenium. The values of liver to body weight in percentage were 7.33 ± 0.3 and 4.40 ± 0.2 for 0.1 ppm selenium plus AAF and 6.0 ppm selenium plus AAF, respectively. A liver/body weight ratio of 4.0% is typical for control adult male F-344 rats in our laboratory. The results at the end of 4 cycles were as follows. Six ppm selenium fed concurrently with AAF had no effect on the number of foci/cm³ of liver but significantly decreased the mean focal volume and focal volume as a percentage of liver volume by 76% and 70%, respectively, compared to 0.1 ppm selenium (Chart 2). Due to the effects of selenium on liver size by the end of 4 cycles, the total number of foci/liver were calculated. A trend towards fewer foci per liver was observed with 6.0 ppm selenium by the end of 4 cycles, but this difference was not significant. The values were 9,835 ± 1,855 and 11,776 ± 1,013 foci per liver for 6.0 and 0.1 ppm selenium, respectively. By the end of 3 cycles, foci in the livers from the 0.1-ppm selenium plus AAF-treated rats, foci were accurately quantifiable in the 6.0-ppm selenium plus AAF treatment group due to less focal involvement in these livers as indicated by the decreased focal volume as a percentage of liver volume with 6.0 ppm selenium. Animals fed either 0.1 ppm selenium or 6.0 ppm selenium without AAF through 3 cycles had no foci.

AAF:Selenium Experiment 2 (Chart 1, c2; High Selenium Subsequent to AAF Feeding). After 4 cycles of AAF feeding in a diet containing 0.1 ppm selenium, livers from these rats showed a number of neoplastic nodules, several exhibiting markedly atypical cellular hyperplasia in addition to a few small hepatocellular carcinomas as diagnosed histologically. Selenium at 0.1, 3.0, or 6.0 ppm was subsequently fed for 21 wk to rats which had received 0.1 ppm selenium plus AAF. Dietary selenium concentration had no effect on the percentage of animals in each group diagnosed histologically to have hepatocellular carcinoma (Table 4). Evaluation of standard liver sections and nodule cross-sections indicated the presence of hepatocellular carcinoma, cholangioma, cholangiocarcinoma, mixed cell type hepatocellular carcinoma, and neoplastic nodules. Six ppm selenium had no significant effect on the number of nodules per liver or the mean nodule volume, while causing a trend towards a decrease compared to 0.1 ppm selenium. A significant reduction of 35% in nodule volume as percentage of liver volume was observed in 6.0 ppm selenium compared to 0.1 (Table 4). Three ppm selenium was without effect. Dietary selenium concentration subsequent to AAF had no effect on body or liver weight (data not shown). Survival of rats from the cessation of AAF feeding until the termination of the experiment was 84%, 79%, and 89% for 0.1, 3.0, and 6.0 ppm selenium, respectively, therefore without treatment effect.

DISCUSSION

In our studies, enzyme-altered foci were used as a quantitative index to assess the effects of dietary selenium concentration on early stages of hepatocarcinogenesis. While unequivocal evidence is lacking that foci are precursors of carcinomas, existing data strongly suggest that foci represent relevant cells in the lineage of hepatocellular carcinoma development and can provide information as to the carcinogenic potential of an experimental system (7, 8, 42, 44, 45) due to the consistent nature in which foci appear during the postinitiation stages of hepatocarcinogenesis. Because of the strong association between foci and hepatocellular carcinoma, the results from these experiments indicate that high dietary selenium can affect postinitiation stages of hepatocarcinogenesis. The concentrations of selenium used in our studies have previously been shown (by others) to alter tumor development in a number of experimental systems. In the experiments using DEN, 100 mg/kg body weight, high dietary selenium slowed the growth of foci as indicated by a smaller mean focal volume compared to 0.1 ppm selenium. Inhibition of cell proliferation as a mechanism of the anticarcinogenic effect of selenium has been previously suggested using [³H]thymidine incorporation into colon (12) and colon weights (9) as indicators of proliferation in vivo. Studies in vitro have also indicated an effect of selenium on cell proliferation (29, 31, 32). Depending on the dietary selenium concentration, in the present experiments, selenium effects on mean focal volume were temporary and reversible as indicated by an initial decrease at 8 wk and subsequent return to control values by 16 wk in the 6.0-ppm selenium treatment group. The difference in the persistent effect...
of 3.0 ppm selenium and the temporary effect of 6.0 ppm selenium on mean focal volume may be explained by chronic selenium caused by 6.0 ppm selenium, as was indicated histologically, and subsequent adaptive liver growth. Increased cell proliferation during adaptive liver growth has been implicated as a stimulator of hepatocarcinogenesis (38, 39, 48). A promoting effect of 6.0 ppm selenium at longer time points also seems plausible. An important finding in these studies was that dietary selenium concentration had no effect on the number of foci/cm³ of liver, suggesting that high selenium does not enhance "repair" or "remodeling" (7) of preneoplastic lesions but merely decreases their growth rate. Experimental evidence by others supporting this finding exists.

In studies in which selenium has been shown to decrease the number of animals with hepatocellular carcinoma or total numbers of carcinoma following 3'-methyl-1arminoazobenzene or AAF administration, the precursor lesions may still have been present in the liver, but their growth rate slowed such that carcinoma appearance was delayed beyond the experimental end point (6, 10, 13). This interpretation is supported by an increased latency period in carcinoma appearance following concurrent feeding of high selenium and AAF (13). Similar results have been reported with dimethylhydrazine-induced colon cancer (1, 22), where initially high selenium caused a decrease in tumor incidence of the colon but at longer time points had no effect. The number of colon tumors per animal followed a similar pattern.

Similar inhibitory effects of high selenium on focal growth induced by DEN were observed when selenium was fed concurrently with AAF in the cyclic protocol. While the cyclic AAF protocol is less accurate for foci quantitation than the DEN protocol in our laboratory, the results indicate that high selenium decreased mean focal volume without affecting the number of foci in the liver similar to results obtained using DEN. In the DEN experiment, selenium effects must be independent of alterations in carcinogen metabolism. In the first AAF experiment, it is uncertain whether high selenium is directly affecting the growth of cells or altering the metabolism of AAF as has been previously described (5, 23, 30). The lack of effect of selenium on the number of foci/liver induced by AAF suggests that selenium is not affecting the efficiency at which AAF can initiate foci appear-
Selenium effects on liver foci and carcinoma development

In summary, effects of selenium on focal growth may represent a “selective toxicity” to proliferating cells by virtue of the fact that they are proliferating compared to a relatively nonproliferating background and thereby decrease carcinogenesis. A separate report (27) describes studies in which selenium has been shown to decrease proliferation in both regenerating liver and in vitro with minimal deviation hepatoma cells and fibroblasts, thus supporting our hypothesis that selenium effects on carcinogenesis are at least, in part, mediated through inhibition of cell proliferation. If, however, adaptive cell proliferation occurs in response to high selenium in an organ, an enhancement of carcinogenesis is likely, resulting in the development of cells that are resistant to high doses of selenium. A selenium interaction with tumor promoters may also enhance promoting activity. In addition, our results suggest that the continued administration of selenium is necessary for the anticarcinogenic effects of selenium, because removal or repair of preneoplastic lesions did not occur with high selenium.

ACKNOWLEDGMENTS

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REFERENCES


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