Antimitogenic and Proapoptotic Activities of Methylseleninic Acid in Vascular Endothelial Cells and Associated Effects on PI3K-AKT, ERK, JNK and p38 MAPK Signaling

Zaizen Wang, Cheng Jiang, Howard Ganther, and Junxuan Lu

AMC Cancer Research Center, Denver, Colorado 80214 [Z. W., C. J., J. L.], and University of Wisconsin, Madison, Wisconsin 53706 [H. G.]

ABSTRACT

Inhibiting the mitogenic response of vascular endothelial cells may in part mediate the antiangiogenic and anticancer activity of supranutritional selenium supplements. Our previous work had shown that methylseleninic acid (MSeA), a precursor of the critical anticancer methylselenol metabolite pool, was a potent inhibitor of the growth and survival of human umbilical vein endothelial cells (HUVECs). Here we investigated the effects of MSeA on selected protein kinase signaling transduction pathways to characterize their role in methylselenium induction of HUVEC cell cycle arrest and apoptosis. Exposure of asynchronous HUVECs for 30 h to 3–5 μM MSeA led to a prolonged G1 arrest, and exposure to higher levels of MSeA not only led to G1 arrest but also to DNA fragmentation and caspase-mediated cleavage of poly(ADP-ribose)polymerase, both biochemical hallmarks of apoptosis. Immunoblot analyses indicated that G1 arrest induced by the sublethal doses of MSeA was associated with dose-dependent reductions of the levels of phospho-protein kinase B (also known as AKT or PKB), phospho-extracellular signal regulated kinase signal regulated kinase (ERK) 1/2, and phospho-Jun NH2 -terminal kinases 1/2 in the absence of any change in p38 mitogen-activated protein kinase (MAPK) phosphorylation. Apoptosis induced by MSeA was associated with an increased phosphorylation of p38 MAPK in addition to the dephosphorylation of the above kinases. In HUVECs deprived of endothelial cell growth supplement (ECGS) for 48 h, resumption of ECGS stimulation resulted in an ~10-fold increase in mitogenic response, as indicated by [3H]thymidine incorporation into DNA. The ECGS-stimulated mitogenic response was inhibited in a dose-dependent manner by MSeA exposure with an IC50 ~1 μM and a complete blockage at 3 μM. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K) upstream of AKT, potently inhibited the ECGS-stimulated DNA synthesis (IC50 ~ 40 nM). Combining MSeA with Wortmannin showed an additive antimitogenic effect. An inhibitor of MAPK/ERK kinase 1, PD98059, also inhibited ECGS-stimulated DNA synthesis (IC50 ~ 55 μM), but combining PD98059 with MSeA had an effect similar to that when PD98059 was used alone. A time-course experiment indicated that PI3K (AKT and ribosomal protein S6 kinase) activation occurred between 6 and 12 h of ECGS stimulation, and 3 μM MSeA exposure decreased AKT phosphorylation after 12 h of exposure, whereas no inhibitory effect was observed for ERK1/2 phosphorylation throughout the 30-h exposure duration. Additional experiments indicated that MSeA, Wortmannin, or a more specific PI3K inhibitor, LY294002, seemed to target, in the mid- to late-G1 phase, a common mechanism(s) controlling G1 progression to S while having no inhibitory effect on DNA synthesis once S-phase had initiated. Taken together, the results support a potent inhibitory activity at achievable serum levels of MSeA on ECGS-stimulated mitogenesis in the mid- to late-G1 phase, and the target(s) of this inhibitory activity seems to be PI3K or components of this signal pathway. At pharmacological levels of exposure, modulation of ERK1/2 and other protein kinases may be relevant for the proapoptotic action of MSeA.

INTRODUCTION

Sustained angiogenesis is obligatory for the genesis and progression of solid tumors (1–3). One of the key angiogenic responses upon stimulation of vascular endothelial cells with polypeptide angiogenic factors is signaling through receptor protein kinase pathways leading to cell cycle entry and progression of the normally quiescent vascular endothelial cells to provide sufficient number of cells for the growing capillaries (1–3). Agents that interfere with endothelial cell mitogenesis and survival can therefore be of significant cancer chemopreventive potential and utility. In this regard, we have previously reported (4, 5) that MSeA3, a novel penultimate precursor of the putative active chemopreventive selenium metabolite methylselenol pool (6–8), exerted a potent inhibitory action on the growth and survival (through apoptosis) of HUVECs. Such an inhibitory activity provides a potential mechanism to account for the observed antiangiogenic and cancer chemopreventive activity of selenium (4). However, how the antimitogenic and proapoptotic effects of the methylselenol pool are mediated in the vascular endothelial cells and, more specifically, whether protein kinase signaling pathways are involved in mediating these activities have yet to be investigated.

The primary function of vascular endothelial cells as lining of blood vessels requires that their mitogenic signaling and responses be different from most other cell types with reference to typical polypeptide growth factors such as platelet-derived growth factor and epidermal growth factor. Such specificity ensures the essential quiescent state of vascular endothelial cells in mature individuals or organs until angiogenesis is called for, such as in wound healing or carcinogenesis. The unique mitogenic signaling behavior of vascular endothelial cells is in part attributable to their possession of special receptors for endothelial-specific mitogens such as VEGF (9). Much work has focused on VEGF signaling through its receptors, which belong to the platelet-derived growth factor receptor-family of receptor tyrosine kinases. Upon activation, these receptors dimerize and/or oligomerize, after which autophosphorylation and transphosphorylation of their tyrosine residues in the intracellular domain occur. These phospho-tyrosine molecules act as docking sites for adaptor signaling molecules and nonreceptor tyrosine kinases, generating signal cascades that culminate into vascular endothelial cellular responses such as mitogenesis, hyperpermeability, increased motility, and matrix degradation through matrix metalloproteinases (10).

Several protein kinase cascades (11–14) have been investigated for their involvement in the vascular endothelial mitogenic, apoptotic, and other responses (15–24). The PI3K is a heterodimer of a M1 85,000 (p85) adaptor subunit and a M1 110,000 (p110) catalytic subunit (11). Activated p110 catalyzes the phosphorylation of mem-

1 This work was supported in part by grants from the Department of Defense and National Cancer Institute (to J. L.).

2 To whom requests for reprints should be addressed, at AMC Cancer Research Center, 1600 Pierce Street, Denver, CO 80214. Phone: (303) 239-3348; Fax: (303) 239-3560; E-mail: luj@amc.org.

3 The abbreviations used are: MSeA, methylseleninic acid; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; PI3K, phosphatidylinositol-3-kinase; PDK, phosphatidylinositol-dependent kinase; PKB, protein kinase B (also known as AKT); S6K, ribosomal protein S6 kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; MEK, MAPK/ERK kinase; P38 MAPK, also known as stress-activated protein kinase 2; SAPK, stress-activated protein kinase; ECGS, endothelial cell growth supplement; JNK, Jun NH2-terminal kinase; PARP, poly(ADP-ribose)polymerase; TCA, trichloroacetic acid.
brane phosphatidylinositol 4,5-bisphosphate in the D3 position to generate phosphatidylinositol 3,4,5-trisphosphate. Phosphatidylinositol 3,4,5-trisphosphate and its phospholipid phosphate product, phosphatidylinositol 3,4-bisphosphate, accumulate in the membrane, creating docking sites for two lipid-binding protein kinases, namely PDK1 and Akt, which bind to these lipids via their pleckstrin homology domains. Akt becomes activated as a result of this plasma membrane localization and by its phosphorylation on both Thr308 and Ser473 catalyzed by PDK1 and an unidentified but provisionally named PDK2, respectively. Once activated, Akt can inhibit apoptosis by a number of actions, including phosphorylation and inactivation of the proapoptotic Bcl-2 homologue Bad (20, 21), the apoptosis-initiating enzyme caspase-9 (22), and the forkhead family transcription factor that mediates transcription of proapoptotic gene products (23). The PI3K and its other downstream substrate, S6K, have been shown to mediate the stimulatory effects of VEGF or serum on DNA synthesis in HUVECs and other endothelial cells (15–17, 24).

The classical MAPK/ERK pathway is a key component in the transduction of signals leading to growth and transformation in many cell types (12, 13). It consists of a linear cascade of protein kinases: Raf, MEK, and MAPK/ERK. ERK1/2 are actively autophosphorylated upon growth factor stimulation. The ERK pathway has been shown to contribute to the mitogenic responses of HUVECs to VEGF or serum (15–17). In addition to the PI3K and ERK pathways, the p38 MAPK/SAPK2 pathway seemed to mediate the motility-stimulating effects of VEGF with a concomitant antimitogenic action in HUVECs (16, 18). In numerous cell lines, the JNK/SAPK1 pathway as well as the p38 MAPK pathway are involved in apoptosis signaling and regulation (14). The interplay of the signals from the various pathways in turn command cell cycle entry and progression by modulating the balance of cyclins and cyclin-dependent kinase inhibitors within cyclin-dependent kinase-cyclin complexes, which in turn inactivate retinoblastoma protein by phosphorylation and G1 transition, leading to DNA replication and mitosis (25).

The objective of this study was to define the effects of MSeA on mitogenesis and protein kinase signaling in the HUVEC model to identify potential target pathways/molecules for the methyl selenium action. We have chosen as endothelial mitogen for the present work, ECGS, a bovine pituitary extract probably made up of a mixture of multiple angiogenic factors (26, 27). This was based on the rationale that tumor angiogenesis would likely be driven by multiple angiogenic factors in addition to VEGF.

MATERIALS AND METHODS

Chemicals and Reagents. MSeA was synthesized as described elsewhere (4). MSeA most likely reacts with reduced glutathione intracellularly to form intermediates that can undergo additional reduction to methylselenol (CH3-SeH; 28). Bovine ECGS, heparin, PD98059, Wortmannin, and an antibody for β-actin were purchased from Sigma Chemical Co., St. Louis, MO. LY294002 was purchased from CalBiochem-Novabiochem Corp., La Jolla, CA. [Methyl-3H]thymidine (20 Ci/mmol) was purchased from DuPont-New England Nuclear, Wilmington, DE. Recombinant human VEGF165 was obtained from Becton Dickinson, Bedford, MA. HUVECs were obtained from American Type Culture Collection, Manassas, VA, and used within 15 passages upon receipt. Antibodies specific for cleaved PARP (p89), caspase-3, and cleaved caspase-7 and those for protein kinases and their phosphorylated forms (AKT Ser473, S6K Thr241Ser242, ERK1/2 Thr202/ Tyr204, MEK1 Ser217/221, JNK Thr183/Tyr185, and P38 MAPK Thr180/Tyr182) were purchased from Cell Signaling Technology, Beverly, MA.

Cell Cycle Distribution and Apoptosis Evaluation. HUVECs were propagated in F12K medium containing 10% fetal bovine serum, 2 mm l-glutamine, 100 µg/ml of heparin, and 30 µg/ml of bovine ECGS, as described previously (4, 5). HUVECs were seeded in T25 or T75 flasks at 60–70% confluence and were treated in fresh complete medium with increasing concentrations of MSeA for 30 h or as otherwise specified. Detached floaters and adherent cells were pooled together and analyzed for cell cycle distribution by flow cytometry and for DNA nucleosomal fragmentation. DNA was extracted and analyzed as previously described (29). Cleavage of PARP (30), caspase-3, and caspase-7 as evidence of caspase-mediated apoptosis was analyzed by immunoblotting with antibodies specific for the cleaved products as we have previously described (31). To standardize Se exposure among different cell culture vessels, 0.2 ml of medium was used per cm2 of vessel surface (e.g., 15 ml for a T75 flask, 5 ml for a T25 flask).

HUVEC Synchronization and [3H]thymidine Incorporation into DNA. HUVECs were seeded in T25 flasks in complete growth medium until 70–80% confluent and then were fed the above medium without ECGS for 48 h. To determine the effect of MSeA on ECGS-stimulated cell proliferation, [3H]thymidine (0.4 µCi/ml) and ECGS were added to the synchronized cells simultaneously. The DNA synthetic activity was measured as [3H]thymidine cumulative incorporation into the TCA-precipitable fraction during 30 h of ECGS stimulation, unless otherwise specified as in selected time course experiments.

Immunoblot Analyses. Cell lysates were prepared in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM dithiothreitol, 5 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride and 38 µg aprotinin/ml were added fresh]. Supernatants after centrifugation (14,000 g × 20 min; 4°C) were recovered and the protein content was quantified by the Bradford dye-binding assay (Bio-Rad Laboratories, Richmond, CA). Six or 20 µg of total protein was size-separated by electrophoresis on 10 or 12% SDS-polyacrylamide gels, depending on the size of the target protein being investigated. The proteins were electroblotted onto nitrocellulose membranes and probed with antibodies for the phosphorylated AKT, ERK1/2, S6K, P38 MAPK, or JNK and those for cleaved caspase-3, caspase-7, or cleaved PARP. Membranes were stripped by incubation in Re-Blot 1× antibody stripping solution (Chemicon International, Inc., Temecula, CA) for 20 min at 28°C and reprobed for the respective total protein kinase content or β-actin for verifying loading evenness.

RESULTS

Part I: Effects of MSeA in Asynchronous Cells

MSeA Induced G1 Arrest at Low Exposure Level and Caspase-Associated Apoptosis at High Level. Exposure of asynchronous HUVECs in complete growth medium (10% serum, 30 µg/ml ECGS and other endothelial additives) for 30 h to 3–7 µM MSeA led to an enrichment of cells in G1-G2 phase of the cell cycle and a significant reduction of cells in S and G2-M phases (Fig. 1). The decrease of the fraction of the proliferating cells (i.e., S plus G2-M) was 44, 60, and 52% for the MSeA exposure concentration of 3, 5, and 7 µM, respectively. Exposure of HUVECs to higher levels (e.g., 10 µM or greater) of MSeA led not only to G1 arrest but also to cell death by apoptosis as indicated by an increase in the sub-G1-G2 fraction of apoptotic bodies (Fig. 1). Biochemically, apoptosis was characterized as DNA nucleosomal fragmentation (Fig. 2A) and cleavage of PARP (Fig. 2B), a key substrate of apoptosis executioner caspases (30, 31). The executioner caspases (e.g., caspase-3 and caspase-7) were cleaved (indicative of activation) in a dose-dependent manner to MSeA exposure (Fig. 2B). These results indicated that exposure to a level of MSeA >5 µM induced caspase activation and apoptosis in asynchronous HUVECs.

MSeA Modulated Phosphorylation Status of Multiple Protein Kinases. To determine the potential involvement of protein kinase pathways in MSeA-induction of G1 arrest and caspase-mediated apoptosis, we surveyed the phosphorylation status (indicative of activation states) of the four major protein kinases after asynchronous HUVECs were exposed to increasing concentrations of MSeA for 30 h (Fig. 3). Exposure to subapoptotic doses of MSeA (3–5 µM) led to a dose-dependent reduction of the levels of phospho-AKT (Ser473),

7172

Downloaded from cancerres.aacrjournals.org on January 22, 2018. © 2001 American Association for Cancer Research.
phospho-ERK1/2 (Thr202/Tyr204), and phospho-JNK1/2 (Thr183/Tyr185) and did not change p38 MAPK phosphorylation (Thr180/Tyr182) status. The observed reduction of phosphorylation status in response to MSeA exposure was not caused by a decrease of the total protein content of the respective protein kinases (Fig. 3).

In cells exposed to an apoptogenic level of MSeA (e.g., 10 μM), an increased level of phosphorylation of p38 MAPK was observed in addition to AKT, ERK1/2, and JNK dephosphorylation. These results indicated that MSeA-induced modulation of AKT, ERK1/2, and JNK kinase pathways were associated with G₁ arrest, whereas such alterations in concert with an enhanced p38 MAPK phosphorylation were likely involved in the proapoptotic action of MSeA in HUVECs.

Part II. Antimitogenic Effects of MSeA in ECGS-Stimulated Cells

ECGS-stimulated Cell Cycle Progression Model. To more sensitively define the G₁ arrest activity of MSeA on HUVECs and the signal transduction mechanisms involved, next we examined effects of MSeA in an ECGS-depletion and stimulation model. To verify the mitogenic response of this model, ECGS was omitted from the complete growth medium for ~48 h to partially synchronize HUVECs and its stimulatory effect was compared with that of recombinant VEGF at 20 ng/ml, a level that has been shown to produce maximal mitogenic stimulation on HUVECs (15–17), during a 24-h stimulation period (Fig. 4A). ECGS treatment stimulated DNA synthesis, assessed as [³H]thymidine incorporation into TCA-precipitable DNA, by ~10-fold (Fig. 4A). In comparison, VEGF stimulation increased DNA synthesis by only ~90% (Fig. 4A). These results indicated that ECGS was a much stronger mitogen than VEGF for the HUVECs in vitro.

Flow cytometry analyses of cell cycle distribution after ECGS depletion in the presence of 10% serum and other endothelial supplements showed that 73% cells were in G₀/G₁, 3% in S, and 24% in G₂-M phases, respectively (n = 3 flasks). Upon ECGS stimulation, the distribution pattern remained unchanged for 12 h and subsequently cells entered S-phase between 12 h and 18 h and the percentage of S-phase cells peaked at 24 h (Fig. 5A). The time course of [³H]thymidine incorporation mirrored the cell cycle distribution pattern, showing a sudden rise in DNA synthetic activity between 12 h and 18 h of ECGS stimulation (Fig. 5B). On the basis of these results, we standardized the ECGS-deprivation protocol for 48 h for assessing the MSeA effects in the rest of the work.

MSeA Potently Inhibited ECGS-stimulated G₁ to S Progression. MSeA treatment that was initiated either 3 h before (experiment 1) or simultaneously with ECGS stimulation (experiment 2) decreased...
E.g., ecological levels (independent of the proapoptotic action of MSeA exposure at pharmacologically stimulated HUVEC cell cycle progression and in MSeA-induced G$_1$-S progression of events involving PI3K and ERK1/2 pathways in ECGS-stimulated HUVECs.

In a time-course experiment designed to delineate the likely involvement of serum achievable levels of MSeA (IC$_{50}$ of $>$3 μM) and a complete blockage to the unstimulated basal level at 3 μM (Fig. 4B). Flow cytometry data (Fig. 5A) corroborated the DNA synthesis results (Figs. 4B and 5B) in that exposure to 3 μM MSeA completely blocked the progression of G$_1$ cells into S-phase.

Exposure to 7 μM or higher level of MSeA led to detectable caspase-mediated PARP cleavage in the synchronized HUVECs (Fig. 4C). Taken together, the [$^{3}$H]thymidine incorporation and cell cycle distribution results demonstrated an excellent inhibitory potency of MSeA on ECGS-stimulated cell cycle progression from G$_1$ to S-phase. Furthermore, the data indicated that the primary antimitogenic activity of serum achievable levels of MSeA (IC$_{50}$ of $>$1 μM) was independent of the proapoptotic action of MSeA exposure at pharmacological levels (e.g., >5 μM).

**Effects of MSeA on ECGS-stimulated PI3K and ERK Signaling Events.** In a time-course experiment designed to delineate the likely sequence of events involving PI3K and ERK1/2 pathways in ECGS-stimulated HUVEC cell cycle progression and in MSeA-induced G$_1$ arrest, ECGS stimulation did not significantly increase the phosphorylation of the PI3K targets AKT and S6K within the first 6 h of exposure, but increased AKT phosphorylation ($\sim$5×) and S6K phosphorylation ($\sim$8×) at 12 h (Fig. 6A). ECGS stimulated ERK1/2 phosphorylation by $\sim$4×, $\sim$10× and $\sim$8× after 6, 12, and 30 h, respectively (Fig. 6A). The above phosphorylation changes of AKT, S6K, and ERK1/2 occurred with little change in the total protein content of the respective protein kinases. In an experiment examining the phosphorylating status of MEK1 and ERK1/2 during acute exposure to ECGS, it was observed that MEK1 phosphorylation already peaked at 5 min of ECGS stimulation and declined gradually after 5 min (Fig. 6B). ERK1/2 phosphorylation plateaued within 5 min and was sustained throughout 30 min. ERK1 showed a slight decrease of phosphorylation afterward. Taken together, these data suggest that upon ECGS-stimulation, PI3K activation (indicated by AKT-, S6K-phosphorylation) was likely activated between 6 h and 12 h during mid- to late-G$_1$ progression; whereas MEK1-ERK1/2 activation was likely involved in signaling for G$_1$ entry within a few minutes of ECGS stimulation in this model.

The effects of MSeA exposure on these kinases were dependent on the dosage. At 3 μM, which completely blocked ECGS-stimulated G$_1$-S progression (e.g., [$^{3}$H]thymidine incorporation and flow cytometry data as shown in Figs. 4B and 5), MSeA did not decrease the levels of ERK1/2- or S6K-phosphorylation throughout the 30-h duration (Fig. 6A). This level of MSeA exposure decreased AKT phosphorylation of the PI3K targets AKT and S6K within the first 6 h of exposure, but increased AKT phosphorylation ($\sim$5×) and S6K phosphorylation ($\sim$8×) at 12 h (Fig. 6A). ERK1 showed a slight decrease of phosphorylation afterward. Taken together, these data suggest that upon ECGS-stimulation, PI3K activation (indicated by AKT-, S6K-phosphorylation) was likely activated between 6 h and 12 h during mid- to late-G$_1$ progression; whereas MEK1-ERK1/2 activation was likely involved in signaling for G$_1$ entry within a few minutes of ECGS stimulation in this model.

The effects of MSeA exposure on these kinases were dependent on the dosage. At 3 μM, which completely blocked ECGS-stimulated G$_1$-S progression (e.g., [$^{3}$H]thymidine incorporation and flow cytometry data as shown in Figs. 4B and 5), MSeA did not decrease the levels of ERK1/2- or S6K-phosphorylation throughout the 30-h duration (Fig. 6A). This level of MSeA exposure decreased AKT phosphorylation of the PI3K targets AKT and S6K within the first 6 h of exposure, but increased AKT phosphorylation ($\sim$5×) and S6K phosphorylation ($\sim$8×) at 12 h (Fig. 6A). ERK1 showed a slight decrease of phosphorylation afterward. Taken together, these data suggest that upon ECGS-stimulation, PI3K activation (indicated by AKT-, S6K-phosphorylation) was likely activated between 6 h and 12 h during mid- to late-G$_1$ progression; whereas MEK1-ERK1/2 activation was likely involved in signaling for G$_1$ entry within a few minutes of ECGS stimulation in this model.

The effects of MSeA exposure on these kinases were dependent on the dosage. At 3 μM, which completely blocked ECGS-stimulated G$_1$-S progression (e.g., [$^{3}$H]thymidine incorporation and flow cytometry data as shown in Figs. 4B and 5), MSeA did not decrease the levels of ERK1/2- or S6K-phosphorylation throughout the 30-h duration (Fig. 6A). This level of MSeA exposure decreased AKT phosphorylation of the PI3K targets AKT and S6K within the first 6 h of exposure, but increased AKT phosphorylation ($\sim$5×) and S6K phosphorylation ($\sim$8×) at 12 h (Fig. 6A). ERK1 showed a slight decrease of phosphorylation afterward. Taken together, these data suggest that upon ECGS-stimulation, PI3K activation (indicated by AKT-, S6K-phosphorylation) was likely activated between 6 h and 12 h during mid- to late-G$_1$ progression; whereas MEK1-ERK1/2 activation was likely involved in signaling for G$_1$ entry within a few minutes of ECGS stimulation in this model.

![Fig. 3. Effects of MSeA exposure of asynchronous HUVECs for 30 h on the phosphorylation status and expression level of selected protein kinases as detected by immunoblot analyses. β-Actin expression was reproped to indicate evenness of loading of protein extract from each treatment. Bold arrows, total proteins of expected sizes; thin arrows, respective phosphorylated kinases.](image)

![Fig. 4. A, a comparison of the mitogenic effect of endothelial cell growth supplement (ECGS, 30 μg/ml) versus VEGF (20 ng/ml) on HUVECs previously deprived of ECGS for 52 h. [$^{3}$H]thymidine was added at the time of treatment with ECGS or VEGF (or 24 h. [$^{3}$H]thymidine incorporation into DNA was measured by TCA precipitation and extensive washing and followed by liquid scintillation. Unstimulated basal activity was set as unity. Results represented mean and SD of triplicate flasks. Fresh serum-containing medium was added simultaneously with ECGS stimulation. Each experiment was done in duplicate flasks; results reflect mean values, with SD <10% of respective means. B, dose-dependent inhibitory effect of MSeA on ECGS-stimulated [$^{3}$H]thymidine incorporation into TCA-precipitable DNA. [$^{3}$H]thymidine was added at the time of treatment with ECGS for 30 h. In experiment 1, MSeA was added 3 h before ECGS stimulation. In experiment 2, MSeA was added simultaneously with ECGS stimulation. Each experiment was done in duplicate flasks; results reflect mean values, with SD <10% of respective means. C, immunoblot detection of cleaved PARP in synchronized HUVECs exposed to ECGS and MSeA for 30 h. Arrow, cleaved PARP. β-Actin was reprobed to indicate evenness of loading of protein extract from each treatment.](image)
phosphorylation at 30 h. MSeA exposure at 5 μM decreased ECGS-stimulated phosphorylation of ERK1/2 at 6 h, AKT and S6K (p70) phosphorylation at 12 h (Fig. 6A). During acute exposure (Fig. 6B), 10 μM MSeA treatment simultaneous with ECGS stimulation did not decrease MEK1 phosphorylation until 30 min of exposure and decreased phosphorylation of ERK1/2 at 3 h. These data therefore implicated an involvement of the inactivation of PI3K and/or ERK1/2 pathway activities for the antimitogenic action of MSeA, probably through a mechanism(s) well after the initial MEK-ERK mediated signaling had taken place.

**Effects of PI3K and MEK1 Inhibitors with MSeA on ECGS-stimulated DNA Synthesis.** To test the role of the PI3K and MEK-ERK1/2 pathways in ECGS-stimulated mitogenesis and MSeA-induced G1 arrest, we next examined the impact of a PI3K inhibitor, Wortmannin (Ref. 32) and a MEK1 inhibitor, PD98059. With Wortmannin preloaded for 1.5 h before ECGS stimulation, a potent inhibition of [3H]thymidine incorporation was observed with IC50 ~35 nM (Fig. 7A). PD98059 (preloaded for 1.5 h) also inhibited ECGS-stimulated DNA synthesis, but at relatively high concentrations (IC50, ~55 μM; Fig. 7A).

In a separate experiment (Fig. 7B), Wortmannin (40 nM) alone inhibited [3H]thymidine incorporation by ~60%, and PD98059 (60 μM) alone inhibited by ~72%. The two inhibitors combined completely blocked ECGS-stimulated DNA synthesis. These results indicated that the PI3K and MEK1-ERK pathways could independently contribute to ECGS-stimulated cell cycle entry and/or progression to S phase, during which [3H]thymidine was incorporated into the DNA. Exposure to 1 μM MSeA alone inhibited [3H]thymidine incorporation by ~38% (Fig. 7B). Combining MSeA with Wortmannin had a near additive inhibitory effect on ECGS-stimulated DNA synthesis (81%...
The mitogenic action of MSeA was exerted specifically during mid- to late-G1 phase.

Interestingly, Wortmannin became progressively more inhibitory on ECGS-stimulated DNA synthesis as the lag time between Wortmannin treatment and ECGS stimulation was increased from 0 to 12 h (Fig. 8B). Wortmannin treatment after 24 h of ECGS-stimulation was ineffective on DNA synthesis (Fig. 8B). Because of the known instability of Wortmannin in neutral pH (33), the data suggested that the onset time of PI3K pathway participation in ECGS-stimulated cell cycle progression was at mid- to late-G1 phase. This is because the longer the lag time between Wortmannin addition and ECGS stimulation, the greater the effective concentration of the inhibitor to block the PI3K activity that was required for mediating HUVEC progression from G1 to S phase. This conclusion was supported further by the pattern of inhibitory effect of a more stable and specific PI3K inhibitor, LY294002 (34), which showed a pattern that was identical to that of MSeA (Fig. 8C).

In contrast to the similarities of mid- to late-G1 arresting action shared among MSeA and the PI3K inhibitors, the MEK1 inhibitor PD98059 moderately inhibited DNA synthesis only when given simultaneously (i.e., with preloading for 1.5 h) with ECGS stimulation (Fig. 8D), and lost the inhibitory activity when provided at 6 h or later. The PI3K and MEK1 inhibitor data were consistent with the notion that MEK1-ERK1/2 signaling was an early event during ECGS-stimulated HUVEC cell cycle entry, rather than for mediating G1 progression; whereas PI3K activation was required during mid- to late-G1 to mediate signaling for G1 progression toward S phase.

**DISCUSSION**

Vascular endothelial cell proliferation is an essential component of the angiogenic responses. Inhibiting angiogenic factor-driven mitogenesis will therefore be an important means of achieving selective antiangiogenic action, because vascular endothelial cells in normal adult tissues are essentially quiescent. In this context, we have shown that serum-achievable levels of MSeA potently inhibited ECGS-stimulated HUVEC mitogenesis in vitro. The data established that when cycling HUVECs were exposed to subapoptotic levels of MSeA, they became arrested in the G1 phase (Fig. 1). Furthermore, in an ECGS-depletion/stimulation model, MSeA inhibited, in a dose-
dependent manner, ECGS-stimulated HUVEC cell cycle progression into S phase (measured as [3H]thymidine incorporation into DNA and by flow cytometry) with an IC50 of 1 μM and a complete blockage at 3 μM (Figs. 4B and 5). Such an excellent inhibitory potency was remarkable, considering that, in a recent cancer prevention trial (35), the average plasma selenium level of United States adults was ~1.5 μM, and selenium supplementation that was associated with a >50% reduction of prostate, lung, and colon cancer risks brought that level to ~2.4 μM.

Furthermore, the data show that when the exposure level exceeded the selenium level normally present in human serum, MSeA decreased HUVEC survival by the induction of apoptosis that involved caspase activation, PARP cleavage, and DNA fragmentation (Figs. 2 and 4C). The proapoptotic activity of MSeA reported here and elsewhere in a capillary histogenetic context when cultured on Matrigel (5) may be pharmacologically achievable and relevant for potential therapeutic applications of methylselenium for cancer treatment. The G1-specific antimitogenic activity and the caspase-mediated proapoptotic activity of methylselenium, along with its potent inhibitory action on endothelial matrix metalloproteinase-2 expression (4, 5) and cancer cell expression of VEGF (5), provide plausible and relevant metabolite-specific mechanisms to account for the antiangiogenic action of selenium that we have described recently (4).

An objective of the present work was to explore the role of the protein kinase signaling pathways in the antimitogenic and proapoptotic actions of methylselenium in vascular endothelial cells using HUVECs as a model. To this end, we have shown that MSeA exposure for 30 h dose-dependently modulated all four major mitogenic and survival pathways examined: i.e., AKT, ERK1/2, p38 MAPK, and JNK1/2 (Fig. 3). Specifically, G1 arrest induced by the exposure to subapoptotic doses of MSeA (less or equal to 5 μM) for 30 h was associated with dose-dependent reductions of the levels of phospho-AKT, phospho-ERK1/2, and phospho-JNK1/2. This absence of change in p38 MAPK phosphorylation is noteworthy that JNK1/2 phosphorylation (i.e., activation) was not increased, but rather was decreased, in MSeA-induced HUVEC apoptosis. This finding is in contrast with other well-established apoptosis models in which JNK activation has been shown to be crucial for apoptosis signaling (14).

In this regard, we have shown that MSeA-induced apoptosis of DU-145 prostate carcinoma cells did not involve JNK activation (31), whereas selenite-induced apoptosis was associated with an increased phosphorylation of both JNK and P38 MAPK (31). Furthermore, HUVEC apoptosis induced by higher levels of MSeA exposure (e.g., 10 μM or greater) was accompanied by an increased phosphorylation of p38 MAPK. These results suggest that the inhibition of PI3K, MEK-ERK1/2, and/or JNK pathways might be involved in the HUVEC G1 arrest activity of MSeA, whereas p38 MAPK induction in addition to the above kinase modulations might either be responsible for or a consequence of HUVEC apoptosis induced by MSeA.

Prompted by these observations, we analyzed the effects of MSeA in an ECGS-depletion/stimulation model of HUVEC cell cycle progression to more precisely delineate possible cause-effect relationships among inhibition of PI3K and/or MEK1-ERK1 pathways and G1 arrest activity. After establishing the approximate cell cycle parameters of this model (Fig. 5) and the phosphorylation (activation) and expression profiles of PI3K targets AKT and S6K as well as those for MEK1 and ERK1/2 (Fig. 6), we showed with pharmacological inhibitors of PI3K and MEK1 that these two pathways could independently contribute to ECGS-stimulated HUVEC mitogenesis (Fig. 7A). Furthermore, we showed that two PI3K inhibitors, despite their structural differences and distinct mechanisms of action (32, 34), recapitulated the mid- to late-G1, stage-specific arresting action of MSeA on ECGS-stimulated HUVEC cell cycle progression to S phase (Fig. 8).

To our knowledge, the current work provided several lines of evidence describing for the first time an antimitogenic action of a methylselenol precursor through a common mechanism(s) or target(s) shared with inhibitors of PI3K. First, MSeA exposure that was commenced after the cell cycle had progressed for 12 h was nearly as inhibitory as MSeA exposure that was initiated at the time of ECGS stimulation (Fig. 8A). The 12-h time point corresponded to late-G1 phase before S entry (Fig. 5). However, after 24 h of ECGS-stimulation, when the S phase was at peak occurrence (Fig. 5), MSeA exposure was totally ineffective at decreasing [3H]thymidine incorporation, indicating that MSeA did not inhibit DNA synthesis per se once cells had entered S phase (Fig. 8A). Second, the mid- to late-G1-specific action of MSeA was shared by PI3K inhibitors, Wortmannin (Fig. 8B) and LY294002 (Fig. 8C), but not by an MEK1 inhibitor, PD98059 (Fig. 8D). Specifically, the closer to G1-S boundary when Wortmannin was introduced, the greater its effectiveness at blocking S entry was observed (Fig. 8B). This increasing potency was consistent with the known instability of Wortmannin in neutral aqueous medium (33) and thereby a greater effective concentration of this inhibitor to inhibit PI3K for mediating G1-S transition when introduced at 12 h. The stable PI3K inhibitor LY294002, which is a competitive inhibitor of the ATP binding site (34), showed an identical pattern of inhibitory effect as MSeA (Fig. 8C). Despite a different mechanism of inhibition on PI3K from Wortmannin, which irreversibly binds to the M1, 110,000 catalytic subunit (32), the data based on LY294002 provided additional support for the above assertion based on Wortmannin data. This commonality of target pathway(s) of action during mid- to late G1 provided a plausible explanation of the additive inhibitory action of MSeA and Wortmannin when used together at low concentrations (Fig. 7B). Finally, the delayed onset of AKT and S6K phosphorylation (PI3K targets) after ECGS stimulation had proceeded for longer than 6 h but within 12 h suggested the participation of this pathway(s) in the mid- to late G1 to mediate G1 progression to S phase (Fig. 6A). The observation that MSeA exposure at 3 μM for >12 h decreased AKT phosphorylation (Fig. 6A) was consistent with an inhibition of PI3K itself or its upstream or downstream components by MSeA. The precise nature of the interactions between the monomethylated selenium pool and PI3K itself or other components in its pathways (i.e., methyl selenium targets) merits additional investigation.

The inhibitory activity of MSeA on G1 progression in HUVECs observed in our study seemed to be in good agreement with the finding of Sinha et al. in a synchronized mammary epithelial cell model (28). They have shown that MSeA exposure for a brief period (as short as 15 min) in the mid-G1 phase (6 h after release of G1 block in that model) inhibited subsequent [3H]thymidine incorporation in the mammary epithelial cells. When the exposure was started at 12 h, a time frame in that model corresponding to the start of S phase, MSeA failed to inhibit ongoing DNA synthesis. It was not yet established whether the PI3K pathway or other protein kinase pathways were involved in the mid-G1-specific action of MSeA in the mammary model.

Our results did not support ERK1/2 dephosphorylation as a mediating event for the antimitogenic action of serum-achievable levels of MSeA. Specifically, the time course experiment (Fig. 6A) indicated that the potent antimitogenic effect of 3 μM MSeA was observed in the absence of ERK1/2 phosphorylation change throughout the duration of 30 h. The antimitogenic activity of a low level (e.g., 1 μM) MSeA exposure was not additive with that of an MEK1 inhibitor (Fig. 7B). However, at higher levels of exposure that might be relevant pharmacologically (e.g., 5 μM or greater), MSeA could effectively inhibit the MEK1-ERK1/2 pathway (Fig. 6A), likely with a delayed kinetics of action after MEK1-ERK1/2 signaling had been accom-
ished (Fig. 6B). These observations indicated either an antimitogenic action of a low level of MSEA exposure that was totally independent of ERK1/2 or, if this pathway were involved, that the point of action by MSEA would have to be downstream of ERK1/2 phosphorylation mechanisms. Although nonspecific inhibitory action of kinase inhibitors on enzyme activities other than the purported target enzymes has been reported in other cell types (e.g., Ref. 36), the collective body of evidence, including that generated with these inhibitors in the present study, strongly supports a PI3K pathway-related inhibitory mechanism for the mid- to late-G1-specific arresting action of MSEA observed here.

In summary, the data support a potent antimitogenic action of achievable serum levels of MSEA on human vascular endothelial cells targeting a mechanism controlling G1 progression in the mid- to late-G1 phase of the HUVEC cell cycle. The target(s) seemed to be PI3K itself or other components of this pathway. In addition, the data suggest that the inhibitory effects of MSEA on additional protein kinase pathways such as ERK1/2 and JNK1/2 or activation effects on p38 MAPK could also be involved in vascular endothelial apoptotic responses in a pharmacological or therapeutic context of MSEA exposure.

ACKNOWLEDGMENTS

Cell cycle analyses were performed at the flow cytometry core facility of the University of Colorado Health Sciences Center Comprehensive Cancer Center, of which J. L. is a member. We thank Karen Helm and core facility personnel for their professional service. The authors also thank Dr. Feng Liu of University of Texas, San Antonio, TX, for help with signal transduction pathways, for kinase inhibitors used in initial experiments, and for critical reading of the manuscript.

REFERENCES

Antimitogenic and Proapoptotic Activities of Methylseleninic Acid in Vascular Endothelial Cells and Associated Effects on PI3K-AKT, ERK, JNK and p38 MAPK Signaling


Cancer Res 2001;61:7171-7178.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/19/7171

Cited articles
This article cites 32 articles, 15 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/19/7171.full#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/19/7171.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/61/19/7171. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.