Methyl-Selenium Compounds Inhibit Prostate Carcinogenesis in the Transgenic Adenocarcinoma of Mouse Prostate Model with Survival Benefit

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Abstract
Chemoprevention of prostate cancer by second-generation selenium compounds in reference to selenomethionine holds strong promise to deal with the disease at the root. Here we used the transgenic adenocarcinoma mouse prostate (TRAMP) model to establish the efficacy of methylseleninic acid (MSeA) and methylselenocysteine (MSeC) against prostate carcinogenesis and to characterize potential mechanisms. Eight-week-old male TRAMP mice (C57B/6 background) were given a daily oral dose of water, MSeA, or MSeC at 3 mg Se/kg body weight and were euthanized at either 18 or 26 weeks of age. By 18 weeks of age, the genitourinary tract and dorsolateral prostate weights for the MSeA- and MSeC-treated groups were lower than for the control ($P < 0.01$). At 26 weeks, 4 of 10 control mice had genitourinary weight $>2$ g, and only 1 of 10 in each of the Se groups did. The efficacy was accompanied by delayed lesion progression, increased apoptosis, and decreased proliferation without appreciable changes of T-antigen expression in the dorsolateral prostate of Se-treated mice and decreased serum insulin-like growth factor I when compared with control mice. In another experiment, giving MSeA to TRAMP mice from 10 or 16 weeks of age increased their survival to 50 weeks of age, and delayed the death due to synaptophysin-positive neuroendocrine carcinomas and synaptophysin-negative prostate lesions and seminal vesicle hypertrophy. Wild-type mice receiving MSeA from 10 weeks did not exhibit decreased body weight or genitourinary weight or increased serum alanine aminotransferase compared with the control mice. Therefore, these selenium compounds may effectively inhibit this model of prostate cancer carcinogenesis.

One in six American men will be diagnosed in their lifetime with prostate cancer (PCa), which is the second leading cause of male cancer death in the United States (1). Treatment options by hormone ablation therapy, radiation, and surgery for advanced PCa do not offer cure but delay the inevitable recurrence of the lethal hormone-refractory disease (2). Available chemotherapeutic drugs including docetaxel offer little or no survival benefit to patients with such late-stage disease. Chemoprevention of prostate carcinogenesis is considered a necessary and practical approach to deal with the problem at the root (3, 4).

Previous studies have suggested that selenium (Se) may modify the risk of and prevent PCa (5, 6). A clinical trial led by the late Dr. Larry Clark showed that Se supplementation in the form of selenized yeast significantly decreased PCa incidence in skin cancer patients compared with those receiving placebo in a randomized trial (7, 8). Prompted by these encouraging results, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) tested the PCa preventive efficacy of l-selenomethionine (SeMet), the major species of Se present in selenized yeast, and/or vitamin E (all rac-$\alpha$-tocopheryl acetate) supplementation in North American men (9). The study was a randomized, placebo-controlled trial of 35,533 men from 427 participating sites in the United States, Canada, and Puerto Rico randomly assigned to four groups (Se, vitamin E, Se + vitamin E, and placebo) in a double-blind fashion between August 22, 2001 and June 24, 2004. Baseline eligibility included age 50 years or older (African American men) or 55 years or older (all other men), a serum prostate-specific antigen level of 4 ng/mL or less, and a digital rectal examination not suspicious for PCa. Treatment groups were oral Se (200 $\mu$g/d from l-selenomethionine) and matched vitamin E placebo, vitamin E (400 IU/d of all rac-$\alpha$-tocopheryl acetate) and matched Se placebo, Se + vitamin E, and placebo + placebo for a planned follow-up of a minimum of 7 years and a maximum of 12 years. The National Cancer Institute stopped the trial in late October 2008, several years ahead of the...
Prostate Cancer Chemoprevention by Methyl Selenium

scheduled completion date due to a possible increase of diabetes risk in SeMet-supplemented subjects and an increase in PCa risk by the vitamin E–supplemented subjects (10). Specifically, as of October 23, 2008 (10), median overall follow-up was 5.46 years (range, 4.17–7.33 years). Hazard ratios for PCa were 1.13 (99% confidence interval, 0.95–1.35; n = 473) for vitamin E, 1.04 (99% confidence interval, 0.87–1.24; n = 432) for Se, and 1.05 (99% confidence interval 0.88–1.25; n = 437) for Se + vitamin E, versus 1.00 (n = 416) for placebo. There were no significant differences (all P > 0.15) in any other pre-specified cancer end points. There were statistically nonsignificant increased risks of PCa in the vitamin E group (P = 0.06) and type 2 diabetes mellitus in the Se group (relative risk, 1.07; 99% confidence interval, 0.94–1.22; P = 0.16) but not in the Se + vitamin E group.

In hindsight, experiments with preclinical PCa models conducted before (11) and since SELECT was initiated including our study with xenograft models (12, 13) did not support any in vivo anticancer activity of SeMet. Because of the metabolic and biological differences that have been well documented between SeMet and other Se forms (13), the failure of SeMet in the SELECT should not be equated to all Se forms as “ineffective” for PCa prevention. Whereas the SELECT results convincingly ruled out SeMet for PCa prevention, we argue that the quest for effective Se agents takes on even greater significance and urgency because there is no other clinically proven effective PCa preventive agent other than the controversial 5α-reductase II inhibitor finasteride, which blocks the intraprostatic generation of the active androgen dihydrotestosterone but with significant side effects and questionable survival benefit (14–16).

Cell culture and preclinical animal studies have suggested that the overall efficacy of Se supplementation depends on the specific form and dosage administered as well as the stage of the disease process (17, 18). Methylselenol has been considered an in vivo active anticancer metabolite (17, 18). Studies conducted by us and others have shown that the putative methylselenol precursor compound methylseleninic acid (MSeA) can inhibit the in vitro growth of hormone-refractory advanced human prostate carcinoma DU145 (19, 20) and PC-3 cells (21) and androgen-dependent human prostate carcinoma LNCaP cells (22–24) by cell cycle arrest and induction of apoptosis through caspase activation as well as inhibition of androgen signaling in the latter. We have recently shown that orally administered MSeA and another methylselenol precursor methylselenocysteine (MSeC) inhibit the in vivo growth of DU145 and PC-3 xenograft in nude mice and SeMet was ineffective (13). These studies led us to hypothesize that these “second-generation” Se compounds (in reference to SeMet) may prevent or delay prostate primary carcinogenesis, leading to a survival benefit. To our knowledge, such Se forms have not been rigorously tested in any animal prostate primary carcinogenesis model.

The transgenic adenocarcinoma of mouse prostate (TRAMP) model, originally developed by Dr. N. Greenberg (25), mimics, to some extent, the natural history and progression of human PCa (26). The probasin promoter-driven SV40 T-antigen expression in the dorsolateral prostate (DLP) glands abrogates p53 and Rb tumor suppressor gene functions to spontaneously propel the progression of PCa from prostatic intraepithelial neoplasia (PIN) to more advanced stages of lesions with morphologic features of adenocarcinomas and poorly differentiated carcinomas (26). In this model, metastasis to lymph nodes and other organs is also a notable feature similar to the human disease (26). More recent studies have shown that the poorly differentiated carcinomas in this model are neuroendocrine-like (NE) carcinomas that are androgen insensitive (lack androgen receptor) and express synaptophysin and belong to a distinct lineage from the PIN and well-differentiated and moderately differentiated adenocarcinoma lesions (27, 28).

Furthermore, the incidence of these NE-carcinomas is mouse strain dependent: A lifetime incidence of ~20% NE-carcinomas in the C57B/6 background versus an 87% NE-carcinoma incidence in the FVB background was recorded as early as 16 weeks of age (28). In both strains, these NE-carcinomas mostly arise in the ventral lobes instead of the DLP (27, 28). As is understood today, the TRAMP therefore represents two models of prostate carcinogenesis: one that approximates the androgen-driven glandular prostate epithelial cancer formation in the DLP and another that simulates the development of NE-carcinomas in the ventral prostate. The latter is also important for human PCa in that the number of NE-cells correlates with the stage, Gleason grades, and survival in castration-recurrent PCa in the clinical cases (29, 30). The TRAMP is by far the most widely available and used transgenic model of PCa in cancer chemoprevention studies (e.g., refs. 31–38).

In the present study, we evaluated the efficacy of MSeA and MSeC against early-stage lesion development in the TRAMP model and the associated biomarkers of cell proliferation and apoptosis. We tested the survival benefits of starting MSeA oral treatment at either PIN (10 weeks of age) or more advanced stages of lesions (16 weeks of age).

Materials and Methods

Selenium compounds

Methaneselenenic acid (same as MSeA, >95%, white powder) was purchased from Sigma Chemical Company. Se-methylselenocysteine was purchased from LKT Labs. Stock solutions of each compound were prepared in water, filter sterilized, and then stored in 1-ml aliquots in a −70°C freezer as previously described (13). A fresh vial was thawed for daily use.

Animal breeding

The animal use protocol was approved by the Institutional Animal Care and Use Committee of the University of Minnesota and carried out at the Hormel Institute Associate for Assessment and Accreditation of Laboratory Animal Care–accredited animal facility. The female heterozygous C57BL/10TGN TRAMP mice, Line PB Tag 8247NG, were purchased from The Jackson Laboratory and were cross-bred with nontransgenic C57BL/6 breeder males. Mouse-tail DNA was isolated from the 3-week-old pups for genotyping by PCR-based screening using the following primers (25): primer 1, 5′- CGGTCGCCGCGGACGAGCCCTCAACAAGTCACATT-3′; primer 2, 5′-AGGCATTCCACTGCTCCCATTCATC-3′. As a control for PCR efficiency, multiplex PCR using primers for mouse β-globulin was done concurrently: primer 1 (β-globulin), 5′-CCAATCTGCTCACAGGATAGGAGGCCAGG-3′; primer 2 (β-globulin), 5′-CCTTAGGCTGTCACAATCATTCCAGCCATCG-3′.

Selenium treatments

Two sets of animal experiments were carried out. In the first experiment, we compared the effect of daily (Monday–Friday, 5 times per week) oral administration of MSeA and MSeC starting at 8 wk of age on early-stage disease burden and lesion progression as represented
by the weight of genitourinary (GU) tract and prostate pathology profiles. Male C3HBL/6 TRAMP mice (n = 20 mice per group) received a daily oral dose of water (control), MSeA, or MSeC delivered to the back of the tongue (5 times per week) at a dosage of 3 mg Se/kg body weight, as we recently described for nude mice (13). Briefly, Se was delivered to the back of the tongue in a volume of 1 μL/g body weight (e.g., for a mouse weighing 30 g, 30 μL were delivered with a disposable plastic tip from a 100-μL Pipetman). The mice ingested the small volume easily without physical irritation to the esophagus that might have resulted from intra gastric gavages. The tight range of the measured tissue Se content within a group indicated good consistency of this manner of Se delivery (see ref. 13). The dosing frequency simulated that used in the Clark study (7) and the SELECT study (9). Mice were given ad libitum access to purifiedAIN-93M diet pellets (Harlan Teklad) and water.

At 58 or 68 wk of age, blood was taken before sacrifice for serum preparation for insulin-like growth factor I (IGF-I) assay by a commercially available kit (See below). The lower GU tract including seminal vesicle, prostate, testes, and bladder was dissected and weighed. The DLP was dissected under a dissecting scope on a bed of ice. Approximately half was fixed for 24 h in 10% (ν/ν) neutral buffered formalin (Fisher Scientific Company) and the other portion was frozen on dry ice and stored in −80°C for Western blot assay and other biochemical measurements. The fixed tissues were stored in 70% ethanol for preservation until processing for histology and immunohistochemistry. At processing time, the fixed tissues were dehydrated in ascending grades of ethanol and xylene, then embedded in paraffin. Sections (5 μm) were cut with a microtome and mounted on Superfrost Plus microscope slides (Fisher Scientific Company). Tissues were processed and stained with H&E for routine histopathologic evaluation.

In the second (long-term survival) experiment, the effect of oral administration of MSeA on overall and cancer-specific survival of the TRAMP mice was studied. Groups of TRAMP mice received a daily oral dose of 3 mg Se/kg body weight as MSeA beginning at 10 wk (n = 31) or 16 wk of age (n = 30) or received water as placebo (n = 31). Mice were given ad libitum access to purified AIN-93M diet pellets and water. TRAMP mice were inspected daily and weighed individually once a week. At 16 wk of age, TRAMP mice were palpated daily before Se or water oral treatment for the presence of abdominal mass (tumor). Mice that lost >25% body weight or were weak or moribund (palpable tumor size >3 cm) were euthanized. The GU tract was removed en bloc and weighed. Tumors were dissected and weighed. The organs were inspected for metastasis and, if visible, the lesions were dissected, weighed, and fixed in formalin for H&E confirmation of metastasis. Mice surviving to the predetermined termination age of 50 wk were killed and necropsy was done as above.

To assess the long-term safety of MSeA oral administration, wild-type male littermates were treated with the same dose of MSeA (n = 10) starting at 10 wk of age or were given water (n = 9). Body weight and general health were monitored until 41 wk of age. Blood was collected before euthanasia for serum preparation. Serum alanine aminotransferase (ALT) activity was measured as a marker of liver damage. GU tracts, lungs, kidneys, livers, and spleens as well as any visible tumors were removed, weighed, and fixed in 10% neutral buffered formalin.

**Histopathology**

H&E-stained sections of DLP were examined in a blinded fashion to the treatment status by trained associate L. Wang (D.V.M., Ph.D.) and pathologist J. Liao (M.D., Ph.D.) and classified (26) as (a) low-grade PIN having foci with two or more layers of atypical cells with elongated hyperchromatic nuclei and intact gland profiles; (b) high-grade PIN having increased epithelial stratification, foci of atypical cells fill or almost fill the lumen of the ducts, enlarged diameter of the glands, distorted duct profiles, increased nuclear pleomorphism, hyperchromatic nuclei, and cribriform structures; (c) well-differentiated adenocarcinoma showing invasion of basement membrane, loss of intraductal spaces, and increased quantity of small glands; (d) moderately differentiated adenocarcinoma showing total loss of intraductal spaces and relatively solid growth; and (e) poorly differentiated carcinoma showing sheets of poorly differentiated cells with remnants of trapped glands. For each animal, the highest lesion grade observed for the DLP section or prostate tumors was used for comparison of lesion progression status among groups.

**Immunohistochemical analysis**

Sections (5 μm) were cut from paraffin-embedded DLP tissues, dried, and deparaffinized. Immunostaining was done with antibodies for the proliferation marker protein Ki67 (NeoMarker), T-antigen transgene product (Becton-Dickinson), at 1:100 dilution. Synaptophysin antibody was purchased from BD Biosciences. Normal horse serum was used as negative control. The biotinylated secondary antibody was rabbit anti-mouse antibody IgG (1:200 in 10% normal rabbit serum, DakoCytomation). The slides were developed in diamobenzidine and counterstained with a weak solution of hematoxylin stain. The stained slides were dehydrated and mounted in Permount. Images were captured and analyzed by ImagePro-plus software. Synaptophysin expression in selected carcinomas and prostate tissues was verified by immunoblottting.

**In situ apoptosis detection by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling**

Five-micrometer-thick sections were processed for terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) assay using a commercial kit (Calbiochem). Apoptosis was evaluated by counting the brown-colored positive cells as well as the total number of cells at five randomly selected fields at >400 magnification. The apoptotic index (per >400 microscopic field) was calculated as number of apoptotic cells × 100 / total number of cells.

**Immunoblot analysis of tissue lysates**

Western blot was carried out on pooled DLP tissues (n = 9 or 10 per group) for the 18-wk samples. The tissues were homogenized in non-denaturing lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2 mmol/L sodium orthovanadate, 0.5% NP-40, 5 μL/mL aprotinin, 10 μL/mL phenylmethylsulfonyl fluoride]. Protein concentration in lysates was determined with Bio-Rad detergent-compatible protein assay kit (Bio-Rad Laboratories). For immunoblot analyses, 20 to 80 μg of protein per lysate were denatured in 2 × SDS-PAGE sample buffer and subjected to SDS-PAGE on 10% or 12% Tris-glycine gel as needed. The separated proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences) followed by blocking with 5% nonfat milk powder (w/v) in TBS (10 mmol/L Tris, 100 mmol/L NaCl, 0.1% Tween 20) overnight at 4°C. Membranes were probed with different primary antibodies, including cleaved poly(ADP-ribose) polymerase, proliferating cell nuclear antigen, cyclin E, IGF-I receptor (IGF-IR), p27, AKT, p-AKT, X-linked inhibitor of apoptosis, and p-actin. The membranes were then incubated with appropriate peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and detected by enhanced chemiluminescence.

**Caspase activity assay**

Caspase-3/caspase-7 activity was assessed with a fluorescence assay peptide substrate, DEVD-conjugated to the fluorescent reporter molecule 7-amino-4-trifluoromethyl coumarin (39). Tissue lysates were the same as the Western blot sample. The kit was purchased from R&D.

**IGF-1 ELISA**

DSL-10-2920 Mouse/Rat IGF-I ELISA kits (Diagnostic Systems Laboratories, Inc.) were used to determine serum concentrations of IGF-I. The absorbance was read at 450 nm with dual wavelength...
correction at 620 nm, and concentrations were determined using the standards provided in the kit. All IGF-1 samples were assayed in duplicate. Intra-assay coefficient of variation was 3%. Within each cohort, all samples were run on one plate.

**Serum ALT assay**
For each sample, 50 μL of Infinity ALT (GFT) Liquid Stable Reagent (Thermo Scientific) were warmed to 37°C for 5 min. Five microliters of each sample were added and incubated at 37°C for an additional 30 s. The absorbance was read at 340 nm and was recorded every 60 s for 5 min to measure the change in absorbance over time. All samples were run in duplicate. Within-sample coefficient of variation was ~5%. Normal (Data-Trol N) and abnormal (Data-Trol A) sera from Thermo Scientific were used as internal controls, and concentrations were expressed as units per liter, calculated as ΔAbsorbance/min × factor, where factor = total volume (mL) × 1,000/6.3 × sample volume (mL) × cuvette path length (cm). In this case, factor = 1,746. As a reference, normal untreated mice had serum ALT of ~50 units/L (40).

**Statistical analyses**
Data are presented as means ± SE. Results were analyzed by ANOVA followed by Newman-Keuls tests to determine significance between specific groups. Survival curves (Kaplan-Meier) and percent with metastasis were analyzed by χ² analysis.

**Results**

**Orally administered MSeA and MSeC decreased the indices of prostate tumor burden**
In the TRAMP model, the activation of probasin-driven T-Ag expression leads to the accelerated growth of the DLP and ventral prostate, where most prostate lesions arise, and of the lower GU tract organs including seminal vesicle, testes, and bladder, in addition to the prostate gland (25). The weights of GU and DLP have therefore been used as quantitative indicators of tumor burden in early-stage carcinogenesis (25, 26, 31). In the first experiment, we initiated daily oral treatment (5 times per week) of MSeA, MSeC, or water (vehicle) at 8 weeks of age and euthanized mice at 18 weeks and up to 26 weeks of age. For reference values, we obtained the baseline GU weights of 8-week-old wild-type and TRAMP mice and 18-week-old wild-type mice. The GU weights for the 8-week-old wild-type and TRAMP mice were the same (0.52 versus 0.53 g; n = 10, mice per group; Fig. 1A, columns 1 and 2). Over the next 10 weeks, the GU weight of the wild-type mice grew by 36% (from 0.52 to 0.71 g; n = 10 mice, P < 0.01; column 3 versus column 1), whereas the GU weight of the TRAMP mice increased by 136% (from 0.53 to 1.25 g; n = 10, P < 0.001; column 4 versus column 2). The GU weight of TRAMP mice receiving MSeA or MSeC only increased by 60% and 49% (from 0.53 g to 0.85 and 0.79 g; columns 5 and 6 versus column 2), respectively. Overall, MSeA and MSeC treatment in the 10-week period resulted in a net inhibition of T-Ag–driven expansion of GU weight by 76% and 87%, respectively (P < 0.001 for each compound). There was no statistical difference between MSeA and MSeC treatments.

The DLP weight (Fig. 1B) of the TRAMP mice at 18 weeks (101 mg; n = 9 mice, excluding 1 mouse with a tumor weighing 2.6 g [synaptophysin (+) NE-carcinoma]; column 2) was 237% greater than that of the wild-type littermates (30 mg; n = 10, P < 0.001; column 1). The DLP weight of the TRAMP mice treated with MSeA or MSeC was significantly less than that of the water-treated mice, being 62 and 58 mg, respectively (P < 0.001 for each compound; columns 3 and 4 versus column 2). As a conservative estimate (excluding a 2.6-g tumor in the water-control group), MSeA or MSeC treatment decreased the T-Ag–driven DLP weight gain by 55% and 63%, respectively, after 10 weeks of administration.

At 26 weeks of age, the GU weight data were not normally distributed due to at least one very large tumor in each group and were therefore presented as ranking order, but were not analyzed by ANOVA (Fig. 1C). The mathematical mean GU weights of the water-, MSeA-, and MSeC-treated TRAMP mice were 3.03 g (n = 10), 1.73 g (n = 10), and 1.21 g (n = 9), respectively. The DLP weights of the water-, MSeA-, or MSeC-treated mice were 424 mg (n = 8, excluding 2 mice with large tumors), 119 mg (n = 9, excluding one mouse with a large tumor), and 76 mg (n = 8, excluding one mouse with a large tumor), respectively (Fig. 1E). Together, these data at 26 weeks of age indicated decreased growth of the DLP and GU organs by MSeA and MSeC treatment. Because of the demonstration of different lineages for NE-carcinomas (synaptophysin positive, mostly arising from ventral prostate) and glandular epithelial lesions from the DLP (27, 28), we analyzed synaptophysin expression in the prostate tissue and tumor sections by immunohistochemical staining (see Supplementary Fig. S1 for staining patterns) and immunoblotting (Fig. 1D). As shown in Fig. 1C and D, synaptophysin (+) carcinomas represented the fast-growing cancer in our model and their growth was seemingly inhibited by MSeA or MSeC.

The body weight of TRAMP mice at 18 weeks was not significantly affected by oral administration of 3 mg Se/kg body weight as MSeA, but was decreased with MSeC by 9.4% (Fig. 1F). At 26 weeks of age, the final body weight was significantly lower (compare 34.3 g in water-control TRAMP mice with 30.0 g of MSeA-treated mice and 29.0 g of MSeC-treated mice; Fig. 1F). A portion of the body weight difference, however, was attributable to the heavier GU weight in the control mice at 26 weeks (Fig. 1C). These data indicated that the dosage level of 3 mg Se/kg was marginally tolerated by the TRAMP mice. Because of the complication of GU weight difference in TRAMP mice, the long-term safety issue of MSeA in wild-type male mice was assessed in a second experiment along with TRAMP mouse survival evaluation (see long-term experiment later).

**MSeA and MSeC delayed the lesion progression in DLP and the occurrence of poorly differentiation NE-carcinomas**

As shown in Fig. 2A, at both 18 and 26 weeks of age, the severity of DLP lesions (highest grade based on H&E for each mouse) showed that the MSeA- or MSeC-treated groups had more mice with PIN lesions and fewer mice with more advanced poorly differentiated carcinomas than the control groups. These results suggest that both Se compounds, when administered starting at 8 weeks of age, inhibit lesion progression in the DLP from PIN and well-differentiated lesions to moderately differentiated carcinomas (Fig. 2A) and also decreased the emergence of poorly differentiated carcinomas (Fig. 2A), which are mostly synaptophysin positive (Fig. 1C).

**Effect of MSeA and MSeC on the proliferation and apoptosis indices in DLP**
To provide a mechanistic explanation of the delayed DLP lesion progression, we next analyzed the indices of epithelial
proliferation and apoptosis, focusing on the 18-week sample cohorts. Ki67 is a nuclear protein highly expressed in proliferating cells. Immunohistochemical analysis of DLP (Fig. 2B) showed a virtual absence of Ki67-positive staining in wild-type mice and greatly increased Ki-67 index in TRAMP mice in the water-control group. TRAMP mice receiving either MSeA or MSeC treatment showed decreased Ki67 expression compared with the water group (t test, P < 0.05; Fig. 2E, graph a).

The in vivo apoptotic response of MSeA- or MSeC-treated DLP in the 18-week cohort was analyzed by TUNEL staining (Fig. 2C). Wild-type mice showed minimal apoptosis in the DLP, and TRAMP mice had much increased apoptosis (~4.4%; Fig. 2E, graph b). The TUNEL-positive cells in MSeA- and MSeC-treated TRAMP mice were greater than in the water-treated TRAMP mice, being 8.9% (t test, P < 0.01) and 7.4% (t test, P < 0.05), respectively (Fig. 2E, graph b).

The expression of the oncogenic transgene T-Ag in the DLP was examined by immunohistochemical staining (Fig. 2D). As expected, the wild-type DLP was devoid of this transgene product. The epithelial expression of T-Ag in the TRAMP mice was evident and was not appreciably affected by either MSeA or MSeC treatment. These data support the involvement of antiproliferative as well as proapoptotic effects of MSeA and MSeC in the suppression of DLP lesion growths in the TRAMP mice without affecting transgene product expression.

**Effect of MSeA or MSeC on selected proteins in cell proliferation and apoptosis**

To begin to define potential in vivo molecular mediators or targets associated with the cell proliferation and apoptosis signaling responses in the DLP affected by Se treatments, we focused on the 18-week tissues because these early samples were less complicated by the emergence of large tumors than at later time points. As shown in Fig. 3A, the abundance of cyclin-dependent kinase (CDK)-2, CDK4, cyclin E, c-Jun, and proliferating cell nuclear antigen was greatly increased in the DLP of the TRAMP mice (lane 1) compared with the wild-type mice (lane 4). MSeA and MSeC decreased the abundance of CDK2 and c-Jun and increased the abundance of CDK inhibitory protein p27Kip1, without appreciable effect on CDK4 and proliferating cell nuclear antigen. MSeA treatment seemed to suppress cyclin E abundance (lane 2 versus lane 1) whereas MSeC did not (lane 3 versus lane 1).
Fig. 2. Effect of daily orally administered MSeA and MSeC starting from 8 wk of age on prostate carcinogenesis lesion progression (A), epithelial proliferation index (B and E-a; Ki67 staining), apoptosis (C and E-b; TUNEL staining), and T-antigen transgene expression (D) in the DLP of TRAMP mice at 18 or 26 wk of age. A, lesion grade distribution of water-treated (control), MSeA-treated, or MSeC-treated mice at 18 and 26 wk of age. The highest lesion grade based on H&E staining in the DLP for each mouse was tabulated. n = 10 mice per group. LPIN, low-grade PIN; HPIN, high-grade PIN; WD, well-differentiated carcinoma with epithelial glandular features; MD, moderately differentiated carcinoma with epithelial glandular features; PD, poorly differentiated carcinomas, mostly synaptophysin-expressing NE-carcinomas. B, representative staining patterns of Ki67 in DLP tissues from water-treated, MSeA-treated, or MSeC-treated TRAMP mice as well as from wild-type mice at 18 wk of age (×400). The overall estimated Ki67 indices for the different groups are shown in F-a based on quantification of 5 to 10 fields of each slide from each mouse. C, representative staining patterns of TUNEL apoptosis in DLP tissues of water-treated, MSeA-treated, or MSeC-treated TRAMP mice as well as from wild-type mice at 18 wk of age (×400). The overall estimated TUNEL-positive apoptosis indices for the different groups are shown in F-b based on quantification of 5 to 10 fields of each slide from each mouse. D, representative staining patterns of T-antigen protein in DLP tissues from water-treated, MSeA-treated, or MSeC-treated TRAMP mice as well as from wild-type mice at 18 wk of age (×400). *P < 0.05; **P < 0.01, versus the respective water-treated group.
As far as cell death was concerned, cleavage of poly(ADP-ribose) polymerase was barely detectable in the wild-type DLP (lane 4) and was higher in the water-treated control TRAMP DLP (lane 1; Fig. 3B). MSeA- and MSeC-treated DLP contained elevated level of cleaved poly(ADP-ribose) polymerase in comparison with the water-control group (lanes 2 and 3 versus lane 1). Measurement of caspase-3/caspase-7 enzymatic activity in the tissue lysate confirmed the increased activation of caspase-mediated execution in the MSeA- or MSeC-treated DLP (Fig. 3C). The abundance and phosphorylation level of the survival kinase AKT were lower in both Se groups (lanes 2 and 3) in comparison with the water-control group (lane 1; Fig. 3B). The mitochondria integrity regulatory proteins Bax and Bcl-xL were not altered by the Se treatments. High expression of the caspase inhibitor protein X-linked inhibitor of apoptosis was found to correlate with the low apoptosis in wild-type mice in spite of low AKT and low Bcl-xL abundance in these mice (lane 4). These biochemical data support the possible involvement of CDK2, c-Jun, and p27Kip1 in regulating DLP cell proliferation and AKT inhibition in caspase-mediated apoptosis in vivo by MSeA or MSeC consumption.

MSeA and MSeC caused a significant decrease of serum IGF-I levels in TRAMP mice

The IGF-I axis plays a crucial role in PCa progression (38, 41), and some studies suggest that serum IGF-I level might be a better predictor of PCa risk than serum prostate-specific antigen (42, 43). We found that MSeA or MSeC treatment led to decreased serum IGF-I in 18-week-old TRAMP mice (Fig. 4A; ANOVA, \( P < 0.01 \)) and 26-week-old TRAMP mice (Fig. 4B; ANOVA, \( P < 0.05 \)).

Western blot analyses of the DLP lysates did not reveal any change of IGF-I receptor abundance resulting from Se treatment (Fig. 4C). The phosphorylation of IGF-IR was increased in the water-treated control TRAMP mice (lane 1) when compared with wild-type mice (lane 4). The treatment by either Se compound decreased the phosphorylation modestly (lanes 2 and 3 versus lane 1). These data suggest that MSeA and MSeC might inhibit the IGF-I axis at both the ligand and receptor phosphorylation levels, upstream of AKT phosphorylation.

MSeA improved the overall and cancer-specific survival of TRAMP mice

Prompted by the data from the above experiment supporting a similar in vivo inhibitory efficacy of MSeA and MSeC on early-stage prostate lesion progression in DLP and the emergence of synaptophysin(+) NE-carcinomas, we next did a long-term experiment to establish the survival benefit of oral MSeA administration. Suspecting that the overall efficacy would depend on the stages of the disease when MSeA intervention was started, we therefore compared the MSeA oral treatment at 10 weeks (early PIN stage) versus 16 weeks of age (PIN to organ-confined NE-carcinomas).

Figure 5A showed the Kaplan-Meier survival curves of TRAMP mice due to all causes of mortality (euthanasia or those found dead) among the three groups. The median survival time was 40.5, 40.5, and 45.0 weeks of age for the water group (n = 31), 16-week MSeA cohort (n = 30), and 10-week MSeA cohort (n = 31), respectively. Whereas nearly all water-treated TRAMP mice were dead by 50 weeks of age, approximately one third of mice treated with MSeA starting either at 10 weeks or at 16 weeks of age survived to this age. Log-rank tests: water versus 10-week MSeA cohort, \( P = 0.0225 \); water versus 16-week MSeA cohort, \( P = 0.1793 \).

There was some unexpected non–cancer-related mortality between 20 and 30 weeks in all three groups. Therefore, we excluded those mice that died of verifiable noncancer causes before 30 weeks of age and replotted the survival curves (effective number of mice: water group, n = 28; 10-week MSeA cohort, n = 25; 16-week MSeA cohort, n = 23; Fig. 5B). The median survival time of the corrected cancer-specific mortality
MSeA decreased and delayed not only death due to the synaptophysin(+) carcinomas but also the death of mice bearing synaptophysin(−) staining lesions (Fig. 5C, 10-wk MSeA group). However, when started at 16 weeks, MSeA did not suppress the early death due to synaptophysin(+) carcinomas (20-24 weeks), but delayed the deaths due to the remaining synaptophysin(+) carcinomas and synaptophysin(−) prostate lesions (Fig. 5C, 16-wk MSeA group).

Consistent with improved long-term survival, the visible macrometastases to the lymph nodes, liver, and lung were 43.8% (14 of 32) in the water group and 26.7% (8 of 30) and 22.6% (7 of 31) in the 16-week and 10-week MSeA cohorts, respectively (Fig. 5D). When the MSeA groups were combined for comparison with the water group, \( \chi^2 = 4.51, 1 \) degree of freedom, 0.025 < \( P < 0.05 \).

Lack of growth inhibition and liver damage by long-term MSeA administration in wild-type littermates

The safety of long-term MSeA treatment was a concern and therefore was simultaneously evaluated in wild-type littermates. The mice given daily with oral MSeA treatment from 10 weeks until 41 weeks of age showed similar rate of body weight gain in comparison with the water-treated mice (Fig. 6A). MSeA treatment did not affect the weight of the GU when compared with the water-control mice (Fig. 6B).

As a biochemical indicator of liver damage, we measured the serum ALT level of the non-TRAMP littermates. There was no significant difference between the water- and MSeA-treated groups (Fig. 6C). These data support the well-tolerated nature of MSeA daily oral dosing regimen for long-term chemoprevention use.

Discussion

The lackluster performance of chemotherapy for advanced recurrent PCa that have failed surgical, hormone ablation, and radiation therapies calls for alternative approaches to combat this disease. The long latency period and high prevalence of PCa in men make chemoprevention of prostate carcinogenesis a plausible and cost-effective means to deal with the problem at the root. Although Se has been implicated for its potential for PCa prevention (7,8), there is a void of pertinent literature in preclinical models of prostate carcinogenesis to support their utility for PCa chemoprevention. In retrospect, the paucity of such preclinical data was at least one reason that SeMet was not prevented from being chosen for the failed SELECT study (9, 10).

Here we have shown for the first time, to our knowledge, that daily oral administration of “second-generation” Se compounds MSeA and MSeC starting at 8 weeks of age (puberty) inhibits early-stage prostate carcinogenesis, as reflected by decreased GU and DLP weight at 18 and 26 weeks of age (Fig. 1), delayed prostate lesion progression in the DLP (Fig. 2A), and slowed the emergence of poorly differentiated synaptophysin (+) NE-carcinomas (Fig. 1C) in TRAMP mice. We further showed that long-term use of MSeA resulted in an improvement of the survival from cancer-specific mortality and all causes of mortality (Fig. 5) with no appreciable adverse effects on body weight gain, GU weight, or liver integrity (Fig. 6). Analyses of the synaptophysin expressing status of prostate lesions and carcinomas in the long-term experiment...
supported the inhibitory efficacy of MSeA not only against the very aggressively growing synaptophysin(+) NE-carcinomas but also against deaths from the synaptophysin(−) DLP lesions and seminal vesicle hypertrophy (Fig. 5C). The daily oral Se dosing regimen was designed to mimic the frequency used in the Clark study (7), the SELECT study (9), and the lung cancer prevention trial with selenized yeast (44) and has been used by us in xenograft studies in athymic nude mice (13). Together these findings illustrate that these Se compounds may be effective chemopreventive agents against primary carcinogenesis in the TRAMP model of prostate NE-carcinomas as well as prostate glandular epithelial cancers and merit serious consideration for translational studies in the future.

A number of points are worthy of elaboration. First, the in vivo efficacy of MSeA and MSeC was indistinguishable at the dosage tested in the short-term experiment (Fig. 1). The efficacy was associated with decreased prostate epithelial proliferation (Fig. 2B and E-a), increased apoptosis (Fig. 2C and E-b), and decreased circulating IGF-I levels (Fig. 4). In our biomarker analyses (Fig. 3), many of the molecular changes identified in DLP were associated with caspase-mediated apoptosis regulation, such as poly(ADP-ribose) polymerase cleavage (Fig. 3B) and caspase-3/caspase-7 activity (Fig. 3C), and decreased expression and phosphorylation of AKT, a critical survival protein kinase (Fig. 3B). Antiproliferation was associated with decreased CDK2 and c-Jun and increased...
Subtle differences were occasionally seen for the analyzed biomarkers, such as cyclin E (Fig. 3A), lending some credence to the possibility of unique targets to each Se form. We have observed that whereas MSeA and MSeC were equally efficacious against the growth of human DU145 PCa xenograft model in nude mice, in the human PC-3 prostate xenograft model, MSeA was more efficacious than MSeC (13). Therefore, the pathogenetic background of the PCa cells may also affect the molecular and cellular targets of the different forms of Se and their in vivo efficacy. Although methylselenol has been suggested as a common mediator of the anticancer activity of MSeA and MSeC, further studies will be required to determine whether each Se involves unique in vivo molecular targets or cellular pathways in addition to shared ones through methylselenol.

In addition to molecular and morphologic changes in DLP, our results showed that the circulating IGF-I was lower in the 18-week (P < 0.01) and 26-week (P < 0.05) MSeA- or MSeC-treated cohorts compared with untreated TRAMP mice (Fig. 4A and B). Additionally, Se treatment of TRAMP mice modestly decreased phosphorylated IGF-IR in the DLP (Fig. 4C). The lower serum IGF-I concentrations found in the treated mice suggest a possible systemic action of these Se forms to regulate PCa genesis and progression. Epidemiologic studies indicate that IGF-I axis signaling is deregulated in clinical PCa, and that serum IGF-I level could be a predictor of PCa risk (42, 43). Furthermore, IGF-IR has been reported to be overexpressed in prostate tumor specimens (45). Recent studies with an IGF-IR receptor neutralizing antibody have shown potent inhibition of in vivo PCa growth alone or in combination with chemotherapy (46, 47). However, the issue with IGF-I/IGF-IR signaling in PCa is rather complex, as highlighted by a recent study by the Greenberg group that has shown that selective knockout of IGF-IR in the epithelial cells of TRAMP mice accelerates lesion progression to a more aggressive phenotype (48), contrary to an expected decreased tumorigenesis. It is possible that different thresholds exist for IGF-IR signaling, and therefore a complete IGF-IR knockout may not recapitulate the action of diminished IGF-I/IGF-IR signaling.

In the long-term experiment, starting MSeA treatment at 10 weeks of age, but not at 16 weeks of age, improved the median survival time (40-41 weeks for control TRAMP mice) by 4 to 5 weeks against overall and cancer-specific mortality of TRAMP mice, yet both MSeA cohorts of mice outlived the water-treated TRAMP mice to 50 weeks of age (Fig. 5A and B). Although the results may seem paradoxical on first glance, it is likely that in TRAMP mice dying early from cancer-related causes before the median survival time, the death was due to early aggressive tumors. Indeed, these tumors were mostly synaptophysin(+) NE-carcinomas (Fig. 5C). Starting MSeA after such tumors have formed (i.e., at 16 weeks of age) would be expected to be less effective than if MSeA was started at 10 weeks of age to prolong the life of the mice with these tumors, which was supported by the data in Fig. 5C. This finding reaffirms the importance of early intervention of the carcinogenic processes of both NE-carcinomas and the slow-growing prostate glandular carcinomas by Se to achieve lasting preventive result. As a precedence, it has been shown in a chemically induced mammary carcinogenesis model that Se-enriched garlic given for a 1-month duration right after a single carcinogen exposure was as effective as when the Se was provided throughout the postinitiation period of the study (49, 50), supporting “clonal deletion” of initiated cells or early precursor lesions for such permanent protection. We should remark that in the future, animal models that exclusively develop adenocarcinoma of the prostate should be evaluated for confirming...
The chemopreventive efficacy of these second-generation Se forms without the complication of the NE-carcinomas.

The Se dosage and delivery regimen warrant further comments. The daily single oral dose of 3 mg Se/kg body weight of either MSeA or MSeC was shown to be tolerated by the TRAMP mice in this study (Figs. 1F and 6) and in nude mice in our previous studies (13). To provide the same Se intake by including these compounds in the diet would have required ~30 ppm (assuming each mouse weighing 30 g eats 3 g of diet per day). Experience indicates that such high dietary levels of Se will not be palatable to the mice and cause severe weight loss. In fact, a feeding study1 with MSeA suggests 5 ppm as the maximally tolerated dietary level for the mice. We suggest that the bolus dosage may have the advantage of a transient elevation of the critical Se metabolites without a lasting effect on appetite and therefore more tolerable than when is chronically fed through the diet. In support of this speculation, we have documented a rapid peak spike of Se after an oral dose of MSeA (51).

In summary, our results indicate that the second-generation Se compounds inhibit the PCA lesion progression in the DLP, delay the emergence of synaptophysin(+) NE-carcinomas, decrease visible metastasis to other organs, and improve the survival of TRAMP mice, which represent a very aggressive PCA model. The findings in the present study, together with our earlier findings in human prostate tumor xenograft in athymic nude mice (13), suggest that these Se compounds may prove to be effective dietary supplements for the chemoprevention of PCA.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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1 Unpublished data.

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Cancer Prevention Research

Methyl-Selenium Compounds Inhibit Prostate Carcinogenesis in the Transgenic Adenocarcinoma of Mouse Prostate Model with Survival Benefit

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