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13. ABSTRACT ***(Maximum 200 Words)***

Our overall aim is to understand the role of inhibition of angiogenesis in breast cancer prevention by selenium (Se). During the current funding period, we have focused on establishing the anti-angiogenesis phenomenon of chemopreventive intake of Se in mammary cancer prevention and we have initiated efforts to refine a Matrigel plug angiogenesis model in terms of specificity of angiogenic factors and reproducibility for assessing impact of Se on the endothelial response component of angiogenesis. In the first area, we published in vivo data supporting an association of reduced microvessel density and decreased vascular endothelial growth factor expression with mammary cancer prevention by Se. Furthermore, we discovered a specific inhibitory effect of methylselenium on vascular endothelial expression of matrix metalloproteinases and a potent apoptogenic activity on the endothelial cells. Because endothelial mitogenesis and matrix degradation are essential for angiogenesis, these observations provide plausible mechanisms to account for the in vivo finding that methylselenol is the active chemopreventive Se metabolite for mammary cancer. With respect to the second task, our results implicated immune response as a major obstacle for achieving a reproducible in vivo angiogenesis assay. Syngenic or immunodeficient mice will be used to reduce this variability in the second year of this research.

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Introduction

The overall aim of this project is to understand the role of inhibition of angiogenesis in breast cancer prevention by selenium. During the current funding period, we have focused on establishing the anti-angiogenesis phenomenon of chemopreventive intake of Se in mammary cancer prevention (See appended publication). We have initiated efforts to develop and refine a Matrigel plug angiogenesis model in terms of specificity of angiogenic factors and assay reproducibility for assessing impact of Se on the endothelial response component of the angiogenesis question (Task 1). The following describes progress made in this period.

Body

As reported in the December issue of *Molecular Carcinogenesis*, 1999, we have documented an association of reduction of tumor microvessel density with chemoprevention of mammary cancer in a chemically induced rat mammary carcinogenesis model. We have made the novel observation that such changes were associated with a decreased expression of vascular endothelial growth factor (VEGF), an important in vivo angiogenic cytokine molecule. Furthermore, we have documented a potent apoptogenic activity of methyl-selenium on endothelial cells and discovered a specific inhibitory effect of this Se pool, the putative chemopreventive Se metabolite (1-3), on the expression of matrix metalloproteinases (MMP) by vascular endothelial cells (see appendices). Because angiogenic factor expression as well as endothelial survival and proliferation and matrix degradative activity are essential for initiating and sustaining angiogenesis, these findings provide mechanistic insights into the cellular and biochemical processes that are potentially targeted by Se to exert anti-angiogenic activity.

Our efforts to develop and refine a Matrigel plug in vivo angiogenesis model as initially reported by Passaniti et al (4) have yielded mixed results. We focused our initial effort on using VEGF as the angiogenic factor. Exogenous VEGF proved to be a very poor stimulant for Matrigel plug assay. After consulting with Dr. Passaniti, we experimented with basic FGF and were able to demonstrate much-enhanced angiogenesis. However, the large inter-animal and intra-plug variability of vessel responses has limited our application of this assay to test the Se anti-angiogenic effect in this model. A significant complication appears to be the immune response mounted by the C57B mice to the injected bFGF-Matrigel solution. At the recent American Association for Cancer Research meeting (April 2000), we have discussed the variability issue with a number of researchers familiar with this assay. A consensus that emerged out of this interchange of information was that the angiogenic responses and reproducibility could be improved in syngenic or immunodeficient mice. We will therefore pursue this line of investigation to establish a reliable and reproducible in vivo angiogenesis assay.

Key research accomplishments.

- Published in vivo data supporting an association of reduced microvessel density and decreased VEGF expression with mammary cancer prevention by Se.
- Discovered a specific inhibitory effect of methylselenium on endothelial MMP expression. (patent filing is being considered).
- Observed a potent apoptogenic activity of methylselenium on vascular endothelial cells.
- Matrigel plug angiogenesis model development and refinement implicated immune response as a major complication for achieving a reproducible in vivo angiogenesis assay.

Reportable outcomes:

Manuscripts

Jiang C, Jiang W, Ip C, Ganther H, Lu J. Selenium-induced inhibition of angiogenesis in mammary cancer at chemopreventive levels of intake. Mol Carcinog. 1999 Dec;26(4):213-225.

Lu, J. Apoptosis and angiogenesis in cancer prevention by selenium. Book Chapter for Proceedings of American Institute for Cancer Research 1999 Annual Research Conference. Washington, DC. *Nutrition and Cancer prevention: new insights into the role of phytochemicals*. Kluwer Academic/Plenum Publishers, New York, NY. In press.

Abstracts presented at national meetings

Jiang C., Ganther, H and J. Lu. Methylseleninic acid induces apoptosis and inhibts matrix metalloproteinases of vascular endothelial cells. (Abstract) American Institute for Cancer Research Annual Research Conference. Washington, DC. Sept 2&3, 1999.

Lu, J., Jiang, C., Wang, Z. and Ganther, H. Merthylselenol as a proximal inhibitory selenium metabolite for matrix metalloproteinases and angiogenesis (Abstract) American Association for Cancer Research Annual meeting, San Francisco, April 1-5, 2000.

Symposium and seminar presentations by PI

Sept, 1999. Symposium speaker. Apoptosis and angiogenesis in cancer prevention by selenium. American Institute for Cancer Research 1999 Annual Research Conference. Washington, DC.

Oct, 1999. Seminar, Cornell University, Ithaca, NY. Div of Nutritional Sciences. Mechanisms of cancer prevention by selenium.

Nov, 1999. Seminar, Univ of Colorado Health Sciences Center, Denver, CO. Cancer Center Cell Biology Program. Mechanisms of cancer prevention by selenium.

Sept, 1999. Seminar, AMC Cancer Research Center, Cancer prevention by selenium.

Conclusions

The discovery of potent apoptotic effect and specific MMP inhibitory effect of methylselenium on vascular endothelial cells supports and extends the anti-angiogenic activity of Se. These observations provide a plausible mechanistic explanation of the in vivo findings by Ip and co-workers (1-3) that methylselenol pool is the active cancer chemopreventive Se metabolite. Se agents that selectively increase this pool may be of greater breast cancer preventive benefit in women. The assessment of in vivo anti-angiogenic efficacy of the different Se forms awaits refinement of the Matrigel plug assay.

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Appendices:

1. Re-print:

Jiang C, Jiang W, Ip C, Ganther H, Lu J. Selenium-induced inhibition of angiogenesis in mammary cancer at chemopreventive levels of intake. Mol Carcinog. 1999 Dec;26(4):213-225.

2. Abstract: Jiang C., Ganther, H and J. Lu. Methylseleninic acid induces apoptosis and inhibts matrix metalloproteinases of vascular endothelial cells. (Abstract) American Institute for Cancer Research Annual Research Conference. Washington, DC. Sept 2&3, 1999.

3. Abstract: Lu, J., Jiang, C., Wang, Z. and Ganther, H. Merthylselenol as a proximal inhibitory selenium metabolite for matrix metalloproteinases and angiogenesis (Abstract) American Association for Cancer Research Annual meeting, San Francisco, April 1-5, 2000.

APPENDIX 1

MOLECULAR CARCINOGENESIS 26:213–225 (1999)

BRIEF COMMUNICATION

Selenium-Induced Inhibition of Angiogenesis in Mammary Cancer at Chemopreventive Levels of Intake

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The trace element nutrient selenium (Se) has been shown to possess cancer-preventive activity in both animal models and humans, but the mechanisms by which this occurs remain to be elucidated. Because angiogenesis is obligatory for the genesis and growth of solid cancers, we investigated, in the study presented here, the hypothesis that Se may exert its cancer-preventive activity, at least in part, by inhibiting cancer-associated angiogenesis. The effects of chemopreventive levels of Se on the intra-tumoral microvessel density and the expression of vascular endothelial growth factor in 1-methyl-1-nitrosourea–induced rat mammary carcinomas and on the proliferation and survival and matrix metalloproteinase activity of human umbilical vein endothelial cells in vitro were examined. Increased Se intake as Se-enriched garlic, sodium selenite, or Se-methylselenocysteine led to a significant reduction of intra-tumoral microvessel density in mammary carcinomas, irrespective of the manner by which Se was provided: continuous exposure (7-wk feeding) with a chemoprevention protocol or acute bolus exposure (3 d) after carcinomas had established. Compared with the untreated controls, significantly lower levels of vascular endothelial growth factor expression were observed in a sizeable proportion of the Se-treated carcinomas. In contrast to the mammary carcinomas, the microvessel density of the uninvolved mammary glands was not altered by Se treatment. In cell culture, direct exposure of human umbilical vein endothelial cells to Se induced cell death predominantly through apoptosis, decreased the gelatinolytic activities of matrix metalloproteinase-2, or both. These results indicate a potential for Se metabolites to inhibit key attributes (proliferation, survival, and matrix degradation) of endothelial cells critical for angiogenic sprouting. Therefore, inhibition of angiogenesis associated with cancer may be a novel mechanism for the anticancer activity of Se in vivo, and multiple mechanisms are probably involved in mediating the anti-angiogenic activity. *Mol. Carcinog. 26:213–225, 1999.*

Key words: intratumoral microvessel density; vascular epithelial growth factor; matrix metalloproteinase; apoptosis; 1-methyl-1-nitrosourea

INTRODUCTION

The published results of a prospective, double-blinded, randomized, placebo-controlled trial by Clark and coworkers demonstrate for the first time that selenized yeast, when used as a supra-nutritional supplement, may be a very effective preventive agent for several major human epithelial cancers [1]. This human trial corroborates the findings from studies of animal models that have shown a potent anti-cancer activity of selenium (Se) in many organ sites with differing biochemical, hormonal, and metabolic profiles [reviewed in 2,3]. The studies using animal model systems have provided significant insight into the possible mechanisms of action of Se in this role. Ip and coworkers have shown that a monomethyl Se metabolite such as methylselenol (CH_3SeH) may be the active Se in vivo against chemically induced mammary carcinogenesis and that the chemopreventive efficacy of a given Se compound may depend on the rate of its metabolic conversion to that active form [2,4,5]. Mechanistic studies using cell-culture models have so far focused almost exclusively on the cancerous epithelial cells as the targets of the anti-cancer activity of Se and have shown differential cellular, biochemical, and gene expression responses to different forms of Se [2,3,6–8]. To our knowledge, no published report has examined the nonepithelial components as potential targets through which Se might exert a cancer-

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Abbreviations: Se-garlic, selenium-enriched garlic; MSeC, Se-methylselenocysteine; VEGF, vascular epithelial growth factor; MMP, matrix metalloproteinase; HUVEC, human umbilical vein endothelial cells; MSeA, Se-methylseleninic acid; MNU, 1-methyl-1-nitrosourea; IHC, immunohistochemical; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; ECGS, endothelial cell growth supplement; IMVD, intra-tumoral microvessel density; AP-1, activator protein-1; NF-κB, nuclear protein-kappa B.

preventive effect. Because angiogenesis, that is, the formation of microvessels, is an obligatory component of the carcinogenesis process for supporting clonal expansion, lesion progression, and growth of solid tumors [9–13], we hypothesize that Se may exert its cancer-preventive activity, at least in part, by inhibiting neo-angiogenesis. In this report, we describe animal experiments showing that high levels of Se intake, given either chronically (7-wk feeding) in a chemoprevention setting or acutely (3-d feedings) in bolus doses after mammary carcinomas had reached a certain size, were able to decrease the density of microvessels in chemically induced rat mammary carcinomas. In the chemoprevention experiments, Se-enriched garlic (Se-garlic) and sodium selenite were used. In the acute treatment experiment, selenite and Se-methylselenocysteine (MSeC) were used, the latter being a predominant form of Se identified in Se-garlic [14] and a metabolic methylselenol precursor [4,5]. All three Se forms have been demonstrated to possess potent cancer chemopreventive activity in vivo [2].

Angiogenesis is regulated by the balance between angiogenic factors and inhibitors [10–13]. A primary angiogenic factor is vascular endothelial growth factor (VEGF)/vascular permeability factor [15,16]. The *VEGF* gene is organized into eight exons and, as a result of alternative splicing, at least four transcripts have been detected that encode mature monomeric VEGFs of 121, 165, 189, and 206 amino acid residues in humans [17]. $VEGF_{121}$ and $VEGF_{165}$ are diffusible forms capable of activating angiogenesis in a paracrine/endocrine manner [17]. The role of $VEGF_{189}$ and $VEGF_{206}$ is less well defined, as they are synthesized but apparently not secreted [17]. VEGF forms symmetric homodimers through intermolecular disulfide bonds, and the dimeric forms are biologically active [18]. VEGF is essential for normal vasculogenesis as well as angiogenesis. A loss of even one VEGF allele has been shown to lead to embryonic lethality, and homozygous mutant embryonic stem cells are incapable of forming tumor [19,20]. Hypoxia, a condition that the cancer cells create and are subjected to in their deregulated growth, is a potent inducer of VEGF expression [21]. In addition, many pro-angiogenic factors such as insulin-like growth factor, tumor necrosis factor-α, fibroblast growth factor, and cytokines [22–25] as well as oncogenic mutations [26–30] stimulate the production of VEGF, supporting its primary role as an angiogenic mediator. In this paper, we present data documenting an inhibitory effect of Se on the in vivo expression of VEGF as one possible mechanism for Se's regulation of the angiogenic switch in a sizeable proportion of the chemically induced mammary carcinomas.

The angiogenesis process itself involves a complex sequence of events [9–13]. When the vascular endothelial cells in existing vessels are stimulated to grow, they secrete proteases such as matrix metalloproteinases (MMPs) [31], which digest the basement membrane surrounding the vessels. The junctions between endothelial cells are altered, cell projections pass through the space created, and the newly formed sprout grows toward the source of the angiogenic stimulus. Continued capillary sprouting angiogenesis depends on the following conditions or processes: the angiogenic stimulus (angiogenic factors, hypoxia, etc.) must be maintained, the endothelial cells must secrete MMPs required to break down the adjacent tissue matrix, the cells themselves must be capable of movement and migration, and endothelial cells must proliferate to provide the necessary number of cells for the growing vessels. The crucial role of specific MMPs such as MMP-2 in angiogenesis has been documented by using knockout as well as other approaches [32–34]. To provide insights into Se action in some of these key processes, we examined the effects of direct Se exposure in cell culture on the proliferation, survival, and gelatinolytic activities of human umbilical vein endothelial cells (HUVEC). The results indicate potential inhibitory effects of Se on endothelial proliferation/survival and the matrix degradation activity critical for sprouting angiogenesis.

MATERIALS AND METHODS

Chemicals and Reagents

Sodium selenite pentahydrate was purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Se-garlic was cultivated and prepared as described previously [35,36]. *d,l*-MSeC was synthesized by H. Ganther as described elsewhere [4,5]. Rabbit anti–factor VIII and rabbit anti-mouse antibodies were purchased from DAKO Corporation (Carpenteria, CA). Monoclonal mouse anti-VEGF was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Biotinylated donkey anti-rabbit Fab'2 secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Conjugated alkaline phosphatase–streptavidin was purchased from Biogenex (San Ramon, CA). Protein size markers and enhanced chemoluminescence reagents were purchased from Amersham Life Science (Arlington Heights, IL).

Methylseleninic acid (MSeA; CH_3SeO_2H) was recently synthesized by H. Ganther as a proximal precursor of methylselenol, a putative active chemopreventive Se metabolite [2–5]. Briefly, dimethyldiselenide (Aldrich Chemical Co., Milwaukee, WI) was oxidized with hydrogen peroxide (3%) at 65°C until the yellow color of the diselenide had disappeared. The solution was adjusted to pH 7 with KOH and then applied to a column of Dowex 1 (chloride). After washing with water until a negative starch/iodide test was obtained, the MSeA was eluted with 0.01 N HCl. The main starch/iodide-

positive fractions were pooled, adjusted to pH 7 with KOH, and analyzed for total Se by fluorometric analysis. Thin-layer chromatography on cellulose in butanol:acetic acid:water (5:2:3 vol/vol/vol) showed a single starch/iodide-positive spot of RF = 0.42. Reduction with excess borohydride gave a single ultraviolet peak (252 nm; mM extinction coefficient, 6.35), corresponding in wavelength and intensity to that of aliphatic selenolates.

Upon reaction with thiols or other reducing agents, MSeA is expected to undergo a four-electron reduction to methylselenol by way of an intermediate selenenylsulfide:

$$CH_3SeO_2H + 3RSH = CH_3SeSR + RSSR + 2H_2O$$
$$CH_3SeSR + RSH = CH_3SeH + RSSR$$
$$\text{Overall}: \quad CH_3SeO_2H + 4RSH = CH_3SeH + 2RSSR + 2H_2O$$

Under cellular conditions, it is expected that MSeA initially will react with glutathione to give the glutathioninyl methylselenenylsulfide. This intermediate will undergo further reduction to methylselenol (which ionizes to methyl selenolate at neutral pH) through nonenzymic reduction by excess thiol or by enzymatic reduction through NADPH-linked reductases such as glutathione reductase.

Design of Animal Experiments

The effects of Se provided in different chemical forms on selected angiogenic parameters were assessed in both a conventional chemoprevention setting in which Se was provided continuously after carcinogen treatment and in an acute-exposure setting after palpable mammary carcinomas had established. Female Sprague-Dawley rats were purchased from Taconic Farms (Germantown, NY) at 21 d of age. The rats were fed a modified AIN-76–based purified diet with corn oil (5%) substituted for soybean oil as the fat source and glucose substituted for sucrose. The animals were housed three per cage in an environment-controlled animal room maintained at 22 ± 1°C with 50% relative humidity and a 12-h light/12-hour dark cycle.

Continuous Se Exposure Protocol

Two independent animal experiments identical in design were performed on different occasions. Weanling rats were fed the control diet (containing 0.1 ppm Se) until 50 d of age, at which time each rat was given an intraperitoneal injection of 50 mg/kg 1-methyl-1-nitrosourea (MNU) to induce mammary carcinogenesis [37]. Seven days after the MNU injection, one group remained on the control diet, and the other group was fed a diet supplemented with 3 ppm Se as either Se-garlic (Experiment 1) or sodium selenite (Experiment 2). At 8 wk after the MNU injection (i.e., 7 wk of Se intervention, in contrast to a typical 6-mo protocol for most carcinogenesis experiments), the rats were killed by inhalation of gaseous carbon dioxide and cervical dislocation. Mammary tumors were dissected, and those greater than 0.1 g were frozen in liquid nitrogen. Uninvolved mammary tissue was also obtained from the Se-garlic experiment, fixed in formalin, and processed for microvessel staining. As shown in Table 1, consumption of either Se-garlic or selenite at a chemopreventive level led to decreased tumor multiplicity, decreased tumor burden, or both.

Acute Se Exposure Protocol

In experiment 3, female rats were given an intraperitoneal injection of 50 mg MNU/kg body weight at 21 d of age to induce mammary carcinogenesis [38]. Starting 4 wk after the carcinogen was administered all rats were palpated daily for the detection of mammary tumors. Tumor dimensions were measured using a caliper. When mammary tumors reached or exceeded approximately 1 cm on the longest dimension, the tumor-bearing rats were randomly assigned to one of three groups: (i) gavage control (ii) once-daily gavage of 2 mg Se/kg body weight as MSeC, or (iii) once-daily gavage of 2 mg

Table 1. Effects of Dietary Supplementation with Se-garlic (Experiment 1) or Selenite (Experiment 2) on MNU-Induced Mammary Carcinogenesis*

Supplemental selenium	Supplement level	Number of rats	Number of carcinomas†	Total Tumor burden‡
Experiment 1				
None	0	9	9	5.3 g
Se-garlic	3 ppm	9	6	2.7 g
Experiment 2				
None	0	15	25	ND
Selenite	3 ppm	15	7	ND

*Mammary tumors were collected from Sprague-Dawley rats at the end of the 7-wk feeding intervention.
†In the Se-garlic experiment (Experiment 1), only lesions greater than 0.1 g were collected. In the selenite experiment (Experiment 2), all lesions visible under 7 × magnification were collected, but the tumor weight was not recorded.
‡Tumor burden is the sum of tumor weight for tumor bearing animals for each group. ND, not determined.

Se/kg body weight as sodium selenite. The Se gavage was repeated two more days for a total exposure of 6 mg of Se/kg body weight over 3 d. At 24 h after the last Se dose, the rats were killed, and the mammary tumors were dissected. A portion of each tumor was fixed in formalin for histological analysis and immunohistochemical (IHC) staining, and the rest was frozen in liquid nitrogen for later biochemical analyses.

Microvessel Evaluation

Microvessels were detected by staining for vascular endothelial cells by using an antibody specific for factor VIII/von Willebrand's factor–related antigen, a marker for vascular endothelial cells [39,40]. Formalin-fixed, paraffin-embedded carcinomas were cut into 5-μm sections and placed on 3-amino propyltriethaoxysilane treated slides (X-tra Surgipath, Richmond, IL). The sections were immobilized at 60°C for 20 min and deparaffinized with three changes of xylene for 5 min each. After rehydration in a series of graded ethanols and, finally, distilled water, the sections were treated with 0.4% pepsin (0.4 g+100 mL H_2O+500 μL of 2 N HCl) at 37°C for 15 min to retrieve the antigen. Normal donkey serum (diluted 1:5 in phosphate-buffered saline (PBS)) was added to block nonspecific binding for 20 min at room temperature after the sections had been rinsed with three changes of distilled water and PBS each. Excess serum was drained and primary rabbit anti–factor VIII antibody (diluted 1:800) was applied for 30 min. After three rinses with PBS for 5 min each, biotinylated donkey anti–rabbit Fab'2 secondary antibody (diluted 1:1000) was applied for 30 min, and the sections were rinsed. Conjugated alkaline phosphatase–streptavidin (diluted 1:80) was applied for 30 min. The slides were rinsed with three changes of PBS for 5 min each, incubated with one tablet of fast red chromogen (Biogenex, San Ramon, CA) plus 5 mL of naphthol buffer for 10 min, and counterstained with Harris hematoxylin (diluted 1:10) for 2 min.

The intratumoral microvessels were counted separately for the stromal areas and within the tumor epithelium–rich lobules (intra-lobular), each on 10 hot-spot fields [40] representing a total area of 0.5 mm^2. To facilitate counting, each hot-spot field was projected onto a computer screen (CAS-200; Becton-Dickinson/Cellular Analysis Systems, San Jose, CA), and the contrast was adjusted to highlight the vessels. All vessels within a preset rectangle (equivalent to 0.05 mm^2) were counted. To determine whether Se might exert a differential effect on microvessels of different sizes, we classified the microvessels into three categories: "large," a cross-section diameter of 10 or more cells; "medium," five to nine cells; and "small," one to four cells. For the uninvolved mammary glands, which were composed of mostly adipocytes and a very small fraction of mammary epithelial structures (ducts, lobules), microvessels in 10 random fields were counted according to these three size categories.

VEGF Expression

The pattern of VEGF expression in MNU-induced rat mammary carcinomas was determined by IHC staining on formalin-fixed sections after microwave antigen retrieval in 10 mM sodium citrate buffer, pH 6. Mouse anti-VEGF primary antibody was incubated at room temperature. Conjugated horseradish peroxidase–streptavidin (Dako P397; DAKO corporation) diluted 1:1000 was used for binding to biotinylated rabbit anti-mouse secondary antibody (diluted 1:100; PAKO corporation). After rinsing in PBS, the slides were incubated with diaminobenzidine chromogenic substrates (Sigma #D5637; Sigma Chemical Co., St. Louis, MO, at 3 mg of diaminobenzidine in 5 mL of PBS+20 μL of H_2O_2) for 10 min. The expression level of VEGF protein isoforms was assessed by immunoblot analyses of carcinoma extracts. Histologically classified adenocarcinomas (frozen) were homogenized in nine volumes of RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 50 mM NaF; 1% Triton X-100; 1% Sodium deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 1 mM dithiotratol; 5 mM sodium orthovanadate; and 1 mM phenylmethylsulfonyl fluoride and 38 μg/mL aprotinin were added fresh). After centrifugation (14 000 × g for 20 min) the supernatants were recovered, and the protein content was quantified by the Bradford dye-binding assay (Bio-Rad Laboratories, Richmond, CA). Forty microgram of total protein was size-separated by electrophoresis on 15% SDS-polyacrylamide gels under nonreducing conditions. Equal loading was confirmed by Coomassie staining of replicate gels and by reprobing blots for β-actin expression. Protein size markers were loaded on each gel to estimate the sizes of the proteins being detected. The proteins were electroblotted onto nitrocellulose membranes, and the VEGF proteins were detected by probing with the same antibody used for IHC staining. Lung tissue was used as a positive-control source of in vivo VEGF. The relative protein level was determined by enhanced chemiluminescence and x-ray film detection. The exposure time was adjusted so that the signal intensity remained in the quantifiable range. The x-ray films were digitized with a transmission scanner, and the signal intensity was quantitated with the UN-SCAN-IT gel-scanner software (Silk Scientific, Inc., Orem, UT). The signals (pixels) were normalized to those of the corresponding β-actin. The normalized expression data were used for statistical evaluations.

Cell Culture

HUVEC were obtained from the American Type Culture Collection (Manassas, VA). They were

propagated in F12K medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/mL heparin (Sigma Chemical Co.), and 30 μg/mL bovine endothelial cell growth supplement (ECGS; Sigma Chemical Co.). Experiments were conducted within 15 passages of receipt from the American Type Culture Collection. For growth and survival experiments, HUVEC were seeded into 6-well plates for 24–48 h to reach a confluence of about 50%. Fresh medium was replaced, and Se as either sodium selenite or MSeA was added from 100 × stock solutions prepared in PBS. Morphological responses were monitored over time, the cells remaining adherent were fixed in 1% glutaraldehyde and stained with methylene blue, and the cell number was then counted on three high-power (200 ×) fields. Detached cells (in 500 μL of medium) were centrifuged onto slides, fixed in 1% glutaraldehyde, and stained in hematoxylin and eosin.

Zymogram Analysis

HUVEC were seeded into 6-well plates in complete medium for 24–48 h to reach 70–80% confluence. The cells were washed two times with PBS to remove spent medium and refed serum-free medium supplemented with 100 μg/mL ECGS and treated with selenite or MSeA for 6 h (a time frame that did not result in visible cell death or detachment). Conditioned medium and cell lysates (prepared in 1% Triton X-100; 0.5 M Tris-HCl, pH 7.6; and 200 mM NaCl) were analyzed for gelatinolytic activities on substrate gels [41]. Type I gelatin (Sigma Chemical Co.) was added to the standard acrylamide mixture in the resolving gels at a final concentration of 0.3%. Samples were mixed with 5 × loading buffer (10% SDS; 50% glycerol; 0.4 M Tris-HCl, pH 6.7; and 0.1% bromophenol blue) and loaded onto 5% stacking/10% resolving gels. The gels were run at room temperature with cooling (85 V, 4–5 h). After running, the gels were soaked in 2.5% Triton X-100 with gentle shaking for 20 min. The washing was repeated two more times to remove the SDS. The gels were then washed in water once for 20 min and incubated at 37°C for 24–48 h in substrate buffer (50 mM Tris-HCl, pH 8.0, and 5 mM $CaCl_2$). After incubation, the gels were stained for 1 h in Brilliant Blue R250 and destained until clear bands appeared on a blue background. The gels were digitized with a transmission scanner and band intensity (on inverted images) was quantified by using the UN-SCAN-IT gel-scanner software (Silk Scientific, Inc.).

Statistical Analyses

The microvessel counts among groups were compared by student's *t*-test or analysis of variance, as appropriate. For VEGF expression, for which the variance was not normally distributed, the rank-order test was used.

RESULTS

Effect of Se on Microvessel Density and VEGF Expression in a Chemoprevention Context

In MNU-induced mammary carcinomas, the microvessels were concentrated in the fibro-connective stromal areas and spread along stromal ridges on the periphery of epithelial-rich tumor lobules. Microvessels were occasionally observed within such tumor lobules; these vessels are referred to in this report as intra-lobular microvessels. Table 2 presents the vessel counts in carcinomas obtained from rats consuming either the control diet or Se-supplemented diets for 7 wk. In the Se-garlic experiment (Experiment 1), the total microvessel densities (counts/0.5 mm^2) in the stromal and intra-lobular areas from Se-fed rats were 34% and 61% lower, respectively, than in those of rats fed the control diet. When categorized by the size of the microvessels, small microvessels (with a crosssection diameter of one to four cells) accounted for most of the microvessels in the intra-lobular and stromal areas (Table 2). The observed reduction in intratumoral microvessel density (IMVD) in the Se-garlic–fed group occurred almost exclusively in these small microvessels in both the stromal and intra-lobular areas (Table 2). The microvessel density of the uninvolved mammary glands was not decreased by Se-garlic treatment (Table 2); the vessel size distribution in the normal glands was less in favor of the small vessels in comparison with that seen in carcinomas. In the selenite experiment (Experiment 2), 3 ppm selenite exerted a similar degree of reduction of microvessel density in the stromal area of the mammary carcinomas, and the inhibitory effect was again observed predominantly in the small microvessels (Table 2).

Because large carcinomas were likely to contain larger and more vessels than were their small counterparts, it would be expected that the observed lower IMVD in the Se-treated animals might have been biased by the size of the carcinomas examined. To investigate this issue, we analysed the IMVD data against the sizes of the carcinomas in the Se-garlic experiment (Experiment 1), in which the approximate weight of each individual carcinoma was available (Figure 1). The stromal microvessel density in both the control and Se-garlic groups showed a modest linear regression trend over the size (Figure 1A). However, the regression trend line for the Se-garlic group was downshifted in parallel to that of the control group, indicating a reduction of vessel density across the tumor-size ranges evaluated (Figure 1A). The intra-lobular microvessels displayed a greater degree of difference between the control and Se-treated groups as the tumor size increased (Figure 1B). Albeit limited by the small sample size analyzed, these data indicated that the

Table 2. Effects of Dietary Supplementation with Se-garlic (Experiment 1) or Selenite (Experiment 2) on Microvessel Density (counts/0.5 mm^2) of MNU-Induced Rat Mammary Carcinomas and Non-involved Mammary Glands*

Dietary group	Number of rats	Total microvessels	Large (≥ 10 cells)	Medium (5–9 cells)	Small (1–4 cells)
Experiment 1					
Stromal areas:					
Control	9	69.4 ± 6.0^a	4.8 ± 0.8	9.9 ± 1.2	54.8 ± 5.7^a
Se-garlic, 3 ppm	6	45.8 ± 6.4^b	3.0 ± 1.1	7.7 ± 2.0	35.2 ± 5.7^b
Intra-lobular areas:					
Control	9	16.0 ± 2.2^a	0 ± 0	0.4 ± 0.2	15.6 ± 2.3^a
Se-garlic, 3 ppm	6	6.2 ± 1.2^b	0.2 ± 0.2	0.3 ± 0.3	5.7 ± 1.2^b
Uninvolved mammary tissue:					
Control	6	8.7 ± 0.7	1.8 ± 0.5	2.7 ± 0.4	4.2 ± 0.8
Se-garlic, 3 ppm	6	7.2 ± 0.9	1.3 ± 0.4	2.0 ± 0.7	3.8 ± 0.6
Experiment 2					
Stromal areas:					
Control	8	80.0 ± 4.4^a	0.9 ± 0.4	4.4 ± 1.7	74.8 ± 5.3^a
Selenite, 3 ppm	4	61.0 ± 3.0^b	0.3 ± 0.3	3.8 ± 2.8	57.0 ± 2.1^b

*Values are presented as means ± standard errors of the mean. The values within a column bearing dissimilar superscripts (^{a} and ^{b}) are significantly different ($P < 0.05$).

observed reduction of IMVD as a result of chronic exposure to a chemopreventive level of Se could not be entirely accounted for by tumor-size differences.

IHC staining for VEGF proteins in the MNU-induced mammary carcinomas was confined to the cytosol and was mostly localized in the cancerous epithelial cells, with light staining in some stromal cells. Such a staining pattern indicated that the cancerous epithelial cells were the major source of the in vivo expression of this angiogenic factor in this chemically induced model of mammary cancer. Figure 2 shows representative western immunoblot analyses of VEGF expression in selected carcinomas (i.e., those that were large enough for the biochemical assessment). The rat lung tissue (positive control for VEGF) expressed VEGF proteins with apparent dimeric sizes of about 32 and 36 kDa, presumably corresponding to the $VEGF_{164}$ protein (the rat proteins are one amino acid shorter than their human homologs) of varying glycosylation states [14] or $VEGF_{164}/VEGF_{188}$. The 32 and 36 kDa VEGF proteins were the most predominantly expressed bands in the mammary carcinomas; other smaller bands suggestive of $VEGF_{120}$ were also expressed. Based on the limited number of samples analyzed, two of five carcinomas in the Se-garlic group (Figure 2A) and two of four carcinomas in the selenite group (Figure 2B) showed a marked reduction in VEGF expression to almost undetectable levels. Overall, carcinomas from Se-garlic– or selenite-fed rats displayed 45% and 75% reductions of VEGF expression, respectively, in comparison with the levels from the control rats.

Acute Effects of Se on Microvessel Density and VEGF Expression in Established Carcinomas

As shown in Table 3, acute treatment of established mammary carcinomas with either MSeC or selenite decreased the IMVD by 28–32% in the

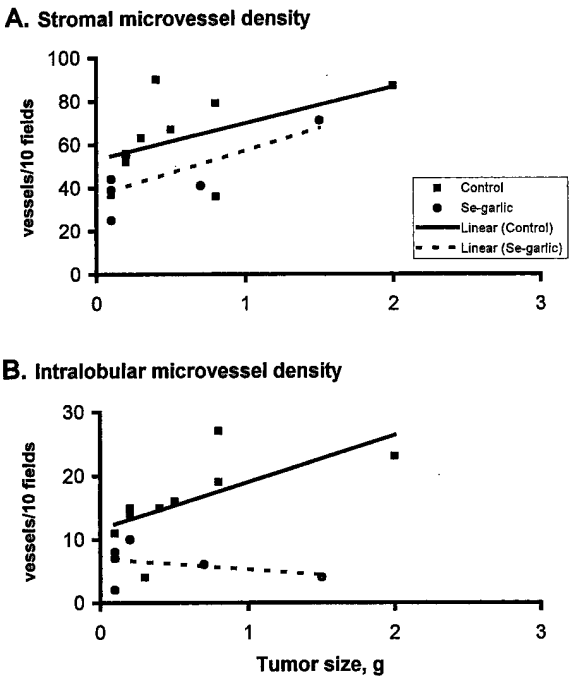

Figure 1. Correlation plots of stromal (A) and intra-lobular (B) microvessel densities against the approximate sizes of the MNU-induced mammary carcinomas examined. The linear regression trend line for each group is shown.

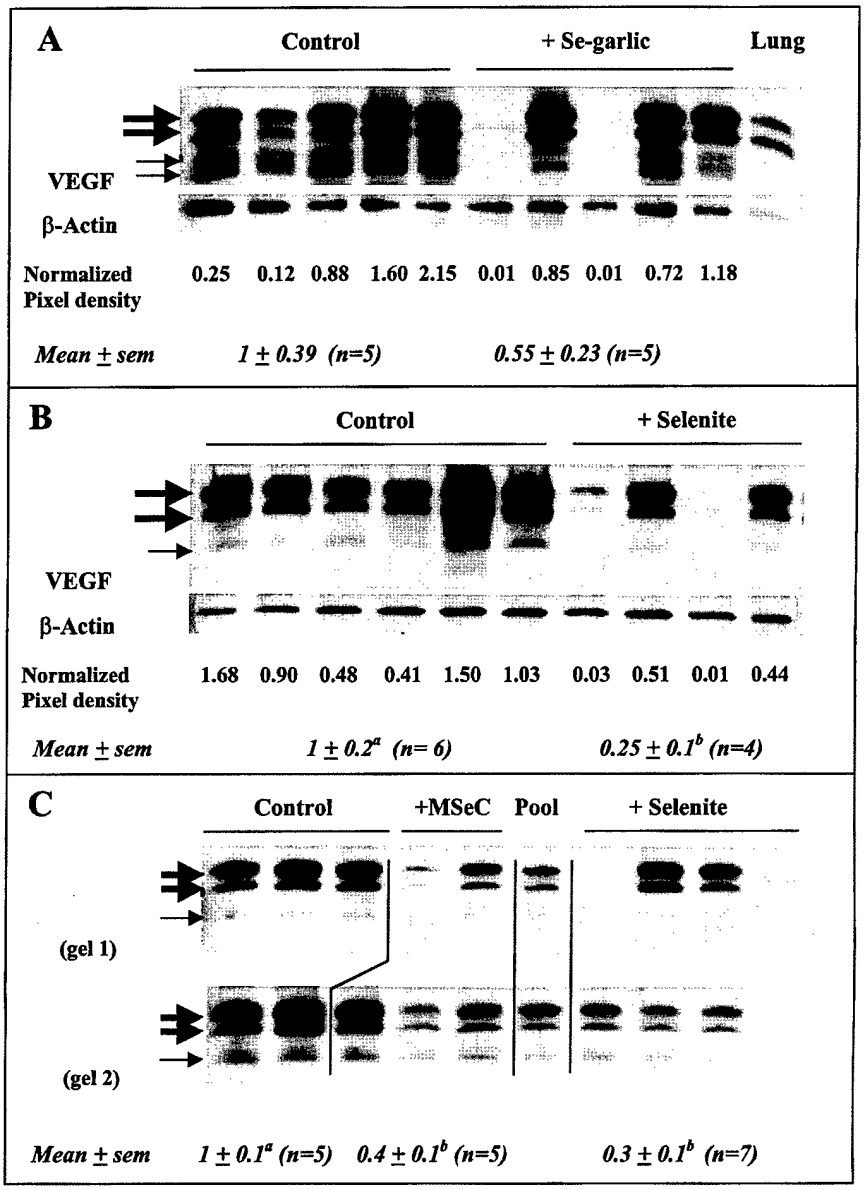

Figure 2. Western blot analyses (nonreducing gel) of the expression level of VEGF proteins in MNU-induced rat mammary adenocarcinomas. Two bands with apparent dimer masses of 32 and 36 kDa (marked by heavy arrows) were presumably $VEGF_{164}$ of varying glycosylation states [14] or $VEGF_{164}$ and $VEGF_{188}$. The additional smaller bands (marked by light arrows) were most likely $VEGF_{120}$. Forty micrograms (by the Bradford dye assay) of carcinoma protein extract was loaded from each sample. After probing for VEGF, the membrane was stripped and reprobed for β-actin expression to correct for loading differences. Rat lung was used as a positive control for VEGF expression. The normalized expression level (pixel intensity) for individual samples is shown below each lane. The group means and number of samples analyzed are shown for each experiment, and those means within the same experiment bearing different superscripts were statistically different. (A) Se-garlic chemoprevention experiment (Experiment 1). (B) Selenite chemoprevention experiment (Experiment 2). (C) Acute Se treatment experiment (Experiment 3). Two separate gels were used in this experiment to accommodate all the samples. Pool = a pooled sampled of the carcinoma extracts from the various groups.

stromal areas; the effect was most profound with the small and medium-size microvessels. Both Se treatments led to a 60–70% reduction in VEGF proteins (Figure 2C). As in the chemoprevention experiments only a sizeable proportion of the mammary carcinomas showed marked reduction of VEGF expression in response to acute Se treatments; the remainder were not altered (Figure 2C).

Effects of Se on Growth/Survival of HUVEC

Treatment of HUVEC with MSeA led to cell retraction (Figure 3A vs. B) and detachment from the flask (Figure 3C). Such changes began to appear 10–12 h after treatment was initiated. Morphologically, most affected cells displayed apoptotic features, as indicated by nuclear condensation and

Table 3. Effects of Acute Se Treatment on Microvessel Density (Counts/0.5 mm^2) in the Stromal Areas of Established Mammary Carcinomas (Experiment 3)*

Treatment group	Number of carcinomas	Total microvessels	Large ($\geq$ 10 cells)	Medium (5–9 cells)	Small (1–4 cells)
Control	5	93.6 ± 4.7^a	2.2 ± 0.6	11.0 ± 0.8^a	80.4 ± 4.5^a
MSeC	7	67.3 ± 5.9^b	4.7 ± 0.9	8.1 ± 0.7^b	54.4 ± 6.3^b
Selenite	6	63.8 ± 3.0^b	3.8 ± 1.0	7.8 ± 1.6^b	52.2 ± 3.4^b

*Values are presented as means ± standard errors of the mean. The values within a column bearing dissimilar superscripts (^{a} and ^{b}) are significantly different ($P < 0.05$).

formation of apoptotic bodies. Replating these detached cells in fresh medium did not result in any cell attachment or growth. After 48 h of MSeA treatment, the adherent cell number was reduced by as much as 80% at 2 μM, and virtually no cell remained attached at 6 μM (Figure 3F). Treatment with selenite at 4 μM or lower for 48 h did not decrease the number of adherent cells and resulted in a concentration-dependent decrease beyond this level (Figure 3F). Morphologically, selenite treatment resulted in both apoptosis and detachment of cells, some of which did not show cell retraction and nuclear condensation (Figure 3D and E).

Effects of Se on Gelatinolytic (MMP) Activities

A brief treatment (6 h, before overt morphological changes) with MSeA led in both the conditioned medium and the cell lysates to a Se concentration–dependent reduction in gelatinolytic activities of a 72 kDa species, corresponding to the latent form of gelatinase A/MMP-2 (Figure 4, lanes 2–5 and 9–12) [31,41]. The gelatinolytic activity of a 53-kDa species [41], which was less abundant than MMP-2, showed a similar pattern of inhibition by MSeA in the medium (Figure 4, lanes 2–5). Incubating the conditioned medium from the untreated cells for 6 h at 37°C with 10 μM MSeA in a test tube did not inhibit the gelatinolytic activities (Figure 4, lane 7 vs. lane 6). This comparison indicated that the inhibitory effect did not result from direct reaction of MSeA with the secreted gelatinases/MMPs and was therefore dependent on cell metabolism to generate the active Se, presumably methylselenol or its derivatives. In contrast to treatment with MSeA, treatment with 10 μM selenite for 6 h did not significantly affect the gelatinolytic activities in either the conditioned medium or the cell lysates (Figure 4, lane 8 vs. lane 2 and lane 13 vs. lane 9).

DISCUSSION

To our knowledge, this communication is the first to report a reduction of the density of microvessels, especially of capillaries with a crosssection diameter of four of fewer cells, in MNU-induced mammary carcinomas by the consumption of chemopreventive levels of Se. This effect was observed with different forms of Se (i.e., Se-garlic, selenite, and MSeC) and with different Se exposure protocols. In the conventional chemoprevention setting, feeding Se for 7 wk (chronic exposure) led to decreased tumor multiplicity, decreased tumor burden, or both (Table 1) and a significant reduction in IMVD in both the fibro-connective stromal areas and the epithelial-rich intra-lobular areas (Table 2). Furthermore, the observed reduction in IMVD could not be entirely accounted for by the tumor-size differences between the groups (Figure 1); this was especially apparent for the intra-lobular microvessels. Because the microvessel density of the uninvolved mammary glands was not decreased by Se-garlic (Table 2), the effect of chemopreventive levels of Se on IMVD reduction appeared to be neoplasia specific. In both stromal areas and intra-lobular areas, the small microvessels with crosssection diameters of one to four cells were most responsive to the Se effect.

However, it is noteworthy that in a chemoprevention setting, carcinomas that emerge in the presence of the selective pressure exerted by an intervening agent would be considered relatively refractory or resistant to the action of that agent. Therefore, the observed reduction in IMVD in carcinomas from the groups chronically fed Se may in fact indicate an underestimation of the true extent of an anti-angiogenic response in the "sensitive" lesions, which would take a longer time to reach a detectable size or would never have reached a detectable size. To seek more direct evidence of anti-angiogenic activity, we examined the effects of acute Se treatment on IMVD in the established mammary carcinomas (Table 3). We observed a one-third reduction in IMVD after 3 d of Se gavage. As in the chemoprevention setting, decreases in the number of the small microvessels accounted for this observed effect on IMVD.

Because growing or newly sprouted vessels are likely to be smaller, the observation of exclusive reduction of small microvessels suggests that chemopreventive intake levels of Se may target regulatory mechanisms governing genesis, survival, or both of the growing microvessels in the tumor environment. In the work reported here, we investigated the impact of Se on the in vivo expression of

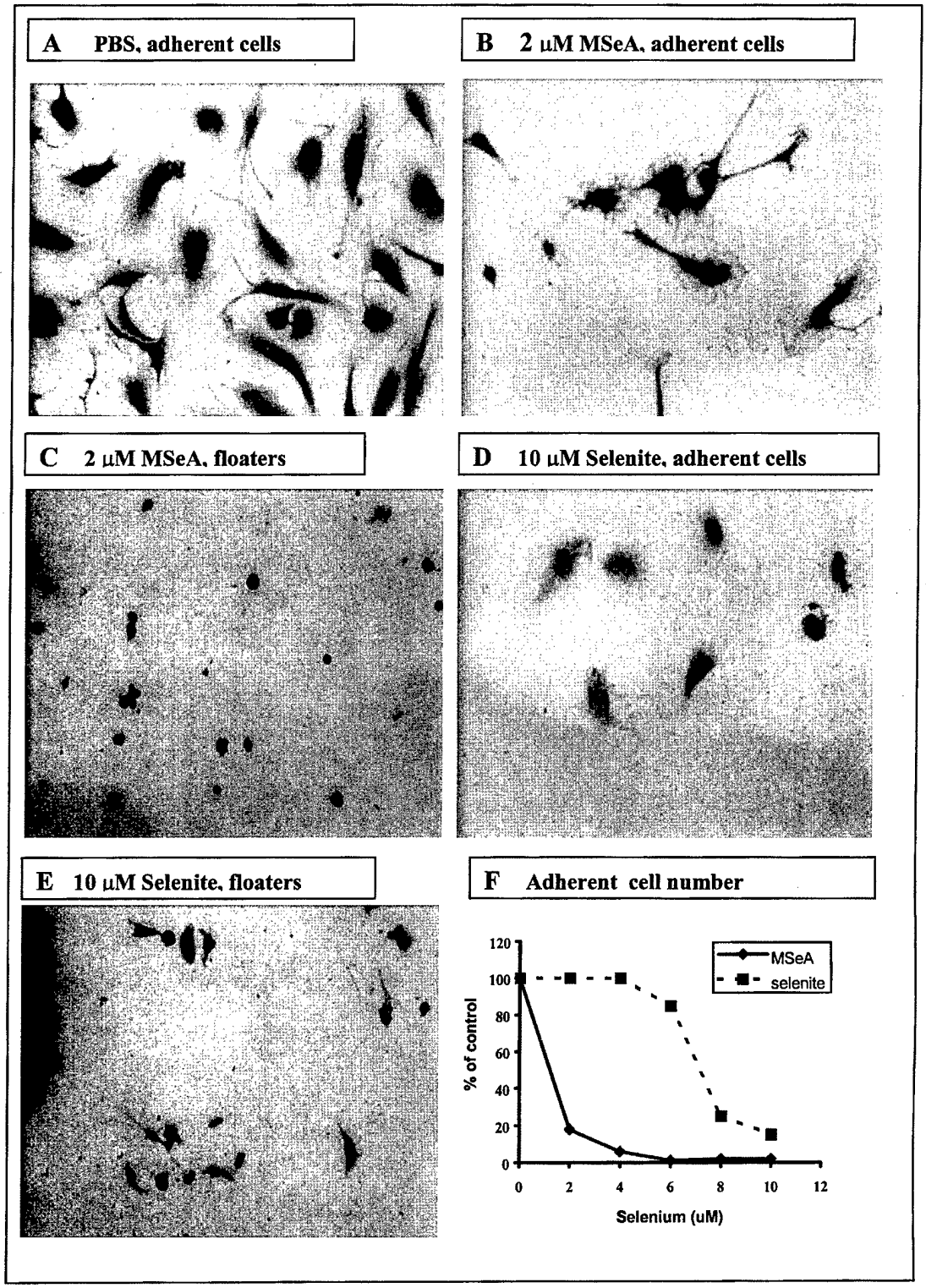

Figure 3. Effects of selenium treatment on the growth and survival of HUVEC. (A) HUVEC treated with PBS (vehicle) for 48 h as controls. (B) Adherent cells after 48 h of treatment with 2 μM MSeA. (C) Detached cells as a result of treatment with 2 μM MSeA for 48 h. Note the extensive nuclear condensation and fragmentation. (D) Adherent cells after 48 h of treatment with 10 μM selenite. (E) Detached cells as a result of treatment with 10 μM selenite for 48 h. Note the extensive nuclear condensation in some but not other cells. (F) Adherent cell number as a function of the Se concentration after 48 h of treatment.

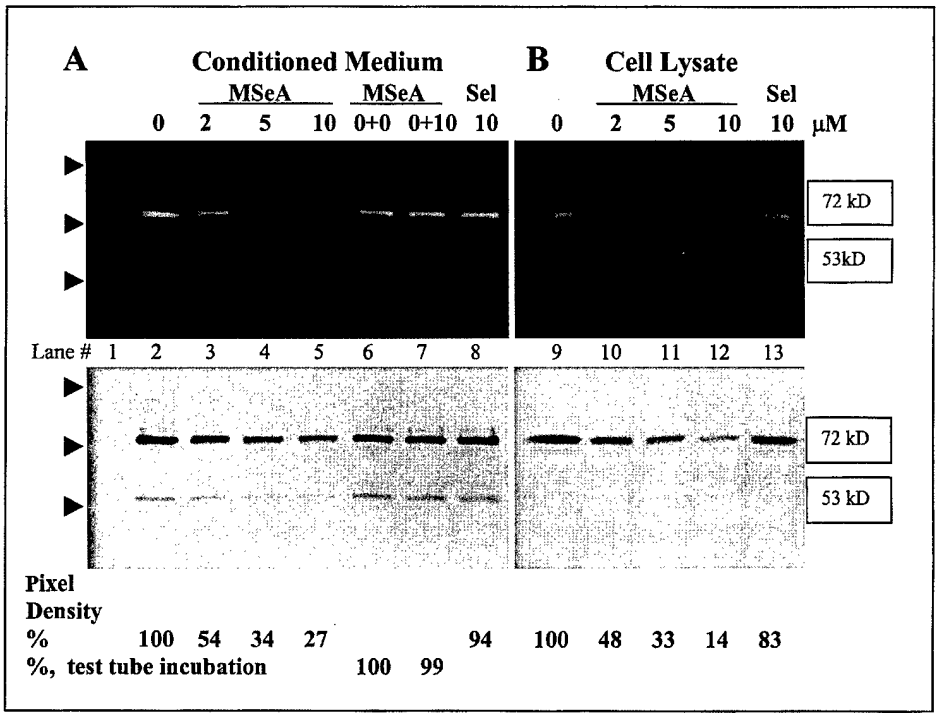

Figure 4. Representative zymographic analyses of the effects of selenium treatment of HUVECs on their secreted (A) and cell-associated (B) gelatinolytic activities. HUVECs were treated with either MSeA or Sel in a 6-well plate for 6 h in serum-free medium supplemented with 100 μg/mL ECGS. The gelatinase activities of the conditioned media (panel A, lanes 2–5 and 8) and cell lysates (panel B) were analyzed on gelatin I–impregnated substrate gels. Conditioned medium from untreated cells was also incubated with 10 μM MSeA (lane 7) or PBS (lane 6) for 6 h and analyzed zymographically to determine whether MMP inactivation was caused by direct reaction between the enzyme proteins with MSeA per se. The inverted images of the zymograms (lower panels) were used for densitometric quantitation. The relative pixel density for the 72-kDa gelatinase A/MMP-2 is shown below each lane. The arrowheads on the left mark the positions of molecular mass standards corresponding to (from top) 97, 66, and 47 kDa. Lane 1 contains serum-free medium as a blank control.

VEGF, a primary angiogenic molecule crucial for the genesis and survival of capillary vessels. IHC staining showed that the cancerous epithelial cells were the major source of VEGF in the mammary carcinomas induced by MNU. This expression pattern agrees with that reported for mammary carcinomas induced by a different mammary chemical carcinogen, 7,12-dimethylbenz[*a*]anthracene [42]. In both the chemopreventive and acute-treatment exposure settings, the VEGF expression level in a sizeable proportion, but not all, of the Se-treated carcinomas was significantly lower than in untreated controls (Figure 2), regardless of the form of Se used. The role of VEGF in initiating and especially in maintaining neo-angiogenic processes and in supporting endothelial survival has been extensively documented. Whereas overexpression of VEGF is linked to increased angiogenesis and more aggressive tumor behavior [43,44], anti-angiogenic interventions, especially those based on VEGF antibodies or interference with signal transduction through its receptors [45–49], have been shown to result in the inhibition of tumor growth and induction of endothelial apoptosis. More profoundly, germline knockout experiments have shown that loss of even one VEGF allele leads to embryonic lethality in heterozygotes and that homozygous mutant embryonic stem cells are incapable of forming tumors [19,20], suggesting a critical threshold effect of VEGF level to mediate and maintain normal vasculogenesis and neo-angiogenesis. In light of the crucial role of VEGF in endothelial proliferation and survival [45–49], it is therefore possible for Se-induced reduction of VEGF production in those affected tumors to lead to reduced endothelial proliferation and survival and consequently a reduction in the density of small microvessels.

In addition to or independent of VEGF inhibition, the direct apoptogenic effect of Se exposure on vascular endothelial cells, the inhibition of the endothelial cells' ability to degrade tissue matrix, or both may also contribute to the anti-angiogenic activity. The consequence of endothelial apoptosis could be collapse of microvessels and reduction in microvessel density. Vascular endothelial cells, by their very nature of lining the blood vessels, are exposed to Se before the delivery of serum Se to the cancerous mammary epithelial cells. The cell-culture data presented in Figure 3 indicate that serum-achievable levels (2 μM or less) of Se as MSeA, a proximal Se precursor of the putative active anti-cancer Se metabolite methylselenol [2–5], induced apoptosis of HUVEC by direct exposure. Selenite

also exerted cytocidal effects on HUVEC but a higher exposure level (fourfold) was needed to achieve the same extent of cell death. Furthermore, a brief treatment (6 h) of HUVEC with MSeA resulted in concentration-dependent inhibition of secreted and/or cell-associated gelatinolytic activities (Figure 4). The inhibitory effect of MSeA on MMPs was dependent on cell/metabolism-activation to yield the putative active Se metabolite methylselenol, because direct incubation of the conditioned medium with MSeA in a test tube did not affect the gelatinase activities (Figure 4). In support of a MSeA/methylselenol-specific inhibitory effect on MMPs, selenite at a level (10 μM) that induced apoptotic and cytolytic effects on HUVEC did not significantly inhibit MMP activity. The crucial role of MMP-2 in capillary angiogenesis was recently established in germline knockout as well as other model systems [32–34]. The MMP inhibitory activity observed here extends the active Se metabolite hypothesis of cancer chemoprevention [2–5] to include a specific action of methylselenol or its derivatives on a key process crucial for angiogenesis, that is, the matrilytic activity of the stimulated endothelial cells to invade the fibro-connective support structures during sprouting. This may offer an explanation for the greater in vivo chemopreventive efficacy of methylselenol precursor compounds (MSeC, Se-garlic, and methylselenocyanate) over selenite and conventional selenoamino acids [2]. That both methylselenol precursors and selenite were observed in this study to reduce VEGF expression and IMVD in the mammary carcinomas in the animal model may reflect the ability of in vivo Se metabolism to generate the active Se metabolites with endothelial apoptogenic and MMP inhibitory activities. This metabolic conversion activity is probably relatively inefficient, if not absent, in cell culture. Future work will determine the significance of and mechanisms involved in Se-induced endothelial apoptosis and MMP inhibition in cancer chemoprevention.

As a matter of speculation on the mechanisms of Se regulation of VEGF expression, MMP activity, and apoptosis, a common thread may rest in redox regulation of the activity of transcriptional factors or redox modification of functional state/activity of redox-sensitive proteins. In the case of VEGF, hypoxia is a potent inducer of VEGF expression [21], and this effect is principally mediated by the hypoxia-inducible factor-1 and activator protein-1 (AP-1) [50–53]. The activity of many nuclear factors such as hypoxia-inducible factor-1 and AP-1 is redox regulated [53,54]. Because hypoxia is commonly experienced by cancerous epithelial cells as the expanding clones strive to grow in size [55], it is possible that Se may inhibit hypoxia induction of VEGF expression by modulating the activity of these transcriptional factors. Thioredoxin has been considered a critical redox mediator for these nuclear factors [53], and its redox state may be controlled by Se through thioredoxin reductase, a newly recognized selenoprotein [56–59]. Indeed, severe Se deficiency has been reported to differentially modulate the DNA binding activity of liver nuclear extract to AP-1 and NF-kB sequences [60]. It is conceivable that the transcriptional activity of these factors, and consequently VEGF expression, may be redox-regulated by thioredoxin reductase in the nutritional range of Se supplementation. On the other hand, AP-1 and NF-kB activities have been shown to be potently inhibited by high levels of Se exposure in cell culture [61,62]. Such an inhibitory effect, which is probably more relevant to chemopreventive levels of Se exposure, may result from the modulation of cysteine residues by Se through formation of Se-S mixed disulfides or selenotrisulfides in these factors and other intracellular proteins. This topic was recently reviewed [63]. As far as MMPs are concerned, the inhibitory effect was not caused by MSeA per se, but most likely through the intracellular generation of methyselenol or its derivatives as described earlier. It remains to be determined whether the inhibitory effect is at the mRNA level or the protein level or whether it results from a direct modification of the MMP enzymatic activity. Relevant to the latter, MMP-2 expression in fibroblasts has been shown to be regulated by thiol antioxidants [64] and oxidation of MMP-2 in the test tube has been found to lead to its inactivation [65]. Because apoptosis initiation and execution in many models involve cysteine proteases (caspases), an additional hypothesis to be tested is that Se somehow exerts apoptogenic effects through modulation of the cysteine in such caspases [63].

Taken together, the in vivo and in vitro data presented are consistent with a potential anti-angiogenic effect of Se at chemopreventive intake levels. If proven true, the anti-angiogenesis effect may warrant a paradigm shift concerning cancer prevention research with Se. The carcinogenesis targets, i.e., the epithelial cells, do not exist in isolation in vivo but instead interact with the extracellular matrix and nonepithelial cells (e.g., fibroblasts, lymphocytes, and blood vessels) that reside in the stroma. By inhibiting neoplasia-driven angiogenesis and inducing growth arrest and apoptosis of cancer epithelial cells, Se may inhibit the conversion of avascular lesions to a vascular phenotype, thereby suppressing the progression and growth of the epithelial lesions. Further work is needed to establish the significance of an anti-angiogenic activity as a mechanism of the cancer-preventive activity of Se, especially when used as a nutritional supplement.

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Poster Abstract Booklet

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Nutrition and Cancer Prevention: New Insights Into the Role of Phytochemicals

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Methylseleninic acid induces apoptosis and inhibits matrix metalloproteinases of vascular endothelial cells

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Earlier work by Ip and Ganther had indicated that a monomethyl selenium (Se) species such as methylselenol may be an, if not the, active cancer chemopreventive Se metabolite in vivo. Methylseleninic acid (MSeA) was synthesized to provide a proximal precursor for methylselenol generation in vitro. Its effects on the proliferation/survival and the matrix metalloproteinase (MMP) activities of human umbilical vein endothelial cells (HUVEC) were examined in cell culture to explore the hypothesis that the cancer chemopreventive activity of Se may in part be mediated through an antiangiogenic effect. Both endothelial cell proliferation and certain MMPs are required for angiogenesis. In growth assays, MSeA at serum achievable levels concentration-dependently decreased cell number predominantly by inducing apoptosis. On an equimolar basis, MSeA was 4 fold more efficacious than the inorganic reference compound sodium selenite for the cyticidal activity. A brief MSeA treatment (6hr) of HUVEC decreased both the secreted (conditioned medium) and cell-associated (lysate) gelatinolytic activity at 72 kD corresponding to gelatinase A/MMP-2 in a concentration dependent manner with 50% inhibition at ~2μM. A 53 kD gelatinolytic activity in the conditioned medium was inhibited by MSeA treatment in the same fashion. However, incubation of the conditioned medium from the control cells with MSeA directly in the test tube for 6hrs did not inhibit these gelatinolytic activities. This observation indicates that the inhibitory effect required cellular metabolism/activation of MSeA. In contrast to MSeA, selenite at a level that exerted cytocidal effects did not inhibit these MMPs. Taken together, the data are consistent with the Se anti-angiogenesis hypothesis and point to a direct endothelial apoptogenic activity and an anti-matrilytic activity as likely mediating processes for the active Se metabolite (methylselenol or related Se species) to achieve an anti-angiogenic effect.

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APPENDIX 3

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Methylselenol as a proximal inhibitory selenium metabolite for matrix metalloproteinases and angiogenesis

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Methylselenol has been implicated as an *in vivo* active selenium (Se) metabolite for cancer chemoprevention by Se. We report here the discovery of a potent inhibitory effect of methylselenol proximal precursor compounds, methylseleninic acid (MSeA) and methylselenocyanate (MSeCN), on the matrix metalloproteinases (MMP-2 and 53kD)of human umbilical vein endothelial cells (HUVEC) that was absent for Se compounds that feed into the hydrogen selenide pool. The MMP inhibitary action occurred at Se levels that were achievable in the human blood ($IC_{50} \sim 2$ μM) and within 30 min. of the initiation of Se treatment. MSeA or MSeCN *per se* did not inhibit MMP when incubated in the test tube, suggesting cellular conversion into methylselenol was required for MMP inhibition. Western blot and ELISA analyses indicated that a reduction of MMP-2 protein level largely accounted for the observed MMP-2 inhibition. Prolonged treatment with MSeA or MSeCN inhibited HUVEC viability and capillary retraction on Matrigel. In addition, inclusion of MSeA in Matrigel plugs in a bFGF-induced mouse angiogenesis model led to decreased capillary formation *in vivo*. Taken together, the *in vitro* and *in vivo* results support methylselenol as a proximal Se metabolite for the inhibition of the extracellular matrix degradation potential of vascular endothelial cells and angigoenesis. These findings also provide a plausible mechanism to account for the greater cancer chemopreventive efficacy of methylselenol precursors *in vivo*. Work is in progress to determine whether the methylselenol inhibitory effect on MMP protein level occurs at the gene transcription, translation or post translational level. Supported by grants (to JL) from AICR and DOD and (to HG) from NCI.

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