Effect of an aqueous extract of selenium-enriched garlic on
in vitro markers and in vivo efficacy in cancer prevention

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Previous work has shown that the efficacy of cancer prevention by selenium-enriched garlic (Se-garlic) is primarily
dependent on the action of selenium. An aqueous extract containing 43 µg Se/ml was prepared from lyophilized
Se-garlic powder by the Soxhlet method. The activity of this Se-garlic extract was evaluated in a transformed
mammary epithelial cell culture model for its effect on cell morphology, cell growth, cell cycle progression and the
induction of single and double stranded breaks in DNA. Comparisons were also made with a similarly prepared
extract from regular garlic, Se-methylselenocysteine (a major water-soluble seleno-amino acid identified in Se-
garlic) and selenium (used for fertilizing Se-garlic). In contrast to the regular garlic extract which produced little
or no modulation of the above parameters, treatment with the Se-garlic extract resulted in growth inhibition, G₁ phase
cell cycle arrest and apoptotic DNA double strand breaks in the absence of DNA single strand breaks. This pattern
of cellular responses was duplicated with exposure to Se-
methylselenocysteine. Selenium, on the other hand, induced
cell cycle blockage in the S/G₂-M phase, and a marked
increase in DNA single strand breaks (a measure of genotoxicity) in addition to growth suppression. The chemopreventive
efficacy of the two garlic extracts was also investigated
in the rat methylotritosourea mammary tumor model. Both extracts were supplemented in the diet for 1 month
immediately following carcinogen administration. Significant
cancer protection was observed with treatment by the
Se-garlic extract (at 3 p.p.m. Se in the diet), while little
benefit was noted with treatment by the regular garlic extract. Based on the above in vitro and in vivo findings, it is
hypothesized that the Se-garlic extract, in part via
the action of Se-methylselenocysteine, is able to inhibit
tumorigenesis by suppressing the proliferation and reduc-
ing the survival of the early transformed cells. Furthermore,
the data also support the concept that the modulation of
certain in vitro markers may be of value in predicting
the effectiveness of novel forms of selenium for cancer
prevention.

Introduction

Plants are capable of converting inorganic selenium in soil to
organic selenium compounds following the sulfur assimilatory
scheme. For example, seleniferous wheat is known to contain
selenomethionine as a major source of selenium (1). Garlic
cultivated with selenium fertilization accumulates high levels
of selenium in an organic form (2). Cai et al. (3) have recently
identified Se-methylselenocysteine as the predominant seleno-
amino acid in the selenium-enriched garlic (Se-garlic*). Their
analytical method involved extracting the lyophilized Se-garlic
powder with a mild acidic solution and then derivatizing
the supernatant for characterization by gas chromatography
coupled to either atomic emission detection or mass spectrometry
detection. Besides Se-methylselenocysteine, seleno-
cysteine was also found in the Se-garlic. However, the latter
compound was present in a much lower proportion.

A number of papers have appeared in the literature describing
the efficacy of cancer prevention by the Se-garlic powder
(2,4,5). Furthermore, it has been reported that the anticarcinogenic
activity of Se-garlic is likely to be accounted for by the
effect of selenium rather than the effect of garlic per se (6).
Past research by Ip and co-workers has documented the ability
of Se-methylselenocysteine to inhibit tumorigenesis (7,8).
Based on the results of several studies, both Se-
methylselenocysteine and Se-garlic are considered superior to either selenium
or selenomethionine in cancer protection (2,4,7,9). The reason
may be related to the facile conversion of Se-
methylselenocysteine to methylselenol (9,10), which might then act as the
immediate signal in instituting events associated with the
suppression of neoplastic development (11). Previous work
from Thompson's laboratory indicated that Se-methylseleno-
cysteine is able to induce a pattern of cellular and molecular responses that is distinct from that caused by selenium (12).
Essentially, the data suggest that different pathways affecting
cell proliferation and cell death are modulated specifically
depending on whether selenium undergoes initial metabolism
predominantly to hydrogen selenide (contributed by selenium) or methylselenol (contributed by Se-
methylselenocysteine).

An objective of this study was to compare the effects of an
aqueous extract of Se-garlic and Se-methylselenocysteine in
order to determine whether they would produce the same
spectrum of in vitro responses with respect to cell morphology,
cell growth, cell cycle progression, selenium retention, as well
as the induction of DNA single strand breaks (a measure of genotoxicity) and DNA double strand breaks (a measure of
apoptosis). This panel of biomarkers has been used successfully
to dissociate the genotoxic and growth inhibitory effects of
various inorganic and organic selenium compounds (12-16).
For the sake of completeness, selenite and an aqueous extract
from regular garlic (grown without selenium fertilization) were
also included in these cell culture experiments. Additionally,
the anticancer efficacy of the Se-garlic extract was evaluated
using the rat methylotritosourea (MNU)-induced mammary
tumor model. The implication of the in vitro findings will be
discussed in relation to the in vivo chemoprevention data.

Abbreviations: Se-garlic, selenium-enriched garlic; MNU, methylni-
trosoareia.

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Materials and methods

Preparation of Se-garlic extract

The Se-garlic that was used to prepare an aqueous extract consisted of 1355 p.p.m. Se dry weight. This particular sample of Se-garlic has been shown to be effective in inhibiting mammary carcinogenesis in a recent publication (6). Pre-weighed 5 g portions of the lyophilized and milled Se-garlic powder were placed in a cellulose thimble and extracted with 100 ml of distilled water for several cycles in a standard Soxhlet extraction apparatus. The aqueous Se-garlic extracts produced by this method contained a concentration of 40-45 μg Se/ml. In contrast, a similar aqueous extract prepared from regular garlic (cultivated without selenium fertilization) contained <0.01 μg Se/ml. Before these garlic extracts were used in the cell culture solution, the selenite was dried through 0.2 μ syringe filters for sterilization and then stored in 1 ml aliquots at -80°C. Selenium measurements of the filtrates indicated no loss of selenium by this process.

Synthesis of Se-methylselenocysteine

The synthesis of Se-methylselenocysteine was described in a previous publication of the authors (7). Briefly, DL-selenocysteine was reduced to selenocysteine with sodium borohydride, then reacted with iodomethane under anaerobic conditions at pH 7. The reaction mixture was adjusted to pH 2 and applied to a column of SP-Sephadex (H⁺). After washing the column with water, Se-methylselenocysteine was eluted with dilute HCl (pH 1.2), while selenocysteine was retained on the column. Fractions were collected and evaluated by thin-layer chromatography; those containing a single ninhydrin-positive spot having an appropriate Rf value were pooled and stored at -20°C.

Design of the in vitro experiments

The biological activities of the two aqueous garlic extracts, Se-methylselenocysteine and sodium selenite (J.T. Baker, Inc., Phillipsburg, NJ) were evaluated in two mouse mammary epithelial cell lines in a step-wise manner. The sequence of work encompassed the following aims: (i) to establish that the filter-sterilized extracts would not result in contamination of the culture system; (ii) to determine the appropriate concentration range for cell morphology and other quantitative studies, (iii) to measure the total cellular selenium content following treatment with the extracts or the other pure selenium compounds; and (iv) to assess the changes in cell growth, cell cycle progression and DNA integrity.

Cell lines and culture conditions

The TM2H mouse mammary preneoplastic epithelial cell line (17,18) and the MOD mouse mammary tumor cell line (15) were used in the in vitro studies. Experiments dealing with cell morphology, cell growth, selenium retention and cell cycle kinetics were carried out in the TM2H cells, while assessment of DNA integrity was carried out in the MOD cells. The reason for choosing a preneoplastic cell line for most of the in vitro marker studies was because of the prior observation that the Se-garlic powder was most active in inhibiting the early stage of mammary carcinogenesis (see companion paper, Ref. 19). On the other hand, the MOD cells have been well-characterized in previous reports (12,15,16) regarding their response to different types of selenium compounds in causing DNA single strand versus double strand breaks. It was our decision to continue this particular aspect of the research with the garlic extracts using the MOD cells.

The TM2H cells were grown at 37°C in 5% CO₂/95% air as previously described (12,15,16). The MOD cells were cultured in DMEM/F12 supplemented with 2% adult bovine serum, 10 mM HEPES, 5 ng/ml of recombinant human epidermal growth factor, rHu-EGF (Intergen, Purchase, NY), 10 μg/ml of insulin and 5 μg/ml of gentamicin. The MOD cells were cultured in DMEM/F12 supplemented with 2.4 g/l of NaHCO₃, 1% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 10 ng/ml of rHu-EGF, at 37°C in 5% CO₂/95% air as previously described (12,15,16).

Assays

Cell number was determined by DNA measurement using a Hoechst dye binding method (20) and verified by counting in a hemocytometer. For flow cytometry analysis, cells were stained with Krishan dye as described elsewhere (21). DNA single strand and double strand breaks were determined by a filter elution assay as detailed in previous publications of the authors (12,15,16). Total selenium was measured by the fluorometric method of Olson et al. (22). Data were evaluated statistically as described previously (15).

Design of the in vivo mammary cancer prevention experiment

Mammary tumors were induced in pathogen-free female Sprague-Dawley rats (Charles River Breeding Laboratories, Raleigh, NC) by intraperitoneal injection of 10 mg of methylN-nitrosourea (MNU) at 50 days of age. Three days after receiving a single dose of MNU, rats were randomly divided into three groups (n = 30/group), which included the following dietary treatments: (a) a continuous feeding of the basal AIN-76A diet containing 0.1 p.p.m. Se (2) as the control; (b) supplementation of the Se-garlic aqueous extract to the AIN-76A diet at a final concentration of 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 remainder of the experiment; and (c) same protocol as in group (b) except an equal volume of the aqueous extract from regular garlic was used instead. The selenium concentration of the regular garlic extract was so low that the dietary selenium level of group (c) was essentially identical to that of the basal diet (i.e. 0.1 p.p.m. Se). The 1-month supplementation period was adopted because, as can be seen from the data presented in the companion paper (19), this length of intervention by the Se-garlic powder was sufficient to produce a marked inhibition of mammary tumorigenesis in the MNU model. The statistical analysis of histologically confirmed adenocarcinomas among groups in the present study was described in the above mentioned paper.

Results

Cell morphology

The effect of increasing concentrations of the aqueous extract from either regular garlic or Se-garlic on the morphology of the TM2H cells was assessed in a preliminary experiment. The concentration of each filter-sterilized extract in the cell culture ranged from 2.5 to 10% of total media volume. Neither extract resulted in the occurrence of culture contamination. At a concentration of 10%, the regular garlic extract had no observable effect on cell morphology or density after 48 h (Figure 1, panel B) when compared to the untreated cells (panel A). In contrast, treatment of cells for 48 h with a concentration of 5% Se-garlic extract (27 μM Se, panel C) resulted in a decrease in cell density which was markedly accentuated by raising the Se-garlic extract concentration to 10% (54 μM Se, panel D). Nonetheless, the attached cells still retained a morphological appearance similar to that of the untreated cells. For comparison, the effects of 50 μM Se-methylselenocysteine or 5 μM selenite were presented in panels E and F, respectively. In general, the changes induced by 50 μM of Se-methylselenocysteine resembled those induced by 5% Se-garlic extract at 27 μM Se. On the other hand, exposure to 5 μM selenite induced extensive cytoplasmic vacuolation and cell detachment. Based on these initial qualitative observations, a decision was made to proceed with the following quantitative experiments using the Se-garlic extract at concentrations of 2.5 and 5% (13 and 27 μM Se).

Cell number and selenium content

The results showing the effects of Se-garlic extract, Se-methylselenocysteine or selenite on cell selenium content and cell number after 24 h of treatment are shown in Table I. Exposure to selenium of all sources increased cell selenium levels and decreased cell number, whereas exposure to the regular garlic extract had no impact on these parameters. The level of cellular selenium, however, appeared to depend on the source. Among the three selenium sources, selenite was present at the lowest concentration in the medium at 5 μM, yet it produced the highest level of selenium accumulation in cells at 41 ng/10⁶ cells. In contrast, Se-garlic extract and Se-methylselenocysteine, even when present at considerably higher concentrations in the culture medium (27 or 50 μM Se, respectively), produced much lower levels of selenium in cells. Overall, the ratio of medium selenium to intracellular selenium for the different selenium sources was in the order of Se-methylselenocysteine >> Se-garlic extract >> selenite. Despite some small differences in selenium uptake and/or retention observed between Se-methylselenocysteine and Se-garlic extract, the growth inhibitory effect of these two reagents was quite comparable. The same magnitude of cell number...
Cancer prevention and aqueous extract of selenium-enriched garlic

Fig. 1. Phase contrast photomicrographs of TM2H cells at 48 h after treatment. (A) Untreated control, (B) 10% regular garlic extract, (C) 5% Se-garlic extract at 27 μM Se, (D) 10% Se-garlic extract at 54 μM Se, (E) 50 μM Se-methylselenocysteine and (F) 5 μM selenite.

Table I. Effect of garlic extracts and different selenium compounds on cellular selenium content and cell number in TM2H cells at 24 h after treatment

<table>
<thead>
<tr>
<th>Source of selenium</th>
<th>Selenium treatment (μM)</th>
<th>Cell selenium (ng/10⁶ cells)</th>
<th>Cell number (× 10⁶/flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>0</td>
<td>1.0 ± 0.1</td>
<td>9.6 ± 0.3</td>
</tr>
<tr>
<td>5% Regular garlic extract</td>
<td>0</td>
<td>1.2 ± 0.1</td>
<td>9.5 ± 0.1</td>
</tr>
<tr>
<td>2.5% Se-garlic extract</td>
<td>13</td>
<td>16.3 ± 2.1</td>
<td>7.5 ± 0.2b</td>
</tr>
<tr>
<td>5% Se-garlic extract</td>
<td>27</td>
<td>27.8 ± 5.0</td>
<td>6.8 ± 0.4b</td>
</tr>
<tr>
<td>Se-methylselenocysteine</td>
<td>20</td>
<td>6.5 ± 0.6</td>
<td>8.4 ± 0.9</td>
</tr>
<tr>
<td>Se-methylselenocysteine</td>
<td>50</td>
<td>12.8 ± 0.8</td>
<td>6.8 ± 0.4b</td>
</tr>
<tr>
<td>Selenite</td>
<td>5</td>
<td>41.0 ± 2.1</td>
<td>5.3 ± 0.3b</td>
</tr>
<tr>
<td>5% Regular garlic extract</td>
<td>50</td>
<td>12.5 ± 0.9</td>
<td>5.8 ± 0.5b</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± SE (n = 3 flasks).

bP < 0.05 compared to the untreated control value.

reduction was achieved at the highest level of Se-garlic extract (27 μM Se) or Se-methylselenocysteine (50 μM Se) tested.

In order to determine if there might be an interaction between the other water-soluble constituents of garlic and Se-methylselenocysteine, cells were treated with a combination of 5% regular garlic extract and 50 μM Se-methylselenocysteine. The results of this combination (Table I), when compared to that of Se-methylselenocysteine alone or regular garlic extract alone, did not support the existence of such an interaction.

Fig. 2. Effect of garlic extracts and different selenium compounds on TM2H cell cycle distribution at 24 h after treatment.

Cell cycle and DNA integrity
Flow cytometry was used to study changes in cell cycle progression. The determinations were made at 24 h after treatment and the results are shown in Figure 2. Compared to the untreated control, regular garlic extract had no discernible effect on cell cycle kinetics. Se-methylselenocysteine, on the
Treatment 'TJNA double strand and single strand breaks were calculated as follows: Values are expressed as mean ± SE (n = 3).

<table>
<thead>
<tr>
<th>Source of selenium</th>
<th>Selenium treatment (µM)</th>
<th>Double strand breaks (%)</th>
<th>Single strand breaks (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>0</td>
<td>2.8 ± 0.2</td>
<td>9.1 ± 0.6</td>
</tr>
<tr>
<td>5% Regular garlic extract</td>
<td>0</td>
<td>2.6 ± 0.1</td>
<td>7.7 ± 0.4</td>
</tr>
<tr>
<td>5% Se-garlic extract</td>
<td>27</td>
<td>6.4 ± 0.2^c</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>Se-methylselenocysteine</td>
<td>50</td>
<td>4.8 ± 0.4^c</td>
<td>5.4 ± 0.4</td>
</tr>
<tr>
<td>Selenite</td>
<td>5</td>
<td>14.6 ± 0.7^c</td>
<td>59.2 ± 2.5^c</td>
</tr>
</tbody>
</table>

*Values are expressed as mean ± SE (n = 3).
^DNA double strand and single strand breaks were calculated as follows: double strand breaks = N/(N + A + F)×100%, single strand breaks = A/(A + F)×100%, where N, A and F were the radioactivity collected in the neutral elutable fraction, alkali elutable fraction and on the filter, respectively (Ref. 12).
^P < 0.05 compared to the untreated control value.

Table III. Efficacy of mammary cancer prevention by treatment with either Se-garlic extract or regular garlic extract

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dietary selenium (p.p.m.)</th>
<th>Tumor incidence</th>
<th>Total no. of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.1</td>
<td>28/30</td>
<td>78</td>
</tr>
<tr>
<td>Regular garlic extract</td>
<td>0.1</td>
<td>25/30</td>
<td>69</td>
</tr>
<tr>
<td>Se-garlic extract</td>
<td>3.0</td>
<td>14/30^b</td>
<td>39^b</td>
</tr>
</tbody>
</table>

^Rats were injected with MNU at 50 days of age. Supplementation of either Se-garlic extract or regular garlic extract was started 3 days later and continued for 1 month. The experiment was terminated 21 weeks post MNU.
^bP < 0.05 compared to the control value.

Discussion

This study shows that an aqueous extract of Se-garlic inhibited cell proliferation, caused cell cycle arrest at the G1 phase, and induced DNA double strand breaks characteristic of apoptosis in neoplastic mammary epithelial cells. Exposure of cells to Se-methylselenocysteine, a water-soluble organic selenium compound identified in the Se-garlic (3), resulted in a similar spectrum of responses, both qualitatively and quantitatively. These findings imply that Se-garlic, in part via the action of Se-methylselenocysteine, may be able to suppress the proliferation and reduce the survival of transformed populations of mammary epithelial cells. The idea is also supported by our observation that treatment of rats with the Se-garlic extract for only 1 month immediately after carcinogen administration was sufficient to confer a lasting protective effect on subsequent mammary tumorigenesis. It is important to note that the above hypothesis does not preclude the possibility that other selenium compounds that remain to be characterized in the Se-garlic extract also play a role in cancer prevention.

Compared to the aqueous extract from Se-garlic, a similarly prepared extract from regular garlic did not produce any perturbation in cell growth, morphology, cell cycle kinetics, or DNA fragmentation. Thus we conclude that the changes observed in our cell culture experiments in response to the Se-garlic extract were most likely due to the effect of extractable selenium from the Se-garlic. More importantly, the active principle in the Se-garlic extract had biological activities akin to that of Se-methylselenocysteine, but unlike that of selenite, which was used to fertilize the garlic. As can be appreciated from the data shown in Tables I and II, the growth inhibitory effect of Se-garlic extract or Se-methylselenocysteine was achieved in the absence of any in vitro genotoxic effect as measured by DNA single strand breaks. Conversely, genotoxicity is an undesirable outcome that has been detected consistently upon selenite exposure (12–16).

The parallelism between the responses to Se-garlic extract and Se-methylselenocysteine was remarkable but not perfect. When equimolar selenium concentrations were present in the media, cells exposed to Se-garlic extract had higher levels of selenium than those exposed to Se-methylselenocysteine (Table I). Part of the difference might be due to the racemic mixture of Se-methylselenocysteine added to the culture media, whereas plants synthesize only the L form (therefore the L form is expected to be present in the Se-garlic extract). In addition to Se-methylselenocysteine, it is likely that there are other unidentified selenium compounds in the Se-garlic extract. These compounds, which might be structurally and functionally distinct from the selenoamino acids, could be taken up more efficiently by the cells or metabolized in a different manner than Se-methylselenocysteine. Such a possibility warrants further examination.

Not to be overlooked is the paradigm highlighted here concerning how the modulation of certain in vitro markers...
could be utilized to forecast the likelihood of successful cancer intervention by selenium. The regular garlic extract did not affect morphology, growth, cell cycle transit, or DNA double strand breaks in the cell culture experiments. It was found to have little or no cancer preventive activity in the animal feeding experiment. This set of data was exactly opposite to that obtained with the Se-garlic extract, which produced a positive (and desired) response in each of the above parameters, both in vitro and in vivo. There is thus a plausible correlation between the relevance of these in vitro markers and the in vivo. There is thus a plausible correlation between the relevance of these in vitro markers and the consequence of in vivo cancer protection. Whether these markers apply only to the biology of selenium chemoprevention or could be extended to other classes of anticancer agents remains to be investigated.

There are a number of attributes associated with the aqueous Se-garlic extract that merit additional comment. First, the extract clearly contains an anticancer factor(s) which is powerful, stable and water-soluble. Therefore it offers a convenient and suitable vehicle for further evaluation in cell culture experiments. Although most interest has been focused on the volatile and lipid-soluble sulfides in garlic, water-soluble organosulfur compounds also have good anticancer activity (23). However, the aqueous extract of Se-garlic has much greater activity than the aqueous extract of regular garlic, as shown in our study. Second, the extract is an appropriate starting place for the purification and characterization of other potentially active compounds in addition to Se-methylselenocysteine. The interaction between various water-soluble selenium compounds in cancer chemoprevention has not been delineated. The ability to prepare an aqueous extract from garlic in a reproducible manner should facilitate future research in this direction. Third, the Se-garlic extract was capable of inhibiting tumorgenesis with an efficacy comparable to that seen with the Se-garlic powder (refer to companion paper, Ref. 19). Instead of using the powder material, the extract conceivably represents an alternative form of delivering selenium for the purpose of cancer prevention. In summary, the continuing investigation of the extract from Se-garlic is expected to open new opportunities in understanding and improving the strategy of cancer intervention by selenium.

Acknowledgement

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References
