Selenium-enriched garlic inhibits the early stage but not the late stage of mammary carcinogenesis

Clement Ip1,4, Donald J.Lisk2 and Henry J.Thompson3

1Department of Surgical Oncology, Elm & Carlton Streets, Roswell Park Cancer Institute, Buffalo, NY 14263, 2Department of Fruit and Vegetable Science, Cornell University, Ithaca, NY 14853, 3Division of Laboratory Research, AMC Cancer Research Center, Denver, CO 80214, USA
4To whom correspondence should be addressed

Previous work has shown that the efficacy of cancer prevention by selenium-enriched garlic (Se-garlic) is primarily dependent on the action of selenium. Additionally, supplementation of Se-garlic inhibited the post-initiation phase of mammary carcinogenesis when it was given continuously to the animals. In this report, experiments were carried out in which treatment with the Se-garlic was started after carcinogen dosing (DMBA or MNU) but was restricted to either the early or late stage of neoplastic progression. The results from these two models showed that a short-term exposure to the Se-garlic for 1 month immediately following carcinogen administration was just as effective in cancer prevention as the continuous exposure regimen (5 months), suggesting that the Se-garlic may irreversibly alter the process of clonal expansion and/or selection of transformed cells during their early stage of development. Plasma and mammary tissue selenium levels essentially returned to basal levels at 1 month after withdrawal of supplementation. These observations imply that the outcome of cancer protection by short-term Se-garlic intervention was not due to a slow turnover, and therefore a lingering presence, of selenium in the target organ or in the circulation. The above finding was in contrast to that of a second study in which Se-garlic was supplemented starting at 13 weeks after carcinogen treatment. With this protocol, the number of new tumors and the number of new tumor-bearing rats found during the intervention period (weeks 13 to 22) were not statistically different between the control and supplemented groups, suggesting that Se-garlic had a minimal effect on the later stages of mammary carcinogenesis.

Introduction

We reported previously that garlic cultivated with inorganic selenium salt fertilization is capable of accumulating high levels of selenium in an organic form (1,2). As detailed in several of our publications (1,3,4), the idea behind this project is to use a food product to deliver a sufficient amount of selenium in a safe and efficient manner for the purpose of cancer prevention. Prior studies have convincingly demonstrated that the anticarcinogenic activity of the selenium-enriched garlic (Se-garlic*) is superior to that of natural garlic (1,4), or other chemically defined sources of selenium such as selenite or selenomethionine (1,5). Based on the results of a number of biological assays, it appears that the ability of the Se-garlic to protect against tumorigenesis is primarily dependent on the action of selenium provided by the vegetable (2).

With the use of the dimethylbenz[a]anthracene (DMBA)-induced mammary tumor model in rats, we reported in a recent paper that treatment with Se-garlic was inhibitory to both the initiation and post-initiation phases of chemical carcinogenesis (2). Part of the suppressive activity in tumor induction during the initiation phase could be accounted for by an interference in DMBA-DNA adduct formation in mammary epithelial cells. However, the mechanism underlying the diminished carcinogenic response during the post-initiation phase has yet to be elucidated. Suffice it to note that significant mammary cancer prevention was consistently observed when supplementation of the Se-garlic was instituted immediately after DMBA administration and continued until the animals were sacrificed 5 months later (2). In an attempt to investigate the mechanism of tumor inhibition during the post-initiation phase, we carried out a series of experiments in which treatment with the Se-garlic was started after carcinogen dosing but was restricted to either the early or late stage of mammmary carcinogenesis. The findings of this study design are reported here.

Materials and methods

The details regarding the growing and processing of the selenium-enriched garlic were described previously (1). The freeze-dried and finely milled material was added to the basal AIN-76A diet to achieve a final concentration of 3 p.p.m. Se (1). The basal AIN-76A diet contained 0.1 p.p.m. Se as sodium selenite. The selenium content of the diets (prepared fresh every week in our laboratory) was analyzed routinely for quality control.

Mammary tumors were induced in pathogen-free female Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) by intragastric intubation of 10 mg of DMBA or by intraperitoneal injection of 10 mg of methylnitrosourea (MNU) at 50 days of age. Animals were palpated weekly to determine the time of appearance and location of tumors. All experiments were terminated at 20–24 weeks post-carcinogen administration, the exact time is indicated in each figure or table presented in the Results section. At necropsy, the mammary glands were exposed for the detection of non-palpable tumors. Only histologically confirmed adenocarcinomas were reported. Tumor incidences at the final time point were compared by \( \chi^2 \) analysis, and the total tumor yield between groups was compared by frequency distribution analysis. These statistical analyses have been described in a previous publication (6).

Two sets of mammary carcinogenesis experiments were carried out according to the goals outlined in the Introduction section. The first set of experiments involved both the DMBA and MNU models and was aimed at investigating the effect of Se-garlic supplementation on the early stage of mammary carcinogenesis. Three days after receiving a single dose of DMBA or MNU, rats in each protocol were divided into three groups (\( n = 30/\)group) which included the following dietary treatments: (a) a continuous feeding of the basal AIN-76A diet (0.1 p.p.m. Se) as the control; (b) supplementation of Se-garlic at a final concentration of 3 p.p.m. Se during the entire post-initiation phase (i.e. until sacrifice); and (c) supplementation of Se-garlic at 3 p.p.m. Se for only 1 month, and a return to the basal diet at 0.1 p.p.m. Se for the remaining duration of the experiment. In order to determine the decay curve of tissue selenium levels upon withdrawal of the Se-garlic, a separate group of rats (not treated with DMBA or MNU, but age-matched to Group C above) were fed the same Se-garlic diet for 1 month and were sacrificed at either 1, 2 or 3 months (\( n = 6 \) per time point) after discontinuing the supplementation. Liver, kidney, mammary gland, and plasma were analyzed for total selenium by the fluorometric method (7).

*Abbreviations: Se-garlic, selenium-enriched garlic; DMBA, 7,12-dimethylbenz[a]anthracene; MNU, methylnitrosourea; IDP, intraductal proliferation.
The second study involved just the DMBA model and was aimed at investigating the effect of Se-garlic supplementation on the late stage of mammary carcinogenesis. Two groups of rats consisting of 20 per group were set up at the same time and in the same animal room. Both were given a single dose of DMBA at 50 days of age and were fed the basal AIN-76 diet (0.1 p.p.m. Se) until 12 weeks after DMBA. At this point, one group was picked randomly to continue to receive the basal diet while the other was switched to the Se-garlic diet (3 p.p.m. Se). Any new tumor appearance was carefully monitored as described above. The experiment was terminated at week 22 after DMBA. Comparisons of tumor incidence and tumor yield between these two groups were made at the beginning of selenium supplementation and at sacrifice.

Results

The result of the effect of Se-garlic supplementation on the early stage of mammary carcinogenesis in the DMBA model is shown in Figure 1. The diagram depicts the time course of cumulative tumor development in three groups of rats: a control group fed the basal diet; a second group receiving the Se-garlic diet continuously; and a third group which was given the Se-garlic for only 1 month after DMBA administration. As expected, an uninterrupted intake of Se-garlic markedly reduced both tumor incidence (see table insert in Figure 1) and tumor yield \( P < 0.05 \). Interestingly, a short-term feeding of Se-garlic was just as effective in inhibiting tumor occurrence as the continuous treatment. There was no statistically significant difference in the tumor data between the two selenium-supplemented groups. Thus it appears that exposure to the Se-garlic during the early stage of carcinogenesis is sufficient to confer a protective effect that is sustained in the absence of a continual administration of the supplement.

The above experiment was repeated with the MNU model. Every detail of the protocol was duplicated with the exception that MNU was used to induce mammary tumors. The results are shown in Figure 2. The format of presentation and the symbols used in Figure 2 are identical to that of Figure 1 so that these two figures can be compared side by side. It is evident that the MNU experiment confirmed the finding that treatment with Se-garlic for 1 month immediately after carcinogen administration significantly reduced tumor occurrence with an efficacy similar to that observed for a continuous treatment.

In order to find out how much selenium remained in the tissues with time following withdrawal of the Se-garlic, age-matched rats were fed the supplement for 1 month and then sacrificed at either 1, 2 or 3 months later. The decay curves of total selenium in various tissues are shown in Figure 3. In each panel, the selenium level from control rats given a basal diet containing 0.1 p.p.m. Se is denoted by an arrow along the Y-axis. As the data indicate clearly, the rate of selenium disappearance was rapid but varied depending on the tissues. Total selenium in plasma, mammary gland and liver returned to basal levels after 1 month or less. This was in contrast to the results in kidney where the selenium levels continued to decrease over the 3-month period.

The second objective of this report was aimed at investigating the effect of Se-garlic on the late stage of mammary carcinogenesis. The DMBA model was used in this experiment which, as explained in the Materials and methods section, involved delaying the supplementation until 12 weeks after DMBA dosing. This was then continued for another 10 weeks when the experiment was terminated. The results are summarized in
Table I. Effect of Se-garlic supplementation on the late stage of mammary carcinogenesis in the DMBA model

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (weeks)</th>
<th>Dietary Se (p.p.m.)</th>
<th>n</th>
<th>No. of tumor-bearing rats</th>
<th>No. of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1-12</td>
<td>0.1</td>
<td>30</td>
<td>17</td>
<td>41</td>
</tr>
<tr>
<td>B</td>
<td>1-12</td>
<td>0.1</td>
<td>30</td>
<td>18</td>
<td>43</td>
</tr>
<tr>
<td>A</td>
<td>13-22</td>
<td>0.1</td>
<td>30</td>
<td>10</td>
<td>24</td>
</tr>
<tr>
<td>B</td>
<td>13-22</td>
<td>3.0</td>
<td>30</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td>A</td>
<td>necropsy</td>
<td>0.1/0.1</td>
<td>30</td>
<td>27</td>
<td>68</td>
</tr>
<tr>
<td>B</td>
<td>necropsy</td>
<td>0.1/3.0</td>
<td>30</td>
<td>26</td>
<td>63</td>
</tr>
</tbody>
</table>

*The data denote the number of new tumor-bearing rats or new tumors detected during a particular period, i.e. weeks 1-12 or weeks 13-22. The necropsy results represent the final tumor data.

Table I. It should be noted that the two groups of rats were treated identically (i.e. they were given DMBA at 50 days of age and fed the basal AIN-76A diet) up to the time of Se-garlic supplementation. At week 12, all the tumor data, including incidence, number and rate of appearance, were very similar between the two groups (see the upper portion of Table I). For all practical purposes, the consistency of the inter-group data prior to the start of intervention minimized the concern of any perceived confounders of the treatment outcome.

Following supplementation of Se-garlic, a number of endpoints of carcinogenesis were carefully monitored. Specifically, these were related to the regression of existing tumors, the appearance of new tumors and changes in the number of tumor-bearing animals. During the intervention period (weeks 13 to 22), three tumors from the control group and four tumors from the Se-garlic group showed some detectable measure of regression (data not shown). The remainder of all the pre-existing tumors either grew larger in size or stayed relatively unchanged, with an approximately equal distribution of these two categories in either the control or supplemented groups. Thus selenium intervention appeared to have no effect on growth (or regression) of the pre-existing tumors. The information on new tumor development detected after the start of intervention was summarized in the middle portion of Table I. It can be seen that the number of new tumors and the number of new tumor-bearing rats found during this period were not statistically different between the two groups, suggesting that Se-garlic had a minimal effect on the late stage of mammary carcinogenesis.

Discussion

A novel and noteworthy finding in this study is that a short-term exposure to the Se-garlic, if implemented immediately after a carcinogenic insult, is able to prevent subsequent tumor development that usually occurs months later on. To our knowledge, this is the first report to show that successful intervention by selenium can be achieved with a short-term regimen in the post-initiation phase that is limited to an interval before any tumor becomes palpable. It is unlikely that the decreased cancer risk under this situation is due to a modulation of carcinogenic activation. In the DMBA model, previous work from our laboratory has shown that maximal binding of DMBA to mammary cell DNA takes place between 24 to 48 h after DMBA dosing (8). As indicated in the Materials and methods section, supplementation of Se-garlic was introduced 3 days after carcinogen administration. This argument is further supported by similar results from the MNU model since MNU is a direct alkylating agent with a half-life of <1 h under physiological conditions (9).

A question that comes to mind at this point is, why are transformed cells in the early stage of carcinogenesis more sensitive to selenium intervention than those in the late stage? More than a decade ago, Russo et al. (10) carefully documented the progression of mammary carcinogenesis in the DMBA model. Within 2 weeks after carcinogen administration, enlargement of the terminal end buds of the mammary gland is detectable in the wholemount preparation. These initial lesions, known as intraductal proliferations (IDPs), are characterized by a multilayered epithelium and are the precursors for the eventual development of palpable tumors. However, the total number of IDPs found in the entire mammary gland far exceeds the number of carcinomas that will arise. This phenomenon suggests that there might be different subsets of IDPs, only some of which will ultimately progress to carcinoma (11). Certain constituent(s) of the Se-garlic could conceivably inhibit or even eliminate specific populations of IDPs, thereby reducing the number of premalignant lesions that are normally present in the early stage of mammary carcinogenesis. A future challenge will be to test this hypothesis under an in vivo condition.

Do we know something about the chemistry of the Se-garlic that might help to support the above proposed concept? Cai et al. (12) have recently identified Se-methylselenocysteine as a major selenoamino acid in the Se-garlic. Using a subline of the MOD mouse mammary tumor cell culture model, Lu et al. (13) have previously reported that Se-methylselenocysteine induces growth inhibition in the absence of DNA single strand breaks (a measure indicative of a lack of genotoxicity). However, cell death upon exposure to Se-methylselenocysteine is accompanied by DNA double strand breaks, producing a pattern of DNA nucleosomal fragmentation characteristic of apoptosis. Lu et al. have also compared Se-methylselenocysteine with an aqueous extract of the Se-garlic and found a high degree of similarity in their activities in modulating cell morphology, growth, DNA integrity and other parameters. These findings are described in a companion paper in this issue of the journal (14). In addition to or in conjunction with the induction of apoptosis (thus resulting in the elimination of premalignant clones of cells), Se-methylselenocysteine may inhibit cell growth via down-regulation of cell cycle proteins. With the use of the TM6 mouse mammary tumor cell line, Sinha and coworkers (15) recently showed that Se-methylselenocysteine arrested cell growth in the G1 phase of the cell cycle. The disruption was associated with a 57% drop in cdk2 kinase activity and a 74% decrease in cyclin E-cdk2 content. Such changes in cell cycle progression are known to be related to apoptotic cell death (16).

Based on the above discussion, it is possible that Se-garlic, in part via the action of Se-methylselenocysteine, blocks clonal expansion and/or alters clonal selection of transformed cells by inducing apoptosis and by interfering with cell cycle transit.

A practical question that needs to be addressed in the future is related to the issue of whether the mechanism of apoptosis or cell cycle disruption induced by certain selenium compounds in animal mammary cancer models is applicable to human breast cancer. This is not a trivial problem because the genomic mutations found in rodent chemical-induced mammary tumors and human breast cancers are very different. The ideal approach would be to identify unique markers for specific clones of

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transformed cells and to determine if selenium will selectively induce apoptosis in some but not other clones. Current research in Thompson's laboratory is focused exactly on this kind of investigation. Although progress is tempered by the paucity of relevant markers in mammary tumors from animal sources, good evidence is emerging that aberrant expression of cyclins and other cyclin-related genes occurs frequently in mammary tumors of both rodent and human origin (17). Thus there is optimism to believe that chemical-induced mammary tumor model is a useful paradigm in the study of chemoprevention.

As shown in Figure 3, plasma and mammary tissue selenium levels essentially returned to basal values at 1 month after withdrawal of Se-garlic supplementation. This observation strongly suggests that the outcome of cancer protection by a short-term selenium intervention regimen is not due to a slow turnover, and therefore a lingering presence, of selenium in the target organ or in the circulation. There is an important implication to the above finding with respect to human application. If early transformed cells are indeed more sensitive to the inhibitory effect of selenium intervention, perhaps an intermittent schedule of treatment may still be beneficial to a reduction of cancer risk. A major concern with the continuous administration of any chemopreventive agent is the accumulation of metabolites that can lead to certain undesirable side effects. Reversible symptoms of selenosis associated with a chronic and excessive intake of selenium have been documented (18). Since the decay of total selenium from various tissues is relatively fast upon discontinuation of Se-garlic supplementation, an intermittent schedule of treatment could retain the attribute of cancer protection, while simultaneously alleviate the concern of selenosis due to the accumulation of high levels of tissue selenium.

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References


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