Impact of ascorbic acid on selenium-induced growth inhibition of canine mammary tumor cells *in vitro*

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Ascorbic acid (50 or 125 μmol/L) accentuated the in vitro growth inhibition of canine mammary tumor cell line 13 (CMT-13) induced by sodium selenite (12.6 μmol/L). While selenate was less effective than selenite in inhibiting growth of these cells, its inhibitory effect was also amplified by supplemental ascorbic acid. Ascorbic acid did not modify the anti-proliferative effects of selenomethionine. Both ascorbic and D-isoascorbic acid (an ascorbate analogue without anti-scorbutic properties) were equally effective in increasing the toxic effects of selenite against CMT-13 cells. Ascorbic acid (125 μmol/L) increased cellular selenium retention. These data show that the antitumorigenic effects of selenite and selenate can be enhanced by supplemental ascorbic acid or a related reducing compound.

Keywords: selenite; ascorbic acid; mammary tumors

Introduction

Selenium, an essential dietary component, has been proposed to have both anticarcinogenic and antitumorigenic properties. Epidemiological data indicate higher dietary intakes of selenium are inversely related to cancer incidence.[1-3] Likewise, laboratory investigations have documented that several forms of selenium can inhibit both virally and chemically induced tumors occurring at a variety of sites.[4-7] Although selenium is effective in inhibiting both the initiation and promotion phases of carcinogenesis,[6-8] the exact mechanism by which it alters tumor risk remains unclear. Considerable in vivo and in vitro evidence also reveals that selenium can alter the growth of some tumor cells.[9-15] However, it is evident that all tumor cells are not equally susceptible to the toxic effects of selenium.[9,10,14,15]

Results of epidemiological and laboratory investigations suggest that dietary ascorbic acid could also be an important factor capable of altering cancer risk.[16-18] Although a depression in nitrosamine formation is a well-documented result of the reducing capacity of ascorbic acid, it is clear the biological functions of this vitamin are far more diverse and complex than originally appreciated.[16]

While the bioavailability of selenium is known to be influenced by a variety of nutrients, including heavy metals,[19] little information exists on the impact of these interactions on the anticarcinogenic and antitumorigenic effects of this trace element. The ability of ascorbic acid to influence the oxidation/reduction state of several minerals makes it a prime candidate for possibly influencing selenium nutriture.[20] While several investigators have proposed that a metabolic interaction exists between selenium and ascorbic acid,[21-24] the physiological significance of this interaction in the cancer process remains largely unexplored.

The influence of ascorbic acid on selenium utilization is equivocal and may depend on the concentration or ratio of the nutrients examined. Combs and Pesti[21] showed that dietary ascorbic acid elevated selenium-dependent glutathione peroxidase activity and reduced the dietary selenium requirement of vitamin E-deficient chicks. These investigators suggested that ascorbic acid inhibited the oxidation of dietary selenium and thereby promoted its absorption and utilization. Poovaiah et al.[22] demonstrated that although dietary ascorbic acid

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did not modify tissue selenium concentrations, it did increase plasma, liver, and erythrocyte glutathione peroxidase activities in the guinea pig. There also is evidence to suggest that ascorbic acid may actually reduce the utilization of selenium. Hill[23] reported that very high intakes of ascorbic acid reduced the toxicity of selenium in chicks, while moderate dosages inhibited intestinal absorption of this trace element.

A comprehensive search of the literature revealed a dearth of information on the interaction of ascorbic acid and selenium in cancer models. Jacobs and Griffin[24] compared the effect of selenium in the presence or absence of supplements of various antioxidants on the incidence of dimethylhydrazine-induced colon tumors in rats. This study revealed that supplying either selenium or ascorbic acid alone significantly decreased the incidence of tumors. However, the simultaneous treatment of rats with both of these nutrients significantly increased the tumor incidence compared with treatment with either nutrient alone.

The widespread use of vitamin C supplements by Americans emphasizes the need to more thoroughly understand the physiological effects of this essential nutrient. Likewise, the widespread belief by many that both selenium and ascorbic acid can prevent or reduce the incidence of some types of tumors emphasizes the need to critically evaluate both of these essential nutrients in the cancer process. The object of the present research was to characterize the interactions of selenium and ascorbic acid using the in vitro growth of a transplantable mammary tumor as a cancer model.

Methods and materials

Neoplastic cells

The present experiments were conducted with cultures of canine mammary tumor cells (CMT-13). This cell line was established and characterized by Dr. J. Watrach at the University of Illinois (Champaign-Urbana, IL USA) and utilized extensively in our laboratory.[10,13,14] The CMT-13 cell line was obtained from a primary mammary tumor of an eleven year old mixed Terrier and had morphologic properties of a cystic adenocarcinoma.

Chemicals

Ringer's Phosphate Medium Inclusive-1640 (RPMI-1640), fetal bovine serum, sodium selenite (Na_2SeO_3), selenomethionine, bovine pancreas insulin, penicillin-streptomycin, trypsin, and L-ascorbic acid were obtained from Sigma Chemical Company (St. Louis, MO USA) and were used without additional purification.

Culture conditions

Cells were grown in RPMI-1640 medium supplemented with insulin, penicillin-streptomycin, and fetal bovine serum. Cultures were maintained at 37° C with 5.0% CO_2. In each experiment, cells were plated at approximately 4.0×10^3 cells/cm^2 and incubated in complete medium for 24 hours before initiation of all experimental treatment. All test solutions were prepared separately in deionized water and added to cultures in a maximum volume of 0.1 mL. This quantity of ascorbic acid was found not to modify the pH of the incubation medium. At the time of treatment all control cultures received an equivalent volume of water. At the termination of each experiment, cells were harvested with 0.025% trypsin-EDTA, diluted with RPMI-1640 medium, scraped, and centrifuged at 500*g* for 10 minutes. The pellet was then resuspended in medium and cell counts determined by trypan blue exclusion using a hemocytometer. For determination of change in growth, the original mean count is defined as the cell count at the time of treatment (generally 24 hr after plating).

Statistical analysis

In all cases, the values reported herein are based on replicates of four flasks. Significant differences among treatments means were determined by analysis of variance statistics and the application of the least significant difference test. The value of $P \leq 0.05$ was considered statistically significant.

Description of experiments

Experiment 1 examined the temporal effects of ascorbic acid (50 μmol/L) and selenium (12.6 μmol/L), either separately or in combination, on the in vitro growth of CMT-13 cells. Cell numbers were determined 24, 48, 72, and 96 hours after treatment. Experiment 2 compared the effects of ascorbic acid (125 μmol/L) on the growth inhibition caused by 12.6 μmol/L selenium as selenate or selenite. The comparative effects of ascorbic acid (125 μmol/L) on selenite (12.6 μmol/L) and selenomethionine (62.5 μmol/L) induced growth inhibition of CMT-13 cells was examined in experiment 3. Experiment 4 compared the effects of ascorbic acid (125 μmol/L) and its structural analogue, D-isoascorbic acid (125 μmol/L), on the growth inhibition of these canine cells induced by supplemental selenite (12.6 μmol/L). Experiment 5 investigated the effect of ascorbic acid on cellular selenium retention in cells exposed to ^{75}Se-selenite (125 μmol/L; 0.8 mCi/mmole). Cellular retention of selenium was determined by detecting the gamma emissions of ^{75}Se (Gamma 5500; Beckman Instruments, Fullerton, CA USA).

Results

In experiment 1, growth of CMT-13 cells increased logarithmetically during the 4-day study in the presence or absence of 50 μmol/L supplemental ascorbic acid *(Figure 1)*. Treatment with selenite resulted in a marked and persistent growth inhibition. Addition of ascorbic acid to cells exposed to selenite resulted in a greater growth inhibition than occurred with cells treated with only selenite *(Figure 1)*. Combined treatment with selenite and ascorbic acid virtually eliminated growth of these cells throughout the 4-day study.

Experiment 2 demonstrated that the synergistic effect of ascorbic acid in inhibiting growth was not unique to selenite *(Figure 2)*. While selenite was more effective in inhibiting the growth of this cell line than isomolar selenate (80% versus 10%, respectively), the addition of ascorbic acid (125 μmol/L) enhanced the growth depression caused by either form of selenium *(Figure 2)*. The influence of ascorbic acid was slightly more pronounced in cultures treated with selenite than selenate (50% versus 33%, respectively). In contrast, experiment 3 revealed that ascorbic acid did not alter the growth inhibitory effects of selenomethionine on CMT-

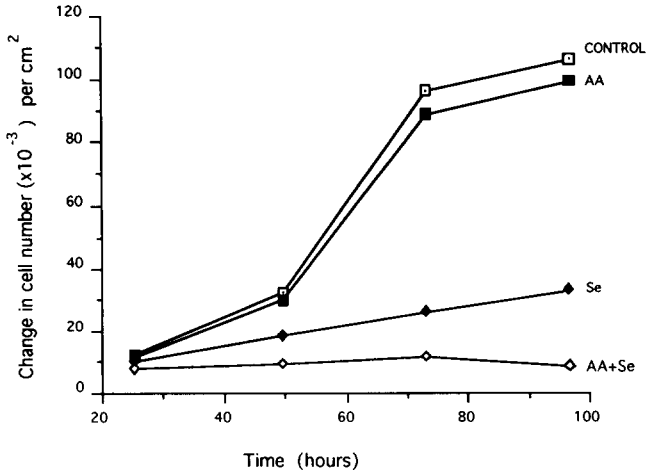

Figure 1 Growth of CMT-13 cells treated with selenite (Se) and ascorbic acid (AA). Cells were treated with either 12.6 μmol/L Se or 50 μmol/L ascorbic acid or the combination of both. Mean standard errors within groups were 0.075 at 24 hours, 0.052 at 48 hours, 1.07 at 72 hours, and 1.86 at 96 hours. Selenite significantly inhibited growth at all times examined ($P < 0.01$). Combining selenite and ascorbate resulted in a greater inhibition of growth than selenite alone at all times examined ($P < 0.01$).

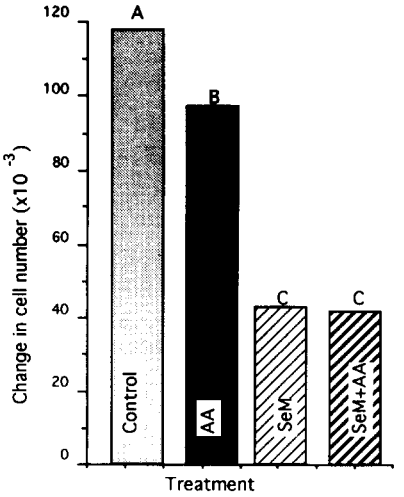

Figure 3 Growth of CMT-13 cells following selenomethionine (SeM) (62.5 μmol/L) or ascorbic acid (AA) (125 μmol/L) treatment, either singly or in combination. Cell numbers were determined 72 hours after treatment. The pooled standard error across treatments was 1.21. Columns not sharing a common letter differ $P < 0.05$.

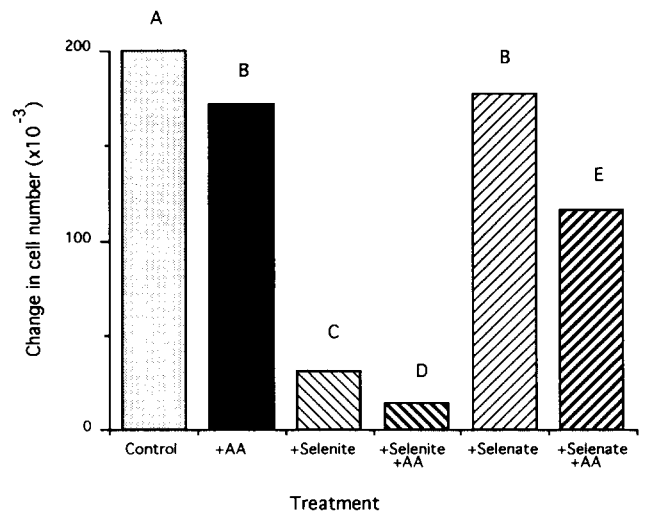

Figure 2 Influence of selenium (12.6 μmol/L) treatment, as selenite or selenate, in the presence or absence of ascorbic acid (AA) (125 μmol/L) on the growth of CMT-13 cells. Cell numbers were determined 72 hours after treatment. The pooled standard error of the mean across treatments was 2.04. Columns not sharing a common letter differ $P < 0.05$.

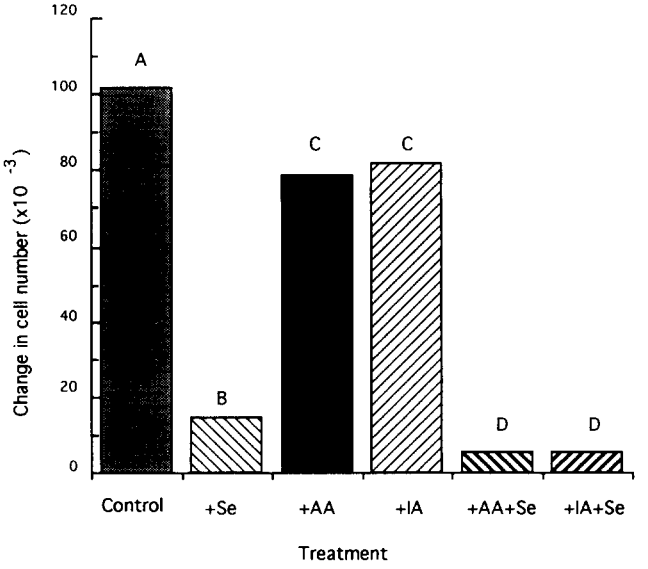

Figure 4 Growth of CMT-13 cells following selenite (Se) treatment (12.6 μmol/L) in the presence or absence of ascorbic acid (AA) or D-isoascorbic acid (IA) (125 μmol/L). Cell numbers were assessed at 72 hours after treatment. The pooled standard error was 5.92. Columns not sharing a common letter differ $P < 0.05$.

13 cells *(Figure 3)*. While selenomethionine was added at a five-fold higher molar concentration than selenite, it caused a minimal, although significant, depression in growth of CMT-13 cells. No interaction between selenomethionine and ascorbic acid was detected.

Experiment 4 demonstrated that ascorbic acid and D-isoascorbic acid had similar effects on the growth of CMT-13 cells, whether administered alone or in combination with sodium selenite *(Figure 4)*. Both substances inhibit cell growth slightly when added to the medium. Combined treatment with either ascorbic acid or D-isoascorbic acid and selenite resulted in comparable accentuation of the growth inhibition compared with selenite supplementation alone.

Experiment 5 revealed that ascorbic acid supplementation increased selenium retention by 60% during the 72-hour incubation period *(Table 1)*. A slight, but nonsignificant, increase in selenium retention was noted within the first 24 hours after treatment.

Table 1 Influence of ascorbic acid on selenium retention by CMT-13 cells

Treatment	Selenium retention (nmoles/cell) 24 Hrs	72 Hrs
Selenite (6.2 μmol/L)	30.0 ± 4.5[a]	1125 ± 78.0[b]
Selenite + ascorbic acid (100 μmol/L)	38.2 ± 4.1[a]	1793 ± 23.0[c]

Mean ± SEM for four observations per treatment.
Means not sharing a common superscript differ $P < 0.05$.

Discussion

Results of the present studies confirm that selenite supplementation inhibits the in vitro growth of CMT-13. The magnitude of this growth inhibition increased with the duration of exposure to selenite from approximately 45% at 24 hours to over 75% by 72 hours. Similar results have been reported for other, but not all, tumor cells.[9,10,13,14] Consistent with other observations,[10,11] these data also document that all forms of selenium are not equally effective in inhibiting cell growth. Selenomethionine and selenate were significantly less effective than selenite in inhibiting the in vitro growth of this cell line. The reason for these differences in growth inhibition is unknown, but may relate to the ability of the compounds examined to be transformed to a particular ionic species during metabolism. Fico et al.[10] showed that cellular selenium uptake could not account for the differences in growth inhibition caused by different forms of selenium. Recent results from our laboratory suggest that the ratio of selenium to intracellular glutathione may be a critical factor in determining at what point cells succumb to the toxic effects of this trace element.[24]

Ascorbic acid was also found in the present studies to inhibit the growth of CMT-13 cells by about 25% when supplied at concentrations of 125 μmol/L. Data of Gardner and Duncan[17] revealed that ascorbic acid suppressed both the in vivo and in vitro growth of BL6 mouse melanomas. Other reports suggest the effects of ascorbic acid are extremely complex and likely influenced by the cell type examined. Park[18] noted considerable variability in the impact of ascorbic acid on bone cell aspirates from 163 humans with acute non-lymphocytic leukemia. In their in vitro studies growth was enhanced in 33%, suppressed in 17%, and unaltered in 50% of the human leukemia colony forming cells examined. Nevertheless, ascorbic acid and a structural analogue were effective in reducing the growth of this canine-derived neoplastic cell line.

The present studies reveal that ascorbic acid can enhance the growth depression caused by some, but not all, forms of selenium. Ascorbic acid accentuated the growth inhibition caused by both selenite and selenate, but not that caused by selenomethionine treatment. D-Isoascorbic acid, a compound with similar oxidation-reduction properties to ascorbic acid also proved to be effective in enhancing selenite-induced growth inhibition. These investigations suggest the redox state of selenium preserved by ascorbic acid is critical for the observed growth inhibition.

Ascorbic acid treatment increased selenium retention in the present studies. Martin et al.[25] reported that ascorbic acid supplements aided in intestinal absorption of selenite compared with controls not receiving ascorbic acid. In this human study, Martin et al.[25] also noted a concentration dependent in selenium absorption from supplemental ascorbic acid with 1 g/day resulting in greater selenite absorption than 20 mg ascorbic acid per day. The greater cellular growth depression caused by selenite in the presence of ascorbic acid likely relates to enhanced cellular uptake of this trace element.

In conclusion, these investigations clearly document the ability of ascorbic acid and a structural analogue to modify the antiproliferative effects of some inorganic forms of selenium. Additional studies are needed to determine the usefulness of combined nutritional supplements of ascorbic acid and selenium on the growth of other neoplasms, especially those of human origin.

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