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PRINCIPAL INVESTIGATOR: Junxuan Lu, Ph.D.

CONTRACTING ORGANIZATION: AMC Cancer Research Center
Denver, Colorado 80214

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13. ABSTRACT (<i>Maximum 200 Words</i>) Our overall aim is to understand the role of inhibition of angiogenesis in breast cancer prevention by selenium (Se). We have during the current funding period focused on following up on our discovery of a specific selenium metabolite inhibitory effect on vascular endothelial expression of matrix metalloproteinases and have extended that specificity to the inhibition of vascular endothelial growth factor VEGF by breast cancer epithelial cells. Our paper describing these findings received cover story honor in Mol. Carcinogenesis, 2000. We have also developed methodologies for evaluating anti-angiogenic attributes (pro-angiogenic factors, mitogenesis, apoptosis, capillary differentiation etc) of Se and validated with other agents and these activities resulted in 4 publications and reviews. Because endothelial matrix degradation, mitogenesis and survival are essential for angiogenesis response and because VEGF is crucial for stimulating angiogenesis, these observations provide plausible mechanisms to account for how methylselenol metabolite pool regulates angiogenic switch during chemoprevention of mammary and other cancers. For the next year, we will pursue the metabolite-specific effects on endothelial mitogenesis and cell cycle regulation and apoptosis and the role of JNK, p38MAPK, ERKs and PI3K signaling in angiogenesis regulation by methylselenium.				
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FOREWORD

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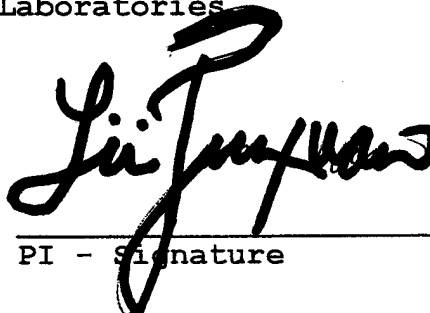
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(4 reprints)

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 developing methodologies for studying selenium metabolite specific effects on angiogenesis switch regulation, in terms of expression of positive factors in epithelial and endothelial cells, endothelial mitogenesis and apoptosis. These activities have resulted in 3 peer-reviewed publications and 1 book chapter on the subject of selenium and angiogenesis regulation (See appendices 1-4). The following describes progress made in this period.

Body- Key research accomplishments.

- As reported in the December issue of *Molecular Carcinogenesis*, 2000, we have discovered a methylselenium specific inhibitory effects on the expression of vascular endothelial growth factor (VEGF), an important in vivo angiogenic cytokine molecule, by breast cancer epithelial cells. This is in addition to methyl selenium specific inhibitory effect on the expression of matrix metalloproteinases (MMP) by vascular endothelial cells (see Appendix 1). They effects these findings provide mechanistic insights into the cellular and biochemical processes that are potentially targeted by Se to regulate the angiogenic switch during mammary carcinogenesis. This article was featured as cover story for that issue.
- As reported in October, 2000 in *Biochemical and Biophysical Research Communications*, we have developed and validated the various assays for angiogenic attributes by extending investigations into silymarin which is an antioxidant polyphenolic compound extracted from milk thistle (See appendix 2). The results indicate that this compound possesses anti-angiogenic attributes in terms of inhibiting VEGF and MMP-2 expression and inhibiting in vitro capillary differentiation. They also showed mechanistic similarities and differences between this class of agents from methyl Se, as expected of the structural differences.
- As reported in April, 2001 in *Cancer Research*, we have invested in developing the expertise to study apoptosis execution and signaling, using the DU-145 prostate cancer cells as our model (Appendix 3). The results support methyl selenium specific induction of caspases for apoptosis execution. Furthermore, we have linked apoptosis induced by methylselenium to anoikis, a form of apoptosis that was originated when adherent cells are derived of matrix attachment. We are in the process of extending our investigation into vascular endothelial cells.
- We have written 1 book chapter on the subject of selenium in apoptosis and angiogenesis (Appendix 4).

Reportable outcomes:

Manuscripts

1. Jiang, C. Ganther H and Lu, JX. Monomethyl selenium-specific inhibition of MMP-2 and VEGF expression: Implications for angiogenic switch regulation. *Mol Carcinogenesis*. 29: 236-250, 2000. (cover story)
2. Jiang C. Agarwal, R and Lu, JX. Anti-angiogenic potential of a cancer chemopreventive flavonoid antioxidant, silymarin: inhibition of key attributes of vascular endothelial cells and

- angiogenic cytokine secretion by cancer epithelial cells. *Biochem Biophys Res Comm.* 276, 371-378, 2000.
- 3. Jiang C, Wang, Z, Ganther, H and Lu, JX. Caspases as key executors of methyl selenium induced apoptosis (anoikis) of DU-145 human prostate cancer cells. *Cancer Res.* 61: 3062-3070, 2001.
- 4. Lu, JX Apoptosis and angiogenesis in cancer prevention by selenium. Book chapter 11. *Nutrition and Cancer Prevention*, Proc. AICR Res. Conf. Pg131-145. Kluwer/ Plenum, New York. 2000.

Abstracts presented at national meetings

J Lu, C Jiang and H. Ganther Methylselenium specific inhibition of cancer epithelial expression of vascular endothelial growth factor. American Association for Cancer Research 92st Annual meeting, New Orleans, 2001 (Abstract. Poster presentation).

C. Jiang, Z. Wang, H. Ganther and J. Lu. Methylselenol-induced cancer cell anoikis is executed through a caspase-dependent mechanism. American Association for Cancer Research 92st Annual meeting, New Orleans, 2001 (Abstract. Poster presentation).

Symposium and seminar presentations by PI

May, 2001 "Selenium and cancer prevention: metabolite-specific effects on apoptosis, angiogenesis and cell cycle" Department of Pathology, University of Tennessee, Knoxville, TN.

May, 2001 "Selenium and cancer prevention: metabolite-specific effects on apoptosis, angiogenesis and cell cycle" Department of Nutrition. Oklahoma State University, Stillwater, OK.

April, 2001 "Selenium and cancer prevention: metabolite-specific effects on apoptosis and angiogenesis" Eppley Institute for Cancer Research, University of Nebraska, Omaha, NE.

April, 2001 "Selenium and cancer prevention: metabolite-specific effects on apoptosis and angiogenesis" Department of Animal Science, University of Connecticut. Storrs, CT.

March. 2001 "Selenium and cancer prevention: metabolite-specific effects on apoptosis and angiogenesis. Hormel Institute, University of Minnesota, Austin, MN.

Oct, 2000. "Selenium and selenium-enriched agricultural and food products for cancer prevention". US Expert Delegation to Jiangsu Provincial Conference on Agri/Biotechnology. Nanjing, China.

Oct , 2000. Seminar, Nanjing University of Chinese Medicine and Pharmacy. Nanjing, China "Selenium and cancer prevention"

Oct, 2000. Seminar, Nanjing Agriculture University, Nanjing, China. "Selenium and selenium enriched products for cancer prevention".

- Nov. 2000. Seminar, Chinese Academy of Agricultural Sciences, Beijing. "Cancer chemoprevention mechanisms of selenium".

Conclusions

The specific inhibitory effects of methylselenium on vascular endothelial cell expression of MMP-2 and cancer epithelial expression of VEGF and potent anti-mitogenic and apoptosis activities (through caspases) support and extend 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. For next year, we will pursue further studies of the biochemical and molecular mechanisms underlying the methyl Se specific activities on MMP-2 and VEGF expression and on endothelial proliferation and survival and the role of JNKs, ERK and PI3K signaling pathways. The results will offer greater insights into the target pathways for developing novel Se agents and biomarkers for their evaluation of breast cancer preventive activities in preclinical and clinical trials.

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1. Ip C, Ganther HE. Activity of methylated forms of selenium in cancer prevention. *Cancer Res.* 1990 Feb 15;50(4):1206-11.
2. Ip C, Hayes C, Budnick RM, Ganther HE Chemical form of selenium, critical metabolites, and cancer prevention. *Cancer Res.* 1991 Jan 15;51(2):595-600.
3. Ip, C. Lessons from basic research in selenium and cancer prevention. *J Nutr.* 1998. Nov;128(11):1845-54. Review.

Appendices:

1. Jiang, C. Ganther H and Lu, JX. Monomethyl selenium-specific inhibition of MMP-2 and VEGF expression: Implications for angiogenic switch regulation. *Mol Carcinogenesis.* 29: 236-250, 2000. (cover story)
2. Jiang C. Agarwal, R and Lu, JX. Anti-angiogenic potential of a cancer chemopreventive flavonoid antioxidant, silymarin: inhibition of key attributes of vascular endothelial cells and angiogenic cytokine secretion by cancer epithelial cells. *Biochem Biophys Res Comm.* 276, 371-378, 2000.
3. Jiang C. Wang, Z. Ganther, H and Lu, JX. Caspases as key executors of methyl selenium induced apoptosis (anoikis) of DU-145 human prostate cancer cells. *Cancer Res.* 61: 3062-3070, 2001.
4. Lu, JX Apoptosis and angiogenesis in cancer prevention by selenium. Book chapter 11. *Nutrition and Cancer Prevention*, Proc. AICR Res. Conf. Pg131-145. Kluwer/ Plenum, New York. 2000.

Monomethyl Selenium-Specific Inhibition of MMP-2 and VEGF Expression: Implications for Angiogenic Switch Regulation

Cheng Jiang,¹ Howard Ganther,² and Junxuan Lu^{1*}

¹AMC Cancer Research Center, Denver, Colorado

²University of Wisconsin, Madison, Wisconsin

Previous work suggested that antiangiogenic activity may be a novel mechanism contributing to the cancer chemopreventive activity of selenium (Se). Because methylselenol has been implicated as an *in vivo* active chemopreventive Se metabolite, experiments were conducted to test the hypothesis that this metabolite pool might inhibit the expression of matrix metalloproteinase-2 (MMP-2) by vascular endothelial cells and of vascular endothelial growth factor (VEGF) by cancer epithelial cells, two proteins critical for angiogenesis and its regulation. In human umbilical vein endothelial cells (HUVECs), zymographic analyses showed that short-term exposure to methylseleninic acid (MSeA) and methylselenocyanate (MSeCN), both immediate methylselenol precursors, decreased the MMP-2 gelatinolytic activity in a concentration-dependent manner. In contrast, Se forms that enter the hydrogen selenide pool lacked any inhibitory effect. The methyl Se inhibitory effect on MMP-2 was cell dependent because direct incubation with Se compounds in the test tube did not result in its inactivation. Immunoblot and enzyme-linked immunosorbent assay analyses showed that a decrease of the MMP-2 protein level largely accounted for the methyl Se-induced reduction of gelatinolytic activity. The effect of MSeA on MMP-2 expression occurred within 0.5 h of exposure and preceded MSeA-induced reduction of the phosphorylation level of mitogen-activated protein kinases (MAPKs) 1 and 2 (~3 h) and endothelial apoptosis (~25 h). In addition to these biochemical effects in monolayer culture, MSeA and MSeCN exposure decreased HUVEC viability and cell retraction in a three-dimensional context of capillary tubes formed on Matrigel, whereas comparable or higher concentrations of selenite failed to exert such effects. In human prostate cancer (DU145) and breast cancer (MCF-7 and MDA-MB-468) cell lines, exposure to MSeA but not to selenite led to a rapid and sustained decrease of cellular (lysate) and secreted (conditioned medium) VEGF protein levels irrespective of the serum level (serum-free medium vs. 10% fetal bovine serum) in which Se treatments were carried out. The concentration of MSeA required for suppressing VEGF expression was much lower than that needed for apoptosis induction. Taken together, the data support the hypothesis that the monomethyl Se pool is a proximal Se for inhibiting the expression of MMP-2 and VEGF and of angiogenesis. The data also indicate that the methyl Se-specific inhibitory effects on these proteins are rapid and primary actions, preceding or independent of inhibitory effects on mitogenic signaling at the level of MAPK1/2 and on cell growth and survival. *Mol. Carcinog.* 29:236–250, 2000. © 2000 Wiley-Liss, Inc.

Key words: selenium; methylselenol; matrix metalloproteinase-2; vascular endothelial growth factor; mitogen-activated protein kinase; angiogenesis

INTRODUCTION

The results of recent human prevention trials using selenium (Se) alone [1,2] or in combination with other agents [3] have demonstrated potential cancer chemopreventive utility for multiple organ sites. Such efficacy has supported by the potent chemopreventive activity of Se in most animal carcinogenesis models when its intake exceeds that required for meeting the nutritional requirement for normal physiologic functions [4,5]. Although several hypotheses have been advanced to account for the anticarcinogenic effects of Se [4,5], the underlying mechanisms remain to be elucidated. We recently reported data that were consistent with an

inhibitory effect of Se at chemopreventive intake levels on tumor angiogenesis [6]. Because angiogenesis is obligatory for early lesion growth and

*Correspondence to: AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214.

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Abbreviations: Se, selenium; MMP, matrix metalloproteinase; MSeA, methylseleninic acid; HUVEC, human umbilical vein endothelial cell; MSeCN, methylselenocyanate; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; ELISA, enzyme-linked immunosorbance assay; ATCC, American Type Culture Collection; FBS, fetal bovine serum; ECGS, endothelial cell growth supplement; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

progression [7-9] as well as metastasis [10], an antiangiogenic activity may be a novel mechanism contributing to the cancer chemopreventive activity of Se. Because methylselenol has been implicated as a critical Se metabolite pool for cancer chemopreventive activity [4,11,12], the present study was designed to test the hypothesis that this Se pool might exert specific inhibitory activities on angiogenic switch mechanisms.

Angiogenic switch in epithelial lesions is controlled through at least two principal cell compartments, i.e., the transformed epithelial cells that serve as a main source of angiogenic factors and the vascular endothelial cells that constitute the targets for the angiogenic signals [8,9]. With angiogenic stimulation, the vascular endothelial cells increase their expression and secretion of matrix metalloproteinases (MMPs) to break down the extracellular and tissue matrix, increase cell motility, and undergo cell division to provide the necessary number of cells for the growing vessels. The essential role of MMP-2 in angiogenesis has been well documented [13-16]. Consistent with a methyl Se-specific hypothesis for Se regulation of angiogenesis, we previously reported a potent inhibitory activity of methylseleninic acid (MSeA), a novel penultimate methylselenol precursor, on MMP-2 gelatinolytic activity (zymographic analyses) in human umbilical vein endothelial cells (HUVECs) that was absent for selenite at an exposure level that produced equivalent inhibitory effect on HUVEC growth and survival [6]. In the present study we extended that work by delineating the Se metabolite specificity for MMP-2 inhibition by comparing Se compounds known to enter the hydrogen selenide pool (sodium selenide and selenite) or methylselenol pool (MSeA and methylselenocyanate (MSeCN)). We characterized the temporal relation of methyl Se inhibition of MMP-2 expression with its effects on HUVEC mitogenic signaling and endothelial growth and survival. In addition, we contrasted the effects of methyl Se versus selenite on HUVEC cell viability and retraction in the three-dimensional context of capillary tubes formed on Matrigel, an extracellular matrix extract of EHS sarcoma.

A key for initiating and sustaining angiogenic responses is increased production of angiogenic stimulators [8,9]. A positive, primary angiogenic factor is vascular endothelial growth factor (VEGF) or vascular permeability factor [17,18]. Transformed epithelial cells are the major source of VEGF expression in many types of solid cancers [19-23]; however, recent data have suggested that stromal cells and even vascular endothelial cells may also express VEGF in the angiogenic microenvironment of tumors [24]. Previously, we documented a decrease of VEGF protein level in a sizable proportion of the chemically induced mammary carcino-

mas treated with Se in vivo [6], suggesting inhibition of VEGF expression as one possible mechanism for Se to regulate the angiogenic switch. In the present study, we investigated the Se metabolite specificity for this effect and report a rapid and sustained methyl Se-specific inhibitory effect on VEGF expression in prostate and breast cancer epithelial cells. We extended our work to a prostate cancer cell line for comparison with breast cancer cell lines because of the results of the trial by Clark et al. [1] in which the prostate appeared to be the most responsive organ site for Se cancer-preventive activity. In addition, a recent study has linked higher Se intake as indicated by the Se content in toe-nail clippings to lower prostate cancer risk in U.S. men [25].

Cell growth, function, and survival are regulated through multiple signaling pathways. Receptor tyrosine kinases as well as mitogen-activated protein kinase (MAPK) or extracellular regulated kinase pathways transduce signals initiated extracellularly by way of cascades of protein kinases to the nucleus [26,27]. The phosphorylated MAPK1 and 2 (phospho-MAPK1/2) are the active forms that translocate to the nucleus, where they phosphorylate protein substrates, leading to cell-type-specific responses including in many cells the activation of the cell-cycle machinery for mitogenesis. MAPK1/2 (44 and 42 kDa, respectively) and p38^{MAPK} have been shown to regulate the expression of some MMPs [28,29] and mediate VEGF-induced endothelial responses such as hyperpermeability and cell motility and proliferation [30-33], although little is known about their involvement in MMP-2 expression in vascular endothelial cells. Similarly, the MAPK cascade has been implicated in VEGF expression regulation by proangiogenic factors such as basic fibroblast growth factor [34], and nothing is known about its role, if any, in methyl Se inhibition of VEGF expression in cancer epithelial cells. Therefore, the phosphorylation state of MAPK1/2 was characterized in relation to the methyl Se-specific inhibitory effects on MMP-2 and VEGF expression in vascular endothelial and cancer epithelial cells, respectively.

MATERIALS AND METHODS

Chemicals and Reagents

Sodium selenite pentahydrate was purchased from J. T. Baker, Inc. (Phillipsburg, NJ). Sodium selenide was purchased from Alfa Products (Danvers, MA). MSeA (CH₃SeO₂H) and MSeCN (CH₃SeCN) were synthesized as described elsewhere [6,35]. Intracellularly, MSeCN and MSeA most likely react with reduced glutathione to generate methylselenol. A VEGF enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN). Human recombinant proMMP-

2 protein and MMP-2 ELISA kits were purchased from Oncogene Research Products (Cambridge, MA). Antibodies to MMP-2 for immunoblots were purchased from Lab Vision Corp. (Fremont, CA). Antibodies to MAPK1/2 and phospho-MAPK1/2 were purchased from New England Biolaboratories (Beverly, MA). Matrigel, a reconstituted extracellular matrix preparation of the EHS sarcoma, was purchased from Becton-Dickinson Labware (Bedford, MA).

Cell Lines

HUVECs were obtained from American Type Culture Collection (ATCC; Manassas, VA) and were propagated in F12K medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 µg/mL heparin (Sigma Chemical Co., St. Louis, MO), and 30 µg/mL bovine endothelial cell growth supplement (ECGS; Sigma Chemical Co.) as described previously [6]. DU145 prostate cancer cells and MDA-MB-468 breast cancer cells were kindly provided by Dr. Rajesh Agarwal, who originally obtained these cells from ATCC. MCF-7 breast cancer cells were obtained from ATCC. DU145 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 2 mM L-glutamine. MCF-7 and MDA-MB-468 breast cancer cells were cultured in Dulbecco's minimum essential medium supplemented with 10% FBS and 2 mM L-glutamine.

Zymogram Analysis for HUVEC MMP-2

Unless otherwise specified, HUVECs were seeded into six-well plates in complete medium (10% FBS, 2 mM L-glutamine, 100 µg/mL heparin, and 30 µg/mL ECGS) for 24–48 h to reach near confluence. The cells were washed three times with phosphate-buffered saline (PBS) to remove spent medium, refed serum-free medium supplemented with 100 µg ECGS/mL, and treated with the various forms of Se for 6 h (a time frame that did not result in visible cellular changes). For time-course experiments, aliquots were taken from the medium at different durations of exposure to MSeA. To evaluate the reversibility of MMP-2 inhibition, HUVECs were treated for 3 h with MSeA or PBS as described above, and the conditioned media were harvested. The cells were washed three times with PBS and were refed serum-free fresh medium supplemented with 100 µg ECGS/mL. Aliquots were taken from conditioned media at 1, 3, 6, and 12 h after MSeA withdrawal. Gelatinolytic activities were analyzed on substrate gels as described previously [6]. Each experiment was replicated at least once.

Western and ELISA Analyses for MMP-2 Protein Quantitation

HUVECs were seeded in T75 flasks in complete medium and grown until near confluence. After

spent medium was removed and cells were washed three times with PBS, the cells were treated with MSeA for different durations in serum-free medium supplemented with 100 µg ECGS/mL. Conditioned media were collected, aliquots were saved for zymographic analyses, and the remainder portion was concentrated with Centricon spin filters (30-kDa cutoff; Millipore Corp., Bedford, MA) for ~50-fold. The concentrated samples were used for MMP-2 quantitation by western blotting [6]. Recombinant human proMMP-2 (72 kDa) was loaded on the gels as a positive standard. In a separate experiment, conditioned media were concentrated (approximately fivefold) by evaporation in a Speedvac Concentrator and used for ELISA according to the manufacturer's instructions (Oncogene Research Products).

Capillary Tube Formation on Matrigel (In Vitro Angiogenesis)

Kubota et al. [36] showed that, when seeded on Matrigel, an extracellular matrix extract from the EHS sarcoma, vascular endothelial cells undergo rapid differentiation into capillarylike structures. This affords a simple assay for assessing the impact of agents such as Se compounds on capillary differentiation in vitro and cell viability and function in a simulated histogenic three-dimensional context. HUVECs were harvested by trypsinization, and ~40 000 cells per well were seeded onto 24-well plates coated with 300 µL of Matrigel (solidified at 37°C for 1 h before the addition of Se stock solutions). Before cell seeding, 0.5 mL of medium was added to each well, and Se stock solution was added at two times the desired concentration. HUVECs were added in 0.5 mL of medium per well. Tube formation was observed periodically over time under a phase-contrast microscope and photographed with a Polaroid camera. At 72 h after seeding, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [37] was added to test the metabolic viability of Se-exposed HUVECs.

ELISA Analyses of VEGF Expression in Cancer Epithelial Cells

In short-term dose-response experiments, DU145 prostate cancer cells and MCF-7 (estrogen dependent) and MDA-MB-468 (estrogen independent) breast cancer cells were seeded in T25 flasks in complete medium until near confluence. The spent medium was removed, and flasks (cells) were washed three times with PBS. Cells were treated in either serum-rich complete medium (10% FBS) or serum-free medium with increasing concentrations of MSeA or selenite for up to 6 h. VEGF content in conditioned media and cell lysates (prepared with calibrator diluent buffer RD5K provided with the ELISA kit) was analyzed with the use of an ELISA kit according to the manufacturer's instructions (R&D

Systems). In time-course experiments, near-confluent DU145 cells were treated with PBS, selenite, or MSeA, and aliquots of the culture media were taken at hourly intervals for VEGF ELISA. Samples were measured in duplicate or triplicate, and experiments were replicated at least once.

In long-term experiments to examine whether the methyl Se-inhibitory effect on VEGF expression was transient, MSeA doses that led to growth arrest without significant induction of apoptosis were applied daily to DU145 prostate cells, starting in log-phase growing (~40–50% confluence) cells, in fresh complete medium. DNA content in cell lysate was measured by Hoechst dye binding to approximate cell number. VEGF content in cell lysate was measured by ELISA and normalized to DNA content to estimate VEGF expression on a per-cell basis.

Cell Growth and Apoptosis Evaluation

Cells were seeded in six-well plates until 50% confluence and were treated in fresh serum-rich (10% FBS) medium with increasing concentrations of MSeA or selenite for 48 h or as specified. Adherent cells were fixed in 1% glutaldehyde and stained with crystal violet for cell enumeration as described previously [6]. To verify that DU145 cells underwent apoptotic cell death with Se treatment, cells were treated in T75 flasks with MSeA or selenite for 24 h, and DNA from both adherent and detached cells was extracted and analyzed as previously described [38]. To standardize Se exposure across different cell culture vessels, 0.2 mL of medium per square centimeter of vessel surface (e.g., 15 mL for a T75 flask, 5 mL for a T25 flask, and 2 mL for each well of a six-well plate) was used.

Immunoblot Analyses of Phospho-MAPK1/2 and MAPK1/2

To determine whether the MAPK signaling cascade was associated with the inhibition of MMP-2 and VEGF expression by methyl Se exposure, cell lysates were prepared in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 50 mM sodium fluoride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM dithiothreitol, 5 mM sodium orthovanadate; 1 mM phenylmethylsulfonyl fluoride, and 38 µg/mL aprotinin were added fresh). Supernatants were recovered after centrifugation (14 000g for 20 min, 4°C), and the protein content was quantified by the Bradford dye-binding assay (Bio-Rad Laboratories, Richmond, CA). Forty micrograms of total protein was size separated by electrophoresis on 15% sodium dodecyl sulfate-polyacrylamide gels. The proteins were electroblotted onto nitrocellulose membranes and probed for phospho-MAPK1/2 (pp44/pp42) and MAPK1/2 (p44/p42).

RESULTS

Part I: HUVEC MMP-2 Expression and In Vitro Capillary Tube Formation

Exposure to methylselenol precursors led to decreased endothelial MMP-2 expression

Treatment of HUVECs for 6 h with MSeA led to a concentration-dependent reduction of the secreted 72-kDa MMP-2 gelatinolytic activity in the conditioned medium (Figure 1A) and in cell lysate, as previously reported [6]. The inhibitory efficacy was remarkable, with an IC_{50} of ~2 µM, which is within the upper range of plasma Se concentration in most U.S. residents [1]. Similarly, treatment with MSeCN resulted in a concentration-dependent decrease of MMP-2, and the inhibitory efficacy was comparable to that of MSeA (Figure 1A). In contrast, treatment with hydrogen selenide precursors (up to 20 µM sodium selenite or 50 µM sodium selenide) did not significantly decrease MMP-2 in the conditioned medium (Figure 1A).

Incubation of HUVEC-conditioned medium (containing secreted MMP-2) with all four Se forms in the test tube for 6 h at 37°C did not decrease the gelatinolytic activity (Figure 1B), indicating that MSeA or MSeCN per se did not react directly with MMP-2 protein to inactivate its activity. The inhibitory action of these methylselenol precursor compounds was therefore dependent on cellular metabolism.

Reduction of MMP-2 protein level largely accounted for methyl Se inhibition of MMP-2

Western blot analyses of the MMP-2 protein level in conditioned medium of HUVECs treated with MSeA indicated that a reduction in the MMP-2 protein level closely paralleled the observed loss of MMP-2 gelatinolytic activity (Figure 1C), whereas selenite treatment had minimal effect on both the gelatinolytic activity and MMP-2 protein level (Figure 1C). Consistent with the immunoblot results, ELISA quantitation of MMP-2 in the conditioned medium in a separate experiment indicated that 5 µM MSeA treatment for 8 h decreased the MMP-2 level from 1.81 ± 0.24 ng/mL to 0.38 ± 0.02 ng/mL, a reduction of 79%.

Methyl Se inhibition of MMP-2 expression was rapid and sustained

In time-course experiments, the inhibitory effect of MSeA on MMP-2 showed a lag time of 10 min, and by 30 min, ~50% reduction was detected (Figures 2A and 3C). In exposure-and-withdrawal experiments (Figure 2B), the MMP-2 inhibitory action of a 3-h MSeA exposure persisted for at least 12 h because the linear slope of the MMP-2 versus the time plot of the MSeA-exposed cells indicated no exponential recovery once MSeA was withdrawn.

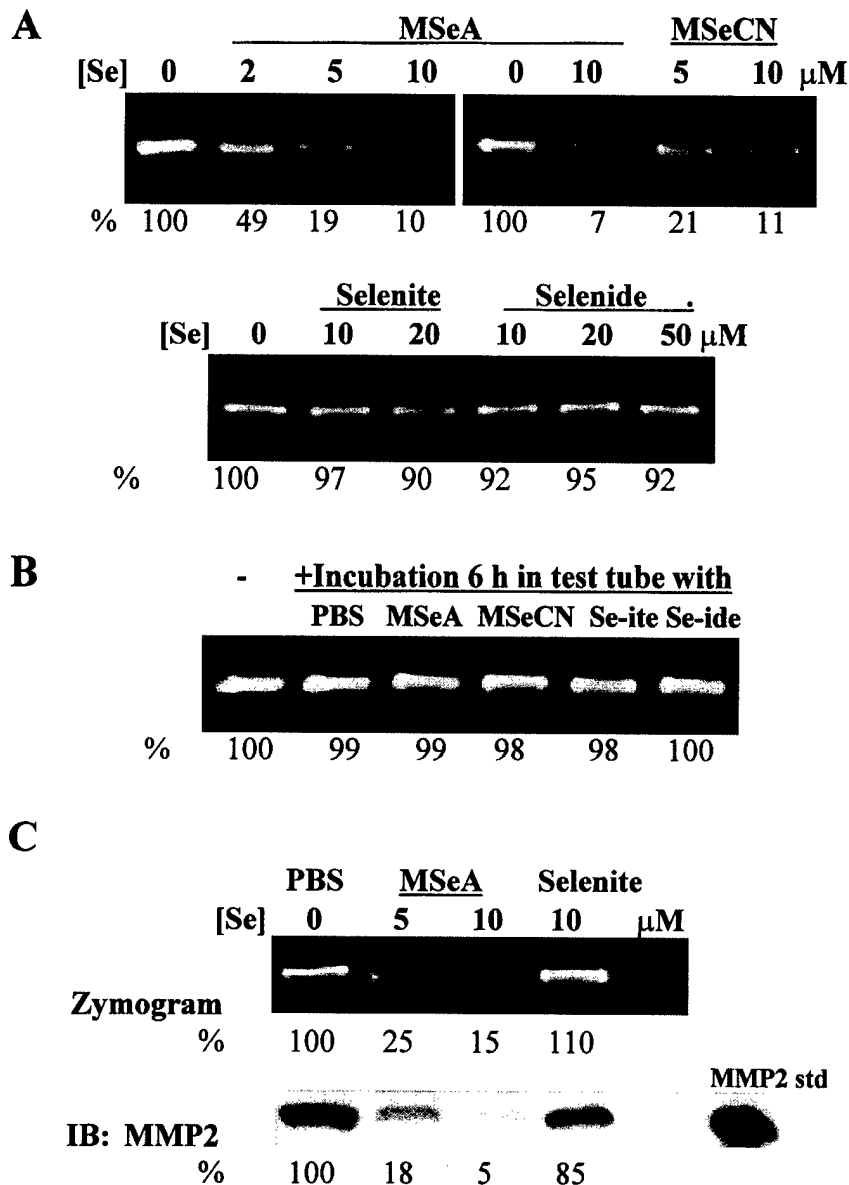


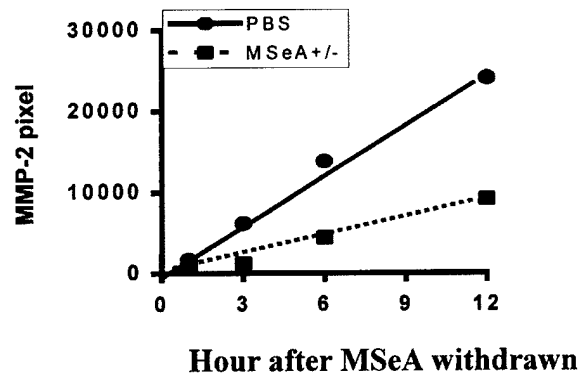
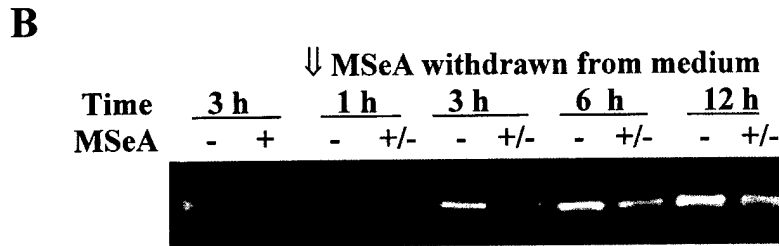
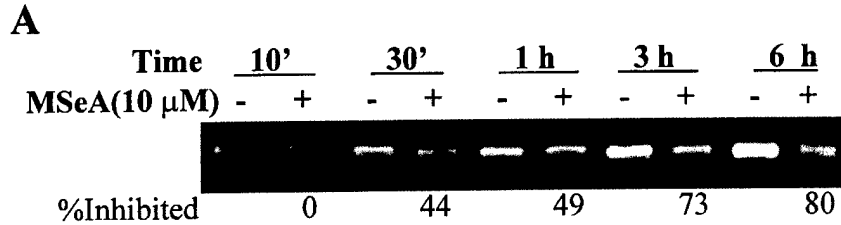
Figure 1. (A) The inhibitory specificity of Se forms on HUVEC MMP-2. Representative zymographic analyses of MMP-2 in conditioned medium of HUVECs treated for 6 h (in separate experiments) with methylseleninic acid (MSeA), methylselenocyanate (MSeCN), sodium selenite, or sodium selenide in serum-free medium supplemented with 100 μg/mL ECGS. Relative pixel density as a percentage of control cells is presented below each lane. (B) Lack of MMP-2 inactivation by direct incubation of HUVEC conditioned medium with Se compounds in test tubes at 37°C for 6 h. Each Se was added to

10 μM. Se-ite and Se-ide denote sodium selenite and sodium selenide, respectively. (C) Immunoblot analyses of MMP-2 protein in conditioned medium. HUVECs (in T75 flasks) were treated with MSeA or selenite for 3 h in the presence of 100 μg/mL ECGS. Aliquots of the conditioned medium were analyzed by zymography for gelatinolytic activity. The bulk of the conditioned media was concentrated ~50-fold using Centricon spin filters (Millipore Corp.) and analyzed by western blotting, with recombinant 72-kD proMMP-2 protein (10 ng) as the standard.

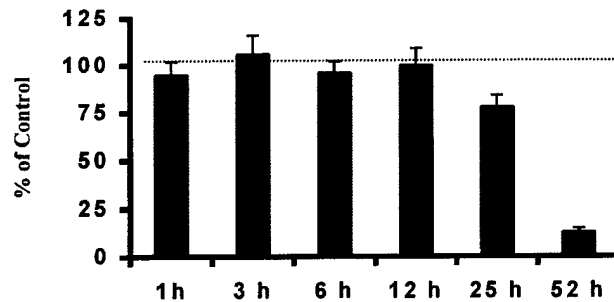
In similar exposure-and-withdrawal experiments, MSeA treatment for 12 h or shorter duration did not decrease the number of HUVECs surviving to 52 h, in contrast to the fast action of MSeA on MMP-2 (Figure 2C). It took 25 h or longer of continued exposure to MSeA to result in cell number reduction (Figure 2C), predominantly through induction of apoptosis, as previously reported [6].

Methyl Se inhibition of MMP-2 occurred irrespective of ECGS stimulation and preceded phospho-MAPK1/2 reduction

Two known responses of vascular endothelial cells with angiogenic stimulation are increased expression of MMP-2 and increased mitogenic signaling leading to cell proliferation. As expected, ECGS supplementation for 6 h to ECGS-starved (~48 h)



C Cell number at 52 hours



Duration of MSeA treatment before withdrawal

Figure 2. (A) Time course of MMP-2 inhibition by MSeA (10 μ M) exposure of HUVECs. Aliquots of medium taken at the different time points were analyzed by zymography. Percentages of inhibition relative to the PBS-treated control at each time point are presented below the treated lanes. (B) Reversibility of MSeA-induced MMP-2 inhibition. HUVECs were treated for 3 h with PBS or MSeA (10 μ M), and the cells were washed three times with PBS and re-fed serum-free medium supplemented with 100 μ g/mL ECGS. Aliquots of medium taken at the different time points were analyzed by zymography. The MMP-2 pixel density was plotted against time to

evaluate the kinetics of MMP-2 recovery in cells exposed once to MSeA. A linear slope of the curve indicated a lasting inhibitory effect after MSeA withdrawal. (C) Effect of duration of MSeA (5 μ M) exposure on number of HUVECs surviving in MSeA-free medium to 52 h. HUVECs were treated with either PBS or MSeA for the time indicated, the medium was removed, cells were washed three times with PBS, and fresh medium was fed until cells were fixed at 52 h. The bar graph represents the number of MSeA-treated cells as a percentage of the respective PBS treated-controls. Error bars indicate SEM of six random fields counted.

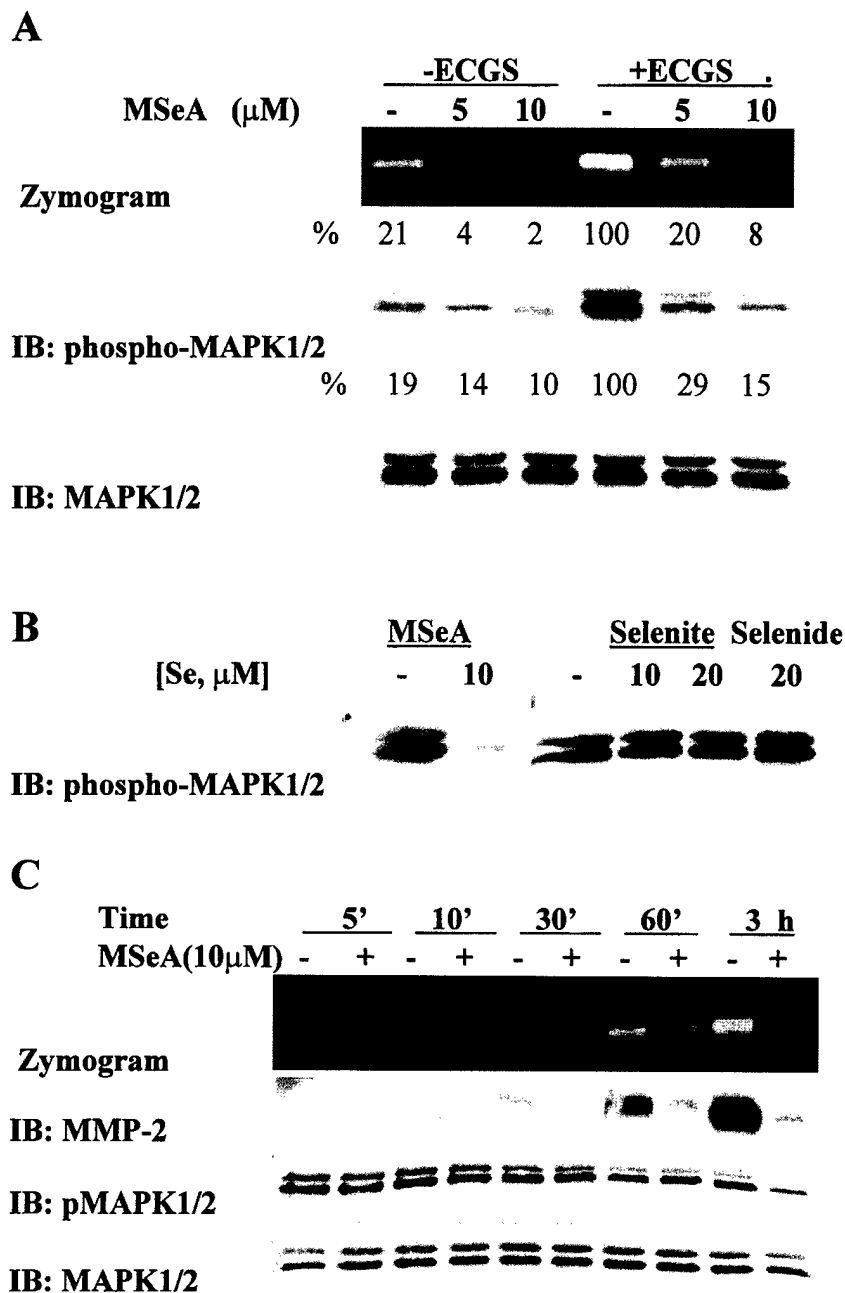


Figure 3. (A) Effects of MSeA exposure (6 h) on MMP-2 expression and MAPK1/2 phosphorylation status in synchronized HUVECs. HUVECs (T75 flasks) were deprived (synchronized) of ECGS for ~48 h and, after PBS washing, were treated with MSeA for 6 h in the presence or absence of 100 μ g/mL ECGS. Conditioned medium was used for zymography. Forty micrograms of cell lysate was

immunoblotted for phospho-MAPK1/2 and total MAPK1/2. (B) Lack of inhibitory effect of selenite and selenide exposure for 6 h on HUVEC phospho-MAPK1/2 level. (C) Temporal relation between MSeA inhibition of MMP-2 expression (zymogram and immunoblot) and phospho-MAPK1/2 inactivation in synchronized HUVECs (T75 flasks) treated in the presence of 100 μ g/mL ECGS.

HUVECs increased secreted MMP-2 by 4.8-fold (Figure 3A). Western blot analyses of HUVEC lysates showed that a 5.3-fold increase of MAPK1/2 phosphorylation was associated with the ECGS-stimulated MMP-2 expression. Exposure to MSeA decreased MMP-2 in a concentration-dependent manner, not only in ECGS-stimulated cells but also in ECGS-starved cells (basal expression). Further,

exposure to MSeA led to a reduction of phospho-MAPK1/2 level in a concentration-dependent manner, and this inhibition closely paralleled that for MMP-2, especially in the ECGS-stimulated cells. The expression level of total MAPK1/2 was not affected by treatment with either ECGS or MSeA. In contrast to MSeA, exposure to selenite and selenide, which lacked inhibitory effect on MMP-2 expression, did

not decrease the level of MAPK1/2 phosphorylation (Figure 3B).

Given the close parallel changes observed between MMP-2 expression and phospho-MAPK1/2 levels, a time-course experiment examined the temporal relation between these MSeA-induced events in ECGS-stimulated HUVECs that had been deprived of ECGS for ~48 h (Figure 3C). Whereas MMP-2 expression was significantly lowered at 30 min, a significant reduction of phospho-MAPK1/2 levels occurred at 3 h. These results indicated that the effect of methyl Se on MMP-2 expression took place before its inhibition of phospho-MAPK1/2.

Methylselenol precursors inhibited HUVEC capillary retraction and survival on Matrigel

When seeded on Matrigel, HUVECs underwent rapid reorganization (visible within 1–2 h) and subsequently formed capillarylike structures (in vitro differentiation). When added simultaneously with cell seeding, none of the Se compounds at the concentration ranges tested negatively affected the initial tube formation, i.e., differentiation, program within the first 28 h (Figure 4A–D). As time progressed, the capillary structures in the PBS control wells started to retract into spheroids (Figure 4E and I) that were metabolically viable at 72 h as measured by their ability to metabolize MTT (Figure 4M). Exposure to 5 μ M MSeA (Figure 4F and J) or MSeCN (Figure 4G and K) resulted in a blockage of the capillary tubes from retracting into such spheroids. By 72 h of exposure to either MSeA or MSeCN, only a minor proportion of the cells in the protracted capillaries were still metabolically viable, as judged by their ability to metabolize MTT (Figure 4N and O). Most cells displayed morphologic apoptotic characteristics. In contrast to these methylselenol precursors, selenite at concentrations as high as 10 μ M did not inhibit tube retraction or cell viability (Figure 4H, L, and P). These results indicated that methyl Se was more effective than selenite in a three-dimensional capillary histogenic context at inhibiting HUVEC cell viability and retraction.

Part II: VEGF Expression in Cancer Epithelial Cells

MSeA rapidly decreased VEGF expression in cancer epithelial cells

In short-term experiments, exposure of near-confluent DU145 prostate cancer cells to increasing concentrations of MSeA or selenite in serum-rich medium (10% FBS) for 6 h led to very distinct effects on VEGF expression, as shown in Figure 5A and B. MSeA treatment decreased both cellular (cellular lysate, Figure 5A) and secreted (conditioned medium, Figure 5B) VEGF levels in an Se concentration-dependent manner, with $IC_{50\%}$ ~2 μ M. The inhibitory effect of MSeA on VEGF expression was also

observed in serum-free medium with almost identical patterns (data not shown). In contrast, exposure to selenite in the same dose range did not decrease VEGF expression; in fact, a slight increase (5–10%) in VEGF expression was often observed in selenite-treated cells. The methyl Se inhibitory action was exerted very rapidly in that exposure to 5 μ M MSeA in serum-rich medium decreased the secreted VEGF level by ~50% within 2 h (Figure 5C).

Lower MSeA concentration was required for inhibiting VEGF expression than for inducing apoptosis

Exposure of DU145 prostate cancer cells to either sodium selenite or MSeA above some threshold levels for longer durations led to apoptosis, as indicated by DNA nucleosomal fragmentation (Figure 5D), and decreased the number of cells remaining adherent after 48 h (Figure 5E). MSeA was more efficacious than selenite at inhibiting cell growth and survival. The inhibitory effect of MSeA on VEGF expression was observed at a concentration (2 μ M) that was twofold lower than that needed to induce significant apoptosis (4 μ M and above). Although selenite exposure at 5 μ M achieved a similar extent of DNA fragmentation and cell number reduction as MSeA at 5 μ M, the difference in the potency of the respective Se to inhibit VEGF expression within a few hours of exposure was self-evident. These results indicated that the inhibitory action of MSeA was not a consequence of cell "poisoning".

Sustained inhibition of VEGF expression required continued presence of MSeA

The possibility that the observed decrease of VEGF expression by MSeA was a transient effect was assessed through daily exposure of DU145 cells (starting when cells were in log-phase growth, ~40–50% confluent) to a low dose (3 μ M) that predominantly led to growth arrest (Figure 6). Consistent with the results in confluent cells shown above, greater than 50% decrease of VEGF expression was observed at 6 h of exposure and the effect was sustained throughout the exposure period of 72 h (Figure 6A). The daily exposure to MSeA led to detectable growth inhibition by 48 h as assessed through DNA measurement (Figure 6B). When expressed on a per-cell basis, i.e., normalized to DNA content, this level of MSeA exposure led to a sustained ~50% decrease of VEGF expression level throughout the exposure duration (Figure 6C). Selenite at the same dose level did not affect cell growth (Figure 6B) or VEGF expression (Figure 6A and C). In a separate experiment, withdrawal of MSeA (3 μ M) after 96 h of exposure led to the rebound of VEGF expression (Figure 6D). These results indicate that the mechanism(s) suppressing VEGF expression was reversible and that continued presence of MSeA was necessary to inhibit VEGF expression.

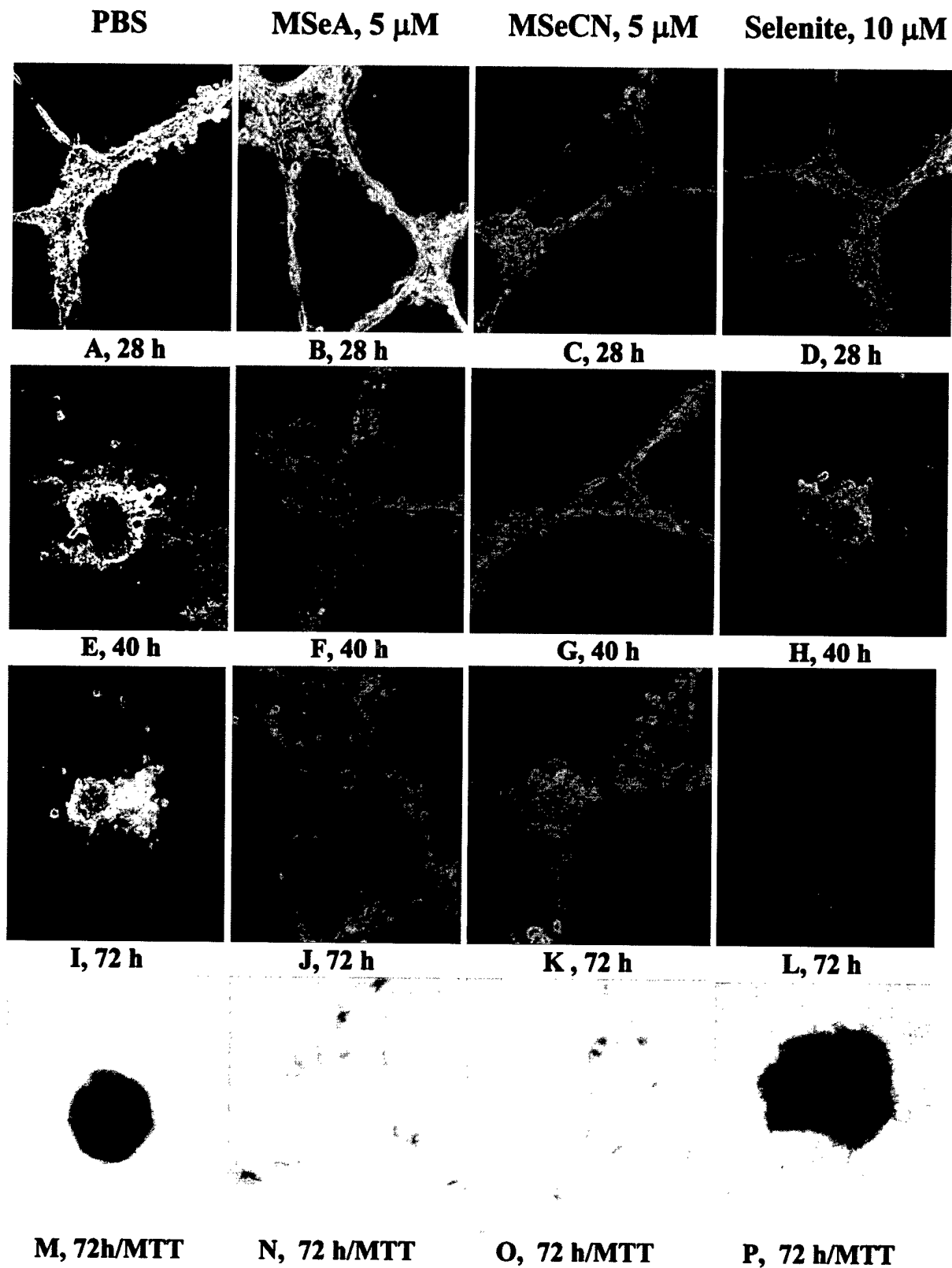


Figure 4. Effects of Se forms on HUVEC retraction and viability in an in vitro capillary differentiation assay. HUVECs ($\sim 40,000$ /well) were seeded into Matrigel-coated, 24-well plates that had been provided with $2 \times$ concentrations of the indicated Se forms, and phase-contrast photomicrographs (magnification, $100 \times$) were

taken at 28 h (A–D), 40 h (E–H), and 72 h (I–L) of treatment. At 72 h (M–P), MTT was added to each well and incubated for an additional 5 h, and brightfield photomicrographs were taken (magnification, $200 \times$). Black products indicate mitochondrial conversion of MTT by metabolically viable cells.

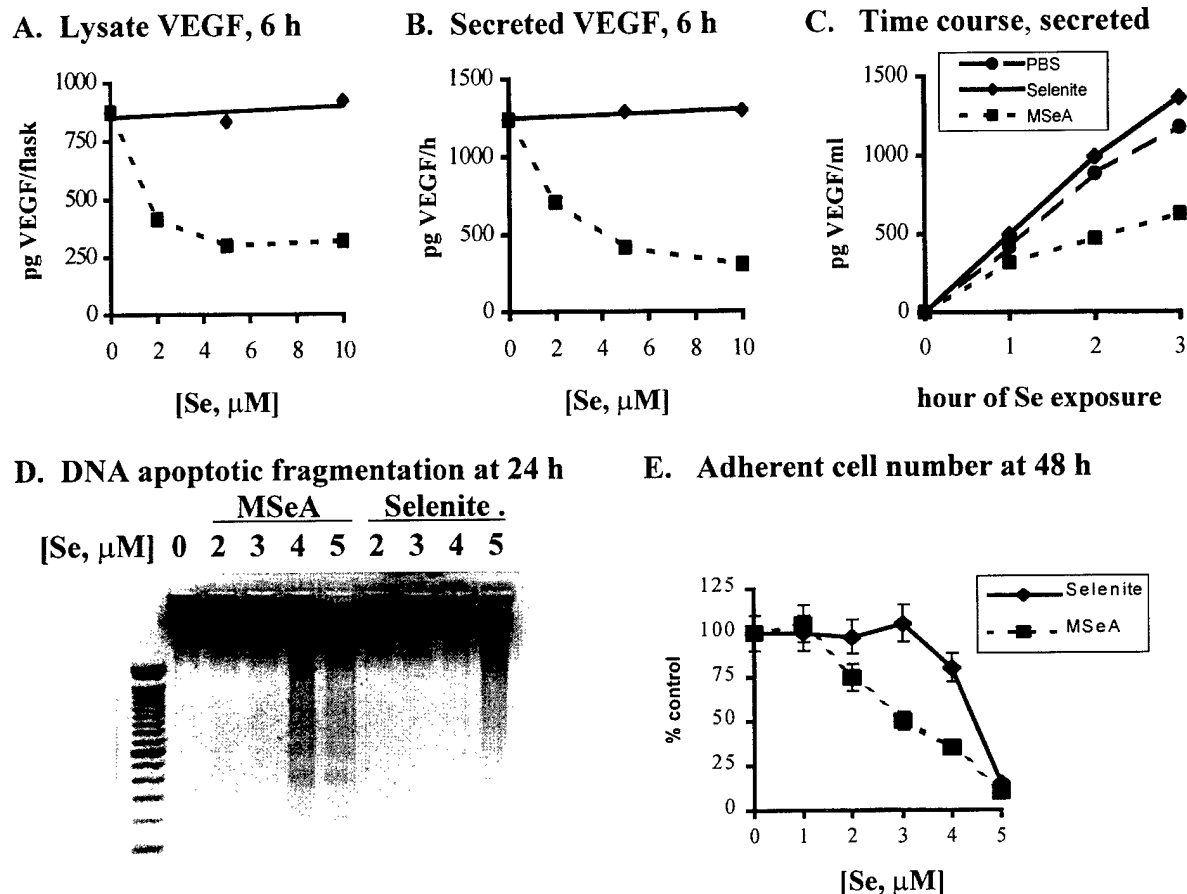


Figure 5. Concentration-dependent effects of selenite (diamonds) and MSeA (squares) on VEGF protein levels in DU145 cell lysate (A) and conditioned medium (B) after exposure for 6 h in serum rich medium. (C) Acute time course of effects of exposure to Se as sodium selenite (10 μM , diamonds) or MSeA (5 μM , squares) in 10% FBS medium on VEGF secretion by DU145 prostate cancer cells. In these short-term exposure experiments, near-confluent DU145 cells were treated with Se in 10% FBS medium or serum-free medium

(identical patterns of responses were observed, data not shown). VEGF was assayed in triplicates by ELISA (R&D Systems, Minneapolis, MN). (D) DNA nucleosomal fragmentation assay after 24 h of exposure to MSeA or selenite (adherent and detached cells were combined for DNA extraction). (E) Effect of exposure to increasing concentrations of selenite or MSeA in 10% FBS medium for 48 h on the number of adherent DU145 cells in six-well plates. Error bars indicate SEM of six random fields counted.

MseA inhibition of VEGF expression was also observed in breast cancer cells and independent of phospho-MAPK1/2

In support of the generality of this inhibitory effect of methyl Se on VEGF expression, two human breast cancer cell lines (MCF-7 and MDA-MB-468) tested thus far showed the same Se metabolite specificity of inhibition irrespective of the serum levels in the treatment medium (Figure 7A and B). It appeared that a greater concentration of MSeA was required to induce a similar extent of inhibition on VEGF expression in the breast cancer cell lines than in the DU145 prostate cancer cell line.

In contrast to vascular endothelial cells in which MSeA exposure led to a decreased level of phospho-MAPK1/2, treatment of DU145 prostate or MDA-MB-468 breast cancer cells with MSeA did not decrease the levels of phospho-MAPK1/2 within the time frame of suppression of VEGF expression (Figure 7C), suggesting a mechanism of inhibi-

tion of VEGF expression independent of MAPK1/2 activity.

DISCUSSION

Although mechanisms underlying the cancer chemopreventive activity of Se are not fully understood, animal and cell culture models have yielded much insight. Of particular significance, the work of Ip and colleagues [4,11,12] has implicated methylselenol as the active *in vivo* Se metabolite pool for anticancer activity. These studies indicated that the mammary cancer-preventive efficacy of a given Se compound appears to depend on the rate of its metabolic conversion to the methylselenol pool. Subsequent studies by us and others had shown that the methyl Se pool induces numerous cellular, biochemical, and gene expression responses that are distinct from those induced by Se forms that entered the hydrogen selenide pool [38-42]. For example, MSeCN and Se-methylselenocysteine,

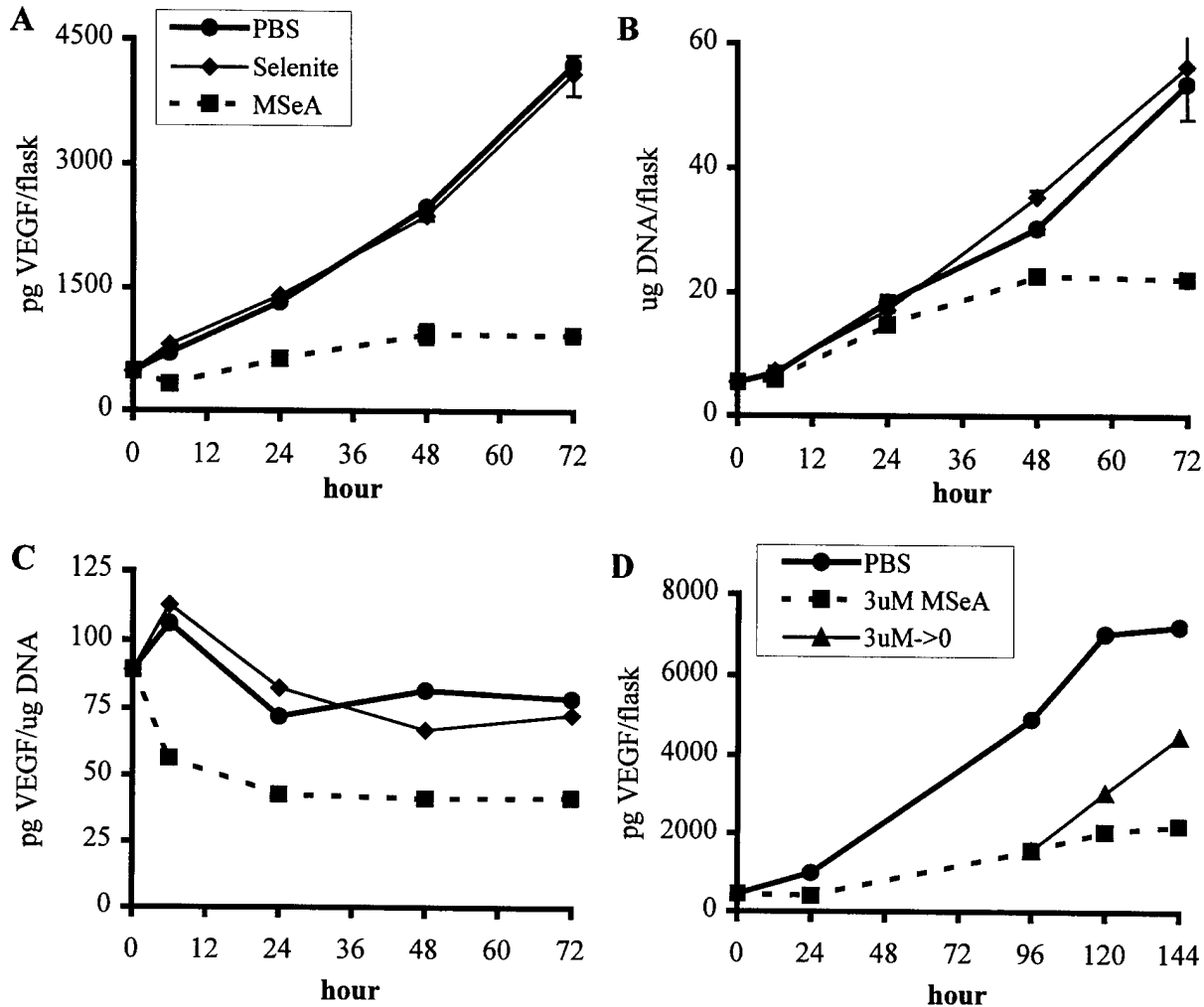


Figure 6. (A–C) Effects of daily exposure to low-dose MSeA (3 μ M, squares) or selenite (3 μ M, diamonds) on cellular VEGF content (A, total; C, normalized to DNA) and DNA content (B) of DU145 cells (two T25 flasks per datum point, each flask assayed in duplicate). Selenium exposure was initiated when cells were 40–50% confluent (log phase). Every 24 h, spent medium was removed and fresh

complete medium (10% FBS) with sodium selenite or MSeA was fed. (D) Reversibility of effects of low-dose exposure to MSeA on VEGF expression in DU145 cells. After 96 h of exposure to 3 μ M MSeA, one group of flasks remained on MSeA treatment, and the other group was washed with fresh medium once and then fed fresh complete medium for 24 and 48 h.

another methylselenol proximal precursor present in Se-enriched garlic and other seleniferous plants, induce exclusively apoptosis of cancerous mammary epithelial cells without induction of DNA single-strand breaks [40–42]. In contrast, sodium selenite and sodium selenide rapidly (within hours of Se exposure) induce DNA single-strand breaks (i.e., genotoxic) and lead to subsequent cell death by a composite of acute lysis and apoptosis [38–40]. These inorganic Se compounds and the methylselenol precursors also exert a differential anti-proliferative effect, as assessed by [3 H]thymidine incorporation into DNA, and arrest the cells at different stages of the cell cycle [40–42]. More recently, specific inhibitory effects on cyclin-dependent kinases [43] and protein kinase C [44] have been attributed to the methylselenol pool. Together these findings indicate the presence of at least two

different pools of Se metabolites that induce distinct types of biochemical and cellular responses. Key features of these differences are schematically illustrated in Figure 8. It is noteworthy that only a single methylation reaction separates the two pools of Se metabolites.

We recently reported data supporting an anti-angiogenic activity of Se at chemopreventive intake levels as a novel mechanism for cancer chemoprevention [6]. The present study extended that work and provided *in vitro* evidence of methyl Se-specific inhibition of the expression of two proteins critical for angiogenesis: MMP-2 by vascular endothelial cells and VEGF by cancer epithelial cells. The data support a rapid, primary, and sustained inhibitory action of the methyl Se pool on these proteins. Specifically, first with respect to MMP-2 expression in HUVECs, exposure to either MSeA or MSeCN led

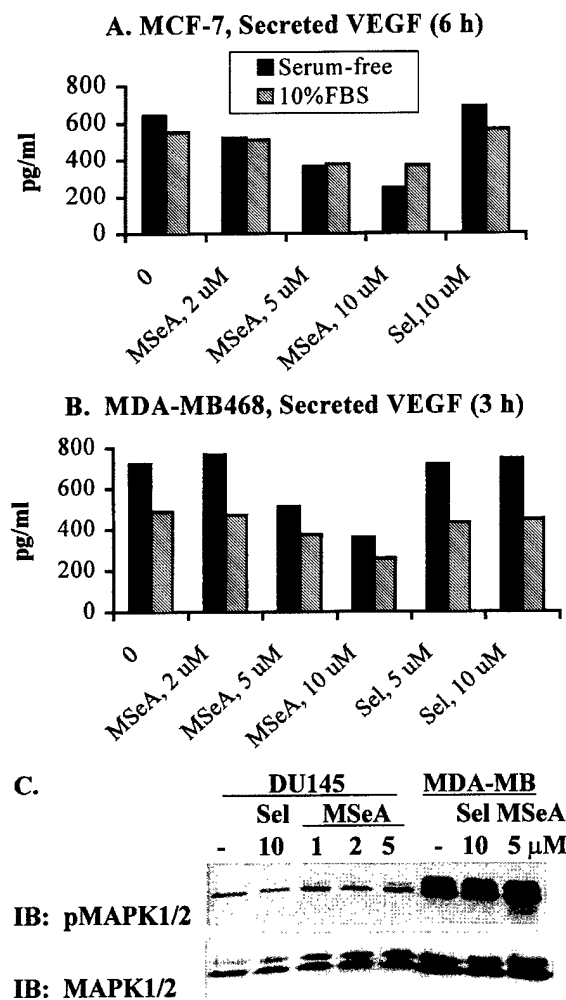


Figure 7. Effects of MSeA or sodium selenite on VEGF protein secreted by MCF-7 (A, 6-h treatment) and MDA-MB-468 breast cancer cells (B, 3-h treatment). Serum levels in the treatment medium were as indicated in panel A. (C) Lack of inhibitory effect by MSeA and selenite exposure (3 h) on phospho-MAPK1/2 levels in DU145 and MDA-MB-468 cancer cell lines.

to an Se concentration-dependent reduction of MMP-2 gelatinolytic activity (Figure 1A). Quantitation of the MMP-2 protein using western blot and ELISA analyses showed that a reduction of the protein level largely accounted for the loss of gelatinolytic activity (Figures 1C and 3C). The methyl Se-specific inhibition occurred rapidly (~0.5 h; Figures 2A and 3C) and appeared to persist well after the Se source was withdrawn (Figure 2B). The onset of inhibitory action on HUVEC MMP-2 level far preceded that of growth arrest and apoptosis responses, which required 25 h or longer exposure to MSeA (Figure 2C). Because neither MSeA nor MSeCN reacted directly in the test tube with MMP-2 protein to lead to its inactivation (Figure 1B), the observed inhibitory effect on MMP-2 expression must be a cell-dependent process or processes. In contrast to these methylselenol

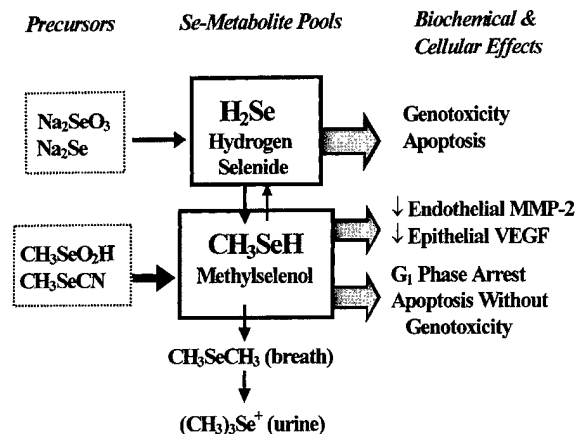


Figure 8. Schematic relation of selenium precursors (dotted rectangles) that enter two metabolite pools that induce distinct biochemical and cellular effects. The genotoxicity (or lack of) and apoptogenic effects were based on studies with mammary cancer epithelial cells and leukemia cells [38–42]. The present study provides data supporting methylselenol-specific inhibitory effects on MMP-2 expression by vascular endothelial cells and VEGF expression by cancer epithelial cells.

immediate precursors, Se forms that enter the hydrogen selenide pool, at least during the time frame of the experiments performed, did not significantly decrease MMP-2 expression. The data, therefore, were consistent with the methylselenol precursors or their common product methylselenol triggering a cellular process or mechanism leading to the reduction of MMP-2 protein level (Figure 8). Technologic advances in quantitating the cellular hydrogen selenide and methylselenol levels should provide more direct support for this hypothesis.

As far as mechanisms are concerned, it will be important to determine whether an inhibition of MMP-2 gene transcription or an increase of MMP-2 protein turnover account for the methyl Se-specific reduction of MMP-2 protein level. Although an inhibition of gene transcription and mRNA translation is possible, the very fast inhibitory action of methyl Se on MMP-2 (~30 min) suggests that a mechanism involving the rapid degradation of cellular MMP-2 protein may also be triggered. It will also be important to determine whether MMP-2 expression in capillary microvascular endothelial cells, the likely targets of angiogenic stimulation, is inhibited by methyl Se with the same specificity and efficacy as in the vein endothelial cells (HUVECs) employed in the current study.

It is noteworthy that exposure to methyl Se induced phospho-MAPK1/2 inactivation (dephosphorylation) in the vascular endothelial cells, an effect that was lacking by either selenite or selenide exposure (Figure 3A and B). To our knowledge, this is the first time an Se metabolite-specific inhibitory effect on MAPK1/2 signaling has been described. However, the onset of the reduction of phospho-

MAPK1/2 level was preceded by the reduction of MMP-2 expression (Figure 3C). Such a temporal relation precluded the methyl Se inhibition of phospho-MAPK1/2 signaling as a mediating event in its acute inhibition of MMP-2 expression.

In addition to the methyl Se-specific inhibitory effects observed in monolayer culture on HUVEC MMP-2 expression and phospho-MAPK1/2, we observed profound inhibitory effects of MSeA and MSeCN on HUVEC retraction and viability in the three-dimensional context of capillary tubes formed on Matrigel (Figure 4). Such effects were methyl Se specific as they were not observed with selenite (Figure 4) or selenide (data not shown) at higher doses. These results suggest that methyl Se was likely more efficacious than selenite or selenide at inhibiting vascular endothelial cell viability and cell motility (for retraction) in a capillary histogenic environment relevant for angiogenic switch regulation. Future work will determine whether methyl Se inhibition of the MAPK signaling cascade and MMP-2 expression contributes to the observed effects on endothelial cell viability and motility in such a three-dimensional context.

With regard to VEGF expression in cancer epithelial cells, we observed a methyl Se-specific inhibition on VEGF protein level in the prostate cancer DU145 cell line and two breast cancer cell lines tested thus far. The inhibitory action on VEGF expression by MSeA was rapid (within 1–2 h; Figure 5C) irrespective of the serum level in which the cells were treated and independent of phosphorylation status of MAPK1/2 in the cancer cells (Figure 7). In addition, the inhibitory effect was elicited by exposure concentrations that did not negatively affect cell viability (Figures 5 and 6). In an attempt to simulate chemopreventive application of Se in a chronic, low-dose exposure context, we observed that daily exposure to MSeA exerted a sustained suppression of VEGF expression without evidence of developing resistance (Figure 6C). Further, withdrawal of MSeA exposure led to the de-repression of VEGF expression (Figure 6D), indicating a reversible mechanism of inhibition of VEGF expression by MSeA under the low-dose exposure context. This observation suggests that continued presence of methyl Se is necessary to inhibit VEGF expression.

VEGF plays a crucial role in vasculogenesis and angiogenesis in normal physiologic and pathologic states as indicated by germline knockout experiments in which a loss of even one *VEGF* allele leads to embryonic lethality in heterozygotes, and homozygous mutant embryonic stem cells are incapable of forming tumor [45,46]. Whereas overexpression of VEGF is linked to increased angiogenesis and more aggressive tumor behavior [47,48], antiangiogenic interventions, especially those based on VEGF antibodies or interference of signal transduction through its receptors [49–53], have been shown to

result in the inhibition of tumor growth and induction of endothelial apoptosis. The methyl Se-specific inhibitory effect on VEGF expression observed in the present work may therefore represent an important mechanism for the regulation of the angiogenic switch in early lesions by chemopreventive intake of Se.

The rapid inhibitory action of methyl Se on the expression of MMP-2 and VEGF suggests a commonality of mechanisms to bring about a reduction of the cellular level of the respective proteins. Further investigation of the biochemical and cellular processes involved in the reduction of their protein levels will shed light on how methyl Se activates such mechanisms. A salient feature of the two molecules is that both are secretory proteins containing intramolecular or intermolecular disulfide bridges critical for their activity or function [13,54]. One attractive hypothesis is that methylselenol generated intracellularly may disrupt such disulfide bridges, leading to destabilization of these proteins and selective proteolysis.

In summary, the present results support the hypothesis that the methyl Se metabolite pool, probably methylselenol, exerts potent and primary inhibitory effects on two proteins, with significant implications for angiogenesis switch regulation (as schematically summarized in Figure 8). Particularly noteworthy is the remarkable inhibitory efficacy, with IC_{50} of $\sim 2 \mu\text{M}$ after just a few hours of exposure. As reference values, the mean plasma Se concentration of subjects without Se supplementation in a recent human trial was $\sim 1.5 \mu\text{M}$, and Se supplementation (200 $\mu\text{g}/\text{d}$ as selenized yeast) brought the mean Se level to $\sim 2.5 \mu\text{M}$ [1]. Therefore, the methyl Se-specific inhibitory activities on these proteins may be physiologically pertinent for angiogenic switch regulation in early transformed lesions in vivo in the context of cancer chemoprevention, which aims at retarding and blocking the growth and progression of early lesions. The antiangiogenic attributes reported in this study and the growth arrest and apoptogenic activities without genotoxicity (Figure 8) make the methylselenol precursors attractive chemopreventive agents for considerations in humans.

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Anti-Angiogenic Potential of a Cancer Chemopreventive Flavonoid Antioxidant, Silymarin: Inhibition of Key Attributes of Vascular Endothelial Cells and Angiogenic Cytokine Secretion by Cancer Epithelial Cells

Cheng Jiang, Rajesh Agarwal, and Junxuan Lü¹

Center for Cancer Causation and Prevention, AMC Cancer Research Center, Denver, Colorado 80214

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In recent studies, we have shown that silymarin, a naturally occurring flavonoid antioxidant, exhibits anti-cancer effects against several epithelial cancers. Here, we assessed its potential as an anti-angiogenic agent employing human umbilical vein endothelial cells (HUVEC) and human prostate and breast cancer epithelial cells. When sub-confluent HUVEC were treated for 48 h, adherent cell number decreased by 50 and 90% at 50 and 100 $\mu\text{g/ml}$ doses, respectively. Apoptotic cell death principally accounted for cell loss at $>50 \mu\text{g/ml}$ doses. In biochemical analysis, silymarin treatment of HUVEC for 6 h resulted in a concentration-dependent decrease in the secretion and cellular content of matrix metalloproteinase (MMP)-2/gelatinase A. Silymarin also inhibited HUVEC tube formation (*in vitro* capillary differentiation) on a reconstituted extracellular matrix, Matrigel. In other studies, 5 to 6 h exposure of DU145 prostate, and MCF-7 and MDA-MB-468 breast cancer cells to silymarin resulted in a dose-dependent decrease in the secreted vascular endothelial growth factor (VEGF) level in conditioned media without any visible change in cell morphology. The inhibitory effect of silymarin on VEGF secretion occurred as early as 1 h. These observations indicate a rapid inhibitory action of silymarin on the secretion of this primary angiogenic cytokine by cancer epithelial cells. Taken together, the results of this study support the hypothesis that silymarin possesses an anti-angiogenic potential that may critically contribute to its cancer chemopreventive efficacy. © 2000 Academic Press

Key Words: silymarin; HUVEC; MMP-2/gelatinase A; *in vitro* capillary differentiation; vascular endothelial growth factor; angiogenesis switch.

It is now well established that angiogenesis, that is, the growth of capillary vessels from existing blood vessels, is obligatory for the growth and progression of solid cancers (1–3). During solid cancer genesis, initiated cells undergo clonal expansion in an avascular state when the expanding lesions are small enough to take in nutrients and to expel metabolic wastes by diffusion. However diffusion is not sufficient to support continued growth of the lesion beyond a certain physical size (estimated ~ 2 mm diameter) because the expanding lesions consume nutrients at a rate proportional to their volume whereas the supply of nutrients is delivered at a rate proportional to their surface area (4, 5). In order for avascular lesions to progress beyond the size limit imposed by simple diffusion, they must turn on their angiogenic switch to form a neo-vasculature. Angiogenesis critically depends on several conditions such as the endothelial cells must proliferate to provide the necessary number of cells for the growing vessels, the activated endothelial cells must secrete matrix metalloproteinases (MMP) required to break down surrounding tissue matrix and the endothelial cells must be capable of movement/migration. In addition, the angiogenic stimuli (for example, hypoxia and production of angiogenic cytokines such as vascular endothelial growth factor [VEGF]) must be sustained. Because of the critical dependence of tumor growth and metastasis on angiogenesis, therapeutic strategies have been developed targeting various aspects of the angiogenic processes, many with promising results. Cancer chemoprevention aims to block or reduce the occurrence or progression of human malignancies by the chronic administration of naturally occurring or synthetic chemical agents. Chemoprevention can be most effective on early lesions, the fate and growth of which are likely to be more critically dependent on angiogenesis. Since the vascular endothelial cells constitute the first line of exposure to blood-borne agents, it is plausible that cancer chemopreventive

¹ To whom correspondence should be addressed at AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214. Fax: 303-239-3560. E-mail: luj@amc.org.

activity of many agents may be attributable, at least in part, to anti-angiogenic properties through an inhibition of one or more of the angiogenic responses of the endothelial cells.

Fruits, vegetables, tea as well as many medicinal herbs and plants have been shown to be rich sources of phytochemicals with chemoprevention potential for some kinds of human cancer (6–9). Naturally occurring polyphenolic antioxidants are among these phytochemicals that have received increasing attention in recent years (6–9). Silymarin is a polyphenolic flavonoid antioxidant isolated from milk thistle (*Silybum marianum* (L.) Gaertn) and is used clinically as a liver detoxicant for almost three decades (10, 11). Several studies in recent past have shown anti-carcinogenic effects of silymarin in short-term bioassays (12–14). More recently, we have shown the cancer preventive efficacy of silymarin in several mouse skin tumorigenesis models (15–19), and its anti-cancer potential for human breast, prostate and cervical cancers (20–24).

Whereas all the mechanistic studies done with silymarin in recent years have focused on the cancer epithelial cells as the targets, the present study was conducted to explore potential inhibitory effects of silymarin on key parameters critical for tumor angiogenesis. In this paper, we report that silymarin treatment of human umbilical vein endothelial cells (HUVEC) inhibits their growth and survival, the secretion and expression of matrix metalloproteinases (MMPs) and capillary tube formation (*in vitro* angiogenesis). In addition, we report a rapid inhibitory action of silymarin on the secretion of a primary angiogenic cytokine VEGF by human prostate and breast cancer epithelial cells. Together, these results support an anti-angiogenic activity of silymarin that may contribute critically to its cancer chemopreventive potential.

MATERIALS AND METHODS

Chemicals and reagents. Silymarin, bovine endothelial cell growth supplement (ECGS) and heparin were purchased from Sigma Chemical Co. (St. Louis, MO). Matrigel was purchased from Becton-Dickinson Labware (Bedford, MA). VEGF ELISA kit was purchased from R&D Systems (Minneapolis, MN).

Cell lines and cell culture. HUVEC cells, DU145 prostate cancer cells, and MCF-7 and MDA-MB-468 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). HUVEC were propagated in F12K medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μ g/ml of heparin (Sigma Chemical Co., St. Louis, MO) and 30 μ g/ml of bovine endothelial cell growth supplement (ECGS) (Sigma Chemical Co.) as described previously (25). DU145 cells were cultured in RPMI1640 medium supplemented with 10% FBS. MCF-7 and MDA-MB-468 breast cancer cells were cultured in DMEM medium supplemented with 10% FBS and 2 mM L-glutamine.

HUVEC growth/survival. Cells were seeded into 6-well plates for 24–48 h to reach ~50% confluence. Fresh medium was replaced and silymarin was added from 100 \times stock solutions prepared in DMSO/

ethanol (20:80). In all the studies, the selection of silymarin doses was based on our earlier studies showing anti-proliferative and differentiation-inducing effects in several human epithelial carcinoma cells (20–24). Morphological responses were monitored over time under a phase contrast microscope. Adherent cells after 48 h of treatment were fixed in 1% glutaraldehyde and stained. The cell number was counted under 100 \times magnification for 5 random fields for each condition. The experiment was repeated at least once.

Zymogram analysis for MMP-2. HUVEC were grown in 6-well plates in complete medium for 24–48 h to near confluence. The cells were washed two times with PBS to remove spent medium and fed serum-free medium supplemented with 100 μ g ECGS/ml and treated with silymarin for 6 h (a time frame that did not result in any visible morphological changes). Conditioned medium and cell lysate (prepared in 1% Triton X-100, 0.5 M Tris-HCl, pH 7.6, 200 mM NaCl) were analyzed for gelatinolytic activities on substrate gels as we previously described (25). The gels were digitized with a transmission scanner and band intensity (on inverted images) was quantified using the UN-SCAN-IT gel scanner software (Silk Scientific, Inc. Orem, UT). As a comparison for the efficacy of silymarin to inhibit HUVEC MMP-2, curcumin, a polyphenolic component of the food flavor turmeric, was included in some experiments. Curcumin has been reported to potently inhibit MMP-2 expression and tube formation in this model (26).

Capillary tube formation on Matrigel (*in vitro* angiogenesis). The method of Kubota *et al.* was used (27). When seeded on Matrigel, a reconstituted extracellular matrix preparation of EHS mouse sarcoma, vascular endothelial cells undergo rapid *in vitro* differentiation into capillary like structures (27), providing a simple assay for assessing impact of agents on endothelial differentiation process which requires cell-matrix interaction, intercellular communication as well as cell motility. To examine the effect of silymarin on this process, HUVEC were treated in two ways in relationship to the time frame of cell seeding onto the Matrigel. (A) Silymarin simultaneous with cell seeding: Twenty-four-well cell culture plates were coated with 0.3 ml of Matrigel and allowed to solidify at 37°C for 1 h. Then 0.5 ml medium was added to each well and silymarin was added at 2 times of the desired concentrations. HUVEC were trypsinized and 20,000 or 40,000 cells were added per well in 0.5 ml medium. Tube formation was observed periodically over time under a phase contrast microscope. Representative Polaroid pictures were taken at 6 or 17 h. (B) Treatment of preformed tubes: HUVEC were seeded onto Matrigel for 6 h to form rudimentary tubes, then the medium was replaced and silymarin was added. Tube morphology was observed over time and representative Polaroid pictures were taken at 20 h after the initiation of silymarin treatment. Curcumin was included in some experiments as a comparison for the efficacy of silymarin to inhibit tube formation. The experiments were repeated twice.

VEGF secretion and expression in cancer epithelial cells. In dose-response experiments, DU145 prostate cancer cells and MCF-7 (estrogen dependent) and MDA-MB-468 (estrogen independent) breast cancer cells were grown in T25 flasks in complete medium until confluence (~48 h). The spent medium was removed, and cells were washed 3 \times with PBS. Cells were treated in serum-free medium with increasing concentrations of silymarin. Conditioned media and cell lysates were analyzed for VEGF protein content by an ELISA kit as per manufacturer's instructions (R&D Systems, Minneapolis, MN). In time course experiments, confluent DU145 or MDA-MB-468 cells were treated in serum-free media with solvent vehicle (DMSO/ethanol), 50 or 100 μ g/ml silymarin. Serial 1-ml aliquots were taken of the culture media for VEGF ELISA. Each sample was measured in triplicate. Experiments were repeated at least once.

RESULTS

HUVEC growth and survival. As shown in Fig. 1 (A–D), treatment with silymarin for 48 h led to a

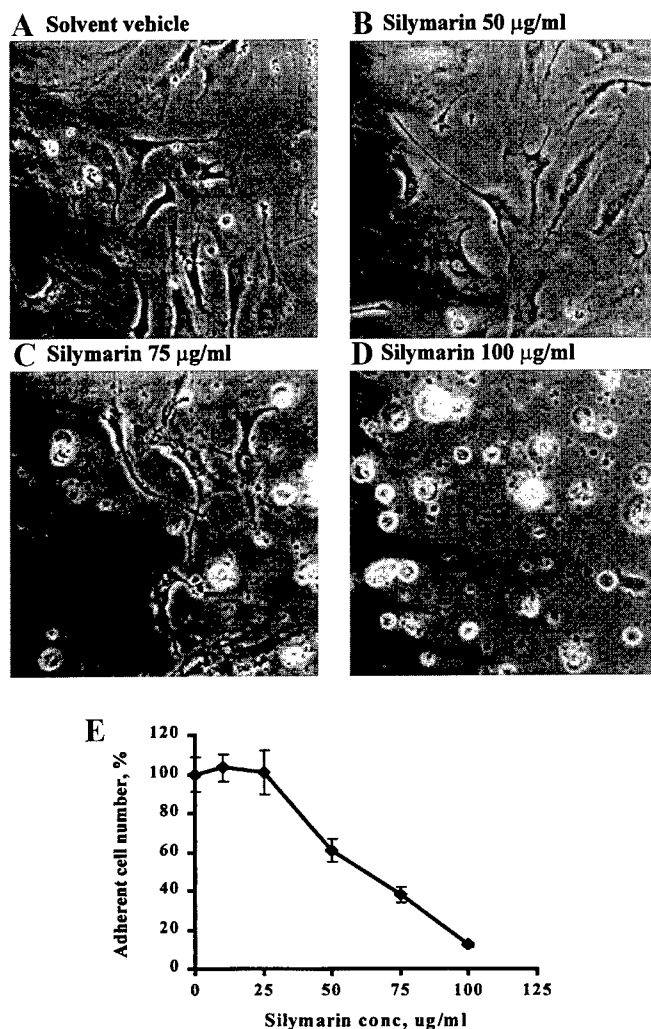


FIG. 1. Effect of silymarin treatment on HUVEC growth and survival. (A–D) Representative phase-contrast photomicrographs of HUVEC at 48 h after treatment was initiated with DMSO/ethanol vehicle (A), 50 (B), 75 (C), and 100 (D) $\mu\text{g/ml}$ of silymarin. Most floaters showed typical apoptotic morphology such as cell retraction, condensation, and fragmentation into apoptotic bodies. (E) Adherent cell number as a function of initial silymarin treatment concentration. Each data point represented the mean \pm SEM of the adherent cells in 5 randomly chosen fields.

concentration-dependent decrease of cells remaining adherent to the culture vessel and an increase of detached floaters. The adherent cell number was inhibited by 50 and 90% at 50 and 100 $\mu\text{g/ml}$ doses of silymarin, respectively (Fig. 1E). The floaters displayed typical apoptotic morphology as indicated by cell condensation and fragmentation into apoptotic bodies. Such floaters did not re-attach or grow upon reseeding into silymarin-free fresh medium (data not shown).

HUVEC MMP-2 expression. Treatment with silymarin for 6 h, an exposure time that did not result in any visible morphological changes, led to a

concentration-dependent decrease of MMP-2 (72 kD gelatinase A) in the conditioned media (i.e., secreted MMP) as detected by gelatin zymogram analyses (Fig. 2A). The extent of inhibition of the secreted MMP-2 by 100 $\mu\text{g/ml}$ of silymarin was comparable to that induced by 25 μM curcumin, which has been shown to inhibit HUVEC MMP-2 and *in vitro* angiogenesis (26). In the cell lysate (Fig. 2B), 100 $\mu\text{g/ml}$ silymarin inhibited MMP-2 by 67% and this effect was greater than that exerted by 25 μM curcumin, even though the secreted MMP-2 was decreased to the same extent by both compounds at the respective concentrations. At 50 $\mu\text{g/ml}$ dose, silymarin did not decrease MMP-2 in the cell lysate even though it decreased the secreted MMP-2 by as much as 63%, indicating that at this level, silymarin might only inhibit the secretion of MMP-2 from the cells but not the cellular level. Incubation of the control medium (MMP-2 containing) with silymarin directly in the test tube did not inhibit its zymographic activity (data not shown), indicating a cellular dependent process for the inhibitory action on MMP-2 secretion and expression by silymarin.

***In vitro* angiogenesis on Matrigel by HUVEC.** In experiments assessing the inhibitory effects on capillary tube formation, silymarin exposure, commenced at the time of seeding HUVEC onto Matrigel,

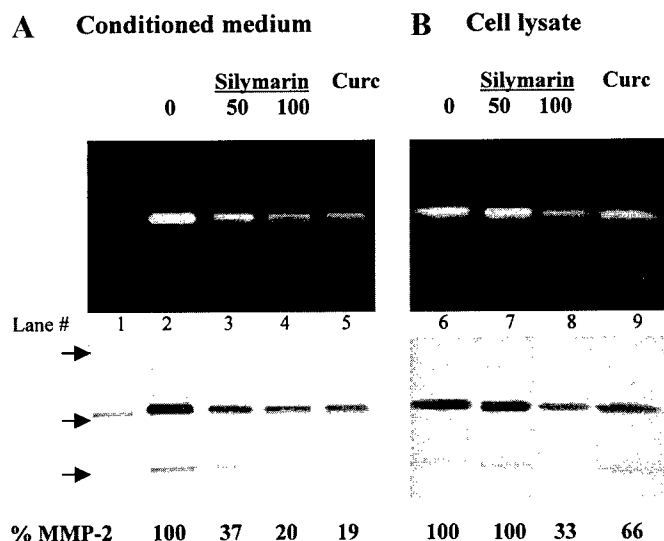


FIG. 2. Effect of silymarin or curcumin on secreted (A) and cell-associated (B) matrix metalloproteinase-2 detected by gelatin substrate gel zymography. HUVEC were treated in serum-free medium supplemented with 100 $\mu\text{g/ml}$ of ECGS with silymarin or curcumin for 6 h. The conditioned media (A, lanes 2–5) and cell lysates (B) were analyzed on gelatin I impregnated substrate gels. Silymarin concentrations were 50 and 100 $\mu\text{g/ml}$. Curcumin treatment concentration was 25 μM . Inverted images of the zymograms (lower panels) were used for densitometric quantitation. The relative pixel density for the 72 kD gelatinase A/MMP-2 was shown below each lane. Arrowheads on the left mark position of molecular weight standards corresponding to (from top) 97, 66, and 47 kD. Lane 1 was serum-free medium as a blank control.

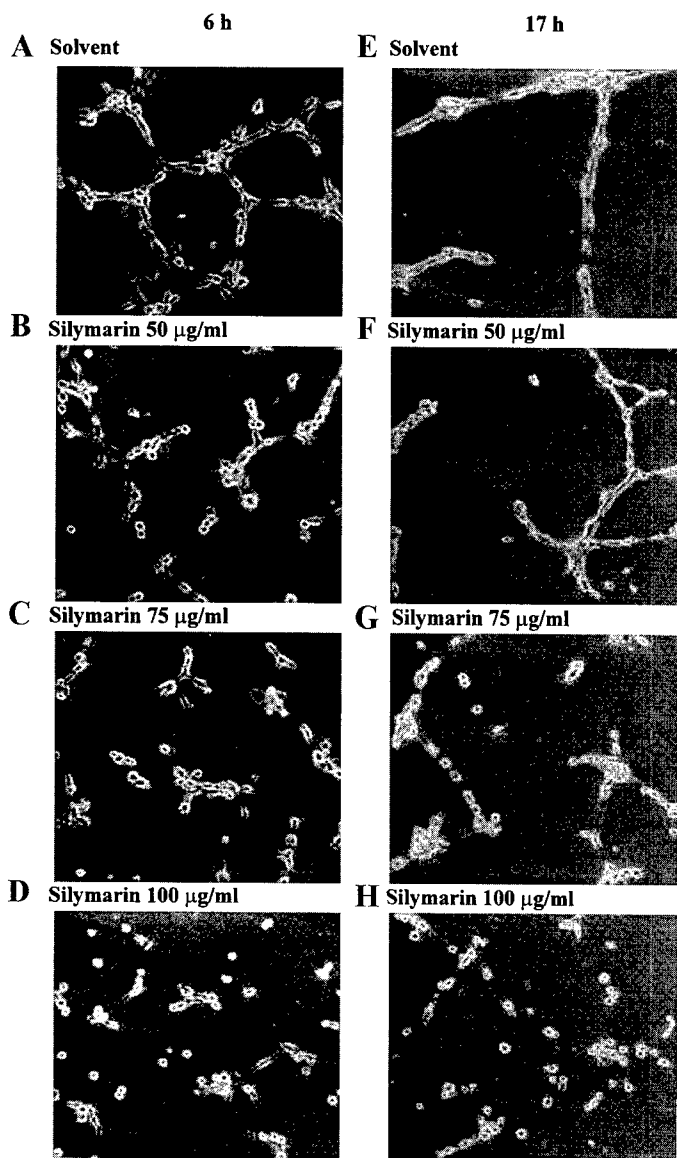


FIG. 3. Effect of silymarin on HUVEC capillary tube formation (*in vitro* differentiation) on Matrigel. HUVEC (20,000 cells/per well) in medium containing 10% serum was seeded into Matrigel pre-coated 24-well plate and treated with DMSO/ethanol solvent vehicle (A, E) or increasing concentrations of silymarin (B–D, F–H). Representative phase contrast photomicrographs (100× magnification) were taken at 6 h (A–D) and 17 h (E–H) after seeding. Each experiment condition was performed in duplicate wells and the experiments were repeated twice.

concentration-dependently inhibited tube formation at both 6 (Figs. 3A–D) and 17 h (Figs. 3E–H), achieving almost a complete block at the 100 µg/ml dose. Silymarin exposure of pre-formed tubes led to the retraction of cells and capillary disintegration (Fig. 4B versus 4A). The efficacy of silymarin at 100 µg/ml was comparable to that of 25 µM curcumin (Fig. 4C).

VEGF secretion by cancer epithelial cells. Silymarin treatment of DU145 human prostate carcinoma

cells for 6 h decreased the secreted (in conditioned medium) VEGF content in a concentration dependent manner, resulting in a complete block by the 100 µg/ml dose (Table 1). Such inhibitory effect was observed in the absence of a reduction of the cell lysate VEGF content (Table 1). In human breast cancer cells, silymarin exposure reduced VEGF level in conditioned media in both MDA-MB 468 and MCF-7 cell lines (Table 1). The impact of silymarin on the cellular VEGF content was similar to that on DU145 cells, i.e., in MCF-7 cells as well as MDA-MB 468 cells at low to intermediate exposure levels, a reduction of secreted VEGF level was not associated with decreased cellular

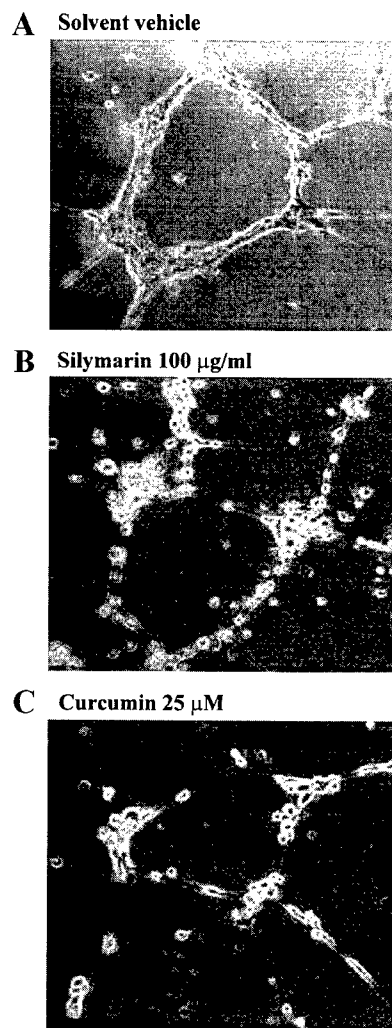


FIG. 4. Effect of silymarin or curcumin treatment on preformed HUVEC tubes. HUVEC (40,000 per well) were seeded into Matrigel pre-coated 24-well plate for 6 h for tube formation. The unattached cells and conditioned medium were removed and the tubes washed in fresh medium. The preformed tubes were treated with DMSO/ethanol vehicle (A), silymarin (B, 100 µg/ml) or curcumin (C, 25 µM). The cells were fixed in 1% glutaraldehyde at 20 h after seeding and representative phase-contrast photomicrographs (100× magnification). Each experiment condition was performed in duplicate wells and the experiment was repeated twice.

TABLE 1

Effects of Silymarin Treatment on Vascular Endothelial Growth Factor (VEGF) Content in Conditioned Media (Secreted) and in Prostate and Breast Cancer Cell Lysates

Cell line	Silymarin $\mu\text{g/ml}$	Exposure time, h	VEGF in medium pg/flask	VEGF in lysate pg/flask
DU145	0	6	4272 \pm 516 ^{a1,2}	640 \pm 54 ^c
	25	6	4128 \pm 480 ^a	760 \pm 16 ^b
	50	6	2658 \pm 264 ^b	894 \pm 10 ^a
	100	6	72 \pm 1 ^c	758 \pm 22 ^b
MDA-MB-468	0	5	7815 \pm 480 ^a	1112 \pm 24 ^a
	25	5	6150 \pm 165 ^b	1222 \pm 42 ^a
	50	5	5300 \pm 105 ^c	1140 \pm 26 ^a
	100	5	2590 \pm 65 ^d	742 \pm 14 ^b
MCF-7	0	6	3420 \pm 120 ^a	455 \pm 24
	50	6	3156 \pm 246 ^a	447 \pm 9
	100	6	2178 \pm 102 ^b	501 \pm 22

¹ Mean \pm sd; $n = 3$ replicates.

² Data were analyzed by one-way ANOVA. Dissimilar superscripts indicate significant difference between means ($P < 0.05$).

VEGF content (Table 1). The exception was MDA-MB cells treated with 100 $\mu\text{g/ml}$ silymarin where cellular VEGF content was decreased. In time course experiments, the secretion of VEGF was significantly decreased at 1 h of exposure to silymarin in both DU145 and MDA-MB 468 cells (Fig. 5). The inhibitory effects in all three cell lines were observed in the absence of morphological changes such as cell retraction, rounding, detachment or cytoplasmic vacuolation.

DISCUSSION

A number of recent studies by Agarwal and associates (15–24) have shown that silymarin possesses significant chemopreventive and anti-cancer activity. Although cell culture studies have revealed many insights concerning the potential direct effects of silymarin exposure on cancer epithelial cells with respect to growth and survival signaling and cell cycle regulation, there has been no published work to address the potential impacts of silymarin on vascular endothelial cells and angiogenesis. The results of the present study support a potential anti-angiogenic activity of silymarin. Because tumor epithelial cells *in vivo* depend on angiogenesis to provide nutrients for their growth and survival, it is plausible that an anti-angiogenic effect may play a primary role in mediating the cancer chemopreventive activity of silymarin.

In the present study, first, silymarin inhibited endothelial cell growth and survival through induction of apoptosis in a concentration dependent manner (Fig. 1). Because angiogenic factor-stimulated proliferation of endothelial cells is crucial for capillary sprouting, growth inhibition and apoptosis induction can be one

mechanism for silymarin to inhibit angiogenic response. Second, silymarin inhibited endothelial MMP-2 secretion and expression (Fig. 2) and such an effect occurred rapidly prior to the onset of any morphological changes. Because matrix activity of angiogenically-stimulated endothelial cells via MMP-2 is another important requirement for capillary sprouting (28–31), the inhibition of MMP-2 secretion and expression by silymarin may provide an inhibitory mechanism on angiogenesis independent of and/or in addition to endothelial growth arrest and apoptosis. Furthermore, silymarin inhibited *in vitro* capillary formation on Matrigel, a process requiring cell-matrix interaction, inter-cellular communications as well as cell motility, to name a few. It was noteworthy that the inhibitory effect on tube formation manifested whether the treatment was initiated simultaneous with seeding cells on the Matrigel (Fig. 3) or when the tubes had preformed (Fig. 4). These results support an anti-

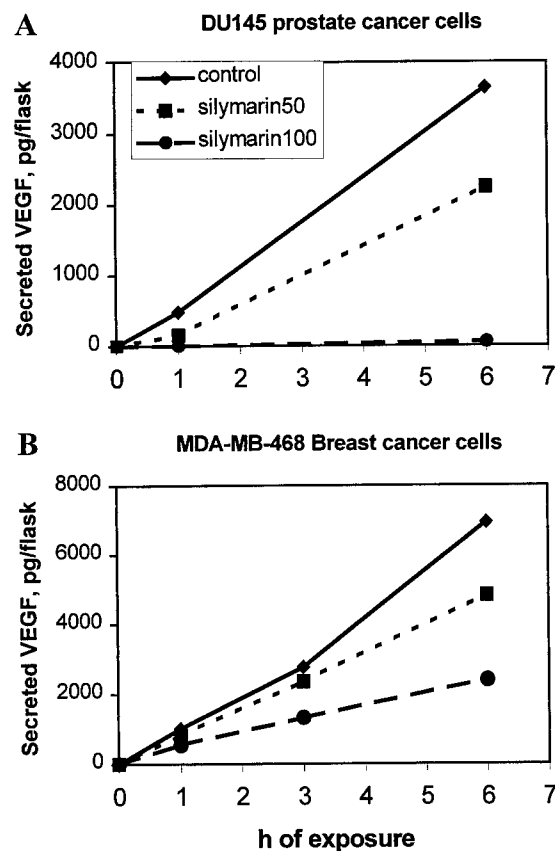


FIG. 5. Representative time course of silymarin effects on VEGF secretion by DU145 prostate cancer cells (A) and MDA-MB-468 breast cancer cells (B) in serum-free media. Confluent cells in T25 flasks were treated with solvent vehicle (DMSO/ethanol) or silymarin in 6 ml serum-free medium. At designated time points, 1-ml aliquots of conditioned media were taken for VEGF assay by ELISA. Each data point represents the mean of triplicate measurements. SD $<$ 5% of respective means.

angiogenic potential for silymarin through multifaceted effects on endothelial proliferation and survival and matrix degradation activity and the capillary differentiation process. Work is in progress to further substantiate an anti-angiogenic effect of silymarin in other human endothelial cells in culture and in *in vivo* models.

In addition to these inhibitory effects on endothelial responses and *in vitro* angiogenesis, silymarin also exerted a rapid inhibitory action on the secretion of VEGF by cancer epithelial cells (Table 1 and Fig. 5). VEGF, also known as vascular permeability factor (VPF) (32, 33), plays several critical roles in vasculogenesis as well as angiogenesis. Its expression is so crucial that germ-line knockout of even one VEGF allele leads to embryonic lethality and homozygous knockout embryonic stem cells are incapable of forming tumors (34, 35). Whereas overexpression of VEGF is linked to increased angiogenesis and more aggressive tumor behavior (36, 37), anti-angiogenic interventions based on VEGF antibodies or interference of signal transduction through its receptors (38–42) have been shown to result in the inhibition of tumor growth and induction of endothelial apoptosis. Transformed epithelial cells have been shown to be the major source of VEGF expression in many types of solid cancers (43–46), however, recent data suggest that stromal cells and even vascular endothelial cells may also express VEGF in the hypoxic angiogenic microenvironment of tumors (47). These findings are supported by the observations that certain oncogenic mutations constitutively upregulate VEGF expression (48–51), and that cancer epithelial hypoxia, as a result of dysregulated cellular proliferation (5), is a potent *in vivo* inducer of VEGF expression (52, 53). The inhibitory effect of silymarin on secretion of VEGF in cancer epithelial cells, therefore, may be an important mechanism to negatively regulate the angiogenic switch of avascular lesions, further contributing to the overall control of lesion growth and progression.

The manners by which epithelial VEGF and endothelial MMP-2 were inhibited by silymarin are noteworthy and suggestive of a commonality with regard to the mechanisms of action by silymarin on these secretory proteins. In DU145 and MCF-7 cancer cell lines, silymarin exposure decreased secreted VEGF in the conditioned media without a reduction of cellular VEGF protein level (Table 1). In the MDA-MB468 cell line, exposure at low to intermediate levels of silymarin (25 or 50 $\mu\text{g/ml}$) decreased secreted VEGF level without lowering the cellular VEGF content, and only at the higher exposure level (100 $\mu\text{g/ml}$) a reduction of cellular VEGF level was observed (Table 1). This pattern was similarly to that observed for HUVEC MMP-2 expression in that an intermediate level of silymarin exposure (50 $\mu\text{g/ml}$) significantly decreased secreted MMP-2 level without a change in cellular MMP-2 (Fig.

2). These results from both epithelial and endothelial cells suggest that a primary action of silymarin may involve preferential targeting of the secretion and/or export (exocytosis) of these proteins critical for angiogenic switch regulation. We are currently investigating such mechanisms.

When the results of the present study showing cell death effect of silymarin on HUVEC were compared to those published by us showing anti-proliferative, but not cytotoxic and apoptotic effects, in several different human carcinoma and normal epithelial cells (20–24), it is important to emphasize here that apoptotic effect of silymarin is possibly specific to vascular endothelial cells. Based on these results, there is a possibility that on one hand silymarin is an anti-proliferative and a differentiation-inducing agent for cancer epithelial cells and on the other hand is both an anti-proliferative and an apoptogenic agent for vascular endothelial cells that are involved in neo-vascularization. These dual effects of silymarin possibly make it a useful agent for the prevention and therapy of epithelial cancers in humans.

In summary, this study, for the first time, documents the inhibitory actions of silymarin on several angiogenic responses, including growth and survival, MMP-2 expression and *in vitro* angiogenesis, of vascular endothelial cells as well as an inhibitory effect on the secretion of a primary angiogenic cytokine VEGF by cancer epithelial cells. The anti-angiogenic activity reported in this paper combined with the previously published multi-faceted broad spectrum anti-cancer effects of silymarin support the merit of further investigations to assess and define its cancer chemopreventive and/or therapeutic potential for humans.

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APOPTOSIS AND ANGIOGENESIS IN CANCER PREVENTION BY SELENIUM

Junxuan Lu, Ph.D.

Center for Cancer Causation and Prevention
AMC Cancer Research Center
Denver, CO 80214

INTRODUCTION

Epidemiological and laboratory findings have long implicated a potential anti-cancer activity of the trace element nutrient selenium (Se). Note that it is often for convenience reasons to describe such anti-cancer activity in terms of the element. A vast volume of data have been accumulated supporting the theme that cancer preventive activity is expressed as a function of the dose and chemical form in which the element resides, not elemental Se *per se* (1,2). The landmark cancer prevention trial by Clark, Combs and coworkers demonstrated for the first time that a supra-nutritional Se supplement (provided as selenized yeast) to a US skin cancer patient population otherwise adequate in Se nutrition might be an effective preventive agent for several major human epithelial cancers (3). With profound public health implications at stake, many serious issues demand a clear understanding of the mechanisms through which Se exerts anti-cancer activity. Some of these pressing questions, for example, include what form(s) and what doses of Se should be used? What populations should be given the intervention? How long should the intervention be given?

With respect to “mechanisms”, a number of them have been investigated: antioxidant protection (via SeCys-glutathione peroxidases), altered carcinogen metabolism, enhanced immune surveillance, cell cycle effects, enhanced apoptosis (1,2) and more recently inhibition of neo-angiogenesis (4). The mechanisms that are actually involved in cancer prevention by Se will likely depend on the dose and form of Se-compounds, the Se status of the individual and perhaps the type and etiology of malignancy to be prevented. It is probable that Se supplementation of individuals with relatively low or frankly deficient Se intakes can be expected to support enhanced antioxidant protection due to increased expression of the SeCys-enzymes or enhanced immune surveillance (2). On the other hand, in animal models and in clinical studies, anti-tumorigenic activities have been associated with Se intakes that are more than sufficient to correct nutritional deficiency. That is, Se appears to be anti-tumorigenic at intakes that are substantially greater than those associated with maximal expression of the known SeCys-containing glutathione peroxidase enzymes (1,2). Therefore, a favored hypothesis that has enjoyed considerable experimental support is that cancer chemopreventive functions of Se are attributable to active Se-metabolite(s) produced in significant amounts at high Se intakes (1,2,5). Specifically methylselenol has been implicated as a candidate *in vivo* active Se metabolite (5-7).

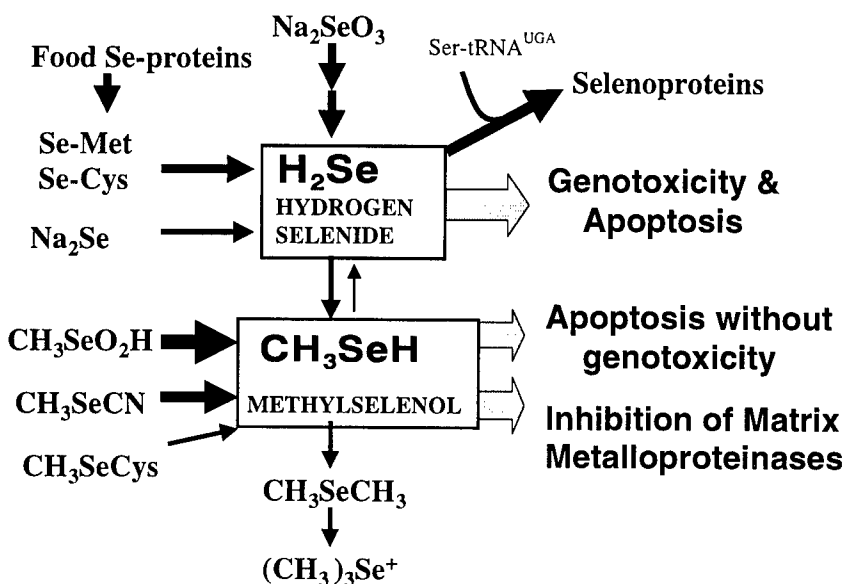


Figure 1. Schematic relationship of Se precursors feeding into two pools of proximal Se metabolites that exert distinct biochemical and cellular effects. The fate of Se amino acids derived from food Se-proteins was also indicated. The genotoxicity (or lack of) effect of the two Se pools was based on studies with mammary cancer epithelial cells and leukemia cells (ref. 15,16, 23-25). Abbreviations: Se-cys, selenocysteine; Se-Met, selenomethionine; CH_3SeCN , methylselenocyanate; CH_3SeCys , Se-methylselenocysteine; CH_3SeO_2H , methylselenenic acid.

In this chemopreventive context, we have used cell culture and animal models to seek a better understanding of how Se confer growth/fate regulation of transformed cells (15,16, 23-25) and more recently the role of angiogenesis in mammary cancer and its prevention by Se (4).

In this chapter, I will focus on the following questions: Does Se induce apoptotic death of cancerous cells? What pathways mediate such an effect? Does Se inhibit neo-angiogenesis and if so, how? The scope of discussion will be limited to those forms of Se that are related to *in vivo* Se metabolism (Figure 1) (8). Interested readers are referred to recent review articles concerning the cancer preventive activity of novel synthetic aromatic organo-selenium compounds (1, 9). I will review pertinent data that support two pools of proximal Se metabolites, namely hydrogen selenide and methylselenol, that induce apoptosis with distinct biochemical/cellular pathways and a specific inhibitory activity of methylselenol on endothelial matrix metalloproteinases (MMPs) which are required for the angiogenic process (Figure 1). In the discussion section, I will propose an integrated (albeit speculative) model of cancer prevention by Se based on the interaction of epithelial lesions and the vasculature that supports such lesions.

SELENIUM AND CELL DEATH/APOPTOSIS

Selenite-induced apoptosis is causally linked to genotoxicity

Treatment of fibroblasts and other cell lines with selenite, a commonly used reference Se compound, has been reported to lead to DNA single strand breaks (defined as genotoxicity) in the late 1980s and early 90s (10-12). Generation of reactive oxygen species (ROS) such as superoxide was demonstrated in *in vitro* models by the reaction of selenite with glutathione and other thiol compounds (13,14). These observations suggested that ROS and genotoxicity might be causally involved in the cytostatic and cytotoxic effects of Se.

To test this hypothesis, we examined the sequence of events in the induction of apoptotic cell death (as evidenced by morphology and DNA apoptotic fragmentation) by sodium selenite using murine L1210 leukemia cells as our model (15). Cell death and DNA apoptotic fragmentation as well as double stranded breaks (DSBs) were preceded by the occurrence of DNA single strand breaks (SSBs) as measured using a filter elution assay (15,16). Much insight was gained by using inhibitors of key biochemical processes to probe the likely sequence of events (Table 1). Copper diisopropylsalicylate (CuDIPS), a superoxide dismutase (SOD) mimetic compound that blocks the generation of hydrogen selenide from selenite (11), completely blocked the effect of selenite on DNA integrity and cell viability. Because the free radical quencher mannitol failed to modulate the treatment effect of selenite, hydroxy

Table 1. Modulation of selenite-induced DNA strand breaks and cell viability of L1210 murine leukemia cells (Modified based on ref. 15)

Treatment	DNA SSBs	DNA DSBs	Cell Viability,%
None	5.7 ± 0.7	5.0 ± 0.1	99
+ 50 µM CuDIPS ¹	4.8 ± 0.4	5.8 ± 0.6	98
+ 5 mM mannitol ²	5.2 ± 0.4	4.8 ± 0.2	98
+ 0.25 mM ATA ²	4.3 ± 0.4	3.4 ± 0.2	99
+ 39 µM ZnSO ₄ ²	6.0 ± 1.1	5.9 ± 0.1	99
10 µM selenite	93.4 ± 0.4	27.1 ± 1.6	72
+ 50 µM CuDIPS	5.6 ± 0.5	4.8 ± 0.2	97
+ 5 mM mannitol	89.0 ± 0.6	25.5 ± 0.9	65
+ 0.25 mM ATA	54.9 ± 0.9	3.7 ± 0.1	97
+ 39 µM ZnSO ₄	62.4 ± 1.9	8.8 ± 0.6	95

Mean ± sem (n=4).

¹CuDIPS, copper diisopropylsalicylate, is a superoxide dismutase mimetic.

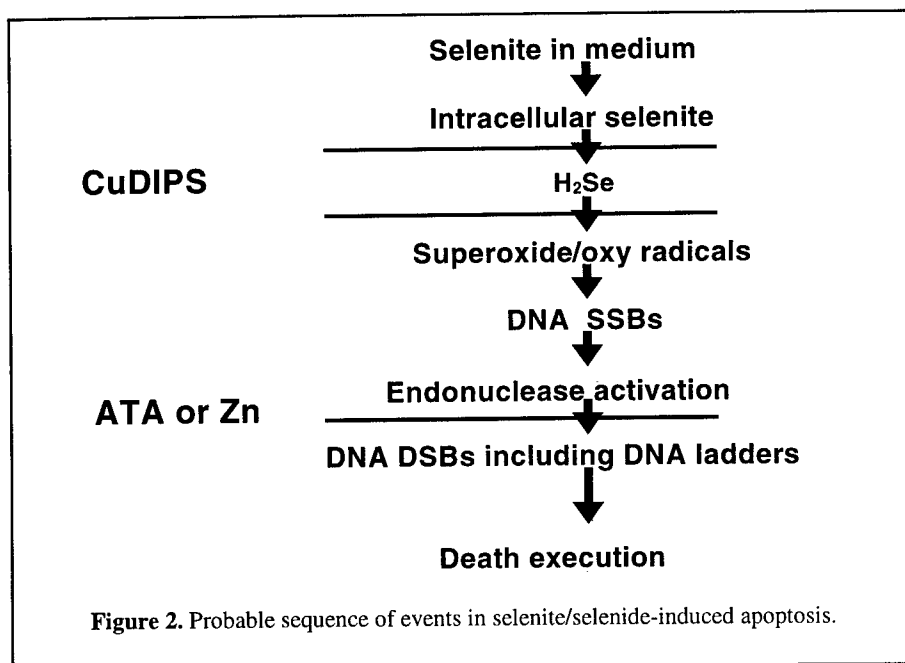
²ATA, aurintricarboxylic acid. ATA and zinc are inhibitors of Ca²⁺/Mg²⁺-dependent endonucleases. Mannitol is a hydroxy free radical quencher.

free radicals were not likely involved as mediators of DNA strand breaks and apoptosis. These data indicated that metabolism of selenite, not selenite *per se*, was required for the induction of DNA damage and subsequent apoptosis and implicated superoxide and/or hydrogen selenide as likely proximal mediators of the observed effects. Co-treatment of L1210 cells with aurintricarboxylic acid (ATA) and zinc, known inhibitors of Ca²⁺/Mg²⁺-dependent endonucleases responsible for nucleosomal apoptotic DNA fragmentation, prevented DNA DSBs and cell viability loss, attenuated but did not block the occurrence of DNA SSBs.

These data indicated that the occurrence of DNA SSBs preceded and was likely causal to Ca²⁺/Mg²⁺-dependent endonuclease activation and apoptotic fragmentation of the DNA. The likely sequence of events in apoptosis induction by selenite treatment is shown schematically in Figure 2. Results from other laboratories published recently have provided confirmation of growth inhibition and apoptosis induction by selenite and related Se forms through genotoxicity mediated by ROS (17-22). The generality of this mechanism appears to extend to human leukemia (17), hepatoma (18), colon carcinoma (20,21), glioma (22) and mouse keratinocytes (19).

Methylselenol induces apoptosis without inducing DNA SSBs (23)

To further define the Se metabolite(s) responsible for induction of DNA damage and apoptosis, we took advantage of Se compounds that enter the Se metabolic pathways at different points (Figure 1). Selenite treatment of



MOD mouse mammary cancer epithelial cells led to a rapid induction of DNA SSBs (Figure 3A) and DSBs (Figure 3B), and subsequent cell death by a composite of acute lysis and apoptosis (Figure 3C). Sodium selenide, which metabolizes to hydrogen selenide (H_2Se), recapitulated the effects of selenite treatment on DNA SSBs and DSBs and cellular morphological responses (23). In contrast, methylselenocyanate (MSeCN or CH_3SeCN) and methylselenocysteine (MSeC or CH_3SeCys) induced exclusively apoptosis of cancerous mammary epithelial cells (Figure 3D) without induction of DNA SSBs (Figure 3, A and B). *Therefore, hydrogen selenide is likely the proximal Se metabolite involved in the induction of DNA SSBs and apoptosis by Se compounds that feed into this Se pool. Methylselenol, on the other hand, is the proximal Se metabolite for induction of apoptosis without the genotoxicity elicited by hydrogen selenide* (Figure 1). It is striking that only a single methylation step separates the two forms of Se metabolites that are responsible for eliciting distinct biochemical and cellular responses.

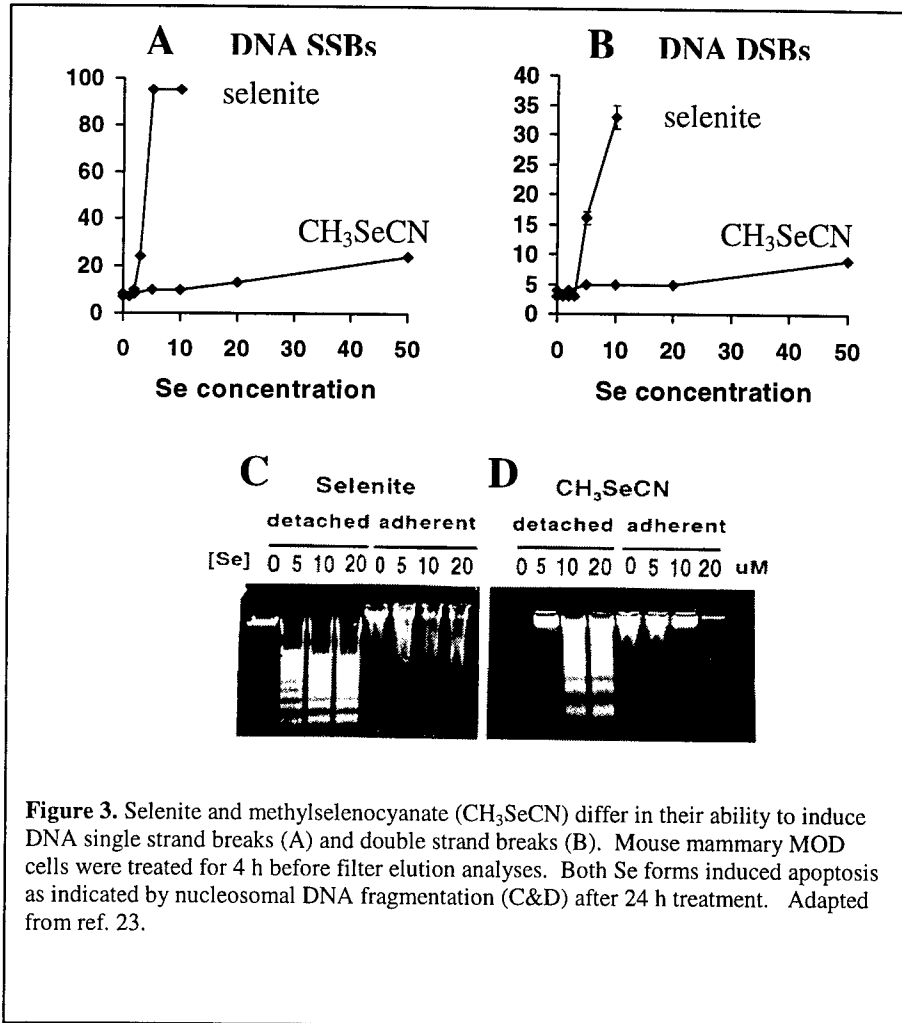


Figure 3. Selenite and methylselenocyanate (CH₃SeCN) differ in their ability to induce DNA single strand breaks (A) and double strand breaks (B). Mouse mammary MOD cells were treated for 4 h before filter elution analyses. Both Se forms induced apoptosis as indicated by nucleosomal DNA fragmentation (C&D) after 24 h treatment. Adapted from ref. 23.

How does methylselenol induce apoptosis?

Very little is known at the present concerning the underlying mechanisms triggering apoptosis by methylselenol. Some insights are suggested by additional differences, besides genotoxicity, in the biochemical and cellular consequences of treatment with the two types of Se compounds (Table 2). The methylselenol precursors MSeCN, MSeC and MSeA exert a moderate anti-proliferative effect as assessed by ³H-thymidine incorporation into DNA and arrest cells in the G₁ phase of the cell cycle, whereas selenite

rapidly blocks DNA synthesis and arrests cells in the S phase (24,25 and unpublished data). Consistent with G₁ arrest by methylselenol, MSeC has been shown to inhibit key cyclin dependent kinases (cdks) whereas selenite lacks such an effect (26). We have reported a differential induction of the *growth arrest and DNA damage inducible (gadd)* genes by selenite and MSeCN in mammary cancer cells (25), although no induction of *gadd* genes was detected in mammary tumors induced to regress by Se *in vivo* (unpublished data).

Table 2. Representative data illustrating the distinct response patterns of MOD cells to selenite (5 μ M) and methylselenocyanate (CH₃SeCN, 5 μ M)

Parameters	Selenite		CH ₃ SeCN	
	4 h	24 h	4 h	24 h
Cellular Se, ng/10 ⁶ cells	24	36	4	4
Thymidine incorporation ¹	16	7	59	45
Adherent Cell number ¹	93	44	98	41
DNA single strand breaks ²	12.5	5.3	1	0.7
DNA double strand breaks ²	6.4	3.5	1	2.4
Membrane leakage ²	3.8	7.4	1.3	3.3
Cell cycle disturbance	No	S-arrest	No	G ₁ arrest
<i>gadd153</i> gene expression	-	+++	+++	-
<i>gadd34</i> gene expression	+	+++	++	+
<i>gadd45</i> gene expression	+	+++	++	+
Morphological cell death	Acute lysis	Apoptosis	No change	Apoptosis

¹untreated control=100

²untreated control = 1

In a recent review article, Ganther proposed several potential chemical reactions in which methylselenol may participate that can either directly modify protein activities or through regulation of gene expression (5). The relevance of these reactions remains to be established. Much work is needed to elucidate the underlying mechanism(s) through which methylselenol regulates cancer cell growth and survival.

SELENIUM AND ANGIOGENESIS REGULATION

It is now well recognized that angiogenesis, i.e., the growth of capillary vessels from existing ones, is obligatory for the growth, progression and metastasis of solid cancer (27,28). During solid cancer carcinogenesis, initiated cells undergo clonal expansion in an avascular state when the expanding lesions are small enough to take in nutrients and to expel metabolic

wastes by diffusion. However diffusion is not sufficient to support continued growth of the lesion beyond a certain physical size (estimated ~2 mm diameter) as the expanding lesions consume nutrients at a rate proportional to their volume whereas the supply of nutrients is delivered at a rate proportional to their surface area (34,35). In order for avascular lesions to progress beyond the size limit imposed by simple diffusion, they must turn on the angiogenesis switch to form a neo-vasculature (29). Because of the critical dependence of tumor growth and metastasis on angiogenesis, therapeutic strategies are being developed targeting various aspects of the angiogenic processes, many with promising results. We have recently initiated work to explore the hypothesis that Se may exert cancer chemopreventive activity, at least in part, through an anti-angiogenic mechanism (4).

Mammary cancer prevention by Se is associated with decreased microvessel density (4)

In a chemoprevention setting, Se (3 ppm) as either Se-garlic (Experiment 1) or selenite (Experiment 2) was fed for ~2 months to Sprague-Dawley rats that were given a single i.p. injection of methylnitrosourea (MNU) to initiate mammary carcinogenesis a week earlier. The microvessels in the mammary tumors were visualized with immunohistochemical staining for Factor VIII and the microvessel number (counts/0.5 mm², 10 fields) in "hotspot" stromal areas was counted. Mammary carcinomas in the Se-fed rats was 34% (Expt 1) and 24% (Expt 2) lower than in those of rats fed the control diet (Table 3).

Table 3. Effects of a chemopreventive level of dietary Se as either Se-garlic (EXPT 1) or selenite (EXPT 2) on the microvessel density (counts/0.5 mm²) of 1-methyl-1-nitrosourea-induced rat mammary carcinomas and non-involved mammary glands. Adapted from Ref. 4.

Dietary group		Large φ>9	Medium φ5-9	Small φ1-4 cells	Total vessels
Experiment 1					
Carcinomas					
Control	n=9	5±1	10±1	55±6	69±6
Se-garlic	n=6	3±1	8±2	35±6	46±6
Mammary glands					
Control	n=6	1.8±0.5	2.7±0.4	4.2±0.8	8.7±0.7
Se-garlic	n=6	1.3±0.4	2.0±0.7	3.8±0.6	7.2±0.9
Experiment 2					
Carcinomas					
Control	n=8	0.9±0.4	4±2	75±5	80±4
Selenite	n=4	0.3±0.3	4±3	57±2	61±3

Mean ± sem. Bold face pairs are significantly different (p<0.05).

When categorized by the size of the microvessels, the observed reduction of microvessel density in the Se-fed groups was almost exclusively confined to the small microvessels (1-4 cells in diameter). The microvessel density of the uninvolved mammary glands was not decreased by Se-garlic treatment (Table 3). Similar results were obtained when established mammary carcinomas were treated acutely through bolus doses of Se (4). These results indicated a potential anti-angiogenic effect of chemopreventive intake of Se and that the effect was neoplasia-specific. Because growing and newly sprout microvessels are likely to be smaller, the observed reduction of small vessels by Se treatment indicated that mechanism(s) governing the genesis of new vessels might be inhibited.

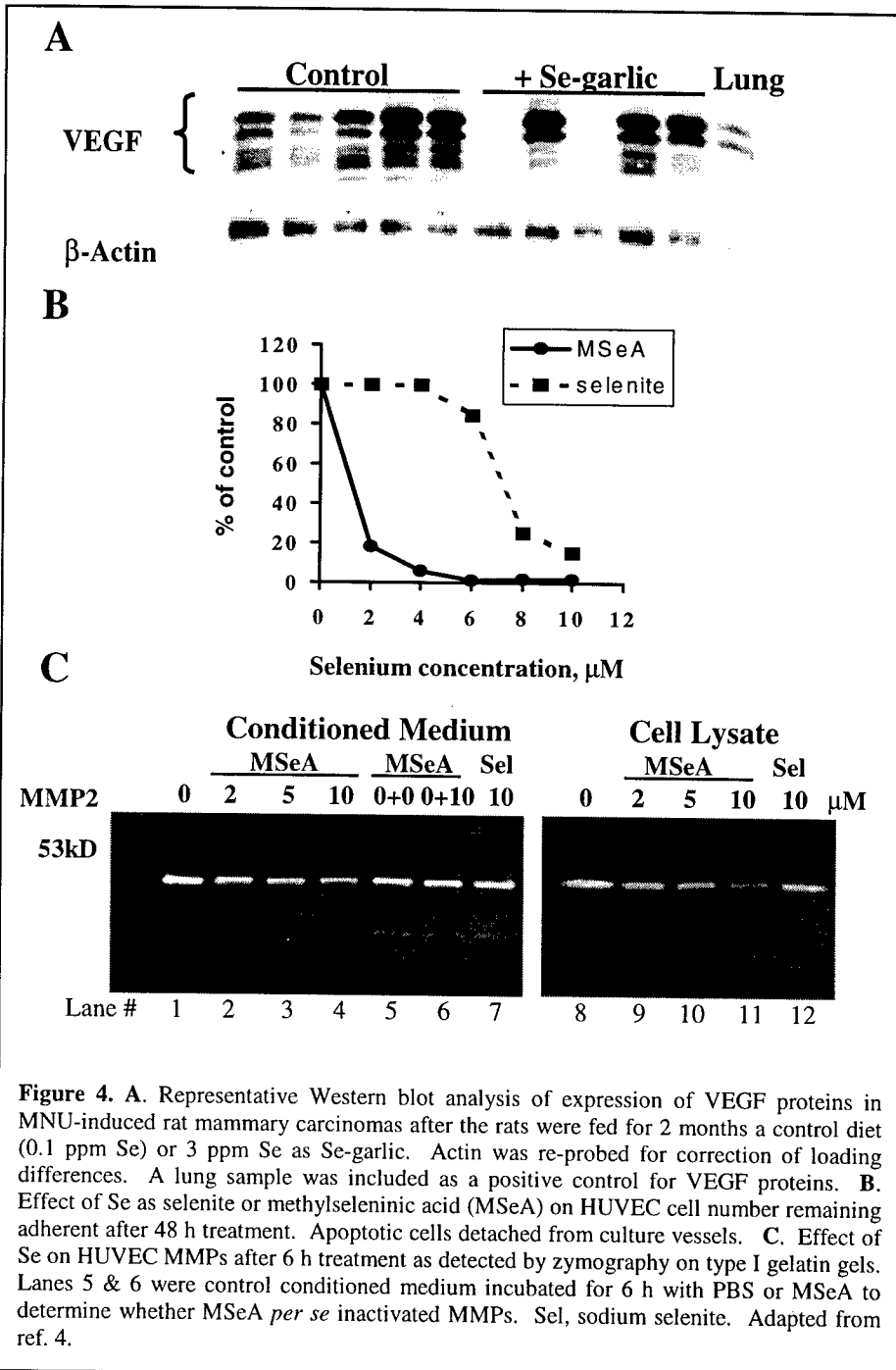
Multiple mechanisms are likely for the anti-angiogenic activity (4)

Sustained angiogenesis depends on the concerted coordination and participation of the following processes (27-29). The angiogenic stimulus (angiogenic factors such as VEGF, FGF, hypoxia, etc.) must be maintained; the endothelial cells must secrete MMPs required to break down the extracellular and adjacent tissue matrix; the endothelial cells must be capable of movement/migration; and endothelial cells must proliferate to provide the necessary number of cells for the growing vessels. To define the potential contribution of these elements, we have examined the expression of VEGF, a primary angiogenic mediator, in Se-treated tumors and also the effects of direct Se exposure in cell culture on the proliferation and survival and MMPs of human umbilical vein endothelial cells (HUVEC). Preliminary results are summarized below.

(A) *Selenium decreases expression of VEGF in some carcinomas.* The expression level of VEGF in mammary carcinomas was measured by Western blot analyses (Figure 4A). Based on the limited number of samples analyzed, 2 out of 5 carcinomas in the Se-garlic group and 2 out of 4 carcinomas in the selenite group (4) showed a marked reduction in VEGF expression to almost non-detectable levels. These results indicated that VEGF down regulation might be involved in some, but not all, tumors. Similar to the chemoprevention setting, acute Se treatment of established mammary carcinomas showed a marked reduction of VEGF expression in some, but not all, treated carcinomas (4).

(B) *Selenium induces apoptosis of endothelial cells.* Treatment of HUVEC with MSeA in monolayer culture led to cell retraction and detachment from flask and such changes started to appear 12 h after treatment was initiated. Most affected cells displayed morphological apoptotic features as indicated by nuclear condensation and formation of apoptotic bodies. Re-plating these detached cells in fresh medium did not result in any cell attachment or growth. By 48 h of MSeA treatment, adherent cell number was reduced by as much as 80% at 2 μ M (a level achievable in human blood) and

virtually no cell remained attached at 6 μM MSeA (Figure 4B). The cytotoxic effect of MSeA was ~4 fold more efficacious than selenite.



This comparison indicated that the inhibitory effect did not result from a direct reaction of MSeA *per se* with the secreted MMPs and was therefore dependent on cell activation to generate methylselenol. This (C)

(C) *Methylselenol specifically inhibits endothelial MMPs.* Treatment with MSeA led to a Se concentration-dependent inhibition of the gelatinolytic activity in both the conditioned medium and the cell lysate (Figure 4C) of the 72kD gelatinase A/MMP-2 and a 53 kD MMP. Methylselenocyanate (MSeCN) treatment had a comparable inhibitory potency as MSeA (unpublished data). 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 MMPs (lanes 7 vs. 6). This comparison indicated that the inhibitory effect did not result from a direct reaction of MSeA *per se* with the secreted MMPs and was therefore dependent on cell activation to generate methylselenol. This postulation was consistent with a time course experiment in which the inhibitory action of MSeA on MMPs followed a 10 min delay, probably a reflection of time needed for this active Se metabolite to reach a critical intracellular level (unpublished data).

In contrast to methylselenol precursors, treatment with 10 µM selenite for 6 h did not inhibit the MMPs (Figure 4C, lanes 7 vs. 1 and 12 vs. 8). Na₂Se had no inhibitory effect when provided at a concentration (~25 µM) that produced a similar extent of cell number reduction as MSeA (unpublished data). These results indicate that the proximal Se metabolite for MMP inhibition is methylselenol and that the MMP inhibitory effect is independent of apoptosis, which requires continued exposure of greater than 12 h. This discovery not only provides a specific mechanism for methylselenol to inhibit angiogenic sprouting (30,31), but also has mechanistic implications on processes, such as lesion progression and metastasis, that require MMPs for extracellular matrix degradation and remodeling (32,33).

CONCLUSIONS AND IMPLICATIONS

The data reviewed above support the apoptogenic activities of two pools of proximal Se metabolites, hydrogen selenide and methylselenol, that are mediated by distinct biochemical and cellular pathways. Compared to our understanding of the sequence of events in selenite/selenide induced growth arrest and apoptosis, much work is needed to elucidate the biochemical and molecular mechanisms underlying the apoptogenic activity of the methylselenol pool. Furthermore, the data support a methylselenol-specific inhibitory activity on endothelial MMPs. In addition to its involvement in angiogenesis regulation, methylselenol may therefore have a potential inhibitory activity on lesion progression and metastasis as they represent processes that require MMPs for extracellular matrix degradation and

remodeling. The MMP inhibitory activity of methylselenol may underlie the increased *in vivo* efficacy of forms of Se that are precursors of this Se pool in comparison to selenite and other Se-amino acids (1). Considering the potential mutagenic side effects of genotoxicity on normal cells, Se compounds that feed into the hydrogen selenide pool may be less desirable for chemoprevention use by humans. Conversely, Se compounds that enter the methylselenol pool may be more desirable Se forms for human applications.

An Integrated Model for Cancer Prevention by Se

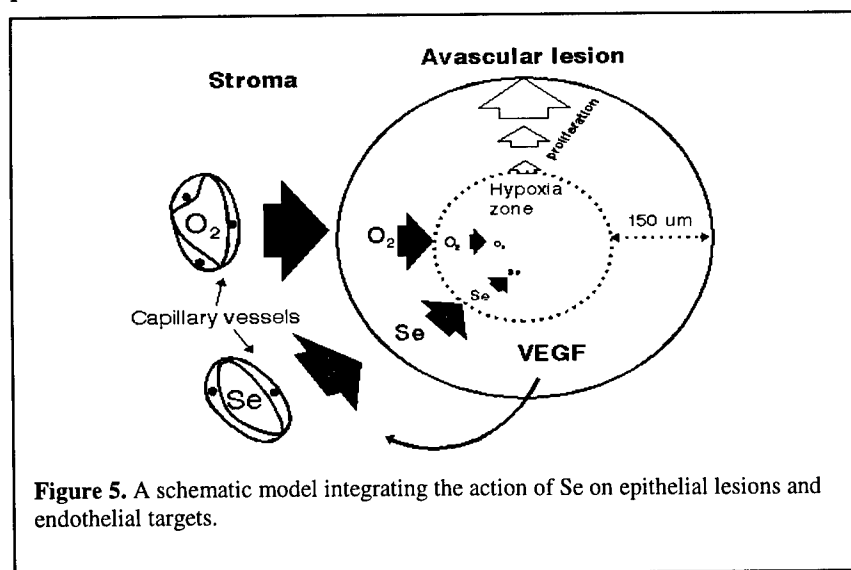
Much mechanistic research effort has so far focused on how Se affects the cancer epithelial cells. Because epithelial lesions do not exist in isolation *in vivo*, but instead intimately interact with the stroma and vasculature, cancer prevention mechanisms by Se should and must integrate the actions of Se on epithelial as well as non-epithelial targets. Figure 5 schematically illustrates a model based on this thesis. The angiogenesis aspect of the work reviewed here is consistent with a potential for Se to regulate non-epithelial targets.

It is probable that apoptogenic activity in the cancer epithelial cells may be triggered by Se through mechanisms reviewed above, if the Se metabolites (hydrogen selenide or methylselenol) can reach critical concentrations in the target cells. However, the physio-chemistry of Se delivery to transformed epithelial cells in *in vivo* lesions may be a major determinant of the actual mechanism(s) as well as the process(es) that are invoked to regulate the epithelial cell fate and growth. It is speculated that Se delivery to epithelial cells in the avascular lesions may follow a concentration gradient similar to oxygen tension that has been known to decline precipitously as the distance to the nearby microvessel increases, resulting in hypoxic state in the interior of such lesions (34,35) (Figure 5). Should such a declining gradient exist for Se, a "conditional Se deficiency" state may be created inside expanding avascular epithelial lesions even when the Se supply is sufficient to saturate selenoprotein activities in the serum or normal tissues. This model predicts that more Se is required to enrich the Se metabolite pools in the avascular lesions in order to elicit the anti-proliferative and apoptotic pathways in the transformed epithelial cells. This model may warrant a re-evaluation of the current paradigm that discounts the likelihood of involvement of selenoproteins for the chemopreventive activity of Se (1,2). For example, is it possible that Se intake much higher than that is required to saturate serum/tissue selenoproteins will be needed to optimize the activity of key selenoproteins such as thioredoxin reductase and Se-Gpx's in the epithelial lesions so as to re-regulate their transformation status/physiology?

This model also highlights the need for investigations that incorporate hypoxia as a feature of the solid lesions when evaluating the efficacy of Se to induce growth arrest and cell death responses. Hypoxia is known to affect cellular energy metabolism (34,35) and therefore it may affect the cellular redox status. It would be important to address whether a hypoxic state alters the apoptogenic efficacy of the different pools of Se metabolites. Hypoxia is also a known potent stimulus for the angiogenic switch. An inhibitory activity by Se on the ability of the epithelial lesions to produce angiogenic factors is an avenue through which Se may regulate angiogenesis and cancer. The observed effect of Se on VEGF expression is consistent with this thesis.

In contrast to the Se delivery physics in epithelial lesions, the endothelial cells in the vasculature are the first line of exposure to blood Se. Direct anti-proliferative and apoptogenic action on vascular endothelial cells as well as inhibitory activity on their MMPs by methylselenol may therefore present a likely and perhaps more significant avenue for Se to inhibit avascular lesion expansion/growth through blocking angiogenesis, provided that the Se actions are preferential against tumor/neo-angiogenesis. The angiogenic microenvironment during clonal expansion and lesion progression may provide a plausible biological basis for such desired selectivity. Sustained angiogenic stimulation provided by the transformed cells in the lesion sites will elicit concerted actions of MMPs, motility and cell division for angiogenic sprouting. It is possible and probable that angiogenically-stimulated endothelial cells, not the quiescent ones in the normal vessels/tissues, may be preferentially targeted for action by blood Se.

It is my hope that the model presented here can serve as a new paradigm to stimulate Se research in both the non-epithelial and the epithelial targets. With such an integrated approach, a more comprehensive mechanistic understanding may be achieved on how Se exerts its cancer preventive activity.



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