Consequences of selenite supplementation on the growth and metabolism of cultures of canine mammary cells

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Previous studies with cultures of canine mammary cells revealed differences in the degree of growth inhibition caused by selenite supplementation, with canine mammary tumor cell line 13 > 11 ≫ non-neoplastic canine mammary cells. The present studies show this variation in growth retardation cannot be explained by selenium retention. Intracellular glutathione related inversely to the degree of growth inhibition resulting from the addition of selenite. Dimethyl selenide formation by S-9 preparations corresponded to the sensitivity of the culture to supplemental selenite. DL-buthionine-SR-sulfoximine, a specific inhibitor of glutathione biosynthesis, accentuated the growth inhibition and prevented the increase in intracellular glutathione caused by supplemental selenite. Treatment of canine mammary tumor cell line 13 cultures with DL-buthionine-SR-sulfoximine resulted in a persistent depletion of intracellular glutathione without affecting growth. Glutathione reductase activity, before and following selenite, was inversely related to the degree of growth inhibition, with canine mammary tumor cell line 13 > 11 > non-neoplastic canine mammary tumor cell line. Selenite addition increased the activity of γ-glutamylcysteine synthetase in canine mammary tumor cell line 11 and non-neoplastic canine mammary cells, but not in canine mammary tumor cell line 13 cells. The present data suggest the differences in the growth inhibition caused by selenite among these mammary cells is related to glutathione regulation and ultimately to selenium detoxification.

Keywords: canine mammary cells; selenite; glutathione; DL-buthionine-SR-sulfoximine

Introduction

Broghamer et al.[1] have reported a negative correlation between serum selenium concentrations and the number of multiple primaries, tumor recurrences, and distant metastases. Subsequent retrospective studies suggested that all tumors are not equally sensitive to higher serum selenium concentrations since a similar relationship was not observed in patients with reticuloendothelial cancers.[2] Increasing evidence has revealed that some, but not all, tumor cells are sensitive to growth inhibition caused by supplemental selenium.[3–8] Fico et al.[8] observed that selenium supplementation caused a marked *in vitro* growth inhibition of two canine mammary tumor (CMT) cell lines without significantly modifying the growth of a culture of non-neoplastic canine mammary (NCM) cells. Based on these studies, the relative sensitivity to growth inhibition following increasing quantities of selenium was: CMT-13 > CMT-11 ≫ NCM cells. The continued examination of the response of these cells may help reveal the basis by which selenium is capable of inhibiting the growth of some tumors.

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Excess selenium is known to influence the sulfhydryl status of various tissues and this response depends on the form and mode of selenium administration and the time of glutathione analysis.[9-12] Alterations in the sulfhydryl status of the cell probably reflect the absolute requirement of glutathione (GSH) and nicotinamide adenine dinucleotide phosphate (NADPH) for the reductive elimination of selenium.[13,14] The sensitivity of cells to selenium supplementation may therefore depend on cellular glutathione concentration and/or other factors (e.g., glutathione reductase) necessary for the regulation of cell sulfhydryl status. In cells less able to regulate these factors a toxic entity may accumulate resulting in the inhibition of growth.

Differences in sensitivity to supplemental selenium may also relate to the ability of the cell to reductively detoxify selenium. Selenite (Na_2SeO_3) reduction by glutathione reductase, NADPH and glutathione leads to the formation of selenodiglutathione (SDG), which is further reduced by glutathione reductase, NADPH and glutathione to H_2Se and is ultimately converted to dimethyl selenide ($[CH_3]_2Se$) or the selenonium ion.[13] Dimethyl selenide has been shown to be less effective than selenite in causing an inhibition of Ehrlich ascites tumor growth.[15] Glutathione and glutathione reductase may therefore be important determinants of the degree of sensitivity observed. A compromised ability to regulate either glutathione or glutathione reductase could result in a decreased cellular capacity to reduce selenite to dimethyl selenide and may result in the accumulation of an active, toxic metabolite.

The present studies were designed to determine if differences in the sensitivity of cultures of canine mammary cells correlated with their ability to accumulate and metabolize selenite. Additional studies were designed to evaluate the ability of these cultures to respond to supplemental selenite by modifying cellular glutathione concentration and/or by modifying the activity of specific enzymes.

Materials and methods

Two cell lines, CMT-13 and CMT-11, and NCM cells were grown and maintained in Ringer Phosphate Media Inclusive (RPMI-1640) (Sigma Chemicals, St. Louis, MO, USA) containing 10% fetal bovine serum as previously described.[8] The characteristics of each tumor cell line and the non-neoplastic cells were previously described.[8] The basal concentration of selenium in the incubation medium was analyzed by the method of McCarthy et al.[16] and found to be negligible, ~ 7 ng/ml.

In general, cultures were allowed to grow for 24 hours before the initiation of any experimental treatments. Sodium selenite at a final concentration of 9.6 μM was added for a 24-hour treatment period. This concentration of selenite was previously observed to cause growth inhibition of CMT-13 cells without the loss of viability between 24 and 96 hours.[8] Thus, any cellular alterations resulting from supplemental selenite at this concentration should be evident at 24 hours.

Cultures were harvested by washing twice with phosphate buffered saline (PBS) followed by gentle scraping and centrifugation at 500 x *g* for 10 minutes. Control cultures had generally grown to subconfluency (ca 1×10^7 cells per 150 cm^2 flask) at the end of the experimental period. Cellular protein was determined by the method of Lowry et al.,[17] and cell numbers were determined electronically (Coulter Electronics, Hialeah, FL, USA). At least 3 flasks per treatment per mammary cell were utilized for analysis in each experiment. Differences were detected by one-way analysis of variance followed by Scheffe's F-test.[18] Specific conditions used for each experiment are described below.

Experiment 1 monitored cellular retention of selenium from selenite as a function of time. CMT-13, CMT-11, and NCM cells were grown in the presence of $[^{75}Se]Na_2SeO_3$ (sodium selenite)(Amersham Corporation, Arlington Heights, IL, USA) at a concentration of 9.6 μM (3 μCi/μg Se) and the amount of selenium retained was determined after 5, 10, 30, and 60 minutes and 6 and 24 hours. Selenium retention was expressed per mg cellular protein and per cell. The ^{75}Se radioactivity was counted using a multi-channel analyzer (Spectrum 88, The Nucleus, Oak Ridge, TN, USA).

The production of dimethyl selenide was examined in experiment 2 by the method of Ganther *et al.*[19] Preliminary studies revealed that the formation of $(CH_3)_2Se$ was linear with respect to protein and maximal when selenium was present at a concentration of 40 μM. Cells were rapidly homogenized in a phosphate buffer (pH 6.25) and centrifuged at $9000 \times g$ for 20 minutes. The resultant supernatant (S-9) was incubated anaerobically under a continuous stream of nitrogen gas at 37°C. Dimethyl selenide formation was defined as the quantity of ^{75}Se lost from the flask after a 20 minute incubation.

Cells grown in the presence or absence of selenite (9.6 μM) for 24 hours were used for the analysis of oxidized and reduced glutathione by the procedure of Teitze (20) in experiment 3. Glutathione was determined for 6 minutes after the addition of Ellman's reagent by measurement of the increase in absorbance at 412 nm using a molar extinction coefficient of 13,600.

In experiment 4, glutathione reductase was determined in the $27,000 \times g$ supernatant from cells grown in the presence or absence of sodium selenite (9.6 μM). The activity from three flasks per treatment was determined using oxidized glutathione as the substrate by measuring the oxidation of NADPH at 340 nm.[21]

Experiment 5 examined the influence of selenite (9.6 μM for 24 hours) on the activity of γ-glutamylcysteine synthetase as measured by the method of Richman and Meister.[22] The reaction was initiated by the addition of a $27,000 \times g$ enzyme supernatant and the quantity of liberated inorganic phosphate determined by the method of Taussky and Shorr.[23]

Experiment 6 examined the influence of DL-

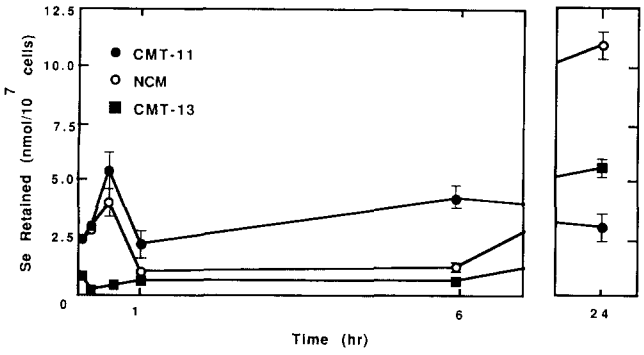

Figure 1 Retention of [^{75}Se]-Na_2SeO_3 by canine mammary cells incubated for various times. Points represent means ± SEM for four observations per treatment per mammary cell.

buthionine-SR-sulfoximine (BSO) on the intracellular glutathione concentration of CMT-13 cells exposed or not exposed to supplemental selenite. Cultures plated at 0.5×10^5 cells/25 cm^2 were grown in the presence or absence of 50 μM BSO for 24 hours before the addition of complete growth medium with or without supplemental selenite (9.6 μM). Cultures were harvested in triplicate and analyzed for cellular glutathione just before and 24 hours after the addition of selenite.

The influence of DL-buthionine-SR-sulfoximine (BSO) on the growth of CMT-13 and CMT-11 cells exposed or not exposed to supplemental selenite was examined in experiment 7. Cultures plated at 1.0 and 0.7×10^5 cells/25 cm^2 flask for CMT-13 and CMT-11 cells, respectively, were incubated in the presence or absence of 50 μM BSO for 24 hours prior to the addition of complete medium with or without supplemental selenite (9.6 μM). Cell numbers for triplicate flasks per treatment were determined 24 hours following the addition of supplemental selenite.

Results

The cellular retention of Se as a function of time is illustrated in *Figure 1*. During the first 30 minutes of incubation the Se content of NCM and CMT-11 cells increased markedly followed by a rapid decline between 30 and 60 minutes and a gradual increase during the next 23 hours. Although no similar initial increase was detected in CMT-13 cells, the Se content increased ~ 10-fold from 5 minutes to 24 hours of incubation. After 24 hours, the retention of Se was significantly greater in NCM cells than either tumor line *(Figure 1)*. Selenite supplementation did not significantly alter the protein content of any of the cultures. Although non-neoplastic cells were observed to contain significantly greater protein than either neoplastic line (4.65 mg, 2.54 and 1.01 mg protein per 10^7 cells for NCM, CMT-11, and CMT-13 cells, respectively), no direct relationship between cellular protein content and selenium retention was evident.

Addition of selenite to S-9 preparations from NCM, CMT-11, and CMT-13 cells resulted in the formation of 9.03 ± 0.4, 4.62 ± 0.2, and 3.46 ± 0.2 nmol dimethyl selenide/mg cellular protein, respectively. The quantity of selenium volatilized was found to be 0.1 to 0.2 nmol when a comparable quantity of heat inactivated S-9 was used. When dimethyl selenide formation was expressed per cell, the average formation was 42, 12, and 3.5 nmol/10^7 cells/20 minutes for NCM, CMT-11, and CMT-13 cells, respectively.

The basal concentration of glutathione was significantly lower in both neoplastic cell lines than in NCM cells *(Table 1)*. Selenite increased the content of reduced and oxidized glutathione in all cultures. The order of cellular glutathione concentration following selenite was NCM > CMT-11 > CMT-13. The order of incremental increase in cellular GSH was NCM ≫ CMT-11 > CMT-13. Only NCM cells maintained the basal ratio of reduced to oxidized glutathione following selenite supplementation, with both neoplastic cells becoming more reduced.

The increase in cellular glutathione per unit of selenium retained varied among the three mammary cells, with NCM > CMT-11 > CMT-13 *(Table 2)*. The magnitude of the increase in glutathione did not relate to the quantity of selenium retained. The molar ratio of cellular glutathione to selenium retained also varied among the mammary cells, with NCM > CMT-11 > CMT-13 *(Table 2)*.

The activity of glutathione reductase before and after selenite was significantly higher in both neoplastic lines than NCM cells *(Table 3)* and was inversely related to sensitivity of the cells to selenite-mediated growth inhibition. Addition of selenite to the incubation medium increased glutathione reductase activities in all cells. Although the magnitude of the increase was greater in NCM (twofold) than in CMT-11 or CMT-13 cells (1.3 and 1.5 fold), the incremental increase was larger in both neoplastic cells. Expressing these data

Table 1 Consequences of sodium selenite on cellular glutathione of canine mammary cells*

	Selenite added (μM)	Mammary cell		
		NCM	CMT-11	CMT-13
Total glutathione (nmol/10^7 cells)	0.0	725 ± 78^d	59 ± 8^a	69 ± 1^a
	9.6	3526 ± 47^e	454 ± 16^c	240 ± 14^b
Oxidized glutathione (nmol/10^7 cells)	0.0	11 ± 1^d	1 ± 0^a	2 ± 1^b
	9.6	56 ± 9^e	3 ± 0^c	3 ± 0^c

* Values represent means ± SEM for 3 flasks per treatment.
$^{a-e}$ Mean concentrations of GSH or GSSG not sharing a common superscript differ ($P < 0.05$).

Table 2 Relationship between intracellular glutathione concentration and the quantity of selenium retained in mammary cells

	Mammary cell		
	NCM	CMT-11	CMT-13
Se retention (nmol/10^7 cells)	112.5	33.4	59.4
Induction of glutathione (nmol/10^7 cells)	280.1	39.5	17.1
Cellular GSH (nmol/10^7 cells)	3526	454	240
Induction of GSH/Se (nmol Glutathione/nmol Se)	2.5	1.2	0.3
Cellular GSH/Se (mole/mole)	31	13	4

Values represent combined data from experiments 1 and 2.

per milligram of protein magnified the differences in activities among the cultures.

The basal activity of γ-glutamylcysteine synthetase was 1.5 and 3.0 times greater in CMT-13 and CMT-11, respectively, than in NCM cells (*Table 3*). The significantly higher basal activity in both neoplastic lines was more apparent when activities were expressed per milligram protein. Incubation with selenite for 24 hours caused an approximate 2.6-fold increase in enzymatic activity in both NCM and CMT-11 cells (*Table 3*). However, selenite supplementation did not alter the activity of this enzyme in CMT-13 cells.

Treatment of CMT-13 cells with 50 μM BSO for 24 hours resulted in a 90% depletion of cellular glutathione (*Table 4*). Cells treated or not treated with BSO, then refed fresh growth medium, increased cellular glutathione by 15 and 23 nmol per 10^6 cells, respectively. Refeeding control cultures with fresh medium containing supplemental selenite resulted in an additional 1.6-fold stimulation of intracellular glutathione concentration. Exposure to BSO followed by refeeding with selenite prevented the additional stimulation caused by supplemental selenite.

Addition of BSO did not significantly alter the growth of CMT-13 or CMT-11 cells replenished with control medium (*Table 5*). The addition of selenite did not influence the growth of either cell line after 24 hours. Addition of selenite to BSO treated CMT-13 and CMT-11 cells resulted in an enhanced growth depression compared to cells exposed to selenite alone (*Table 5*). The percent depression in BSO-selenite treated cells was similar, with the growth of CMT-13 and CMT-11 cells being inhibited by 43 and 39%, respectively.

Table 4 Influence of buthionine sulfoximine (BSO) and selenite on the cellular glutathione concentration of CMT-13 cells*

Treatment	Days after plating	Selenite added (μM Se)	Cellular glutathione (nmol/10^6 cells)
Control	2	0	21.4 ± 1.5[b]
BSO	2	0	2.0 ± 0.2[a]
Control	3	0	43.5 ± 2.9[c]
BSO	3	0	16.9 ± 0.2[b]
Selenite	3	9.6	68.3 ± 7.1[d]
BSO + Selenite	3	9.6	15.2 ± 0.6[b]

* Values are means ± SEM for 3 flasks per treatment. BSO was added on day 1 and the growth medium was replenished with or without supplemental selenite on day 2. Glutathione was measured on the day indicated above.
[a-d] Vertical means with unlike superscripts differ ($P < 0.05$).

Table 5 Influence of buthionine sulfoximine (BSO) and selenite on the growth of CMT-13 and CMT-11 cells*

Treatment	Selenite added (μM Se)	Cell Number (×10^5)	
		CMT-13	CMT-11
Control	0	6.6 ± 0.4[b]	3.6 ± 0.1[b]
BSO	0	5.5 ± 0.4[b]	3.5 ± 0.1[b]
Selenite	9.6	5.2 ± 0.1[b]	3.3 ± 0.1[b]
BSO + Selenite	9.6	3.8 ± 0.1[a]	2.2 ± 0.1[a]

* Values are means ± SEM for 3 flasks per treatment. BSO was added for 24 hours before replenishing medium with or without supplemental selenite.
[a-b] Vertical means with unlike superscripts differ ($P < 0.05$).

Discussion

Previous studies have demonstrated that the growth inhibition caused by selenium supplementation varied among the cell cultures used in the present studies.[8] These differences in sensitivity were attributed to reduced cellular growth and not to reduced viability.[8] In the present studies the rate of growth of each cell culture was similar to those previously reported.[8] The

Table 3 Effect of selenite on the activities of γ-glutamylcysteine synthetase and glutathione reductase in mammary cells

Enzyme	Se(μM)	Mammary cell		
		NCM	CMT-11	CMT-13
γ-GCS	0	67 ± 0.1[a]	20.1 ± 2.6[b]	10.4 ± 1.3[a]
(U/10^7 cells)[1]	9.6	18.7 ± 0.5[b]	52.3 ± 5.9[c]	8.8 ± 1.6[a]
GR	0	23.9 ± 1.7[a]	50.5 ± 0.1[b]	68.5 ± 1.8[c]
(mU/10^7 cells)[2]	9.6	48.0 ± 0.4[b]	64.1 ± 4.5[c]	102.9 ± 1.8[d]

γ-GCS and GR refer to γ-glutamylcysteine synthetase and glutathione reductase, respectively.
[1] One unit of activity liberates 1 μg Pi per hour.
[2] One mU of activity oxidizes a nmol of NADPH per minute.
Data represent means ± SEM for 3 flasks per treatment.
[a-b] Means for each enzyme with unlike superscripts differ ($P < 0.05$).

doubling times for the three mammary cultures were 18–24 hours for CMT-13 and CMT-11 cells and 24–32 hours for NCM cells. Although NCM cultures grew at a slightly slower rate compared to both neoplastic cultures, growth inhibition was not observed in these cells even after 3 doubling times.[8] Thus, the differential growth inhibition caused by selenite in these mammary cells does not appear to relate to the rate of cell growth.

The present studies show that selenium retention cannot account for these differences in growth inhibition after 24 hours of exposure to selenite. In agreement with other investigations,[24] these data suggest that no simple correlation exists between selenium retention and cell growth. At least a portion of the differences in retention of selenium observed among the mammary cells may relate to the formation of various seleno-protein complexes. However, correction for cellular protein content did not account for the differences observed in response to supplemental selenium. Thus, the selenium retention data suggest that factors other than cellular concentration of selenium are critical for cell survival.

Cellular retention of selenium consists of a balance between the rates of uptake and reduction and subsequent export from the cell and assumes uniform distribution of selenium within the cell. Thus, the lack of a relationship between selenium retention and growth inhibition does not exclude the possible importance of the ability of the cell to reductively detoxify selenium. The ability of S-9 preparations to generate dimethyl selenide, a non-toxic reductive end product of selenium, was significantly greater in non-neoplastic mammary cells than in either neoplastic cell line. Since the method used to monitor dimethyl selenide formation was performed at optimal substrate concentrations, these measurements reflect the combined activities of glutathione reductase and the methyl transferases. The S-9 preparations of both neoplastic cell lines formed less dimethyl selenide, despite higher glutathione reductase activities, compared to NCM cells. This observation may indicate that glutathione content or the activity of methyl transferase limited selenite reduction in CMT-13 and CMT-11 cells.

Both neoplastic cells examined in the present studies had a far lower glutathione concentration than non-neoplastic mammary cells. Selenite supplementation increased the concentration of glutathione (both oxidized and reduced) in all cultures. Although the most dramatic increase in glutathione occurred in NCM cells, the quantity of selenium retained and the increase in cellular glutathione concentrations were not directly related in the present studies. Nonetheless, the induction of glutathione per unit of selenium retained did relate inversely to selenite sensitivity. The decreased ability to induce glutathione per μg of selenium retained may indicate a limited capacity to detoxify selenium. Ganther observed that a cell-free system containing glutathione at 40 times the molar concentration of selenium reduced selenite to hydrogen selenide at twice the rate of a system with a molar ratio of 4. The same trend was apparent in the presence of glutathione reductase.[25] The present studies indicate that the cellular glutathione concentrations in the neoplastic cells may be limiting the rate of selenite reduction. A large difference was observed in the glutathione to selenium molar ratios among NCM and CMT-11 and CMT-13 cells. Only NCM cells maintained control glutathione redox state after treatment with selenite. The content of reduced glutathione increased in both neoplastic cell lines following treatment with selenite. Glutathione redox state has been reported to have impact upon many enzymatic systems.[26]

Chung and Maines[9] reported that hepatic glutathione reductase activity increased in a dose dependent manner following a single injection of selenite. In the present studies, the percentage, but not the absolute, increase in glutathione reductase activity related directly to selenium retention in all cultures. Since glutathione reductase is responsible for the regeneration of reduced glutathione, the induction of this enzyme may reflect the cell's attempt to regulate the ratio of intracellular GSH to glutathione (GSSG).

Hepatic γ-glutamylcysteine synthetase activity is known to increase in a dose-dependent manner following a single injection of selenite.[9] In the present studies, increased synthetase activity in CMT-11 and NCM cells may partially account for the observed increase in the glutathione pool and for their decreased sensitivity to supplemental selenite compared to CMT-13 cells. Either decreased degradation of this tripeptide by γ-glutamyltranspeptidase, or an increased flux of substrates through the γ-glutamylcysteine synthetase reaction may also account for the observed increase in GSH in all three mammary cells.

BSO has been shown to be a potent and specific inhibitor of γ-glutamylcysteine synthetase, but not to perturb the activity of glutamine synthetase.[27] The BSO-mediated depletion reported here was similar to those observed by other investigators in cultured tumor cells.[28,29] The present data emphasized the importance of glutathione in selenite-mediated toxicity. BSO depressed basal glutathione concentrations and limited the ability of CMT-13 cells to increase cellular glutathione in response to supplemental selenite. The present studies, however, did not distinguish which of these effects was most important. Depressed glutathione before and following selenite supplementation was accompanied by enhanced selenite-mediated growth inhibition in both CMT-11 and CMT-13 cultures. These data support the importance of cellular glutathione in modulating the toxicity of supplemental selenite.

In summary, differences in sensitivity among the mammary cell cultures used here provide evidence that intracellular factors determine the ability of a cell to withstand a challenge of excess selenium. Although one can not easily extrapolate from *in vitro* studies to studies in humans, these findings may be useful in evaluating which, if any, cancer patients might be expected to benefit most from dietary selenium sup-

plementation. The present data suggest those cells that are least able to modify intracellular glutathione concentrations would be the most susceptible to selenium supplementation.

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