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Dietary selenium supplementation modifies breast tumor growth and metastasis

Yu-Chi Chen[1], K. Sandeep Prabhu[2], Arunangshu Das[3] and Andrea M. Mastro[1]

[1] Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA
[2] Department of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA
[3] Department of Biochemistry and Molecular Biology, Penn State Hershey College of Medicine, Hershey, PA

The survival rate for breast cancer drops dramatically once the disease progresses to the metastatic stage. Selenium (Se) is an essential micronutrient credited with having high anticancer and chemopreventive properties. In our study, we investigated if dietary Se supplementation modified breast cancer development *in vivo*. Three diets supplemented with sodium selenite, methylseleninic acid (MSA) or selenomethionine (SeMet), as well as a Se-deficient and a Se-adequate diet were fed to mice before mammary gland inoculation of 4T1.2 cells. The primary tumor growth, the numbers of cancer cells present in lungs, hearts, livers, kidneys and femurs and several proinflammatory cytokines were measured. We found that inorganic selenite supplementation provided only short-term delay of tumor growth, whereas the two organic SeMet and MSA supplements provided more potent growth inhibition. These diets also affected cancer metastasis differently. Mice fed selenite developed the most extensive metastasis and had an increased incidence of kidney and bone metastasis. On the other hand, mice fed the SeMet diet showed the least amount of cancer growth at metastatic sites. The MSA diet also provided some protection against breast cancer metastasis although the effects were less significant than those of SeMet. The cytokine profiles indicated that serum levels of interlukin-2, interleukin-6, interferon γ and vascular endothelial growth factor were elevated in SeMet-supplemented mice. There was no significant difference in tumor growth and the patterns of metastasis between the Se-deficient and Se-adequate groups. Our data suggest that organic Se supplementation may reduce/delay breast cancer metastasis, while selenite may exacerbate it.

Breast cancer is the second highest cause of cancer death among women following lung and bronchial cancer.[1] After breast cancer metastasizes to secondary organs, the 5-year survival rate drops dramatically.

Because of the strong association between metastasis and poor prognosis, much effort has been focused on early detection. Circulating tumor cells (CTCs) have been used as an early indicator of metastasis.[2] Their presence indicates that metastasis may occur earlier than detectable clinical symptoms.[2] These CTCs or disseminated tumor cells in the bone may remain dormant for years. These findings illustrate the difficulty in determining when metastasis occurs and how to prevent it. One approach may be to use a dietary supplement as a preventive treatment.

Selenium (Se) is a micronutrient important to human health, primarily through antioxidant, anti-inflammatory and antiviral mechanisms.[3] Se compounds and selenoproteins are thought to have important anticancer activity and chemopreventive properties.[4,5] Organic Se is present in foods in the forms of selenomethionine (SeMet), selenocysteine (Sec), γ-

Key words: dietary nutrient supplementation, breast cancer, metastasis

Abbreviations: 400Sel: 0.4 ppm sodium selenite; 80Sel: 0.08 ppm sodium selenite, selenium-adequate; CTC: circulating tumor cells; G-CSF: granulocyte-colony stimulating factor; GPX: glutathione peroxidase; IFNγ: interferon γ; IL-2: interlukin-2; IL-6: interleukin-6; M-CSF: macrophage-colony stimulating factor; MCP-1: monocyte chemotactic protein 1; MMP: matrix metalloproteinase; MSA: methylseleninic acid; NF-κB: nuclear factor-κB; Se: selenium; Se-def: selenium-deficient; SeC: selenocysteine; SeMet: selenomethionine; TNFα: tumor necrosis factor α; TR: thioredoxin reductase; VEGF: vascular endothelial growth factor

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Correspondence to: K. Sandeep Prabhu, Department of Veterinary and Biomedical Sciences, Penn State University, 115 Henning Building, University Park, PA 16802, USA, Tel.: +1-814-863-8976, Fax: +1-814-863-6140, E-mail: ksp4@psu.edu or Andrea M. Mastro, Department of Biochemistry and Molecular Biology, Penn State University, 308 Althouse, University Park, PA 16802, USA, Tel.: +1-814-863-0152, Fax: +1-814-863-7024, E-mail: a36@psu.edu

What's new?

While previous studies have suggested that selenium may possess anticancer and chemopreventative properties, whether its dietary intake has any effect on late stages of cancer, especially metastasis, is largely unknown. Here, comparison of different types of dietary selenium supplements in mice reveals that selenomethionine (SeMet), a form of the mineral used in foods, may reduce or delay breast cancer metastasis, whereas selenite, a form found in soil, may encourage the development of extensive metastases. The results shed light on the application of selenium in cancer treatment and research.

glutamyl-Se-methyl-selenocysteine and Se-methylselenocysteine, while inorganic Se is usually found as selenate or selenite in the soil.[6] Cellular glutathione is essential for the conversion of inorganic Se to selenide,[7] a precursor of selenophosphate. Besides, some organic Se compounds, including SeMet, γ-glutamyl-Se-methyl-selenocysteine and Se-methylselenocysteine, are able to form methylselenol (CH_3SeH).[7] When cells generate too much selenide, it can react with oxygen to produce toxic O_2^-.[8] It is well accepted that methylselenol is involved in the anticancer properties of Se.[8,9] In our study, we used several Se compounds to determine their suitability as antimetastatic agents.

For decades, epidemiological and preclinical evidence supported the belief that a higher dietary intake of Se decreases the incidence and alter biological behaviors of several types of cancers.[10] Generally, Se has been shown to prevent or to express anticancer activity.[9] The results of the majority of animal studies indicate that the preventive properties of Se occur at supranutritional levels. It is believed that the active Se metabolite is a monomethylated Se species, such as methylselenol. The chemoprevention efficacy of any given Se compound may rely on how efficiently it can be converted to this active Se pool, including the synthesis of selenoproteins.

In addition to Se compounds, the involvement of selenoproteins in cancer progression has also been noted.[9] For example, the genetic variants and the allelic loss of glutathione peroxidase-1 (GPX1) or selenoprotein 15 have been linked with breast cancer.[9] Other mechanisms of prevention by Se may be *via* the regulation of redox-active proteins, controlling the redox status of proteins, maintaining intracellular redox balance, monitoring inflammatory and immune responses, enhancing DNA stability, causing cell cycle arrest, promoting apoptosis, blocking cancer cell invasion and migration, inhibiting angiogenesis, controlling crucial regulatory proteins of cell growth and promoting phase II carcinogen-detoxifying enzymes.[4] In addition to chemoprevention, Se may be used in cancer therapy. Organic and inorganic Se compounds can induce apoptosis in cancer cells although through different mechanisms.[11] Some Se compounds such as methylseleninic acid (MSA) can inhibit angiogenesis.[12] These data combined with the inhibitory effects of Se compounds on cancer cell growth[13] have offered new directions for Se studies.[9]

Given mixed epidemiological results, the association between Se intake and breast cancer incidence is still unclear. The results of one study among Japanese women showed a significant difference in Se levels between newly diagnosed breast cancer patients and healthy counterparts[14]; whereas in other studies there was no relationship between Se levels and breast cancer risk or incidence.[15–17] Despite the lack of a strong association between Se intake and human breast cancer incidence, the effects of Se on mammary gland tumorigenesis have been studied extensively. The inhibition of tumorigenesis by different Se compounds was demonstrated in several models including mouse virus-induced, chemical carcinogen-induced and spontaneous mammary tumors.[18] Ip *et al.* showed that *in vitro*, MSA inhibited cell growth and induced apoptosis in mouse mammary hyperplastic epithelial cells. *In vivo*, methylselenocysteine and MSA both reduced the incidence of chemical carcinogen-induced breast cancer by nearly 50%.[19] Li *et al.* demonstrated in an MCF7 human breast cancer cell/nude mice xenograft system that methylselenocysteine reduced breast cancer tumor growth by inducing apoptosis and inhibiting angiogenesis.[20] Nevertheless, most investigations of Se and cancer are focused on chemoprevention and inhibition of the early events of tumor progression. There are very few investigations of the role of Se in later stages of tumor development including metastasis. Song *et al.* showed that melanoma metastasis was suppressed in a C57BL/6 mouse model supplemented with Se.[21] Another report suggested that a deficiency of selenoprotein 15 in colon cancer cells reduced both cancer incidence and lung metastases in a BALB/c mouse model.[22] So far, there are no reports regarding the role of Se in spontaneous metastasis of breast cancer.

In our study, we used a 4T1.2/BALB/c mouse model to investigate how the status of dietary Se affected primary mammary tumor growth and metastasis. We asked further if different Se compounds showed distinct effects. Three Se-supplemented diets containing either sodium selenite, SeMet or MSA were fed to mice for 3 months before cancer inoculation. Primary tumor growth and metastatic severity for each group were compared to those receiving a Se-deficient or a Se-adequate diet. Previously, we had determined that MSA, but not selenite supplementation mitigated an osteoblast inflammatory response to cancer cells (Ref. 23 and data not shown), which implies that Se compounds differently modulate immune responses. Therefore, we assayed the mice sera for cytokines important for the regulation of immunity and cancer growth: granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), interlukin-2 (IL-2), interleukin-6 (IL-6), monocyte chemotactic protein 1 (MCP-1), interferon γ (IFNγ), tumor necrosis factor α (TNFα) and vascular endothelial growth factor

(VEGF).[24–27] We found that the various diets affected breast cancer metastasis differently. Inorganic Se, sodium selenite, only provided short-term inhibition of primary tumor growth, failed to reduce and possibly exacerbated metastasis. Conversely, the organic Se supplements, especially SeMet, resulted in significantly reduced primary tumor growth and in the least metastatic burden. IL-2, Il-6, IFNγ and VEGF elevation in the sera of SeMet-supplemented mice emphasized the differences among Se compounds and the importance of utilizing the most suitable one.

Material and Methods

Cells

4T1.2, a murine metastatic breast cancer line derived from a spontaneously arising mammary tumor in BALB/cfC3H mice, mimics the metastatic pattern of human breast cancer with a higher tendency to metastasize to bone when inoculated orthotopically.[28] The cells, provided by Dr. Erica Sloan and Dr. Robin Anderson (Peter Mac Callum Cancer Institute, Melbourne, Australia), were maintained in alpha minimum essential medium containing 10% fetal bovine serum plus penicillin 100 U/ml and streptomycin 100 μg/ml. 4T1.2luc cells stably expressing luciferase were gifted by Dr. Yuan Mei Lou and Dr. Shoukat Dedhar (British Columbia Cancer Research Centre, Canada) and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, nonessential amino acid, penicillin 100 U/ml, streptomycin 100 μg/ml and 5 μg/ml puromycin (Sigma, St. Louis, MO). Proportionally mixed 4T1.2luc and 4T1.2 cells (0.01–40%) were used to extract genomic DNA and to generate a standard curve for luciferase quantitative PCR as described below.

Animal care

Three-week-old female BALB/c (Jackson Laboratory, Bar Harbor, ME) were kept on Se-supplemented diets for 3 months before inoculation with cancer cells. They were observed daily and weighed once a week throughout the experiment. All the procedures were conducted under the approval of the Pennsylvania State University, the Institutional Animal Care and Use Committee (IACUC).

Se diet

L-SeMet was a kind gift from Sabinsa Corporation (East Windsor, NJ); whereas MSA was purchased from Sigma for the preparation of custom diets (Harlan Teklad Laboratories, Indianapolis, IN). Sodium selenite is the Se source used by the Harlan Teklad Laboratories and its concentration was adjusted accordingly. Mice were fed one of five different Se-containing diets. The Se-deficient chow contained less than 0.01 ppm Se (Se-def); the Se-adequate contained 0.08 ppm sodium selenite (80Sel); the selenite-supplemented contained 0.4 ppm sodium selenite (400Sel); the SeMet-supplemented contained 3 ppm L-SeMet and the MSA-supplemented contained 3 ppm MSA. Concentrations of Se selected were based on previous literature.[19,29–32] Mice were provided with double distilled water. Mice were kept on the same diet for the duration of the experiment.

Cancer cell inoculation

4T1.2luc cells were maintained without antibiotics for 2 weeks before inoculation. The intensity of luciferase expression was confirmed by using the luciferase assay system (Promega, Madison, WI) as the manual instructed. Cells (5×10^4/25 μl PBS) were injected into the left fourth mammary gland of mice under anesthesia by isoflurane inhalation. Once the primary tumor was palpable, tumor size was measured weekly with an electric caliper. The equation, tumor volume = (length × width^2)/2, was used to calculate primary tumor size. Animals showing signs of distress before the end point, such as rapid loss of weight, decreased response to stimulation and hindered limb movement, were removed from the experiment. A total of four mice were removed before the end of the experiment.

In vivo imaging (IVIS)

Tumor growth and metastatic patterns were monitored by IVIS® Lumina II (Caliper, Hopkinton, MA) weekly. Mice were injected intraperitoneally with 150 μl D-luciferin (15 mg/ml) and placed under isoflurane for 10 min. Animals were imaged for 1 min. Because the respiratory function was weakened by the development of lung metastases in some mice, total isoflurane inhalation time was limited to 15 min.

Animal dissection and genomic DNA extraction

Thirty days after cancer cell inoculation, mice were euthanized by CO_2 inhalation. Lung, heart, liver, kidney, spleen and both femurs were removed, and metastatic nodules on organ surfaces were counted. Organs were stored at −80°C until genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA). Organs were homogenized in liquid nitrogen into fine powders to eliminate uneven distribution of cancer cells. About 20 mg of tissue powder from each organ was used to extract DNA. Serum was stored at −80°C before cytokine analysis.

Luciferase quantitative PCR

Real-time PCR was used to quantify tumor burden. This assay was modified from Havens *et al.*[33] Genomic DNA (1 μg) was subjected to qPCR to detect the cycle threshold (Ct) of luciferase (cancer cells) and GAPDH (all tissue). This Ct value was normalized using GAPDH to measure total sample DNA. A standard curve was established by using DNA from 4T1.2 and 4T1.2luc mixed proportionally. A comparison of the Ct values of luciferase and GAPDH was used to calculate the amount of DNA from cancer cells. The PCR reactions were done by a StepOnePlus™ Real-Time PCR themocycler (Applied Biosystems, Carlsbad, CA) with standard cycling methods. SYBR Green supermix, ROX was purchased from Quanta Biosciences (Gaithersburg, MD). The PCR reaction contained 1 μg genomic DNA, 12.5 μl SYBR

Green/ROX, 100 nM luciferase primers (360 nM GAPDH primers) and distilled water to a final volume of 30 μl. The luciferase primers were F′ AGCAGCTGCACAAAGCCATG AA and R′ ATGTCCACCTCGATATGTGCGT. The GAPDH primers were F′ GCCCCCAACACTGAGCAT and R′ CTAG GCCCCTCCTGTTGT. The detection limitation of luciferase real-time PCR in our study was 0.01%.

Cytokine analysis

To determine the impact of Se on the cytokine profile, serum IL-2, IL-6, MCP-1, G-CSF, M-CSF, VEGF, TNFα and IFNγ were measured by a MILLIPLEX 8-plex mouse cytokine array (Millipore, Billerica, MA), which allowed for the simultaneous quantitation of eight mouse cytokines. Every sample was measured in duplicate. The sensitivities and precision of the assay depend on the cytokines. Generally, the detection limit of each cytokine was less than 3.5 pg/ml except MCP-1, which was 6.7 pg/ml. The coefficient of variation was less than 3%.

Se analysis

The assay for elemental Se was modified from Crampsie *et al.*[34] Briefly, ~0.5 g of liver tissue was homogenized in 0.9% KCl. The tissue homogenate was digested in 50% nitric acid in a MARS Xpress microwave digestion system (CEM Corp, Matthews, NC). The nitric acid in the digested solution was diluted to 20% before Se analysis by Atomic Absorption Spectroscopy using an AAnalyst 600 spectrometer (PerkinElmer, Norwalk, CT) with graphite furnace. Total Se was analyzed by measuring the absorption at 196 nm. Each sample was measured in duplicate. Livers from five mice from each dietary group were measured.

Western blot

Liver tissues from each dietary group were homogenized in T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL). A total of 20 μg homogenate was used for GPX1, TR1 and GAPDH detection.

Statistics

Statistics analysis was carried out using SAS and Prism. Main effects were evaluated by Fisher's test, Turkey multiple comparison and chi-squared test using one-way analysis of variance model. Statistical significance was defined as the probability of at least $p < 0.05$ in all analyses.

Results

Se supplementation affected primary tumor growth

In a pilot study, we found that dietary Se supplementation did not result in a longer survival time compared to either a Se-def or a Se-adequate diet (data not shown). Therefore, to reduce any unnecessary discomfort to animals, the endpoint was set at 30 days after cancer cell inoculation. Generally, mice developed a palpable tumor mass by 2 weeks. The diameters of primary tumors were measured and used to calculate tumor volumes (Table 1). On day 16, tumor volume was clearly affected by the Se status. All mice on Se-supplemented diets, 400Sel, SeMet and MSA, showed significantly less primary tumor growth ($p < 0.001$) compared to those on the Se-def diet. Tumor volumes of mice fed a Se-def diet were 2.4- to 3.9-fold greater than those of mice fed Se-supplemented diets. The difference between the Se-def and the Se adequate (80Sel) diets was also significant (1.8-fold, $p < 0.001$). However, not every Se-supplemented diet maintained the suppression of tumor growth. By day 23, the 400Sel diet was no longer effective. Mice supplemented with SeMet or MSA still showed reduced tumor volumes compared to mice on either Se-def or 80Sel diets. By day 30, only mice on the SeMet diet showed significantly reduced tumor growth ($p < 0.05$). Taken together, these data suggested that Se deficiency may be permissive to the initiation of tumor development. Although mice on all three Se-supplemented diets showed delayed tumor growth at the earlier stages of tumor development, only the SeMet diet maintained this property by the endpoint.

Table 1. Primary tumor volume (mean ± SD) of each group

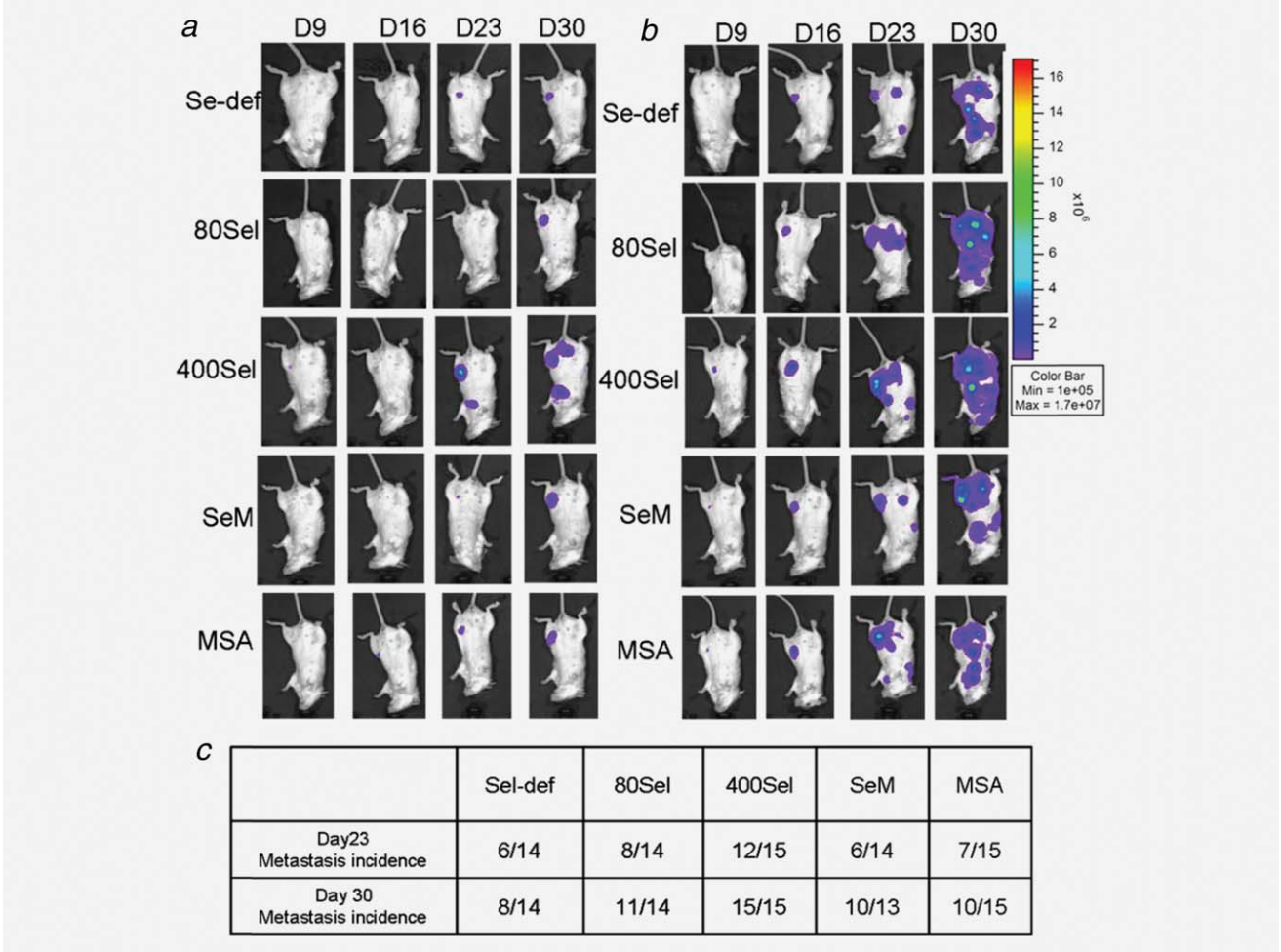
Tumor volume, mm^3 (mean ± SD)	Day 16	Day 23	Day 30
Se-def	149.2 ± 93.5	203.3 ± 122.4	389.4 ± 197.1
80Sel	81.1 ± 45.0	220.2 ± 79.3	370.3 ± 130.3
400Sel	61.2 ± 24.2[1]	193.0 ± 80.1	377.3 ± 153.3
SeMet	38.4 ± 30.4[1]	120.1 ± 74.9[2]	242.0 ± 133.9[2]
MSA	50.8 ± 27.2[1]	135.3 ± 57.5[2]	278.9 ± 148.5

Primary tumors were palpable by 2 weeks after cancer cell inoculation. Tumor measurement was done with an electric caliper on the same day as IVIS imaging. Tumor volume was calculated using the equation: volume = (length × width^2)/2.
[1] $p < 0.001$,
[2] $p < 0.05$.

To test the Se levels in each dietary group, we measured the total Se content and the expression of GPX1 and TR1 in liver (Supporting Information Fig. 1). Mice were randomly selected to represent each group. There was no Se detectable in the liver of Se-def mice. All Se-containing diets (80Sel and all three Se-supplemented) resulted in a significant amount of Se in the liver (Supporting Information Fig. 1a). There was a nonsignificant increase of Se content in 400Sel and MSA groups compared to 80Sel group. Mice fed with SeMet contained significantly more Se than other groups, which may be because of SeMet nonspecific incorporation into polypeptides. Similar results were observed in GPX1 and TR1 detection (Supporting Information Fig. 1b). No GPX1 and very little TR1 were present in the livers of Se-def mice; all Se-containing diets sufficiently saturated the expression of both selenoproteins.

Cancer metastasis varied within each diet group

Tumor development and metastasis were monitored by IVIS imaging on days 9, 16, 23 and 30 after cancer cell inoculation. All mice developed primary tumors. However, the pattern and severity of metastasis detected by this method were



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	Sel-def	80Sel	400Sel	SeM	MSA
Day23 Metastasis incidence	6/14	8/14	12/15	6/14	7/15
Day 30 Metastasis incidence	8/14	11/14	15/15	10/13	10/15

Figure 1. The effect of Se supplementation on breast cancer development. At various times after inoculation of 4T1.2luc cells into the mammary gland, the mice were imaged for the presence of luciferase expression. After i.p. injection with 150 μl of 15 mg/ml luciferin, mice were held for 20 min (10 min in the presence of isoflurane) before imaging. The exposure time was set at 1 min. All pictures were adjusted to the same scale for comparison. The color purple to red corresponded to a weak to strong intensity of chemiluminescence. Shown are images from two mice per diet group. (*a*) Mice with the least metastases from each group. (*b*) Mice with extensive metastases from each group. Pictures of the same row represent tumor development in the same mouse through time. D, days postinoculation of cancer cells. (*c*) Metastatic incidence on days 23 and 30. The severity of metastasis varied with each dietary group. The numbers of mice with IVIS-detectable metastasis were documented.

diverse even within the same diet group. Some mice appeared to develop primary tumors without any IVIS-detectable metastasis (Fig. 1*a*); some developed extensive metastases in multiple organs (Fig. 1*b*). On day 23, less than half of mice on Se-def, SeMet or MSA diets showed signs of luciferase-expressing metastases. However, mice with selenite supplementation had a greater incidence of metastasis than the mice supplemented with SeMet or MSA (Fig. 1*c*). There were 12 mice in the 400Sel diet group, eight in the 80Sel group, six in the Se-def group, six in the SeMet group and seven in the MSA group, which showed detectable metastasis (Fig. 1*c*). Based on IVIS imaging (Fig. 1 and data not shown) nearly half of the mice on the 400Sel diet (7/15) showed extensive metastasis on day 30 compared to other diets (about 3/15). Also, all mice in the 400Sel diet group developed metastasis on day 30. There were no significant differences among other diet groups. These data suggested that supplementation with sodium selenite may promote breast cancer metastasis.

Se supplementation and lung metastasis

Among all organs investigated, lungs showed the most extensive metastases. There were more metastatic nodules and cancer cells present than in other organs. We used two methods to determine the severity of metastasis. One was by counting the metastatic nodules on the organ surface; the other was by measuring the amount of the luciferase gene (cancer cells only) by qPCR in genomic DNA extracted from organs. Some mice in the Se-def, 80Sel and 400Sel diets showed extensive lung metastasis, *i.e.*, more than 20% of the genomic DNA was from cancer cells (Fig. 2). In contrast, the DNA from lungs of mice bearing the most extensive lung metastases in the SeMet and MSA diet groups contained only 4.4 and 6.3%, respectively, of

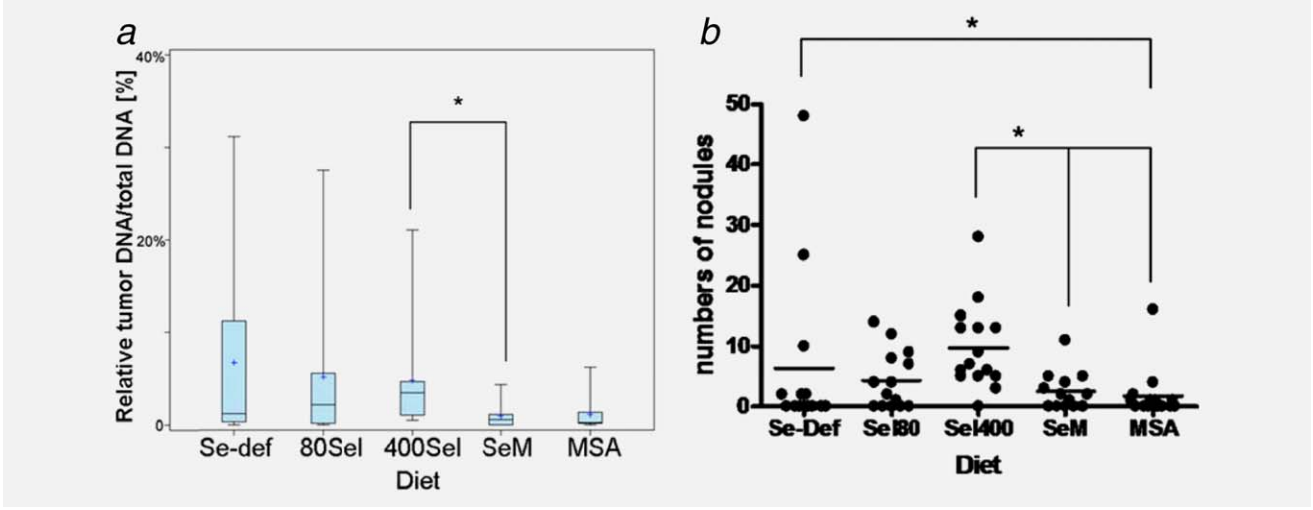

Figure 2. The quantification of breast cancer lung metastasis. At the time of sacrifice, the lungs were removed and the numbers of nodules were counted. The lungs were then frozen in liquid nitrogen, homogenized and the DNA extracted as described in the Material and Methods section. Real-time PCR was used to quantify the amount of luciferase DNA in the lung as an indication of tumor cell burden. (*a*) Quantification of 4T1.2luc cancer cells in lungs. A box plot was used to represent the number of cancer cells in lungs measured by luciferase and GAPDH real-time PCR. A standard curve was generated by mixing 4T1.2luc and 4T1.2 cells at the ratio from 0.01 to 40%. The $\Delta CT_{GAPDH\text{-}luciferase}$ from each sample was used to compare the $\Delta CT_{GAPDH\text{-}luciferase}$ from the standard to calculate the amount of cancer cells present in each sample (shown as percentage). The bottom and upper bar indicates the minimum and maximum values. The box includes the range of values from 25th to 75th percentile. "+" represents the average and the line in the box indicates the median. Mice fed the 400Sel diet showed significantly more breast cancer lung metastasis compared to mice on the SeMet diet. $*p < 0.05$. (*b*) Numbers of nodules in the lungs of each mouse. A dot plot was used to show the distribution of the numbers of nodules in each lung, where each dot represents one mouse and the lines indicated the means. Compared to the mice on the SeMet and MSA diets, those on the 400Sel diet contained significantly more lung nodules. $*p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

total genomic DNA from cancer cells. With this method, nearly all mice had detectable luciferase DNA in their lung tissues (Supporting Information Table 1b). However, the only statistically significant difference was between mice fed the 400Sel and SeMet diets ($p < 0.05$) (Fig. 2*a*). The difference in sensitivity between this method and imaging may be owing to the size of the metastases and the intensity of the luciferase signal.

In accordance with the results with the genomic DNA analysis, the numbers of nodules varied within and among groups. Some mice had more than ten lung nodules, whereas some had none (Fig. 2*b*). More mice in the 400Sel diet (14/15) developed nodules in the lung compared to mice fed with Se-Def or MSA diets ($p < 0.05$) (Supporting Information Table 1a). Significantly, mice in the 400Sel diet group contained more nodules in the lung than mice in either SeMet or MSA diet group ($p < 0.05$). Another statistically significant difference was found between the Se-Def and MSA groups ($p < 0.05$). Taken together, these data suggested that sodium selenite supplementation did not suppress and may have enhanced the numbers of cancer cells present in the lung. Conversely, the other two Se-supplemented diets appeared to have somewhat been protective.

Se and heart metastasis

Heart metastases were less prevalent across all diet groups. Fewer mice showed metastatic nodules (Supporting Information Table 1a); many of them had a single nodule (date not shown) compared to multiple nodules in lungs (Fig. 2*b*). Using the same methods to investigate the metastatic status in the heart, we found that there were no statistical significant differences in the severity of heart metastasis among the Se diets. Even so, we noticed that more mice in the 400Sel diet group (12/15) had more visible nodules than mice in the Se-def and 80Sel groups (4/14, $p < 0.05$) (Supporting Information Table 1a).

Se supplementation and liver metastasis

Eckhardt *et al.* reported that 4T1.2 cells metastasized to the liver far less than to other major organs.[35] Our results corresponded with their observation. We found that less than 1% of the DNA was from cancer cells for most liver samples (Fig. 3*a*). Nonetheless, the data indicated that mice on a Se-Def diet had significantly more liver metastases than those on the SeMet diet ($p < 0.05$). On the other hand, only two mice had visible nodules in the liver (Supporting Information Table 1a), which made the determination of tumor burden based on the counts of nodules inappropriate.

Se supplementation and kidney metastasis

With the exception of those in selenite-fed groups, about half of the mice had no detectable cancer DNA in the kidney (Fig. 4*a*), which suggested that kidney was not a major metastatic target of 4T1.2 cells. Interestingly, we found that both sodium

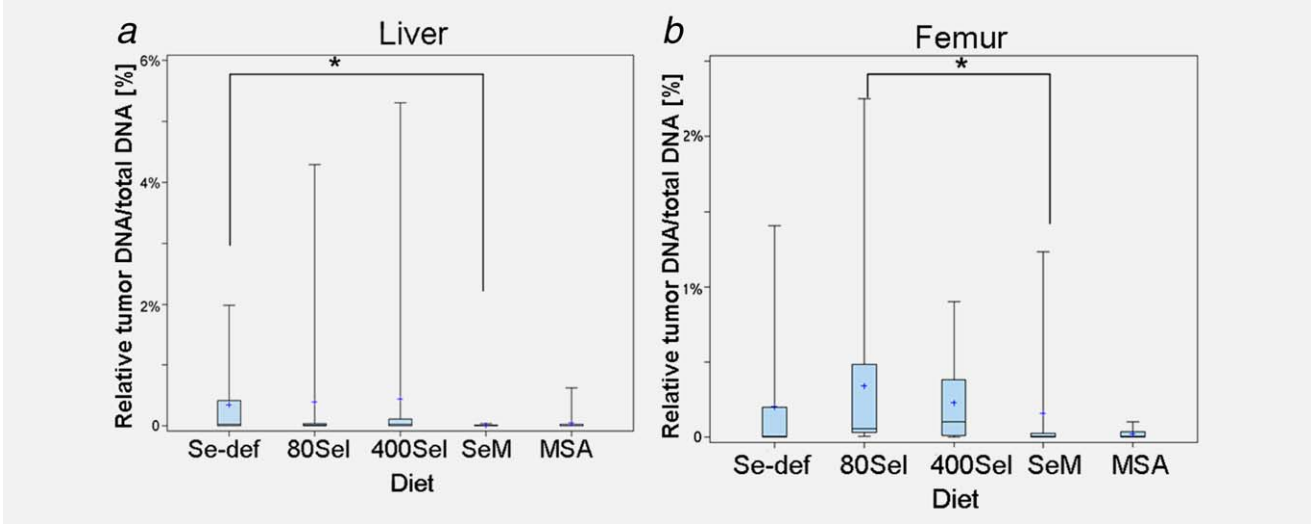

Figure 3. The SeMet-supplemented diet protected against breast cancer liver and bone metastasis. Livers and femurs were removed and treated as described in the legend to Figure 2 and in the Material and Methods section. (*a*) Quantification of cancer cells in livers. A box plot was used to demonstrate the amount of cancer cells in livers measured by luciferase and GAPDH real-time PCR as described. The bottom and upper bar indicates the minimum and maximum values. The box includes the range of values from 25th to 75th percentile. "+" represents the average and the line in the box indicates the median. The SeMet diet leads to a significantly reduced presence of breast cancer cells in livers compared to those in the Se-def diet group. *$p < 0.05$. (*b*) Quantification of cancer cells in femurs. The difference between two diet groups, 80Sel and SeMet, was significant. *$p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

selenite diets had a tendency to increase the occurrence of kidney metastasis. Eleven of 14 mice in both 80Sel and 400Sel diet groups had detectable cancer DNA in the kidney, whereas fewer mice in the other diet groups had cancer DNA (7/14 in the Se-def diet, 5/13 in the SeMet diet and 8/15 in the MSA diet, Supporting Information Table 1b). There was a significant difference in tumor burden in the kidney between the 400Sel and SeMet diets ($p < 0.05$, Fig. 4*a*).

Nodule formation on the kidneys was higher in mice maintained on either of the sodium selenite diets (Supporting

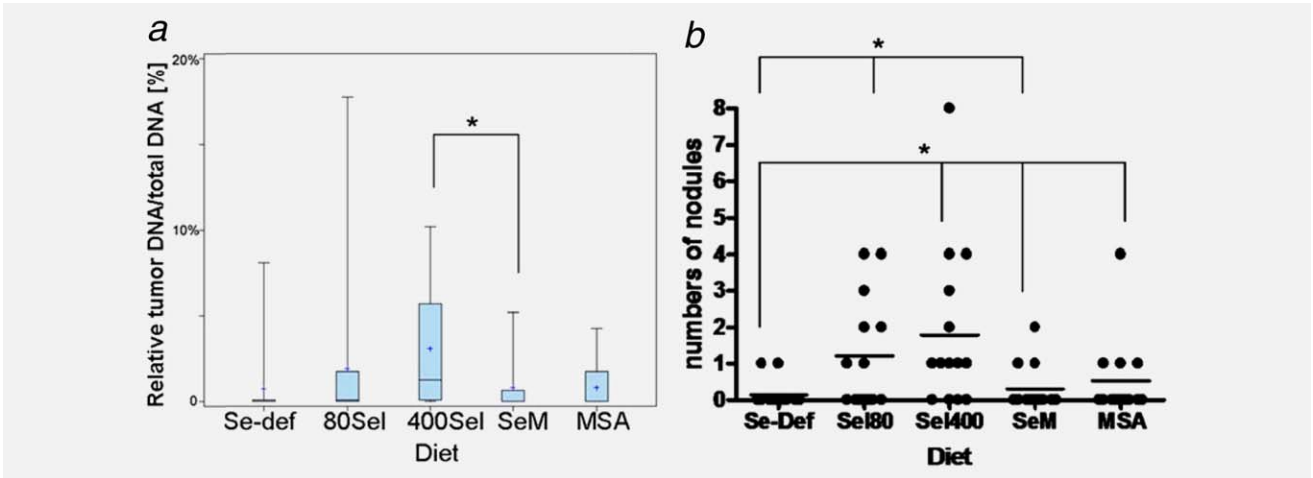

Figure 4. SeMet supplementation reduced breast cancer kidney metastasis. Kidneys were removed and treated as the lungs as described in Figure 2. (*a*) Quantification of cancer cells in kidneys. A box plot was used to illustrate the amount of cancer cells in kidneys measured by luciferase and GAPDH real-time PCR. The bottom and upper bar indicates the minimum and maximum values. The box includes the range of values from 25th to 75th percentile. "+" represents the average and the line in the box indicates the median. Different Se dietary supplementation showed various degrees of protection against breast cancer kidney metastasis. Compared to the 400Sel diet group, mice in the SeMet diet group had significantly fewer cancer cells present in kidneys. *$p < 0.05$. (*b*) Numbers of nodules in the kidney of each mouse. Metastatic nodules in the kidney were significantly different among Se diet groups. A dot plot was used to show the distribution of the numbers of nodules in the lung. Each dot represents an individual mouse and the lines indicate the means. The 400Sel diet resulted in more nodules compared to the Se-def, SeMet and MSA diets; the 80Sel also caused significantly more nodules compared to the Se-def and MSA diets. *$p < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Information Table 1a) (Fig. 4*b*). More mice in the 400Sel diet group developed visible nodules than mice in Se-def and SeMet groups ($p < 0.05$). These findings also supported the tendency for kidney metastasis with the sodium selenite diets. Mice fed the 400Sel diet showed significantly increased nodules in the kidney compared to those on the Se-def, SeMet and MSA diets (400Sel-MSA, $p < 0.05$; others, $p < 0.01$). Compared to the Se-def and SeMet diets, kidneys of mice on the 80Sel diet also showed significantly increased nodules ($p < 0.05$).

Se supplementation and bone metastasis

To investigate breast cancer metastasis to bone, we used the femurs as indicators of the skeleton (Fig. 3*b*). Although we did not observe significant amounts of cancer cells in bone by IVIS imaging, the DNA analysis showed that cancer cells were present in the femurs. Similar to what was seen with kidney, mice on sodium selenite diets had a higher incidence of bone metastasis. 4T1.2luc cancer cell DNA was detectable in all samples from mice on the 80Sel diet and 13 of 14 on the 400Sel diet, whereas it was only detectable in seven of 13 on the Se-def diet, eight of 13 on the SeMet diet and eight of 15 on the MSA diet (Supporting Information Table 1b, 80Sel-Se-def, SeMet, MSA and 400Sel-MSA, $p < 0.05$). As for the severity of bone metastasis, mice fed the SeMet diet showed a significantly reduced tumor burden in the femur compared to the 80Sel diet ($p < 0.05$).

Se supplementation triggered different cytokine responses

Finally, we measured IL-2, IL-6, IFNγ, TNFα, MCP-1, G-CSF, M-CSF and VEGF to see if Se supplementation could alter an inflammatory response. Similar to the metastatic patterns, cytokine production varied widely among and within each Se dietary group (Fig. 5). IL-2 and IFNγ were not detected in the sera of mice from the Se-def and 80Sel groups, whereas they were generated in mice fed SeMet and MSA. The levels of IL-6 and TNFα were generally higher in Se-supplemented mice, especially in SeMet and MSA groups. For IL-6, there was a significant difference between SeMet and 80Sel mice ($p < 0.05$). For TNFα, a significant difference existed between MSA and Se-Def and MSA and 80-Sel mice. We also observed an increase expression of VEGF in the SeMet groups. No difference was found in the amount of G-CSF, M-CSF and MCP-1.

Discussion

In our study, we investigated whether dietary Se affected breast cancer growth and metastasis in the 4T1.2luc/BALB/c mouse model. Furthermore, we compared three different Se supplements: sodium selenite, MSA and SeMet. Although no Se compound tested was the "magic cure" for preventing cancer growth or metastasis in this aggressive tumor animal model, our data suggested that of the diets, sodium selenite supplementation (400Sel) exhibited only a short-term delay of tumor growth, which was overcome at later stages. Conversely, two organic Se compounds (SeMet and MSA) were more potent in inhibiting primary tumor growth. Despite the noticeable variation within groups (Table 1), the primary tumors of SeMet-supplemented mice were significantly reduced compared to those of Se-def mice. Moreover, these Se compounds affected breast cancer development diversely with different efficiencies. Among three diets, the 400Sel diet resulted in the most extensive metastasis based on IVIS imaging and tumor burden quantification in lungs, livers, kidneys and femurs. Mice fed selenite also exhibited increased metastasis in the kidneys and the femurs. Mice fed the SeMet diet showed less metastases in the lungs and the kidneys compared to the 400Sel diet, less in the liver compared to the Se-def group and less in the femur compared to the 80Sel diet. The MSA diet also resulted in less metastasis although not to the same extent as the SeMet diet. We found no significant difference in cancer development between the Se-deficient and Se-adequate groups, which supports the theory that the anticancer benefits of Se supplementation take place at supranutritional levels.[36] Taken together, among three Se compounds used in our study, SeMet provided the most protection against primary tumor growth and metastasis.

It has been noted that the plasma or serum Se levels are low in breast cancer patients[18] at the time of diagnosis and throughout treatment compared to their healthy counterparts.[18,37,38] There is another decrease after radiotherapy.[39] However, whether this reduction was caused by low Se intake or was a result of metabolism is unknown. Holmes *et al.* observed a nonsignificant inverse association between Se intake and mortality,[40] whereas Saquib *et al.* reported a nonsignificant increase in mortality with breast cancer patients with either an intake of more than 400 μg/day or less than 55 μg/day Se compared to patients with an adequate intake.[41] Interestingly, a study by Harris *et al.* in Sweden, a naturally low-Se country, showed a significant positive association between Se intake (more than 27.7 μg/day, median 31.6 μg/day) and cancer-specific and overall mortality in breast cancer patients compared to patients with less than 20 μg/day Se intake.[42] These data suggest that Se supplementation is more effective in a population with lower Se levels.[43]

The relevance of Se in breast cancer prevention and treatment has been studied extensively using *in vitro* and animal models. It has been reported that Se effectively inhibited cancer cell growth, caused cell cycle arrest, induced apoptosis, reduced angiogenesis and enhanced the efficacy of the anticancer drug, paclitaxel.[18–204445] The majority of current research is focused on deciphering the involvement of Se in early stages of cancers. There are very few researchers investigating the association between Se and later stages of cancer. As far as we know, there is no previous report focused on the effect of Se on spontaneous breast cancer metastasis. Our data suggest that Se may inhibit breast tumor development. However, the efficacy of Se may depend on the formulation. None of the Se compounds used in our study with an aggressive tumor model was totally effective.

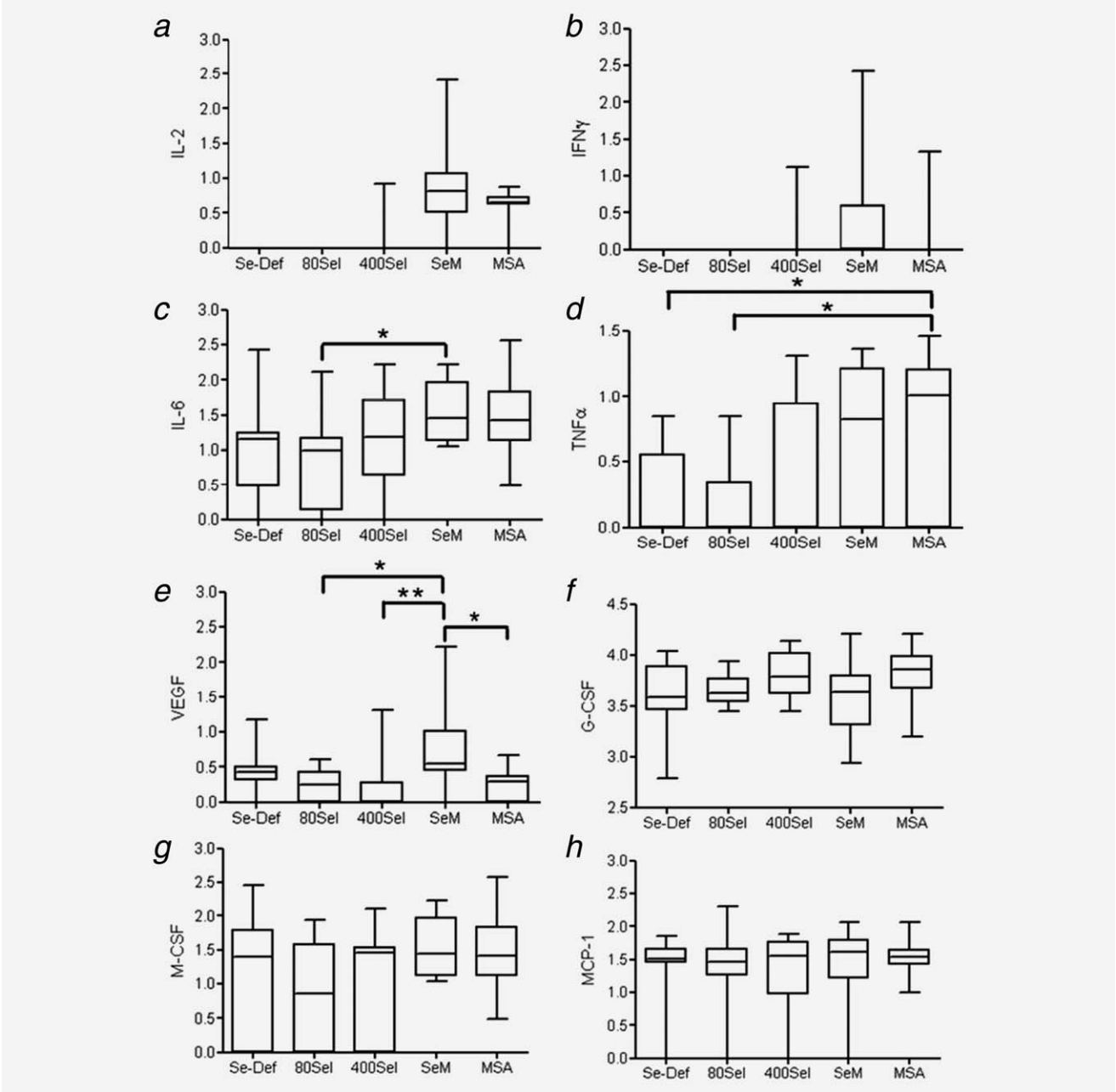

Figure 5. Cytokine concentrations in serum. Sera were collected and measured with a multiplex cytokine array as described in the Material and Methods section. Values were $\log_{10}$ transformed for analysis. Each box plot represents the concentration of a particular cytokine measured in five Se dietary groups. (*a*) IL-2 and (*b*) IFNγ were nondetectable in the Se-Def and 80Sel groups and were detected in only one and two mice in the 400Sel group, respectively (out of 14 mice). (*c*) IL-6 and (*d*) TNFα were generally higher in the Se-supplemented groups (400Sel, SeMet and MSA). The level of IL-6 was significantly different between SeMet and 80Sel mice. The level of TNFα was significantly different between MSA and Se-def and MSA and 80Sel mice. (*e*) VEGF was significantly higher in the SeMet mice. There was no significant difference among mice fed the various Se diet (*f*) G-CSF, (*g*) M-CSF and (*h*) MCP-1. Each sample was measured in duplicate. $*p < 0.05$, $**p < 0.01$.

We measured serum cytokines to seek insights about the diverse results among Se-supplemented groups. Compared to the Se-def or 80Sel groups, several cytokines were detected or elevated in both SeMet and MSA groups, including IL-2, IFNγ, TNFα and IL-6. IL-2 and IFNγ are involved in NK cell activation, the first-line immune barrier against tumor cells.[25] It has been reported that IL-2 and IFNγ induce the antitumor activity of NK cells,[46] which may contribute to the decreased primary tumor and lower metastatic burdens in SeMet and MSA groups. TNFα secreted by macrophages and NK cells can induce cancer cell death. On the other hand, TNFα is a multifunctional cytokine that can stimulate the production of many other cytokines, including IL-6 and VEGF,[47] which may explain their observed increase.

Considering that the serum levels of IL-6 and VEGF increased but the tumor burdens were lower in mice fed SeMet or MSA, we speculate that Se may act through other mechanisms to mitigate metastasis. The short-term growth-inhibitory effect of selenite may be explained by its ability to induce apoptosis.[11] We must also consider the fact that the levels of circulating cytokines can differ widely from local sites and may affect different types of cells differently. It is very likely that Se alters the interactions between breast cancer cells and local host cells to make the microenvironment more or less favorable for the cancer cells, which could result in different metastatic burdens in the Se-supplemented groups. In a previous *in vitro* study, we found that selenite did not decrease the inflammatory response of osteoblasts caused by breast cancer cells, and slightly increased the production of some proinflammatory cytokines, IL-6 and MCP-1 (data not shown). These cytokines are osteoclastogenic and promote osteolysis, which releases growth factors into the bone microenvironment to support tumor cell growth.[48] We believe that the strong local inflammation may account for the more aggressive metastasis pattern in the 400Sel group. Additionally, we reported that MSA inhibited NF-κB and decreased the inflammatory response of osteoblasts to breast cancer cells.[23] MSA can directly generate methylselenol, which is believed to be responsible for the anticancer effect of organic Se. SeMet aided by methioninase can also rapidly generate methylselenol to induce apoptosis.[49] Moreover, methylselenol appeared to exhibit some antiangiogenesis activity by downregulating key angiogenic molecules or by inducing apoptosis of endothelial cells.[26,27] Taken together, these organic Se compounds (SeMet and MSA) had the ability to induce cancer cell apoptosis, inhibit angiogenesis and reduce inflammation, all of which could contribute to the inhibition of primary tumor growth and the reduction of the severity of metastasis.

One concern is tissue storage and distribution. The amount of Se varies among tissues. In non-Se-deficient humans, generally 30% of total body Se is found in the liver, 15% in the kidney, 30% in the muscle, 10% in the plasma and the remaining 15% throughout the body.[18] However, in a Se-deficient state, some organs, such as liver, rapidly lose Se (Supporting Information Fig. 1) while in other organs, such as brain, Se is more well maintained.[50] The distribution and concentration of Se in each organ may affect the efficacy of Se protection. It might be worthwhile to investigate delivery routes other than diet in order to increase Se availability in specific organs.

In summary, SeMet supplementation provided more protection from breast cancer metastasis than selenite and MSA supplementations in a mouse model. We showed the unsuitability of selenite to prevent or decrease breast cancer development and metastasis. Although the 400Sel diet provided short-term protection against tumor growth, these same mice exhibited extensive metastasis. Our data suggested that the selenite diet may increase breast cancer metastasis to the kidney and to bone. Our results also indicated that organic Se compounds inhibited tumor growth more efficiently and had a greater effect on metastasis. An assay of serum cytokines indicated that mice fed SeMet and MSA had higher levels IL-6, IL-2, IFNγ, TNFα and VEGF. In particular, SeMet provided the strongest defense against breast cancer development.

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