Effects of Selenium on 7,12-Dimethylbenz(a)anthracene-induced Mammary Carcinogenesis and DNA Adduct Formation

Clement Ip* and F. Bernard Daniel³

Department of Breast Surgery and Breast Cancer Research Unit, Roswell Park Memorial Institute, Buffalo, New York 14263 [C. I.], and Health Effects Research Laboratory, United States Environmental Protection Agency, Cincinnati, Ohio 45268 [F. B. D.]

ABSTRACT

The purpose of the present investigation was to determine the effects of dietary selenium deficiency or excess on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary neoplasia in rats and to delineate whether selenium-mediated modification of mammary carcinogenesis was associated with changes in carcinogen:DNA adduct formation and activities of liver microsomal enzymes that are involved in xenobiotic metabolism. Female Sprague-Dawley rats were divided into three groups from weaning and were maintained on one of three synthetic diets designated as follows: (a) selenium deficient (<0.02 ppm); (b) selenium adequate (0.2 ppm); or (c) selenium excess (2.5 ppm). For the DMBA binding and DNA adduct studies, rats were given a dose of [³H]DMBA p.o. after 1 month on their respective diets. Results from the liver and the mammary gland indicated that neither selenium deficiency nor excess had any significant effect on the binding levels, which were calculated on the basis of total radioactivity isolated with the purified DNA. Furthermore, it was found that dietary selenium intake did not seem to affect quantitatively or qualitatively the formation of DMBA:DNA adducts in the liver. Similarly, in a parallel group of rats that did not receive DMBA, the activities of aniline hydroxylase, aminopyrine N-demethylase, and cytochrome c reductase were not significantly altered by dietary selenium levels. Concurrent with the above experiments, the effect of dietary selenium intake on carcinogenesis was also monitored. Results of this experiment indicated that selenium deficiency enhanced mammary carcinogenesis only when this nutritional condition was maintained in the postinitiation phase. Likewise, an excess of selenium intake inhibited neoplastic development only when this regimen was continued after DMBA administration. In either case, deficient or excess selenium at the time of carcinogenic insult failed to produce a significant effect on subsequent tumor yield, if selenium intake was returned to the nutritional condition at 1 month on their respective diets. Results from the liver and the mammary gland indicated that neither selenium deficiency nor excess had any significant effect on the development of tumors or preneoplasia, if the animals are maintained on the selenium-deficient diet (<0.02 ppm) for the entire duration of the experiment. No information is available on the development of tumors or preneoplasia, if the animals are returned to a normal selenium regimen after carcinogen treatment. On the other hand, we have reported that supplementation of selenium at 5 ppm just around the time of DMBA administration was effective in producing a prophylactic response (8), suggesting that selenium may influence certain events in the initiation phase of carcinogenesis. Subsequent investigation in our laboratory (10) has confirmed the results reported by Medina and Oborn (20) that selenium is cytotoxic at high levels. Thus, it is necessary to repeat the in vivo carcinogenesis experiment under similar conditions but with a lower level of selenium. The purpose of the present study therefore was to determine the effects of short-term dietary selenium deficiency or supplementation during the initiation phase on DMBA-induced mammary neoplasia and to delineate whether the selenium-mediated modification of mammary carcinogenesis is associated with changes in either DMBA:DNA adduct formation or in the alteration of the levels of liver microsomal enzymes that are involved in xenobiotic metabolism.

MATERIALS AND METHODS

Animals and Diets. Weanling female Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Wilmington, MA. They were fed one of 3 synthetic diets designated as follows: (a) selenium deficient (<0.02 ppm); (b) selenium adequate (0.2 ppm); or (c) selenium excess (2.5 ppm). Each of these diets contained 25% corn oil. The composition of the basal selenium-deficient diet has been described previously (11). The other 2 diets were obtained by adding 0.2 and 2.5 ppm of selenium, respectively, in the form of sodium selenite, to the basal diet. For the DMBA binding and DNA adduct studies, rats were given a dose of 5 mg of [³H]DMBA (see below) p.o. after an adaptation period of 1 month on their respective diets. They were sacrificed 24 hr after carcinogen administration. Samples of liver and mammary gland were excised and immediately frozen in liquid nitrogen. Rats that were used for enzyme and tissue selenium assays were treated similarly, but without receiving DMBA.

Mammary tumors were induced by i.g. administration of 5 mg of DMBA at 50 days of age (11). Five groups of rats (25 rats/group) were included in the carcinogenesis experiment. Depending on selenium intake

1 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; PBS, phosphate-buffered saline; HPLC, high-pressure liquid chromatography; i.g., intragastric.
either before or after DMBA, these groups were designated as follows: deficient—deficient; deficient—adequate; adequate—adequate; excess—adequate; and excess—excess. The first selenium diet was given from weaning until 1 week past DMBA administration, after which the diets of 2 of the groups were changed as noted above, and the third group continued until the experiment was terminated at 22 weeks. Rats were palpated once a week, and the location of new tumors was recorded. At autopsy, rats were dissected and examined for nonpalpable tumors.

**Hydrocarbon.** Generally tritiated DMBA was purchased from Amer sham (Arlington Heights, IL). It was mixed with unlabeled material (East man Organic Chemicals, Rochester, NY) and purified by preparative HPLC within several days of use to a chemical and radiochemical purity of >98%. The specific radioactivity of the labeled DMBA preparation used in this study was 33.1 mCi/mmole.

**DNA Isolation.** Frozen mammary tissue was pulverized, and the powder was treated with collagenase III (9 mg/g tissue; Worthington Biochemical Corp., Freehold, NJ) at 37° for 60 min in PBS. The digest was then centrifuged at 1000 rpm for 15 min, and the upper layer of lipids and fat cells was removed by aspiration. The cell pellet was washed with PBS, and an aliquot was examined under a Zeiss UPL phase-contrast microscope (x100). These preparations were predominantly pieces of mammary ductal material and were used as the source for the mammary DNA. Five ml of PBS with 1.5% sodium dodecyl sulfate were added to the cell pellet, followed by an aliquot of autodigested Pronase B (Calbiochem, Los Angeles, CA) at a concentration of 100 μg/ml. The pellet was then homogenized using a Tissumizer equipped with a microprobe at 100 rpm for 5 sec. The homogenate was incubated for 60 min at 37°, after which 4-aminoacetaldehyde and NaCl were added to a final concentration of 6% and 1%, respectively. The homogenate was then extracted with 1 volume of Kirby’s reagent (14), and the resulting aqueous phase was further extracted with 1 volume of chloroform:methanol (24:1, v/v). The aqueous phase was transferred to a tube containing 1 g of HTP (DNA grade) hydroxylapatite (Bio-Rad Laboratories, Richmond, CA) at a concentration of 100 μg/ml. After mixing, the HTP was pelleted by centrifugation, and the liquid was decanted. The DNA-containing hydroxylapatite gel was then washed 2 times with 5 ml of 0.05 M sodium phosphate buffer, pH 6.5. After mixing, the HTP was pelleted by centrifugation, and the liquid was decanted. The DNA-containing hydroxylapatite gel was then washed with 2 ml of 0.05 M sodium phosphate buffer, pH 7.1, and dialyzed against 500 volumes of distilled water. The DMBA:DNA binding level was analyzed by quantitation of a small aliquot of the bound hydrocarbon via liquid scintillation counting of a small aliquot of the supernatant. These preparations were predominantly pieces of mammary ductal material and were used as the source for the mammary DNA. Five ml of PBS with 1.5% sodium dodecyl sulfate were added to the cell pellet, followed by an aliquot of autodigested Pronase B (Calbiochem, Los Angeles, CA) at a concentration of 100 μg/ml. After mixing, the HTP was pelleted by centrifugation, and the liquid was decanted. The DNA-containing hydroxylapatite gel was then washed 2 times with 5 ml of 0.05 M sodium phosphate buffer, pH 6.5, and 4 times with 5 ml of 0.05 M sodium phosphate buffer, pH 6.5. The DNA was desorbed with 2 ml of 0.05 M sodium phosphate buffer, pH 7.1, and dialyzed against 500 volumes of distilled water. The DMBA:DNA binding level was analyzed by quantitation of the DNA with 3,5-diaminonaphthalene (15) and determination of the bound hydrocarbon via liquid scintillation counting of a small aliquot which had been enzymatically digested with DNase I. The preparation of the liver DNA has been described previously in detail and is similar to the procedure for the mammary DNA, except that the collagenase step is omitted, and the DNA is isolated by precipitation rather than hydroxyapatite adsorption (12).

**DNA Hydrolysis.** Purified DNA was hydrolyzed by the addition of DNase I and snake venom phosphodiesterase in 0.3 M Tris buffer (pH 8.3) containing 0.01 M MgCl₂, 0.005 M CaCl₂, and 0.001 M ZnCl₂. After incubation in the dark at 37° for 4 to 6 hr, bacterial alkaline phosphatase was added, and the solution was incubated at 22°-25° in the dark for another 14 to 18 hr. Samples were checked by thin-layer chromatography prior to HPLC to ensure that hydrolysis to the deoxyribonucleoside level was complete (12).

**Chromatography.** DMBA:deoxyribonucleoside adducts were separated from the unmodified deoxyribonucleosides by 2 extractions of the hydrolysates with an equal volume of water-saturated normal butyl alcohol. The normal butyl alcohol was evaporated under a nitrogen stream, and the DMBA:deoxyribonucleoside adducts were redissolved in 1 ml of 35% methanol in water for HPLC analysis. The HPLC system consisted of a 45-min linear, 35 to 80%, methanol-in-water gradient, eluted at 1 ml/min from a DuPont-Zorbax octadecyl silane column (4.6 x 250 mm). The LDC (Riviera Beach, FL) chromatographic system used consisted of a LDC chromatographic control module fitted with a LDC Spectromonitor III UV detector set at 290 nm and a LDC Fluorometer III detector set at an excitation of 254 nm with a 470-nm emission filter. Ninety fractions of 0.5 ml were collected via an ISCO (Lincoln, NE) Model 1800 fraction collector fitted with a Model 590 flow stream, and the DMBA:deoxyribonucleoside adducts were redissolved in 1 ml of 35% methanol in water for HPLC analysis.

**SELENIUM AND MAMMARY CARCINOGENESIS**

**RESULTS**

Table 1 shows the effect of different levels of selenium intake on the binding of DMBA to liver and mammary gland DNA. These animals (10/group) were maintained on their respective diets from weaning for 1 month. They were given 5 mg of [3H]DMBA p.o. at 50 days of age and were sacrificed 24 hr later. Neither of the 2 treatments, i.e., prior adaptation of the animals with dietary deficiency or excess of selenium, had any significant effect on the binding levels, which were calculated on the basis of total radioactivity isolated with the purified DNA. In general, the values in both organs agreed with the data obtained previously, using rats of the same strain and sex but fed a laboratory chow diet and given the same dose of DMBA i.v. instead of i.g. (4).

In a previous paper (12), it has been shown that HP6 (Fractions...
SELENIUM AND MAMMARY CARCINOGENESIS

is that product resulting from the addition of the analogous diol-epoxide of 7-hydroxymethyl-12-methylbenza(a)anthracene, a major primary metabolite of DMBA (12). The line structures of the diol-epoxides leading to HP4 and HP6 are shown in Chart 2. The adducts eluting before Fraction 35 are, as yet, unidentified. As can be seen from Chart 1, dietary selenium intake did not seem to have any significant effect on either the quantitative or qualitative aspect of DMBA:DNA adduct formation in the liver. Because of the limited amount of DNA obtained from the mammary gland epithelial cells, a comparable analysis of the DMBA:mammary DNA adduct chromatographic profile was not possible.

The selenium status of the animals was confirmed by tissue selenium levels and the activity of the selenium-dependent glutathione peroxidase enzyme in a separate experiment using rats that were not treated with DMBA, and these results are summarized in Table 2. As expected, selenium deficiency reduced both tissue selenium level and enzyme activity in the liver as well as in the mammary gland. On the other hand, excess selenium supplementation only raised tissue selenium levels but had no effect on the enzyme activity. This observation suggests that, in rats receiving an adequate amount of selenium in the diet (0.2 ppm), the enzyme is already operating at near-maximal capacity. Additional selenium will not further increase its level, since the apoprotein becomes the limiting factor.

We have also examined the activities of 3 hepatic microsomal enzymes that are involved in xenobiotic metabolism. Included in our initial survey were aniline hydroxylase, aminopyrine N-demethylase, and cytochrome c reductase. As shown in Table 3, none of these enzymes responded to changes in dietary selenium intake. These results, although only preliminary in nature, are consistent with our observation that dietary selenium does not influence DMBA metabolism and DNA binding.

Concurrent with the above experiments, the effect of dietary selenium intake on DMBA-induced mammary carcinogenesis was investigated. Before discussing the results which are summarized in Chart 3 and Table 4, it is worthwhile to point out the

### Table 2

<table>
<thead>
<tr>
<th>Dietary selenium (ppm)</th>
<th>Mammary gland</th>
<th>Liver</th>
<th>Mammary gland</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.02</td>
<td>0.02 ± 0.01(^a,b)</td>
<td>0.15 ± 0.06(^b)</td>
<td>18 ± 6(^c)</td>
<td>38 ± 6(^b)</td>
</tr>
<tr>
<td>0.2</td>
<td>0.09 ± 0.03</td>
<td>0.81 ± 0.24</td>
<td>56 ± 13</td>
<td>205 ± 51</td>
</tr>
<tr>
<td>2.5</td>
<td>0.21 ± 0.06(^c)</td>
<td>1.53 ± 0.52(^c)</td>
<td>59 ± 14</td>
<td>217 ± 53</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.D. (n = 10).  
\(^b\) Significantly different from the corresponding value from rats receiving 0.2 ppm selenium.

### Table 3

<table>
<thead>
<tr>
<th>Dietary selenium (ppm)</th>
<th>Aniline hydroxylase (nmol p-aminophenol formed/min/mg protein)</th>
<th>Aminopyrine N-demethylase (nmol formaldehyde formed/min/mg protein)</th>
<th>Cytochrome c reductase (nmol cytochrome c reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.02</td>
<td>0.59 ± 0.15(^a)</td>
<td>0.92 ± 0.30</td>
<td>65 ± 14</td>
</tr>
<tr>
<td>0.2</td>
<td>0.64 ± 0.17</td>
<td>0.87 ± 0.27</td>
<td>59 ± 12</td>
</tr>
<tr>
<td>2.5</td>
<td>0.68 ± 0.18</td>
<td>0.81 ± 0.24</td>
<td>71 ± 16</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± S.D. (n = 10).
emphasize as well as the modifications incorporated into the present study in order to underscore the differences in the specific aims from those of our previous papers (8, 11). First of all, we want to compare the effect of long-term versus short-term selenium deprivation. In the long-term protocol, rats were fed a selenium-deficient diet from weaning until termination of the experiment at 22 weeks after DMBA administration (Group 1). In the short-term protocol, rats were fed a selenium-deficient diet from weaning until 1 week past DMBA administration, after which they were returned to a selenium-deficient diet until sacrifice (Group 2). Next, we would like to reassess the effect of short-term versus long-term supplementation using a design identical to that described above (Groups 4 and 5). However, instead of adding 5 ppm of selenium in the diet as in our previous study (8), the present study used a lower level of supplementation at 2.5 ppm. Based on our prior experience, rats ingesting this amount of selenium in the diet did not show any reduction in growth or changes in circulating hormone concentrations (7).

Results of the carcinogenesis experiment are shown in Chart 3 and Table 4. Both the tumor incidence and tumor yield indicated that selenium deficiency enhanced mammary carcinogenesis only when this nutritional condition was maintained in the postinitiation phase (Group 1 versus Group 3). Likewise, an excess of selenium intake inhibited neoplastic development only when this regimen was continued after DMBA administration (Group 5 versus Group 3). In either case, selenium deficiency or excess at the time of carcinogenic insult alone failed to produce a significant effect on subsequent neoplastic development, if selenium intake was returned to normal during the proliferative phase of tumor growth (Groups 2 and 4). Furthermore, the different selenium treatments were found to have no effect on the latency period of tumor formation (Table 4).

DISCUSSION

Based on the results of the present study, it is suggested that selenium-mediated modification of mammary tumorigenesis is probably not due to perturbations in carcinogenic initiation processes (i.e., metabolism or DNA adduct formation). Rather, some biochemical or biological changes induced by dietary selenium intake during the tumor-proliferative phase are most likely to be responsible for the enhancement or inhibition of mammary cancer. This conclusion is supported by the following observations. (a) Selenium deficiency or excess during the initial phase alone did not affect subsequent carcinogenesis. However, the reverse was true if these nutritional conditions were maintained in the postinitiation (i.e., promotional) phase. (b) Dietary selenium intake had no effect on carcinogen:DNA binding levels, the DNA adduct types, or on a battery of the microosomal enzymes that are involved in xenobiotic metabolism. These data for DMBA, a polycyclic aromatic hydrocarbon, are thus consistent with the finding by Thompson and Becci (23) that selenium was effective in inhibiting mammary carcinogenesis induced by N-methyl-N-nitrosourea. The latter compound is a direct alkylating agent which does not require activation.

Interpretation of the present study is limited in that data obtained from the liver may not always be directly applicable to our understanding of the influence of dietary selenium on mammary carcinogenesis. Nevertheless, even though the liver is refractory to DMBA-induced tumorigenesis, the HPLC profiles of the DMBA:deoxyriboonucleoside adducts obtained from the liver and the mammary gland are quite similar qualitatively (4). For example, a deoxyriboonucleoside adduct of the A-ring diol-epoxide, the supposed ultimate carcinogenic metabolite of DMBA, is present as a major peak (HP6) in both organs. By the same token, since the liver is the primary site for detoxification of xenobiotics, it is not unreasonable to survey selected enzymes of the mixed-function oxidase as an index of the capacity to metabolize carcinogens. Obviously, while a more exhaustive investigation might be desirable, suffice it to say that the enzymes that were selected and evaluated in this study are generally accepted as indicators of the microsomal Phase I detoxification system. In spite of the indications of this study, it might be prudent to rule out conclusively the possibility that selenium may influence other microsomal enzymes. For example, results from Burk and Masters (3) and also from this laboratory (8) have suggested that selenium may be involved in regulating the induction of microsomal hemoprotein(s) by barbiturates.
In the present study, we found that supplementation of selenium at 2.5 ppm prior to and around the time of DMBA administration failed to inhibit tumorigenesis. This is in contrast to our previous finding that 5 ppm of selenium supplementation in the same time frame did suppress neoplastic development (8). Previously, we have observed that mammary explants that were treated with DMBA in the presence of high levels of selenium had a low proliferative rate, and they also had a diminished potential to develop into tumors when grafted to isologous hosts (10). Likewise, Medina et al. (19) have shown that exposure to high concentrations of selenium will inhibit DNA synthesis, blocking the cells in the S-G2 phase of the cell cycle. Thus, it seems reasonable that, when rats were fed 5 ppm of selenium at the time of the carcinogen insult, the subsequently observed inhibition of tumor development is the result of a nonspecific effect which reduces the population of initiated cells. Alternatively, the effect could be due to an overall reduction in DNA synthesis resulting in a lowered rate of damage fixation. In addition, a recent publication by Thompson et al. (24) showed that supplementation of selenium at 2.5 ppm from 22 days before to 14 days after DMBA administration was effective in inhibiting mammary carcinogenesis in Sprague-Dawley rats. Although there are minor differences in terms of DMBA dosage and length of selenium supplementation in the aforementioned study, the protocol is very similar in many respects to that described in this paper. The reason for the discrepancy in the results between these 2 studies is not apparent at the present time.

Earlier, Marshall et al. (18) have shown that supplementation of 4 ppm of selenium in the drinking water of rats increased ring-hydroxylation and decreased N-hydroxylation of 2-acetylaminofluorene, thereby impeding activation and accelerating detoxification of this carcinogen. Grunau and Milner (6) have also demonstrated that selenium inhibited 3,4-oxidation of DMBA but stimulated 12-methyl oxidation. However, since selenium inhibits mammary carcinogenesis after the carcinogen is administered, mechanisms other than those involving alterations of carcinogen metabolism and DNA binding would have to be postulated.

The principal biochemical function of selenium is in regulating the activity of the selenium-dependent glutathione peroxidase, which is responsible for destruction of hydrogen peroxide and organic peroxides in the cell, thereby preventing peroxidative damage to macromolecules. In our study as well as the one reported by Lane and Medina using mice (16), the selenium concentrations in the mammary gland and the liver increased with increasing dietary intake, but glutathione peroxidase activity was unaffected. These results suggest that the protective effect of selenium in mammary tumorigenesis cannot be attributed to changes in glutathione peroxidase activity. Alternatively, the anticarcinogenic action of selenium may be mediated via perturbations of the host defense system. Not only is immune function depressed under selenium deficiency, but more interestingly, supplementation with selenium in quantities in excess of established dietary requirement has been reported to produce immunostimulatory effects (2). Further research is needed to determine the relevance between changes in the primary immune response as influenced by selenium and susceptibility to carcinogenesis. It is most likely that, at high but nontoxic levels of selenium, the mechanism may involve a combination of complex biological and biochemical adaptations which render the host more resistant to the expression of neoplasia.

REFERENCES


CANCER RESEARCH VOL. 45 JANUARY 1985

65

Downloaded from cancerres.aacrjournals.org on April 19, 2017. © 1985 American Association for Cancer Research.
Effects of Selenium on 7,12-Dimethylbenz(a)anthracene-induced Mammary Carcinogenesis and DNA Adduct Formation

Clement Ip and F. Bernard Daniel


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/45/1/61

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.