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Negation of the cancer-preventive actions of selenium by over-expression of protein kinase Cε and selenoprotein thioredoxin reductase

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Selenium prevents cancer in some cases but fails to do so in others. Selenium's failure in this respect may be due to the development of resistance to its chemopreventive actions. Selenocompounds induce a variety of cancer-preventive actions in tumor cells, but these actions may be limited by the low concentrations of free selenocompounds able to reach cells from the plasma. Therefore, we have sought to identify the chemopreventive action requiring the lowest concentration of the redox-active form of selenium, methylseleninic acid (MSA). At submicromolar concentrations, MSA inhibited the malignant transformation of RWPE-1 prostate epithelial cells. In contrast, in already transformed prostate cancer cells, selenium in the micromolar range was required to inhibit cell growth and invasion and to induce apoptosis. The role of protein kinase C (PKC) in these cellular processes, especially the moderately selenium-sensitive PKCε, was demonstrated using PKC-specific inhibitors and small interfering RNA. PKCε levels inversely correlated with cellular sensitivity to MSA. An over-expression of PKCε minimized MSA-induced inhibition of RWPE-1 cell transformation and induction of apoptosis. Thioredoxin reductase (TR), a selenoprotein, reversed the MSA-induced inactivation of PKC isoenzymes. High TR expression in advanced prostate cancer cells correlated with resistance to MSA. Furthermore, inhibition of TR by its specific inhibitor, auranofin, resulted in increased sensitivity of prostate cancer cells to MSA. Collectively, these results suggest that the cancer-preventive actions of selenium may be negated both by an over-expression of PKCε, which is a redox-sensitive target for MSA, and by the selenoprotein TR, which reverses PKC sulfhydryl redox modification.

Introduction

Prostate cancer is the leading cause of cancer morbidity and mortality in American men aged 55 years and older (1). As such, there is a great need for its chemoprevention. The high incidence of prostate cancer and the decades of preneoplasia during its development make it an ideal target for chemoprevention (2). Prostate carcinogenesis is a multistage process that begins with the initiation of normal prostate epithelium by a carcinogen followed by promotion to prostate intraepithelial neoplasia and then subsequent progression to invasive metastatic carcinoma (3). The promotion stage, with its longer duration, is ideal for chemoprevention (4).

Epidemiologic data suggest that cancer mortality inversely correlates with selenium consumption (5). Experimental studies in animals indicate that supplementation with selenium, depending on the form and at a dose well above the dietary requirement, can prevent cancer at various sites (5–7). The trial carried out by Clark *et al.* (8) suggests that selenized yeast may reduce both the incidence of and mortality associated with prostate, lung and colorectal cancers, but not cancers of the breast and skin. However, the recent Selenium and Vitamin E Cancer Prevention Trial did not support the efficacy of selenomethionine in the prevention of prostate cancer (9). Thus, it is important to elucidate the mechanism of action of selenium in order to understand why selenium appears to prevent cancer in some cases but fails to do so in others. The development of resistance to the actions of chemotherapeutic agents is a well-known phenomenon. Whether resistance develops to the cancer-preventive actions of selenium remains to be determined.

Previous elegant studies have demonstrated selenium-induced inhibition of tumor cell growth and induction of apoptosis (10,11), which are considered to be important mechanisms of selenium's cancer-preventive actions. These actions require micromolar concentrations of selenium. However, at cancer-preventive doses, the majority of selenium in circulation is present as selenoproteins or is bound to proteins, and only a limited amount of selenium (<3%) is present as low-molecular weight selenocompounds (12,13). In this context, it is noteworthy to mention that the concentration of selenium required for *in vitro* inhibition of tumor promotion is in the submicromolar range and is lower than that required for growth inhibition or apoptosis of established malignant cells (14,15). Nevertheless, these growth-inhibiting and apoptosis-inducing mechanisms are also important in the tumor promotion stage, in which there is a clonal expansion of preneoplastic cells.

Protein kinase C (PKC) is a potential target involved in both tumor promotion and progression (16,17). Therefore, it is possible that chemopreventive agents such as selenium act on PKC just as tumor promoters do but induce an opposing response (18). In this scenario, the cancer-preventive agent efficiently counteracts the tumor promoter-induced effects on PKC. Direct acting selenocompounds inactivate PKC isoenzymes by inducing the oxidative modification of PKC at its cysteine-rich regions, an action which is reversed by a reductase system involving thioredoxin reductase (TR), a selenoprotein (19). Therefore, the degree of expression of PKC within a cell as well as the ratio of intracellular selenocompounds to TR, particularly in the vicinity of PKC, may determine cellular response to selenium. When methylseleninic acid (MSA) is generated within the vicinity of PKC, it specifically inactivates PKC isoenzymes (19). Moreover, an over-expression of PKCε, an oncogenic, pro-survival and promitogenic isoenzyme, resulted in a relative resistance to selenium-induced apoptosis in DU145 advanced prostate cancer cells (19). However, it remains unknown whether there is any difference in the distribution of PKCε in pre-cancer and early stage cancer cells showing variable degrees of sensitivity to selenium.

Immortalized human prostate epithelial cells (RWPE-1) provide an excellent model for studying *in vitro* transformation and biochemical changes at various stages of carcinogenesis (20,21). These non-tumorigenic cells do not grow in soft agar; however, upon initiation with *N*-methyl-*N*-nitrosourea (MNU), they transform and form colonies in soft agar (22). Previous elegant studies of cancer chemoprevention have utilized RWPE-1 cells and the cancer cell lines derived from it (23).

In this study, we show that submicromolar concentrations of MSA inhibit the transformation of pre-malignant prostate epithelial cells, but not the growth and invasion of already malignant cells at this low concentration. Additionally, we show the role of PKCε in malignant transformation as well as tumor cell growth and invasion. Furthermore, we demonstrate that the over-expression of either PKCε, a redox target for MSA, or TR, an enzyme which reverses PKC redox modification, could lead to development of resistance to the cancer-preventive actions of selenium.

Abbreviations: BIM, bisindolylmaleimide; DAPI, 4′,6-diamidino-2-phenylindole; IC_{50}, 50% inhibitory concentration; KSFM, keratinocyte-serum-free medium; MNU, *N*-methyl-*N*-nitrosourea; MSA, methylseleninic acid; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PKC, protein kinase C; siRNA, small interfering RNA; TR, thioredoxin reductase.

Materials and methods

Chemicals

The MSA used in these experiments was a kind gift from Dr Howard Ganther, University of Wisconsin, Madison, WI. Aprotinin, leupeptin, pepstatin A, 5,5′-dithiobis(2-nitrobenzoic acid) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were from Sigma, St Louis, MO. Bisindolylmaleimide (BIM) and BIM V were from Alexis Biochemicals, Plymouth Meeting, PA. Auranofin and [γ-^{32}P]ATP (specific activity 20 Ci/mmol) were from MP Biochemicals, Irvine, CA. Using a previously published procedure, we raised rabbit polyclonal antibodies against PKCε by injecting hemocyanin coupled with sequence-specific peptide from the variable region in PKCε (19). This peptide and a PKC-specific substrate peptide that corresponds to a neurogranin sequence (residues 25–43) were synthesized at the core facility of Norris Comprehensive Cancer Center.

Cell culture and treatments

The RWPE-1 cell line used in this study was kindly provided by Dr Mukta Webber, University of Michigan, East Lansing, MI. RWPE-1 cells were maintained in complete keratinocyte-serum-free medium (KSFM) containing bovine pituitary extract (50 μg/ml), epidermal growth factor (5 ng/ml) and 1% antibiotic and antimycotic mixture (penicillin 100 U/ml, streptomycin 100 μg/ml and fungizone 0.25 μg/ml). Androgen-independent DU145 prostate carcinoma cells were obtained from the American Type Culture Collection and maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum. MSA was diluted in Hanks' balanced salt solution and added to the culture medium. MNU was dissolved in ethanol and used immediately. When agents were dissolved in organic solvents, appropriate solvent controls were used.

In vitro transformation and anchorage-independent growth in soft agar

RWPE-1 cells were seeded in six-well plates in complete KSFM at a density of 5×10^4 cells per well. After 1 day, cells were treated with MNU at a final concentration of 50 μg/ml for 1 h. Then cells were washed with Hanks' balanced salt solution and maintained in complete KSFM. Clonal expansion of initiated RWPE-1 cells was promoted in soft agar by epidermal growth factor (22). For soft agar growth, 2 ml of 0.6% agar in complete KSFM were placed in 35 mm petri dishes. One day after MNU treatment, RWPE-1 cells derived from each well were seeded separately at 5×10^4 cells per 35 mm petri dish in 2 ml of 0.3% agar over the bottom agar layer in complete KSFM and then incubated in a cell culture incubator. On days 9 and 18, 1 ml of 0.3% agar in complete KSFM with or without MSA was added. After 28 days, colonies consisting of >20 cells were counted.

Isolation of transformed prostate cancer cell line (RW-M)

From a rapidly growing colony of RWPE-1 MNU transformants in soft agar, cells were isolated and allowed to grow in an anchorage-dependent manner. These cells were again subjected to soft agar growth and the rapidly growing cells were isolated. This process of selection was repeated two more times. The transformed cell line thus isolated was referred to as RW-M (M denotes MNU). The soft agar growth and Matrigel invasion of RW-M cells are higher than that of RWPE-1 untransformed cells, but they are lower than those of highly malignant DU145 cells. The RW-M-transformed cell line maintained its intermediate malignant phenotypic properties such as growth in soft agar and Matrigel invasion even after several passages in cell culture.

PKC assay

Cells were treated with MSA and then homogenized in buffer (20 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 0.5 mM phenylmethylsulfonyl fluoride, 150 nM pepstatin A, and 1% Igepal CA-630). Unless otherwise indicated, mercapto compounds were omitted from all the buffers used for cell homogenization and chromatographic isolation of PKC. The cell extracts were subjected to diethylaminoethyl-cellulose chromatography as described previously (24). The assay of PKC was carried out in 96-well plates (24). Briefly, PKC reaction samples containing 20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.33 mM $CaCl_2$, 0.1 mM [γ-^{32}P]ATP (3 million c.p.m.), 5 μM neurogranin substrate polypeptide (residues 25–43), 40 μM leupeptin and 25 μl of PKC sample in a total volume of 125 μl were incubated for 5 min at 30°C. The reaction was arrested with 10 μl of 1 M phosphoric acid, the samples were applied to Whatman P81 paper (2 × 2 cm) and the papers were washed four times with 75 mM phosphoric acid. Radioactivity retained in the washed paper was counted. The basal activity observed in the absence of cofactors was subtracted from the activity observed in the presence of cofactors. The difference was expressed as PKC activity in units, where 1 U enzyme transfers 1 nmol of phosphate to neurogranin polypeptide per minute at 30°C.

Western immunoblotting for PKCε isoenzyme

Cell extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Electrophoretically separated proteins were transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% dry milk and subsequently incubated with PKCε isoenzyme-specific primary antibodies followed by goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase. The immunoreactive bands were visualized by the enhanced chemiluminescence western blot detection kit (Pierce, Rockford, IL). These bands were analyzed by densitometric scanning using the Omega 12 IC Molecular Imaging System and UltraQuant software.

Stable transfection of PKCε

The metallothionein expression vector (25) used in these experiments was a kind gift from Dr Wayne Anderson, National Cancer Institute, Bethesda, MD. The cells were transfected with either a metallothionein-driven PKCε expression vector (to over-express PKCε) or an empty vector (as a control) using Lipofectamine 2000 according to the manufacturer's recommended procedure. One day after transfection, RWPE-1 cells were plated at a lower density and grown in a selection medium containing 450 μg/ml G418. After 4 weeks in the selection medium, single colonies were picked, expanded and screened for the presence of PKCε by using western blot analysis.

Transient transfection of RW-M cells with PKCε small interfering RNA

RW-M cells were plated in a six-well plate. After 24 h, 50 nM PKCε small interfering RNA (siRNA) oligonucleotides (three pre-designed Silencer oligonucleotides from Ambion, Austin, TX) were transfected into RW-M cells with Lipofectamine 2000 according to the manufacturer's instructions. As a negative control, we used scrambled siRNA that did not exhibit homology to any encoding region but had similar guanine and cytosine content. The experiments were continued with the PKCε siRNA oligonucleotide producing the greatest knockout (a decrease of ∼70–80% from the control).

TR assay

TR activity was determined using an insulin–thioredoxin method (26). Sulfhydryls released from insulin were determined by 5,5′-dithiobis(2-nitrobenzoic acid) and the absorbance of the nitrothiobenzoic acid was measured at 405 nm. This method was modified to suit 96-well plates. One unit of the enzyme produces one absorbance at 405 nm/min.

Anchorage-dependent cell growth assay

Cells grown in 96-well plates were treated with various concentrations of MSA for 48 h. Then, cells were fixed in 10% trichloroacetic acid. After washing them with water, the cells were stained with a 0.4% solution of sulforhodamine B for 30 min. The excess dye was removed by washing the cells with 1% acetic acid, and the dye bound to the cells was later dissolved in 10 mM Tris base. The absorbance was read at 550 nm as a growth index (27).

Cell viability assay

We determined the cytotoxicity of MSA using the MTT reduction assay. Initially, cells were grown in 96-well plates and treated with MSA for 24 h. Then, an MTT solution (5 mg/ml) in Hanks' balanced salt solution was added, and the plates were incubated for 4 h. The formazan formed was dissolved in dimethylsulfoxide, and the absorbance was read at 550 nm (28).

Apoptosis assay

To assess morphological changes in the chromatin structure of cells undergoing apoptosis, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were grown on culture slides, treated with MSA for 24 h and then fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min at room temperature. After rinsing them with phosphate-buffered saline, cells were stained with DAPI (10 μg/ml) for 5 min. The morphology of the nuclei was observed using a fluorescence microscope (Nikon Eclipse TE300) at an excitation wavelength of 345 nm. Apoptotic nuclei were identified by chromatin condensation and fragmentation. The incidence of apoptosis in each preparation was analyzed by counting 500 cells and determining the percentage of apoptotic nuclei (29).

Caspase-3 assay

Enzyme activity was determined by using tetrapeptide substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) with an assay kit obtained from BIOMOL (Plymouth Meeting, PA). Cells were seeded in 100 mm petri dishes and allowed to grow for 24 h. The cells were treated with various concentrations of MSA for 24 h. Then, the treated cells were homogenized, and caspase-3 activity was determined according to the manufacturer's instructions.

Cell invasion assay

Approximately 100 μg of reconstituted basement membrane (Matrigel) was used to coat polycarbonate filters (8 μm, pore size) fitted in Transwell inserts (Costar, Cambridge, MA). Prostate cancer cells were grown to confluency and detached by brief trypsinization, and 200 000 cells were added to a Transwell insert in minimum essential medium supplemented with 0.1% fetal calf serum. In the lower well, 1% fetal calf serum was added as a chemoattractant. After 24 h, the cells that invaded the lower chamber were counted (30).

Results

Enhanced sensitivity of promoting cells to MSA

For this study, we used MSA, an oxidation product of methylselenol that has been postulated to mediate the cancer-preventive actions of selenium (6,31). A recent study has revealed superior *in vivo* inhibitory efficacy of MSA against prostate cancer over selenomethionine or selenite (32). Considering that only limited amounts of free selenocompounds are available to tissues from the plasma, we have compared the relative sensitivity of cell transformation, cell growth, cell invasion and apoptosis to MSA in order to identify which of the processes is more susceptible to selenium. Since the precise intracellular concentration of low-molecular weight selenocompounds is not known, we have considered MSA effects occurring at submicromolar concentrations to be sensitive ones and the effects occurring in the micromolar range to be relatively resistant ones. MSA completely inhibited MNU-initiated transformation of RWPE-1 cells with a 50% inhibitory concentration (IC_{50}) of 0.35 μM (Figure 1). This concentration of MSA was significantly lower than that required for the inhibition of soft agar growth of already transformed prostate cancer cells (RW-M), which was in the low micromolar range. Similarly, inhibition of anchorage-independent (soft agar) growth of other prostate cancer cells, such as DU145, required micromolar concentrations of MSA (Figure 1). The greater sensitivity of RWPE-1 cell transformation to MSA is either due to the process of transformation itself being more sensitive to selenium or the initiated RWPE-1 cells being more susceptible to selenium-induced growth inhibition in soft agar. Since RWPE-1 cells cannot grow in soft agar, it is difficult to determine whether anchorage-independent growth of RWPE-1 cells is more sensitive to MSA. Therefore, another set of experiments was performed in which anchorage-dependent growth was compared among the three cell types.

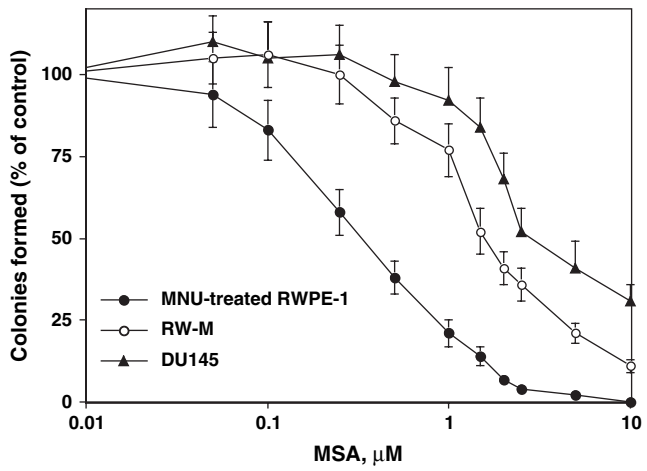

Fig. 1. MSA-induced inhibition of anchorage-independent growth of prostate cells in soft agar. Initially, RWPE-1 cells were treated with MNU (50 μg/ml) for 1 h. The MNU-treated RWPE-1 cells (50 000), RW-M cells (5000) or DU145 cells (5000) were suspended in 0.3% agar in complete KSFM along with the indicated concentrations of MSA. This suspension was layered over the top of 0.6% agar containing the same concentrations of MSA in complete KSFM in 35 mm petri dishes and incubated in a cell culture incubator. After 28 days, colonies consisting of >20 cells were counted. The mean number of colonies formed in the absence of MSA for RWPE-1 (MNU treated), RW-M and DU145 cells were 94, 123 and 228, respectively. These values were expressed as 100%. The data are means ± standard errors of three experiments.

For comparison of MSA sensitivity, all these three cell lines were switched to complete KSFM medium so that the differences in medium constituents would not influence cell sensitivity to MSA. Anchorage-dependent growth was measured by sulforhodamine B assay. RWPE-1 cells (not MNU treated) were most susceptible to MSA-induced growth inhibition with an IC_{50} of 0.75 μM (Figure 2A). This was followed by RW-M cells (IC_{50} = 1.5 μM), which were more susceptible to MSA than DU145 cells (IC_{50} = 2 μM).

Cytotoxicity and cell viability of MSA-treated cells were determined by an MTT assay (28). As shown in Figure 2B, MSA decreased the viability of these cells to a variable extent. Again, RWPE-1 cells were most sensitive to MSA-induced cytotoxicity, followed by RW-M cells and DU145 cells. Next, we assessed whether the loss of viability induced by MSA correlated with the induction of apoptosis, a common property of many cancer-preventive agents (33). Apoptosis was determined by identifying chromatin condensation and fragmentation after DAPI staining (29). At a low concentration (<1 μM), MSA induced apoptosis to an appreciable extent in RWPE-1 cells, whereas a high concentration (>1 μM) was required to induce apoptosis in RW-M cells and DU145 cells (Figure 2C). Under these conditions, the activation of caspase-3 correlated well with the observed apoptosis by DAPI staining method (Figure 2D). Collectively, these results suggest that RWPE-1 cells and intermediate RW-M cells are more sensitive to the cytotoxic effect of MSA than highly malignant DU145 cells.

Invasion is the hallmark of malignancy. Therefore, we measured the invasive ability of these cell types *in vitro*. Parent RWPE-1 cells did not show Matrigel invasion. However, RW-M-transformed cells, derived from RWPE-1 cells, showed invasion of nearly 55% that of the highly malignant DU145 cell line. MSA inhibited Matrigel invasion in both cell types (Figure 2D). However, the invasion of RW-M cells was more sensitive to MSA than that of DU145 cells.

In our studies, selenomethionine at low (1–10 μM) concentrations did not produce any significant effect on cell transformation, apoptosis or invasion. This is not an unexpected finding, as selenomethionine requires metabolism into methylselenol for efficacy, a process that may be slow in cells in culture.

Effect of PKC inhibitors on the clonal expansion of MNU-initiated cells

First, it is important to establish a causal role of PKC isoenzymes in prostate cell transformation before evaluating PKC as a molecular target for selenium in preventing prostate cell transformation. We assessed the role of PKC in cell transformation by using three cell-permeable PKC-specific inhibitors, each acting by a different mechanism. Calphostin C is a PKC-specific inhibitor that irreversibly inactivates PKC by binding to the regulatory domain (34). Chelerythrine is an irreversible inactivator of PKC and covalently binds to cysteine residues at the catalytic site (34). BIM was used at low (100 nM) and high (2 μM) concentrations to preferentially inhibit a subset of PKC isoenzymes.

Both calphostin C (100 nM) and chelerythrine (2 μM) substantially decreased colony formation of MNU-initiated RWPE-1 cells in soft agar (Figure 3A). This supports a critical role for PKC in prostate cell transformation. BIM at 100 nM concentration partially inhibited cell transformation. Since BIM is a highly potent inhibitor for conventional PKC isoenzymes with an IC_{50} of ~10 nM (35), a partial inhibition of cell transformation suggests the role of conventional PKC isoenzymes in RWPE-1 cell transformation. However, a lack of complete inhibition even at 100 nM (a 10-fold higher than IC_{50}) suggests the involvement of other PKC isoenzymes as well whose inhibition requires higher concentration of BIM. Since BIM inhibits novel PKC isoenzymes (δ and ε) with lower affinity (IC_{50} ~0.2 μM), we tested whether BIM at a higher (2 μM) concentration inhibits cell

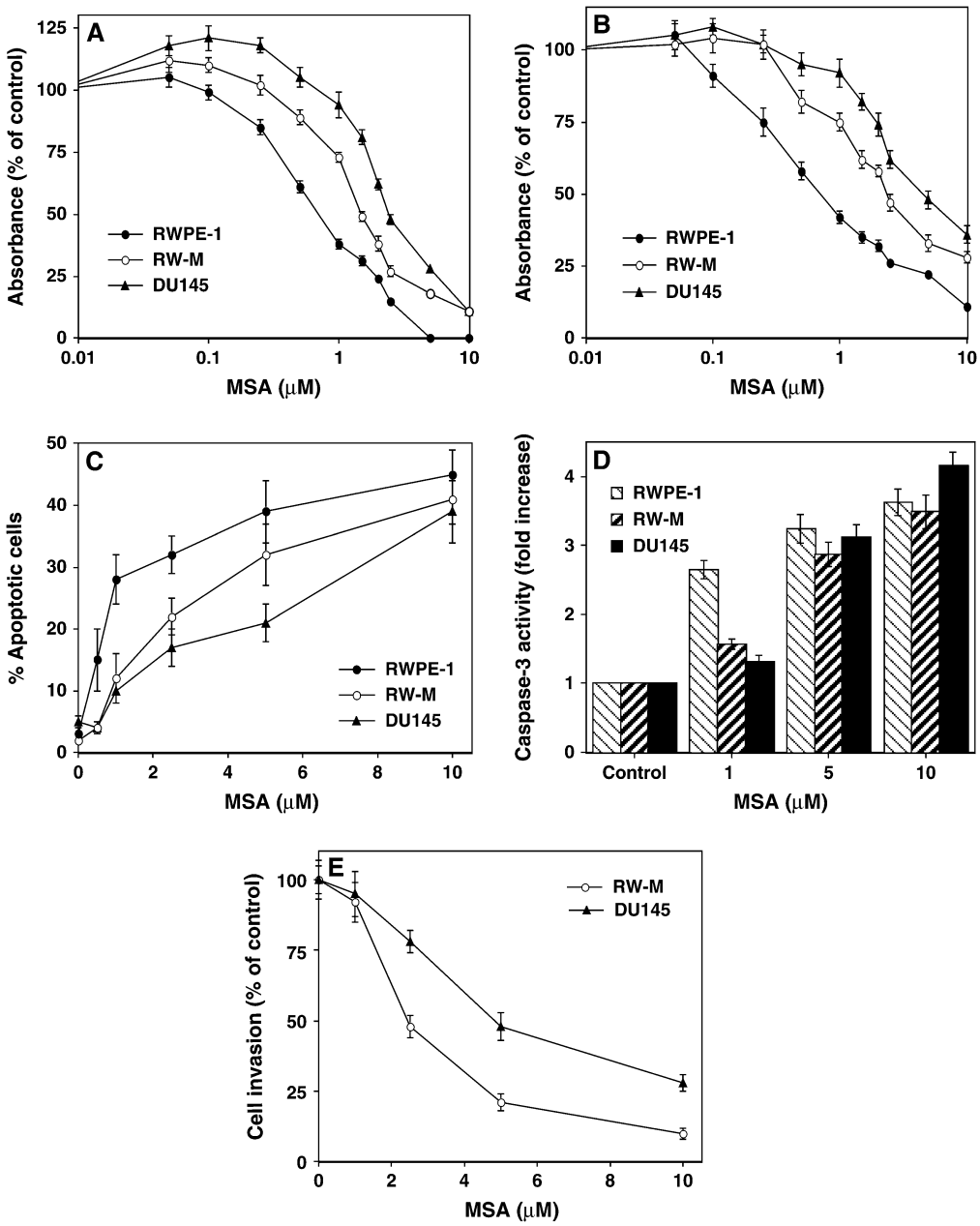

Fig. 2. Differential effects of MSA on cell growth, cytotoxicity, apoptosis, caspase-3 activity and invasion of RWPE-1, RW-M and DU145 cells. (**A**) MSA-induced cell growth inhibition. Cells were seeded in 96-well culture plates, and after 48 h, cells were treated with the indicated concentrations of MSA for 48 h. Then cells were stained with sulforhodamine B, and the absorbance was measured at 550 nm. (**B**) MSA-induced cytotoxicity. Cells grown in 96-well plates were treated with MSA for 24 h and then incubated with MTT for 4 h. The formazan that developed was dissolved, and the absorbance was read at 550 nm. (**C**) MSA-induced apoptosis. Cells, grown on culture slides, were treated with MSA for 24 h and then stained with DAPI. The incidence of apoptosis in each sample was analyzed by counting 500 cells and determining the percentage of apoptotic nuclei. (**D**) MSA-induced activation of caspase-3. Cells were grown in 100 mm petri dishes and treated with indicated concentrations of MSA. The caspase-3 activity was determined as described in Materials and methods. The mean values of caspase-3 activity in the control RWPE-1, RW-M and DU145 cells were 0.67, 0.73 and 0.85 U/μg (*p*-nitroaniline formed per minute) of protein, respectively. (**E**) MSA-induced inhibition of tumor cell invasion. Prostate cancer cells (200 000) were added to Transwell inserts in which filters were coated with Matrigel. These cells were treated with the indicated concentrations of MSA. After 24 h, the cells that invaded Matrigel were counted. Cells that invaded in the absence of MSA were considered as 100%. For A and B, the values are the mean ± standard error of eight replicate estimations. For C, D and E, the values are the mean ± standard error of three estimations.

transformation. At this concentration, BIM further inhibited colony formation in soft agar. In order to exclude the possibility of non-specific inhibition of other enzymes by BIM at a high (2 μM) concentration, we used its inactive analogue BIM V (2 μM) as a negative control. This inactive analogue did not inhibit RWPE-1 cell transformation. Since BIM poorly inhibits PKCζ with a high IC_{50} of ∼5 μM, it is unlikely that PKCζ plays a key role in RWPE-1 prostate cell transformation. These observations support the role of both classic isoenzymes and novel isoenzymes in the RWPE-1 cell transformation.

Role of PKCε in invasion of RW-M prostate cancer cells

As shown in Figure 3B, Matrigel invasion of RW-M cells was substantially inhibited by both calphostin C (100 nM) and chelerythrine (2 μM). BIM was required at a high (2 μM) concentration to inhibit tumor cell invasion, suggesting the involvement of PKCε and/or

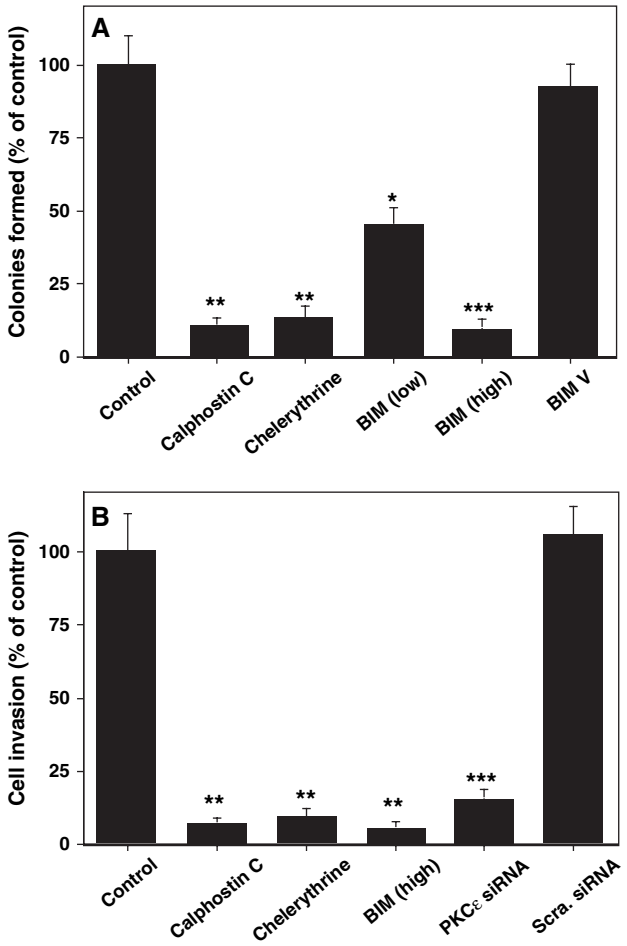

Fig. 3. PKC inhibition blocks cell transformation and Matrigel invasion. (**A**) PKC-specific inhibitors block transformation of RWPE-1 cells. MNU-treated RWPE-1 cells (50 000) were grown in soft agar in the presence of the following PKC inhibitors: 200 nM calphostin C, 2 μM chelerythrine, 100 nM BIM (low), 2 μM BIM (high) and 2 μM BIM V (inactive analogue). After 28 days, colonies consisting of >20 cells were counted. In the absence of inhibitors, the mean number of colonies formed for MNU-treated RWPE-1 cells was 102. This value was expressed as 100%. The data are the means ± standard errors of three experiments. **, Values for calphostin C and chelerythrine are statistically different from control (paired *t*-test $P < 0.01$). *, Value for BIM (low) is statistically different from negative control BIM V (*t*-test $P < 0.05$). ***, Value for BIM (high) is statistically different from its negative control BIM V ($P < 0.01$). (**B**) PKC-specific inhibitors block Matrigel invasion of RW-M prostate carcinoma cells. The PKC inhibitors mentioned above were included in both Transwell inserts and lower chambers and invasion was determined after 24 h. For the siRNA experiment, monolayer cells that were growing in six-well culture plate were treated with siRNA or scrambled siRNA oligonucleotides for 24 h. Then, cells were detached and subjected to Matrigel invasion assay in the presence of siRNA oligonucleotides. The values are the mean ± standard error of three experiments. **, Values for PKC inhibitors (calphostin C, chelerythrine and BIM) are statistically different from the control (paired *t*-test $P < 0.01$). ***, Value for PKCε siRNA is statistically different from its control scrambled siRNA ($P < 0.01$).

PKCδ isoenzymes. PKCε can induce the aggressive behavior of tumors and is involved in invasion of tumor cells (36). Therefore, we determined the role of this PKC isoenzyme in tumor cell invasion by using an siRNA approach. RW-M cells transfected with PKCε siRNA, exhibiting a decrease of >70% in PKCε, showed a substantial decrease in invasion. Scrambled siRNA did not cause a significant decrease in invasion, suggesting the involvement of PKCε in this process.

Inverse relationship between distribution of PKCε and cellular sensitivity to MSA

Since PKCε is an oncogenic, pro-mitogenic and pro-survival enzyme (36), we determined its intracellular levels by western immunoblotting to evaluate whether the intracellular levels of this isoenzyme correlate with cellular sensitivity to MSA. As shown in Figure 4A, PKCε was low in RWPE-1 cells, intermediate in RW-M cells and high in DU145 cells, inversely correlating with their relative sensitivity to MSA.

Over-expression of PKCε decreases cellular sensitivity to MSA

To evaluate the role of PKCε as a cellular resistance factor to MSA, we determined whether elevation of its levels inhibits the cancer-preventive actions of MSA. PKCε was transfected into RWPE-1 cells and then the sensitivity of these cell lines to MSA was evaluated. As shown in Figure 4B, RWPE-1 cells transfected with PKCε showed enhanced transformation as judged by growth in soft agar compared with the control cells transfected with an empty vector. Furthermore, the concentration of MSA needed to inhibit RWPE-1 cell transformation increased by nearly 10-fold after transfection with PKCε. Control RWPE-1 cells transfected with empty vector did not show resistance to MSA. Thus, with over-expression of PKCε, a resistance was developed to MSA-induced inhibition of cell transformation. Furthermore, over-expression of PKCε in RWPE-1 cells also led to resistance of MSA-induced apoptosis (Figure 4C). Similarly, an over-expression of PKCε in the moderately MSA-sensitive RW-M cell line resulted in a decrease in sensitivity to MSA-induced inhibition of cell growth and induction of apoptosis (Figure 4D, E and F). These results suggest that PKCε over-expression during tumor progression makes prostate cancer cells less sensitive to the cancer-preventive actions of MSA.

Inverse relationship between TR activity and cellular sensitivity to MSA

Given that the TR system can reverse MSA-induced modification of purified PKC (19), its intracellular over-expression may counteract those of selenium's actions that are caused by selenium-induced PKC inactivation. Therefore, we determined the activity of this reductase in prostate cells. As shown in Figure 5A, TR activity was low in RWPE-1 cells, intermediate in RW-M cells and high in DU145 cells, inversely correlating with their relative sensitivity to MSA. Furthermore, MSA induced TR activity in RWPE-1, RW-M and DU145 cells by 1.1-, 1.9- and 2.7-fold, respectively, from their base level.

TR counteracts MSA-induced PKC inactivation and cellular actions

When RWPE-1 and RW-M cells were treated with MSA, a substantial decrease in the activity of PKC (all isoenzymes) occurred within 5–15 min, which then partially recovered over a 90 min time period to a level still below that of the control (Figure 5B and C). The extent of inactivation of PKC inversely correlated with TR distribution, suggesting that TR may be reversing the MSA-mediated inactivation of PKC. To assess the role of TR in intact cells, we have used auranofin, a potent specific inhibitor of TR (IC_{50} = 20 nM). A nearly 1000-fold higher concentration of auranofin is required to inhibit other selenoenzymes such as glutathione peroxidase and sulfhydryl enzymes such as glutathione reductase and PKC (37). Thus, it is likely that only TR is inhibited in cells treated with the limited concentration of auranofin (25–100 nM) used in this study. Since the TR system reverses the MSA-induced redox modification of purified PKC (19), we used auranofin to determine whether TR system blockade enhances PKC inactivation by MSA in intact cells. As shown in Figure 5B and C, auranofin pre-treatment increased MSA-induced inactivation of PKC and minimized its reversal, suggesting that TR can reverse MSA-induced PKC inactivation.

In addition to increasing MSA-induced inactivation of PKC in intact cells, auranofin enhanced the cancer-preventive actions of selenium. As shown in Figure 6A, auranofin co-treatment resulted in a reduction in the concentration of MSA required to inhibit

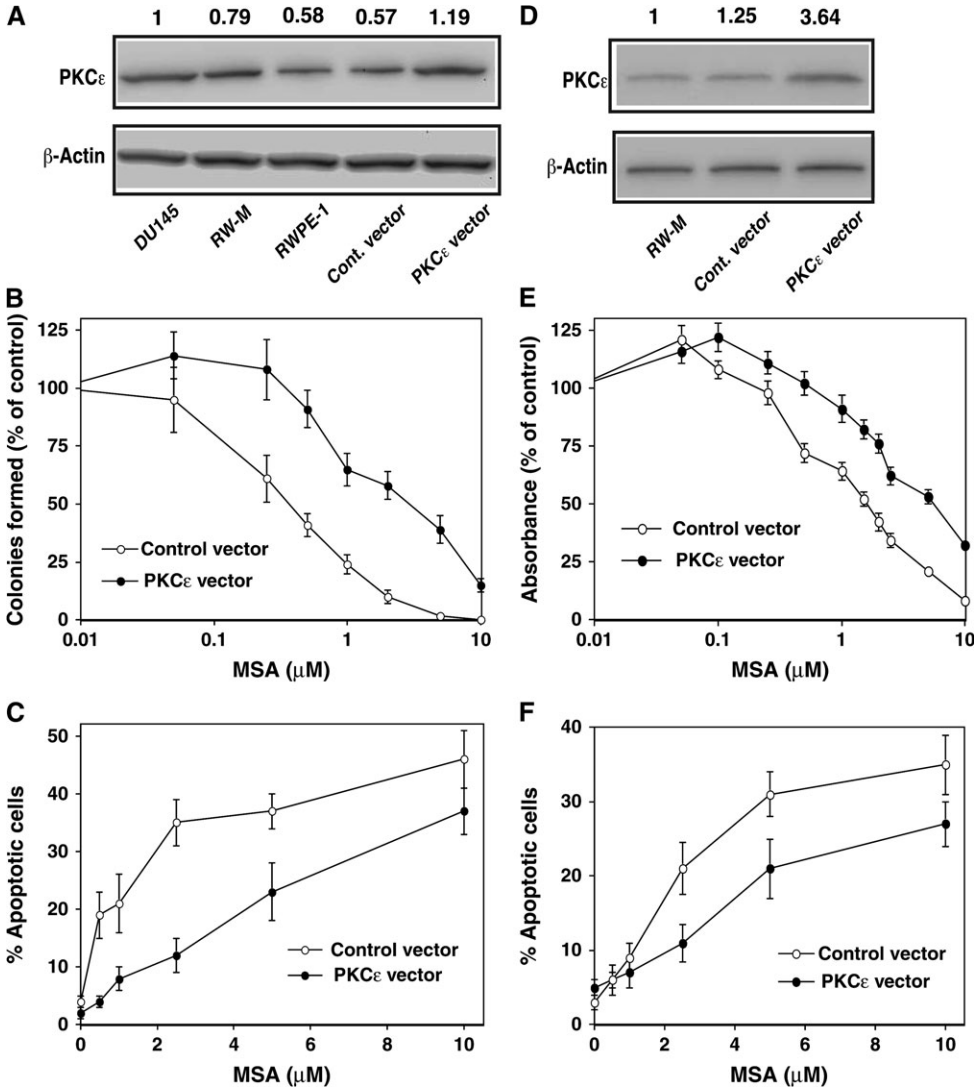

Fig. 4. (**A**) Western immunoblotting of PKCε in prostate cells (DU145, RW-M and RWPE-1) and RWPE-1 cells stably transfected with either a control vector or a metallothioneine-driven expression vector. β-Actin as the loading control was carried out. The band density of PKCε in each sample was quantitated by densitometry and expressed considering the band density of DU145 cells as one. (**B**) Resistance of RWPE-1 cells over-expressing PKCε to MSA-induced inhibition of cell transformation. Growth in soft agar was compared between MNU-treated RWPE-1 cells transfected with PKCε vector and MNU-treated RWPE-1 cells transfected with control vector. In the absence of MSA, the mean values of colonies formed for these two cell types were 164 and 89, respectively. These values were expressed as 100%. (**C**) Resistance of RWPE-1 cells over-expressing PKCε to MSA-induced apoptosis. Apoptosis was measured by identifying chromatin condensation and fragmentation after DAPI staining. (**D**) Western immunoblotting of PKCε in RW-M cells, RW-M cells transfected with control vector and RW-M cells transfected with PKCε vector. The band density was expressed considering the band density of RW-M as one. (**E**) Resistance of RW-M cells over-expressing PKCε to MSA-induced cell growth inhibition. Anchorage-dependent cell growth was determined as described in Materials and methods. (**F**) Resistance of RW-M cells over-expressing PKCε to MSA-induced apoptosis. The results obtained with the control cells transfected with empty vector are not significantly different from that of the wild-type control cells. Therefore, the results obtained with wild-type cells were not shown in (B, C, E and F). For (E), the values are the mean ± standard error of eight replicate estimations. For (B, C and F), the values are the mean ± standard error of three experiments.

RW-M cell growth. Similarly, auranofin sensitized RW-M cells to MSA-mediated induction of apoptosis (Figure 6B). Collectively, these results suggest that TR expression can counteract MSA action and can negate the cancer-preventive actions of selenium in tumor cells.

Discussion

The following key points emerge from this study. First, based on the results of our study, it is clear that clonally expanding initiated cells (promotion) are likely to be the most sensitive to selenium's action, followed by early stage tumor cells and finally by advanced tumor cells. After administration of cancer-preventive doses of selenomethionine, the majority of selenium in circulation is present in selenoproteins and albumin and <3% of selenium exists in the low-molecular weight form in the plasma (12,13). In humans supplemented with selenized yeast (200 μg selenium per day), total plasma concentration of selenium reaches levels of 200 ng/ml that corresponds to a ~2.5 μM (38). Therefore, it is likely that under these conditions, plasma concentrations of low-molecular weight selenium are exceedingly low (<1 μM). Furthermore, the methylated selenometabolites, methylselenol and dimethylselenide are volatile and unlikely to accumulate in high amounts in tissues. Therefore, only cellular processes that are highly sensitive to selenium are likely to be blocked by selenium supplementation. Although at high concentration selenium kills advanced tumor cells in culture, the selenium supplementation in a canine model indicated an intriguing U-shaped dose–response relationship between selenium status and the extent of DNA damage within the prostate (39).

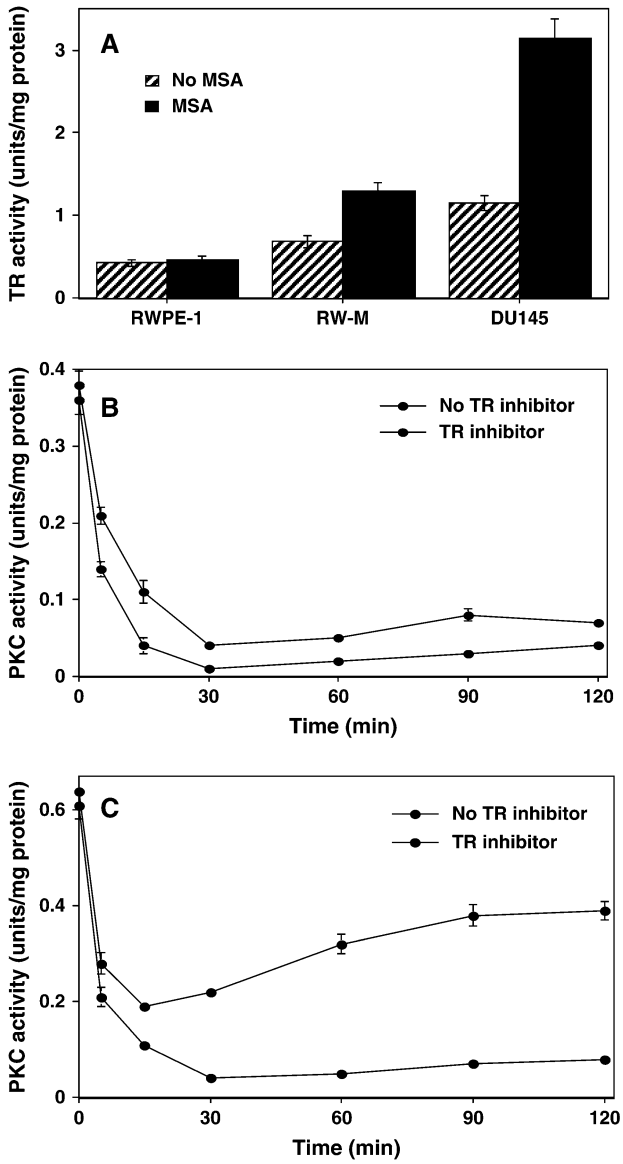

Fig. 5. (**A**) Basal and MSA-induced TR activity in prostate cells. TR activity was determined in RWPE-1, RW-M and DU145 cells incubated for 24 h in a medium without added selenium and in a medium to which MSA (100 nM) was added. MSA-induced inactivation of PKC in RWPE-1 cells (**B**) and RW-M cells (**C**) and its enhancement by TR inhibition. Cells were initially treated with TR-specific inhibitor auranofin (100 nM) or vehicle (ethanol) for 1 h and then treated with MSA (5 μM) for the indicated time periods. Cells were homogenized and total PKC isoenzymes were extracted from both the cytosol and membrane and subjected to diethylaminoethyl-cellulose chromatography (24). PKC activity was determined using neurogranin peptide as a substrate. The values are the mean ± standard error of triplicate estimations.

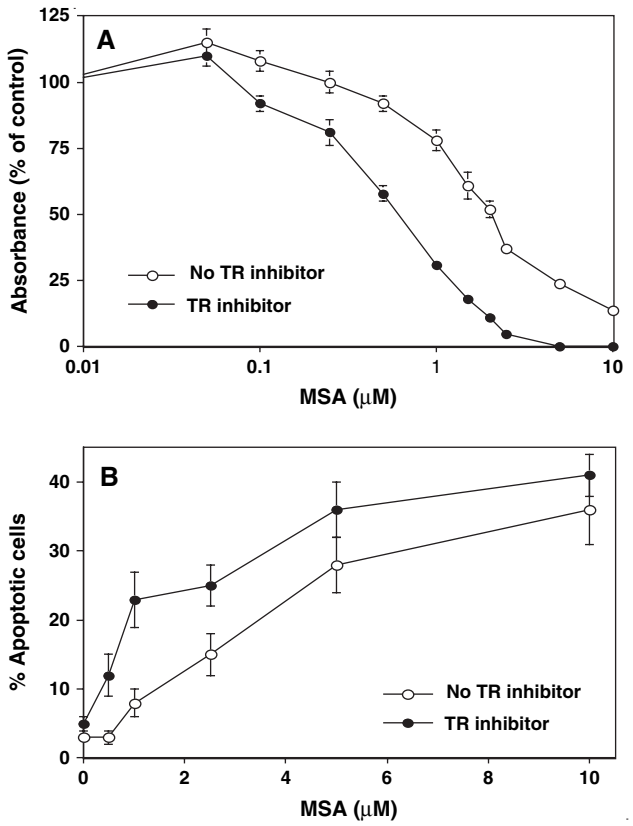

Fig. 6. (**A**) Inhibition of TR in RW-M cells enhances their sensitivity to MSA-induced cell growth inhibition. RW-M cells were seeded in 96-well culture plates, and after 24 h, cells were treated with indicated concentrations of MSA along with auranofin (50 nM) or vehicle (ethanol) for 48 h. Then, cell growth was determined by sulforhodamine B staining. The values are the mean ± standard error of eight replicate estimations. (**B**) Apoptosis induced by MSA in the presence or absence of auranofin. RW-M cells, grown on culture slides, were treated with MSA with or without auranofin and then apoptotic nuclei were analyzed by using DAPI staining. The values are the mean ± standard error of three experiments.

Selenomethionine was detected in trace quantities in the prostate (40). Moreover, various pre-clinical models of prostate cancer did not support any *in vivo* anticancer activity of selenomethionine (32). Therefore, it has been argued that selenomethionine was an inappropriate choice for Selenium and Vitamin E Cancer Prevention Trial, which did not support the efficacy of selenium in the prevention of prostate cancer (9). Selenomethionine non-specifically incorporates into proteins (6). In addition, it needs to be metabolized to methylselenol to exert its cancer-preventive effects, which may be a limiting factor.

Second, the current studies have shown that RWPE-1 cells are more sensitive to MSA than prostate cancer cells. Previous studies also have shown that RWPE-1 cells are more sensitive to selenite than LNCaP and PC3 prostate cancer cell lines (41). Although the conditions used in our study and in others are different, RWPE-1 cells have consistently shown a higher sensitivity to selenium. Conversely, few studies have shown resistance of normal prostate cells to selenium (42). Although RWPE-1 cells possess some features resembling normal prostate epithelial cells such as lack of growth in soft agar or nude mice and lack of Matrigel invasion, they are immortalized by papillomavirus 18, a postulated etiologic factor involved in prostate carcinogenesis (21). Despite RWPE-1 cells being widely used as normal cells, they are likely to be already partially initiated even prior to treatment with MNU. MNU-initiated or promoted RWPE-1 cells are then likely to be even more sensitive than parent RWPE-1 cells to MSA.

Third, selenium may encounter a resistance mechanism to its cancer-preventive actions. Such counteractive resistance may be present in pre-cancer and tumor cells constitutively or induced as a cellular adaptive response to selenium's actions. Elevated levels of redox target PKCε, which is modified at its sulfhydryls by selenium, and elevated levels of selenoprotein TR, the enzyme that reverses this modification, may be involved, at least in part, in the counteractive mechanisms to selenium's cancer-preventive actions. PKCε, an oncogenic, pro-mitogenic and pro-survival enzyme, is involved in tumor cell invasion (36). This isoenzyme is moderately sensitive to MSA (19). An over-expression of this enzyme in RWPE-1 cells caused a loss of their sensitivity to selenium. Selenoproteins as

antioxidants may oppose the actions of redox-active selenocompounds, which are pro-oxidants. Selenometabolites as pro-oxidants produce free radicals and cause lipid peroxidation and DNA damage (43–45). Selenocompounds such as selenite produce hydrogen peroxide and lipid peroxides (43,45), which are removed by the selenoprotein glutathione peroxidase. Similarly, selenometabolite-induced protein thiol oxidation is reversed by TR (19). Thus, selenium itself induces its own adaptive cellular response to counteract its toxic action.

Besides selenium, a variety of cancer-preventive agents induce oxidative stress in tumor cells resulting in cell death (46–48). Induction of antioxidant enzymes such as TR is an adaptive response to cell survival. Thus, pre-cancer cells may escape from pro-oxidant cancer-preventive agents, especially when these agents are given at low doses for many years. It has also been shown that TR inhibition may enhance the ability of anticancer drugs to induce cell death (49). Furthermore, PKC may play a role in the induction of TR, suggesting an interaction between these two enzymes that results in resistance to selenium (50).

The resistance mechanisms we have proposed account for both the success and failure of selenium as a cancer-preventive agent. Understanding these mechanisms is also relevant to the study of other pro-oxidant chemopreventive agents such as vitamin E, vitamin C and polyphenolic agents. Within the class, selenium is an ideal model for study as the molecular players (selenometabolites and selenoproteins) have been well characterized.

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