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PRINCIPAL INVESTIGATOR: James D. Brooks, M.D.

CONTRACTING ORGANIZATION: Stanford University
Stanford, California 94305-5401

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James D. Brooks, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Stanford University
Stanford, California 94305-5401

E-Mail: jdbrooks@stanford.edu

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13. ABSTRACT *(Maximum 200 Words)*

Virtually all human prostate cancers lose expression of glutathione *S*-transferase-π, an enzyme that protects against oxidative electrophiles that attack the DNA and result in cancer. Based on this observation, we proposed identifying compounds effective at inducing other carcinogen defense (phase 2) enzymes. During our Phase I Award, we identified sulforaphane as the most potent inducer of carcinogen defenses in the prostate cell. We have characterized global effects of sulforaphane in prostate cancer cell lines using cDNA microarray technology that allows large-scale determination of changes in gene expression. Our finding has been substantiated by recent epidemiologic findings of a link between high consumption of broccoli (a rich source of sulforaphane) and decreased risk of prostate cancer. These findings argue strongly for a preventive intervention trial involving supplementation with sulforaphane. Furthermore, our work demonstrates the utility of cDNA microarray technology in understanding the mechanisms of action of preventive agents. We plan to exploit this opportunity in future investigations.

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Phase I: Final Progress Report

Prostate Cancer Prevention Through Induction of Phase 2 Enzymes

New Investigator Award
DAMD17-98-1-8555

James D. Brooks, M.D.

Stanford University School of Medicine
Department of Urology, Room S 287
Stanford, CA 94305-5118 USA

Tel. 650-723-3745
Fax: 650-723-0765
jdbrooks@stanford.edu

Introduction

We have identified the earliest and most universal genetic alteration thus far described in human prosdtate cancer: loss of expresson of the enzyme glutathione *S*-transferase-π due to methylation of "CpG islands" in the regulatory regions of the *GSTP1* gene. This enzyme is a member of the class of phase 2 enzymes, know to detoxify carcinogens by conjugation to reduced glutathione. The phase 2 enzymes comprise a large and diverse group of enzymes that are quite labile in expression, and induction of expression by a variety of structurally unrelated compounds can protect against carcinogenesis. Because of this, we have proposed that a mechanistically based prevention strategy for prostate cancer may involve induction of phase 2 enzymes. We have identified sulforaphane as a potent phase 2 inducing agent in prostatic cells. Subsequent epidemiological studies by others have confirmed that individuals who consume high levels of sulforaphane have a decrease risk of developing prostate cancer. We are now working to understand the mechanism of action of sulforaphane and other putative prostate cancer preventive agents using cDNA microarray technology.

Report Body

1. Progress

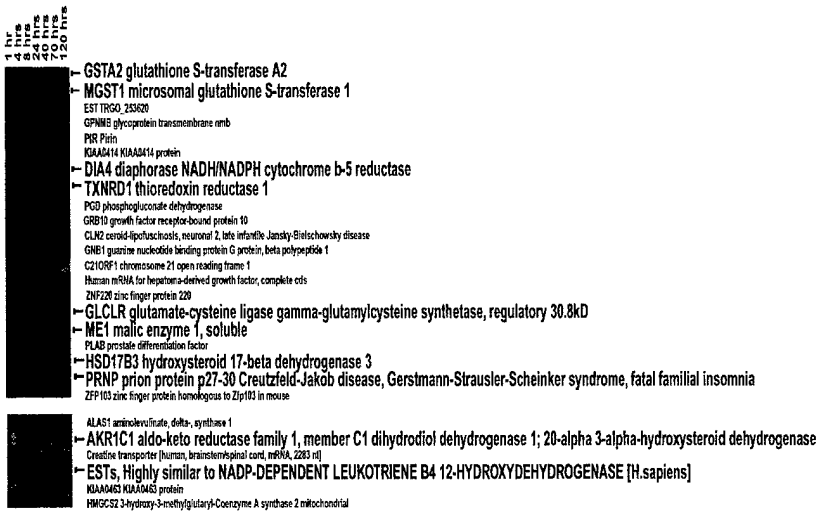
Task 1: To characterize phase 2 enzyme induction in human prostate cells *in vitro*. (mos. 1-15)

We have surpassed our original objectives of this task during the period of funding. Our initial intent was to identify promising preventive compounds and assess a few molecular genetic biomarkers of response to this compound. Early on our investigations, we identified sulforaphane, a isothiocynate found in cruciferous vegetables, as one of the most potent phase 2 enzyme inducing agents in prostate cancer cells *in vitro*. We went on to characterize the response of prostate cells to sulforaphane and our findings are summarized in a paper recently submitted to *Cancer Epidemiology, Biomarkers & Prevention*. Our findings were: 1) Sulforaphane potently induces quinone reductase activity in cultured prostate cells and this induction appears to be mediated by increased transcription of the *NQO-1* gene. 2) Sulforaphane also induces expression of γ-GCS light subunit, but not the heavy subunit, and this induction is associated with moderate increases in intracellular glutathione levels. 3) Microsomal and α-class glutathione transferases were also induced transcriptionally. Our findings demonstrate that regulation of phase 2 enzymes is far more complicated than previously described. In the past, Phase 2 enzyme induction has been attributed to antioxidant response elements (ARE) in the regulatory regions of phase 2 enzyme genes. Our findings clealr call such a simplified model into question. We continue to delve into regulation of these interesting genes.

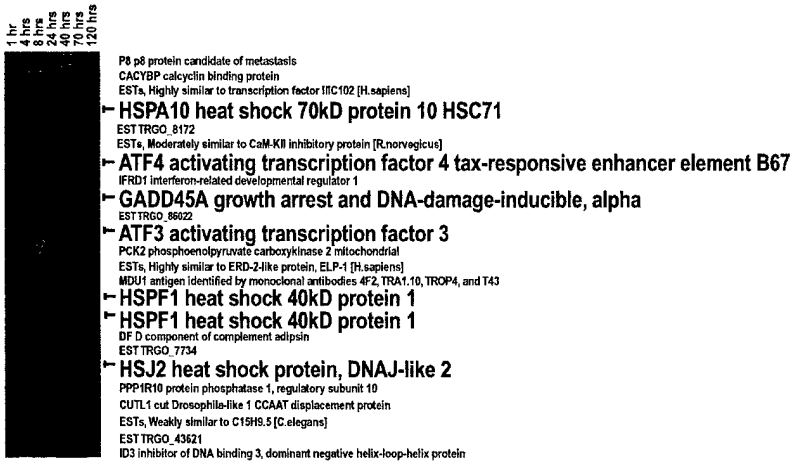
Our finding of sulforaphane's ability to induce phase 2 enzymes in prostatic cells also suggests the possibility of a preventive intervention trial. We have begun to plan a clinical trial in collaboration with William G. Nelson MD, PhD at Hopkins to administer sulforaphane to patients prior to radical prostatectomy and evaluate phase 2 enzyme activity and/or expression in prostatic tissues once removed. The possibility that sulforaphane may exert some effects in the prostate *in vivo* is suggested by two carefully crafted case-control studies in which consumption of cruciferous vegetables was associated with a decreased risk of prostate cancer (1, 2).

Through work funded by my Phase I award, my laboratory is now well versed in cDNA microarray technology. Methods are now well established to measure changes in gene expression for 48,000 gene elements with a single hybridization. We have applied this technique to prostate cancer cells treated with sulforaphane to gain insights into the mechanisms of action of sulforaphane. We are currently preparing a manuscript which summarizes these results. They follow in abbreviated format.

1) *Sulforaphane induces carcinogen defenses in human prostate cancer cells.* To better characterize the effect of sulforaphane, we assessed its effect on global patterns of gene expression in the human prostate cancer cell line LNCaP. After treatment, poly-A RNA was extracted at 0, 2, 4, 8, 16 24, 48, 60 and 96 hrs and arrayed with mRNA from control LNCaP cells treated with vehicle alone and harvested at parallel time points. Analysis of data using hierarchical clustering software developed in the Brown /Botstein laboratories reveals genes that are coregulated in response to sulforaphane (3). The data is displayed in a "hotmap" in which red indicates genes that are up-regulated in response to sulforaphane, and green, genes down-regulated. The degree of color saturation corresponds to the degree of induction or repression. In the figure, a number of phase 2 enzymes (shown in **bold**) are up-regulated in response to treatment of LNCaP with sulforaphane *in vitro*. In addition several poorly characterized genes and ESTs cluster with this set of genes, implying that they too may have a role in defense against oxidative stress. Subsequent experiments refine these observations with the inclusion of additional prostate cell lines and experiments in which LNCaP was treated with other phase 2 inducing compounds (not shown).



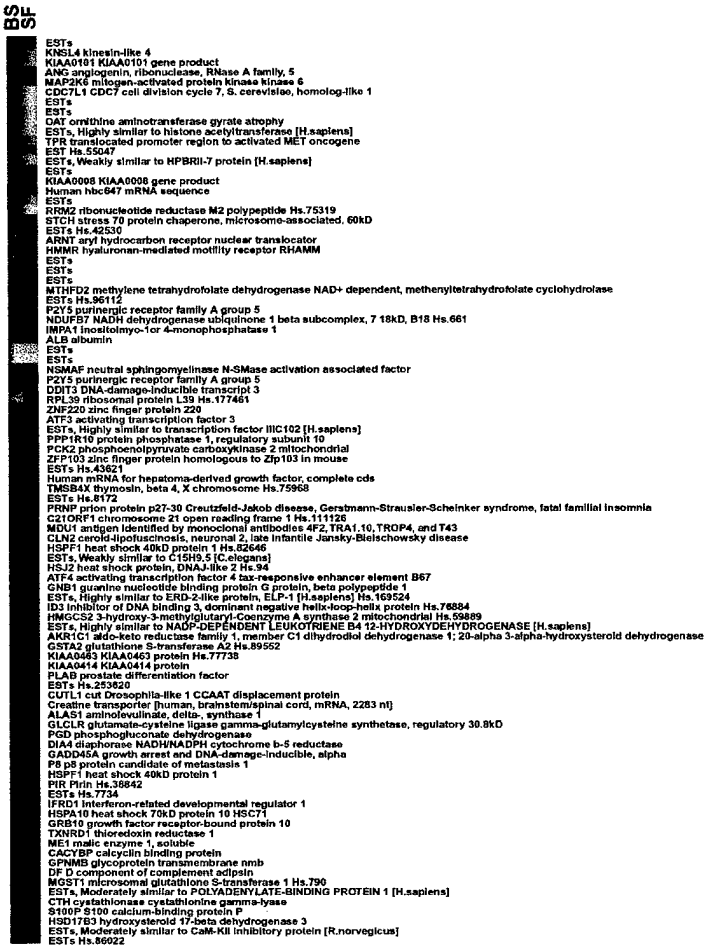
2) *Sulforaphane acts through additional, previously unknown mechanisms that may account for its anticarcinogenic properties.* Sulforaphane has been shown to block DMBA-induced breast tumors in Sprague-Dawley rats. These anticarcinogenic properties had been ascribed to sulforaphane's ability to induce carcinogen defense enzymes. Our data suggests several other pathways through which sulforaphane may exert its effects. For instance, it induces early stress response genes (in bold in the figure). Note that several heat shock protein



transcripts are induced coordinately in this cluster of early-response genes. ATF3, a known regulator of genes activated in stress-response, is also induced in this early cluster. Induction of GADD45A expression in by sulforaphane is somewhat surprising. Several in vitro mutagenesis assays have confirmed that sulforaphane does not cause DNA damage or mutation. Thus induction of GAAD45A may be through other mechanisms and related to stress-response. Certainly, induction can be viewed as exerting beneficial effects on the prostate cell in protecting against other DNA-damaging carcinogens.

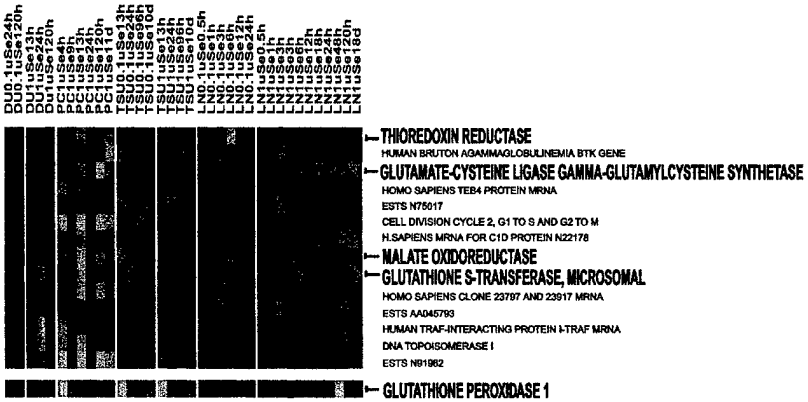
In addition, sulforaphane treatment leads to down-regulation of a number of genes including the master-regulator of carcinogen activation (Phase 1) enzymes, ARNT. ARNT is known to induce expression of enzymes which activate many pro-carcinogens. A number of previously uncharacterized genes are also suppressed in response to sulforaphane treatment. Further data (not shown) demonstrates down-regulation of growth factors and their receptors (the endothelin axis) as well as genes associated with proliferation.

3) *Broccoli sprouts appear to effect the same changes in gene expression in LNCaP.*



Recently, we evaluated expression patterns induced by an aqueous extract of broccoli sprouts, a known natural source of sulforaphane (4). We were delighted to observe that gene expression pattern changes closely matched those seen after treatment with sulforaphane. The figure at left shows almost perfect correspondence between genes induced by broccoli sprouts (left column) to pure sulforaphane (right column). As mentioned above, all foodstuffs are composed of myriad micro- and macronutrients that would be expected to affect cells differently. One concern, as with β-carotene, is that the parent food may exert different effects than one or several micronutrients that it contains. Although not definitive, it is reassuring to see that broccoli sprouts do not appear to induce alterations in gene expression much different than sulforaphane. This suggests that sulforaphane is the principle biologically active compound in broccoli sprouts and suggests that sprouts would be a suitable source of sulforaphane for use in clinical trials.

4) *Selenium, another putative prostate cancer preventive agent, appears to act through mechanisms similar to sulforaphane.* Several epidemiologic studies, including our own, have reported an association between high selenium intake and a decreased risk of prostate cancer.



Selenium is thought to act by inducing enzymes that participate in defense against oxidative stress such as the glutathione peroxidases. Indeed, treatment of selenium deprived LNCaP cells with low dose (100 nM) and high dose (1 mcM selenium) produced modest levels of induction of glutathione peroxidase (bottom row). More interestingly, selenium also produced striking induction of phase 2 enzymes and glutathione synthetic pathways. Thus, cDNA microarray analysis of gene expression can reveal novel pathways through which preventive agents act and heirarchical clustering can expose similarities and differences in the means by which they act.

Task 2: To test whether induction of phase 2 enzymes will attenuate oxidative stress in prostate cancer cell lines *in vitro*. (mos. 12-30)

In our original proposal, we had planned to induce oxidative stress in prostate cells based by treatment with androgen based on the findings of Ripple et al. (5, 6). Unfortunately, we have not been able to measure oxidative stress in response to androgen using the fluoroprobe 2'7'-dichlorofluorescin diacetate (DCF). We have treated LNCaP with androgen and characterized the pattern of gene expression (see references below) have observed induction of a few genes associated with oxidative stress (thioredoxin peroxidase, UDP glucuronosyl transferase). However, few other genes related to oxidative stress appear induced by androgen.

Despite these negative findings, we remain convinced that oxidative stress is one important feature of prostate carcinogenesis. Despite our inability to create stress with androgen, other biochemical pathways (e.g. prostaglandin synthesis and polyamine synthesis) will produce abundant oxygen free radicals. Indeed, other putative prostate cnacer preventive agents are thought to act by quenching free radicals and in our follow up Phase II award, we will be assessing the mechanisms of action of these compounds using microarray technology. During this time, we will devote considerable energy to developing a model of oxidative stress – possibly by interrupting oxidative phosphorylation or by simply treating the cells with peroxide.

Task 3: To investigate the pharmacokinetics of phase 2 inducing agents in human prostate cancer grown in a xenograft model (mos. 1-30).

We had hoped to evaluate phase 2 enzyme induction in an animal model during our period of funding. Unfortunately, these studies have not been completed. In part, we delayed because we had not identified which compound we desired to test. In part, cost proved to be prohibitive since my original funding had been cut substantially leaving me little money for the disposables necessary to carry our these experiments. In future months, I hope to carry out these experiments with sulforaphane. Since sulforaphane is found at high levels in broccoli sprouts, which are available in my local supermarket, I may be able to get similar pharmacokinetic information about delivery of isothiocynate to the prostate through clinical trials outlined above.

Key Research Accomplishments

- Identification of sulforaphane as a potential prostate cancer preventive agent.
- Translation of cDNA microarray technology into my laboratory.
- Evaluation of gene expression induced by sulforaphane using this technology.
- Evaluation of effects of androgen on prostate cnacer cell lines using cDNA microarray technology.
- Establishment of a multidisciplinary research team in Stanford University to evaluate gene expression profiles from tumor samples removed at surgery. This team has now begun to collaborate with other groups nationally and internationally to evaluate gene expression in prostate, renal, testis and bladder cancers.
- Competed successfully for peer-reviewed funding to continue research in prostate cancer prevention.
- Peer reviewed funding to investigate gene expression patterns in prostate and renal cell carcinomas.
- Continued demonstration of the importance of GSTP1 inactivation in prostate carcinogenesis.

Reportable Outcomes

Presentations

James D. Brooks and Vincent Paton: Potent Induction of Carcinogen Defense Enzymes with Sulforaphane, a Putative Prostate Cancer Chemopreventive Agent. Innovators in Urology, Oxford England, July 28-30, 1999.

James D. Brooks: Sulforaphane and Gene Expression in Prostate Cells. Strategies for Developing New Clinical Trials for Prostate Cancer Chemoprevention Workshop. National Cancer Institute, Baltimore, MD, August 8-9, 1999.

James D. Brooks: Nutrition and Gene Expression. CaPCURE Sixth Annual Scientific Retreat. Lake Tahoe, Nevada, October 17, 1999.

James D. Brooks: Defining the mechanisms of prostate cancer chemopreventive agents using cDNA expression arrays. 8th Prouts Neck meeting on Prostate Cancer, Prouts Neck, Maine, October 23, 1999.

James D. Brooks: Genomics of Prostate Cancer Chemoprevention. Keystone Symposium on Advances in Human Breast and Prostate Cancer, Lake Tahoe, NV, March 22, 2000.

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Elizabeth Williams and James D. Brooks: New Molecular Approaches for Identifying Novel Targets, Mechanisms, and Biomarkers for Prostate Cancer Chemopreventive Agents. *Urology* **57** (supplement)**:** 100-102, 2001.

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Rajesh Shinghal, Cheryl Yomoto, Thomas A. Stamey, James D. Brooks: Slow PSA velocity characterizes a subset of late PSA failures following radical prostatectomy. In preparation. 2001.

James D. Brooks, Michael Goldberg, David Wu, and William G. Nelson: Identification of prostate cancer chemopreventative agents through the induction of phase 2 enzymes. In preparation, 2001.

Funding

Doris Duke Foundation Clinical Research Scientist Award, "Prostate Cancer Prevention Through Induction of Phase 2 Enzymes." Principal Investigator, July, 1998-June, 2001, $300,000 direct, $24,000 indirect.

The Bernard Lee Schwartz Foundation, Inc., "Mechanisms of action of prostate cancer preventive agents." Principal Investigator, July, 1999-June 2000, $50,000 direct.

Medical Scholars Program: "Inactivation of the *GSTP1* gene by promoter methylation in MDA PCa 2A and MDA PCa 2B cell lines" Faculty Preceptor for Benjamin Hoehn, January 1999-July 1999, $10,000 direct.

Deans Postdoctoral Fellowship Award: "Prostate Cancer Prevention through Induction of Phase 2 Enzymes." Faculty Preceptor for Samuel DePrimo, August, 1999-July, 2000 $16,000 direct.

National Cancer Institute, NIH: "A Cancer Taxonomy Based on Gene Expression Patterns." (PI: Patrick O. Brown), Co-Investigator: James D. Brooks. October, 1999-September 2004. Total direct costs: $1,664,908 (year 1).

National Cancer Institute, NIH: "Effects of Soy Isoflavones on the Prostate, Breast and Bone" (PI: Jack Farquar) Co-Investigator James D. Brooks. July, 2000-June 2005, $1,195,080 total funding.

Calydon, Inc. "A Phase I/II Dose Finding Trial of the Intraprostatic Injection of Calydon CV787, a Prostate-Specific Antigen Cytolytic Adenovirus, in Patients with Locally Recurrent Prostate Cancer Following Definitive Radiotherapy. December 1999-November 2000.

National Kidney Cancer Association: "Gene expression profiling of renal cell carcinoma: a new cancer taxonomy" Prinicipal Investigator July 1, 2000-June, 2002, $100,000 direct.

Deans Postdoctoral Fellowship Award: "Microarray analysis of the transcriptional program activated by exposure of prostate cancer cells to androgen." Faculty preceptor for Samuel DePrimo. August, 2000-July 2001, $16,000 direct.

Department of Defense New Investigator Award, "Gene expression patterns and prostate cancer prevention." Principal Investigator, April 2001-March 2003, $480,000.

Pfizer Inc. "Effects of Doxazosin on Gene Expression Profiles of Prostatic Stromal Cells Cultured from Normal and BPH Tissues" (P.I. Donna Peehl) Co-investigator James D. Brooks. January 2001-December 2001, $15,000 direct.

National Cancer Institute, NIH PO1: "Determinants of Prostate Cancer Prognosis" (PI: Alice Whittemore) Co-Investigator James D. Brooks, Submitted, 10/1/00, $12,906,703

Doris Duke Foundation Clinical Research Scientist Award, Phase 2 award, "Prostate Cancer Prevention Through Induction of Phase 2 Enzymes." Principal Investigator, July, 2001-June, 2002.

The Oxnard Foundation: Mechanisms of action of the prostate cancer preventive agent selenium. Principal Investigator, July, 2001-June 2003, $150,000.

Conclusion (The so-called "So What Section")

We are pleased with our progress in developing sulforaphane as a potential prostate cancer preventive agent. We decided to investigate sulforaphane on a hunch – since prostate cancer loses expression of a phase 2 enzyme, we need to find something to turn defenses back on. Sulforaphane was know to act this way in mice and prevent breast tumors in these animals. We documented that sulforaphane is great at turning on cancer defense enzymes in prostate cells *in vitro*. At the time we made this observation, we were thrilled to learn that 2 epidemiologic studies had documented that eating cruciferous vegetables was associated with protection against prostate cancer in men. Taken together, this work offers the possibility of rapid translation into clinical trial since sulforaphane, a naturally occurring compound found in the diet, is likely to be safe for evaluation in patients.

We are very excited by the data we are gathering from our work with microarray technology. This research requires a new thinking with regard to experimental design and interpretation of results and will be indispensable in reaping the benefits of the Human Genome Project. The New Investigator Award from the Dept. of Defense has made possible the establishment of this technology in my laboratory, and opening numerous opportunities for continued work. I envision a broadened understanding of the mechanisms of action of cancer preventive agents arising from a systematic application of this technology. I look forward to productive years ahead.

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Potent Induction of Phase 2 Enzymes in Human Prostate Cells by Sulforaphane[1]

James D. Brooks[2], Vincent G. Paton and Genevieve Vidanes

Department of Urology, Stanford University Medical Center

Key Words: Prostate Cancer, Sulforaphane, Prevention, Phase 2 Enzymes

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2. To whom correspondence should be addressed

Department of Urology, Rm S287

Stanford University Medical Center

300 Pasteur Drive

Stanford CA, 94305-5118

jdbrooks@stanford.edu

Abstract

Introduction and Objectives

Two population-based, case control studies have documented reduced risk of prostate cancer in men who consume cruciferous vegetables. Cruciferae contain high levels of the isothiocyanate sulforaphane. Sulforaphane is known to bolster the defenses of cells against carcinogens through up-regulation of enzymes of carcinogen defense (phase 2 enzymes). Prostate cancer is characterized by an early and near universal loss of expression of the phase 2 enzyme glutathione S-transferase-pi. We tested whether sulforaphane may act in prostatic cells by increasing phase 2 enzyme expression.

Materials and Methods

The human prostate cancer cell lines LNCaP, MDA PCa 2a, MDA PCa 2b, PC-3 and TSU-Pr1 were treated with 10 μM sulforaphane *in vitro*. Quinone reductase enzymatic activity, a surrogate of global phase 2 enzyme activity, was assayed by the menadione-coupled reduction of tetrazolium dye. Expression of NQO-1, GST-α, γ-GCS – Heavy and Light chains, and microsomal GST was assessed by northern blot analysis.

Results

Sulforaphane potently induces quinone reductase activity in cultured prostate cells and this induction appears to be mediated by increased transcription of the *NQO-1* gene. Sulforaphane also induces expression of γ-GCS light subunit, but not the heavy subunit, and this induction is associated with moderate increases in intracellular glutathione levels. Microsomal and α-class glutathione transferases were also induced transcriptionally.

Conclusions

Sulforaphane induces phase 2 enzyme expression and activity significantly in human prostatic cells. This induction is accompanied by, but not due to, increased intracellular glutathione synthesis. Our findings may explain the observed inverse correlation between consumption of cruciferae and prostate cancer risk.

Introduction

In the United States prostate cancer is the most prevalent non-cutaneous malignancy and the second leading cause of male cancer death (1). Prostate cancer has a long latency and estimates are that 10 to 12 years are required before prostate cancer becomes clinically manifest (2). Sakr et al. have identified prostatic intraepithelial neoplasia (PIN), a prostate cancer precursor lesion, in 10% of men by age 30 years, and small foci of frank carcinoma in more than 10% of men prior to age 40 (3). Prostate cancer is usually diagnosed clinically in the sixth and seventh decades of life allowing a large window of opportunity for interventions to prevent or slow the progression of the disease.

The most common molecular genetic change in prostate cancer involves silencing of expression of glutathione-S-transferase P1 (GSTP1), a critical enzyme of carcinogen defense, through methylation of deoxycytidine residues in 'CG islands' in the 5' regulatory region of the GSTP1 gene (4, 5). This change appears to occur early in prostate carcinogenesis, as it is found in virtually all cases of high grade PIN and is a near universal finding in clinical prostate cancers regardless of grade or stage (6). The glutathione transferases protect cells against carcinogenic oxidative stress by conjugation of electrophiles to reduced glutathione. Up-regulation of phase 2 enzymes, including the glutathione transferases, can protect cells against carcinogens and has been documented to prevent carcinogen-induced tumors in a variety of animal models (7, 8).

Early loss of GSTP1 may predispose prostatic cells to the damaging effects of endogenous or exogenous carcinogens and may contribute to carcinogenesis. We have

speculated that induction of phase 2 enzymes may offer a mechanistically based prostate cancer prevention strategy by compensating for this loss of GSTP1 expression. Two recent epidemiological studies suggest that such a preventive intervention may be possible. Both studies have found an association between decreased prostate cancer risk and high consumption of cruciferous vegetables (9, 10). Cruciferae are known to contain high levels of the isothiocyanate sulforaphane, the most potent monofunctional phase 2 enzyme inducing agent thus far identified (11).

Phase 2 inducing agents have been reported to increase phase 2 enzyme activity through increased transcription at phase 2 enzyme gene loci (12). A putative antioxidant response element (ARE) in the regulatory regions of these genes is thought to be responsible for enhanced expression of many of these genes (13-19). Sulforaphane, for instance, will increase expression of a reporter gene downstream of promoter constructs containing the ARE consensus sequence and a minimal promoter. Levels of reporter gene induction parallel endogenous quinone reductase induction in the same cell line (20).

Our hypothesis is that induction of phase 2 enzymes the sulforaphane may help explain the association between high consumption of cruciferae and decreased prostate cancer risk. Since prostate cancer lacks expression of GSTP1, induction of other phase 2 enzymes by sulforaphane may offer a mechanistically based prostate cancer preventive strategy. Since little is known about phase 2 enzyme expression, regulation or activity in prostatic epithelial cells, we evaluated the response of four human prostate cancer cell lines and a normal prostate epithelial cell strain to sulforaphane.

Materials and Methods

Cell Culture—LNCaP were obtained from ATCC and grown in RPMI 1640 with L-glutamine, supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin (GIBCO BRL). PC3 and Tsu-Pr1 were a gift from William G. Nelson (Johns Hopkins University) and grown in the same medium. MDA PCa 2a and MDA PCa 2b were kindly provided by Nora Navonne (MD Anderson Cancer Center) and were grown in HPC1 (BRFF) supplemented with 20% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin (21).

Reagents—L-sulforaphane was purchased from LKT Laboratories (St. Paul, MN). All remaining chemicals were purchased from Sigma (St. Louis, MO). Broccoli sprouts were grown from seed on sterile agar and aqueous extracts prepared as described (22). Organic broccoli sprouts purchased from a local supermarket exhibited nearly identical inducer potency to those raised in the laboratory and were therefore used for subsequent experiments.

Northern Blot Analysis—Cells were harvested at approximately 70% confluency and mRNA was isolated using Oligotex Direct mRNA isolation kit (Qiagen). For each lane, 6 μg of poly(A)+ mRNA were electrophoresed through a 1% agarose gel and transferred to Brightstar-Plus nylon membrane (Ambion) using the Stratagene Posiblot pressure blotter and pressure control station (Stratagene). The RNA was cross-linked to the membrane by exposure to 125 mJoules of UV light in GS Gene Linker (BioRad). cDNA probes were labeled with either [^{32}P]dCTP using the Nick Translation System (Promega) or

psoralen-biotin using the BrightStar Psoralen-Biotin Kit (Ambion). Hybridizations were performed at 50° C in a buffer containing 6x SSPE, 5x Denhardt's Reagent, 6% SDS, 25 μg/mL salmon testes DNA, and 50% formamide. Washes were performed at 55° C with 1x SSC and 0.1% SSC. Northern blots hybridized with [^{32}P]dCTP labeled probes were exposed to a Molecular Dynamics Phosphorimager screen and scanned ImageQuant software. Northern blots hybrizided with psoralen-biotin labeled probes were processed using the BrightStar Detection kit (Ambion) according to the recommended instructions. All images were analyzed using ImageQuant software.

Determination of Enzyme Activity in Cell Culture—LNCaP, MDA PCa 2a, and MDA PCa 2b were grown in 96-well plates at a density of 8 x 10^4 cells/mL. PC3 and Tsu-Pr1 were grown in 96-well plates at a density of 4 x 10^4 cells/mL. After 20 hrs of incubation, cells were treated with L-sulforaphane dissolved in DMSO (LKT Laboratories) at the indicated concentrations. Control wells were treated with the corresponding concentration of DMSO. Quinone reductase activity was assessed by the menadione-coupled reduction of tetrazolium dye as modified from Prochaska and Santamaria (23, 24). After 48 hrs of treatment with L-sulforaphane, media was gently aspirated and cells were lysed by incubation at 37° C with 50μl of 0.08% digitonin and 2 mM EDTA (pH 7.8) with gentle agitation. While the cells were incubating, a stock solution was prepared by combining 16.7 mg bovine serum albumin, 7.5 mg 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 0.6 mg NADP, 1.25 ml 0.5 M tris HCl (pH 7.4), 166.7 μl 1.5% Tween-20, 166.7 μl of 150mM glucose 6-phosphate, 16.7 μl of 7.5 mM FAD, 50 U of yeast glucose 6-phosphate dehydrogenase and distilled water to a final

volume of 25 ml for each plate to be assayed. Immediately prior to use, 25 µl of 50 mM menadione dissolved in acetonitrile was added to the stock solution. After 30 minutes incubation, 200 µl of the complete stock solution was added to each well. After 5 minutes, optical absorbance at 610 nm was determined in a LabSystems Multiscan Ascent microplate reader. GST activity was determined by reduction of CDNB in accord with methods described by Habig (25).

Toxicity of L-sulforaphane was assessed in parallel plates treated identically to those used in assays for quinone reductase activity. Cytotoxicities were monitored by the LIVE/DEAD fluorescent assay (Molecular Probes) according to the suggested protocol. Quinone reductase activity, in arbitrary units, was calculated automatically from the mean activity for all 3 wells at each concentration. Activity was corrected for toxicity at each concentration as described by Prochaska et al. (23, 24). Inducer potency is expressed as the ratio of corrected QR activity for treated cells to corrected QR activity for the vehicle controls.

Determination of GSH Levels in Cell Culture— LNCaP were grown in 96-well plates at a density of 8×10^4 cells/mL. After 20 hrs of incubation, cells were treated with L-sulforaphane at the indicated concentrations. After an additional 48 hrs, the medium was removed, and the relative GSH levels were determined as described by Gerhauser et al. (26). GSH levels were determined in triplicate for each dose of sulforaphane and were corrected for toxicity as above. Reported values represent the average of two separate experiments.

Results

Sulforaphane induces quinone reductase activity in cultured prostate cells. Quinone reductase (NADPH menadione: oxidoreductase EC 1.6.99.2) protects cells from quinones and their precursors by obligate two-electron reduction of quinones to hydoquinones thereby preventing generation of highly reactive semiquinones (that arise from single electron transfer). Quinone reductase (QR) is stably expressed *in vitro* and is induced coordinately with other phase 2 enzymes (27). Quinone reductase has been used as a surrogate marker of global phase 2 enzyme activity *in vitro* and *in vivo*. To test whether sulforaphane has the ability to induce QR enzyme activity in prostate cells, we treated four prostate cancer cell lines and one primary prostate cell strain grown from histologically normal prostatic tissue harvested at surgery (courtesy of Donna Peehl). Cells were treated with sulforaphane or DMSO vehicle as control, and QR enzymatic activity was measured using the technique of Prochaska and Santamaria (23). Over a range of concentrations, sulforaphane induced QR activity in all of the prostate cell lines tested (Table 1). Sulforaphane was particularly potent at inducing QR enzymatic activity in the normal prostate cell strain, with maximal induction (2.46-fold) at 1-3 μM and 1.35-fold induction occurring at 100 ηM sulforaphane. Potent induction was also seen at micromolar doses in LNCaP, MDA PCa 2a and MDA PCa 2b. Each of these cell lines resemble human prostatic epithelia in that they express PSA and androgen receptor and possess relatively slow growth kinetics (21, 28). TSU-PR1, on the other hand, lacks these features of prostatic cells, and shows somewhat diminished responsiveness to sulforaphane.

Broccoli sprouts have been reported to contain high levels of sulforaphane and decrease the rate, incidence and multiplicity of mammary tumors in dimthylbenz[a]anthracene-treated rats (22). In order to determine whether broccoli sprout extracts also have the ability to induce QR in human prostate cells, LNCaP cells were treated with water extracts of broccoli and assayed for QR enzyme activity. Table 2 illustrates the dose-dependent increase in QR in LNCaP cells with inducer potencies similar to those observed in cells treated with pure sulforaphane.

Quinone reductase mRNA levels are increased by sulforaphane.

Induction of phase 2 enzymes *in vitro* and *in vivo* is mediated by increased transcription at phase 2 enzyme gene loci. This transciptional induction is thought principally due to binding of specific proteins to an antioxidant response element (ARE) in the 5'-regulatory regions of these genes (13-19). To evaluate whether increased QR enzymatic activity is due to increased transcription of the *NQO-1* gene in human prostatic cells, we treated five prostate cancer cell lines with 10 μM sulforaphane or with DMSO control for 8 hrs, then performed northern blot analysis using the *NQO-1* cDNA as a probe. Hybridizations revealed marked induction of the 1.9 and 2.7 kb transcripts of the *NQO-1* gene. Transcriptional induction closely mirrored enzymatic activity in each of the cell lines. Densitometric measurements revealed that LNCaP, MDA PCa 2a, MDA PCa 2b, PC3, and TSU-Pr1 had a 2.6, 2.2, 1.9, 1.8, and 1.6 fold increase in *NQO-1* mRNA levels, respectively, as compared to control 8 hours after treatment (Figure 1A).

To determine the temporal induction profile of *NQO-1* by sulforaphane, we treated LNCaP cells with 10 μM sulforaphane over a 72 hr time course and performed northern blot analysis. *NQO-1* mRNA levels were measured by densitometry and fold induction was calculated for each time point relative to DMSO treated control cells. After treatment with 10 μM sulforaphane *NQO-1* mRNA levels at 1, 4, 8, 46, and 72 hrs were induced 0.7, 1.9, 4.5, 3.9, and 4.6 fold, respectively (Figure 2A). Thus, sulforaphane produces an early and sustained *NQO-1* transcriptional response. QR enzymatic activity was also induced and sustained over an identical time course (not shown).

Sulforaphane induces glutathione synthetic pathways

The γ-glutamylcysteine synthetase (γGCS) enzyme catalyzes the rate-limiting step in glutathione synthesis and is composed of two subunits - heavy and light chain. The 5' regulatory regions of the heavy (29) and light (30) subunits of γGCS both contain an ARE and their expression is induced coordinately by β-naphthoflavone, a well characterized bifunctional (phase 1 and 2) enzyme inducing agent. Northern blot analysis using the γGCS – Light chain (γGCS-L) cDNA revealed potent transcriptional induction of this subunit similar to that observed with NQO-1. Sustained induction of γGCS-L mRNA levels of 0.5, 6.5, 7.8, 3.6, and 4.3 fold relative to DMSO controls were observed for the respective time points of 1, 4, 8, 46, and 72 hrs (Figure 3A). Somewhat surprisingly, sulforaphane did not induce expression of γGCS – Heavy chain in the LNCaP cell line at 8 hrs although abundant message was expressed (Figure 3B).

Sulforaphane elevates glutathione levels.

Sulforaphane has been shown to decrease intracellular glutathione levels in murine hepatoma cells by direct conjugation to reduced glutathione (31). Since sulforaphane elevated γGCS-L, but not γGCS-H mRNA levels in human prostate cells, we were curious whether it could increase glutathione levels in LNCaP cells. After treatment of LNCaP cells with 10 μM sulforaphane for 48 hours, levels of reduced glutathione were measured and normalized to cell number. Between 5 and 10 μM, the amount of reduced glutathione per cell increased an average of 17% after treatment and this increase appeared to be dose dependent.

Since intracellular glutathione levels increased in conjunction with phase 2 enzyme induction after treatment of the LNCaP cell line with sulforaphane. We wondered whether raising intracellular glutathione levels with n-acetyl cysteine (NAC) could potentiate the effects of sulforaphane. LNCaP cells were pretreated with 10 mM NAC for 2 hours followed by either vehicle control or 8 μM sulforaphane for 48 hours and QR enzymatic activity assayed. QR enzymatic activity was compared to that obtained from cells treated with 8 μM sulforaphane or vehicle control alone (Figure 4). NAC alone did not induce QR activity whereas sulforaphane alone did reproducibly. Intriguingly, pretreatment of LNCaP cells with 10 mM NAC abolished the induction of QR enzymatic activity.

Sulforaphane induces modest increases of expression of glutathione transferases

Unlike several species, the 5'- regulatory regions of most human phase 2 enzyme gene lack an ARE consensus sequence. Both human α-class and microsomal glutathione S-transferases appear to lack this regulatory element (32). We investigated whether absence of this element abrogated the transcriptional response of these genes to sulforaphane. Northern blot analysis showed modest induction of expression of GST-α in LNCaP, MDA PCa 2a, and MDA PCa 2b (1.7, 1.7, and 1.4 fold, respectively) (Figure 1B); yet the 0.9 kb *GSTA1* band was unchanged in PC3 and TSU-Pr1. Microsomal GST was induced similarly in LNCaP, MDA PCa 2a, MDA PCa 2b, and PC3 (1.7, 1.8, 1.3, and 1.4 fold, respectively) (Figure 1C) and again TSU-Pr1 was essentially unaffected. Global glutathione transferase activity was evaluated in all cell lines by reduction of CDNB. Unfortunately, like many cells *in vitro*, the prostate cell lines exhibited no measurable GST activity (not shown) (33).

Discussion

Sulforaphane is a potent phase 2 enzyme inducing agent in human prostate cells *in vitro*. Sulforaphane produced robust and sustained transcriptional induction of NQO-1 gene expression that was accompanied by similar increases in quinone reductase enzymatic activity. Other members of the class of phase 2 enzymes were also induced transcriptionally. Intracellular levels of reduced glutathione increased after sulforaphane treatment, likely due to increased expression of the γ-GCS-L gene, an enzyme that catalyzes the rate-limiting step in glutathione synthesis. Together, the changes induced by sulforaphane buttress cellular defenses against carcinogens by increasing reductive capacity of the cell.

All prostatic cells tested *in vitro* were affected similarly by sulforaphane, including a normal prostate cell strain, three hormone responsive immortalized cell lines and an androgen insensitive cell line. Each of the cell lines demonstrated the same pattern of phase 2 enzyme response and glutathione induction although there were quantitative differences. Regulation of the response to sulforaphane in these cell lines, therefore, appears to remain intact, although somewhat complicated. The light and heavy subunits of γ-GCS were not induced coordinately, despite the presence of a stereotypical ARE in the 5'-regulatory regions of both genes. Other investigators have reported coordinate induction of these subunits in response to phase 2 inducing agents (29, 30). Furthermore, sulforaphane was capable of inducing expression of phase 2 enzymes known to lack AREs, namely GST-α and microsomal GST. Thus, regulation of these enzymes, at least in prostatic cells, is likely to involve more than binding of a protein complex to the ARE

enhancer element. Detailed study of the regulatory regions of these genes will be necessary to understand the complex regulatory pathways that modulate the cellular response to sulforaphane.

Alteration of intracellular redox status may be one means by which sulforaphane acts to increase phase 2 enzyme expression. Pretreatment of prostatic cells with NAC, which is known to increase intracellular levels of reduced glutathione, completely ablated the effects of sulforaphane. While it is possible that NAC acts directly on sulforaphane, we suspect that NAC reduces intracellular proteins mediating the phase 2 enzyme response. Understanding the role of intracellular redox in the regulation of phase 2 enzyme response has implications in the design of future clinical trials in cancer prevention. For example, one proposed intervention strategy for prostate cancer combines NAC (to increase intracellular reduced glutathione – a GST substrate) with a phase 2 enzyme inducing agent such as sulforaphane (34). Our results suggest that such an approach may ablate the response to sulforaphane, at least in prostatic cells. Indeed, since sulforaphane increases intracellular glutathione pools by itself, such combined therapy may be unnecessary.

Our findings may help explain the recent observation that consumption of cruciferae, naturally rich sources of sulforaphane, may lower the risk of later development of prostate cancer (9, 10). Since the loss of one phase 2 enzyme, namely π-class glutathione transferase, is an early and near universal finding in human prostate cancer, sulforaphane may help compensate for this loss by increasing global phase 2 enzyme activity. At first

glance, it seems somewhat surprising that loss of expression of a single GST could increase risk of prostate cancer. Glutathione transferases comprise a family of enzymes with broad and overlapping substrate specificity; thus, loss of any single member should be compensated by the activity of the remaining GSTs (32). However, several epidemiological studies have suggested that loss of individual GSTs (e.g. GSTM1 – null phenotype) can confer increased susceptibility to cancer at several organ sites (35-38). Low activity GSTP1 alleles have been associated with increased prostate cancer risk (39, 40). Indeed, mice engineered to lack π-class GST expression are more susceptible to carcinogen induced tumors (41). Thus, loss of expression of a single GST appears to increase cancer risk – either from global decreases in GST activity or from loss of protection against a carcinogen inactivated solely by the lost enzyme.

Could sulforaphane's capacity to induce phase 2 enzymes compensate for or prevent loss of GSTP1 expression? An intriguing study by Lin and associates suggests that induction of phase 2 enzymes may be particularly pertinent in the setting of GST enzymatic deficiency (42). Patients with a previous history of colonic polyps were stratified for their subsequent risk of developing colorectal polyps based on levels of consumption of cruciferous vegetables. Compared to subjects that never consumed broccoli, those in the highest quartile of broccoli consumption had an odds ratio of 0.47 (95% confidence interval 0.30-0.73), and this protective effect was only observed in subjects with the *GSTM1* null genotype. No protection was conferred in subjects with wild-type GSTM-1 alleles. A similar interaction between GSTM1 genotype and broccoli consumption has been observed in lung cancer (43). Since GSTP1 is lost in all human prostate cancers,

induction of global phase 2 enzyme activity and increasing intracellular reduced glutathione may be have great relevance in preventing this disease.

In summary, sulforaphane is a potent inducer of phase 2 enzymes in human prostatic cells. Induction of phase 2 enzymes is one possible explanation for the association between high consumption of cruciferae and decreased prostate cancer risk. Based on these findings, intervention trials may be warranted, and broccoli sprouts, a rich natural source of sulforaphane, may be appropriate for use in such a trial. Additional work will be necessary to elucidate the mechanisms of phase 2 enzyme induction in human prostate cells.

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Table 1. Dose dependent induction of quinone reductase activity in response to sulforaphane.

μM sulforaphane	15	10	8	5	3	1	0.5	0.1
LNCaP	1.98	2.29	1.93	2.11	1.39	1.28	1.11	1.00
MDA Pca 2a	1.67	1.60	2.10	1.99	1.89	1.31	0.94	1.04
MDA Pca 2b	1.52	2.47	2.10	1.92	1.90	1.70	1.25	1.04
TSU-Pr1	1.86	1.28	1.39	1.14	1.21	1.01	0.95	0.92
normal strain	1.81	1.86	1.80	1.95	2.46	2.08	1.57	1.35

Table 2. Dose dependent induction of quinone reductase activity in response to broccoli sprout extract.

% broccoli sprout extract	1.250	0.625	0.313	0.156	0.078	0.039	0.020
LNCaP	1.138	1.974	2.134	1.462	1.176	1.044	0.957

Table 3. Dose dependent induction of cellular glutathione by sulforaphane.

μM sulforaphane	15	10	8	5	3	1	0.5	0.1
LNCaP	1.103	1.179	1.152	1.172	1.122	1.051	1.034	1.009

Figure Legends

Figure 1. **Transcriptional response of phase 2 enzymes to sulforaphane in various prostate cancer cell lines.** The cell lines LNCaP, MDA PCa 2A, MDA PCa 2B, PC3 and TSU-Pr1 were treated for 8 hours with 10 mM sulforaphane or with the DMSO control. Northern blot analyses were performed using (A) *NQO-1*, (B) *GSTA1*, (C) microsomal GST, and (D) GAPDH cDNA probes.

Figure 2. **Northern blot analysis of the transcriptional response to sulforaphane.** LNCaP cells were incubated for 1 to 72 hours with 10 mM sulforaphane or the DMSO control before the RNA was harvested. The membrane was probed with labeled (A) *NQO-1*, and (B) GAPDH cDNAs.

Figure 3. **Differential effects of sulforaphane on γ-glutamylcysteine synthase subunits.** Northern blot analyses of (A) sulforaphane induced γ-GCS light chain expression over time and (B) unchanged γ-GCS heavy chain expression treated with 9 h of 10 μM sulforaphane treatment in LNCaP cells.

Figure 4. **QR response to sulforaphane is abolished in a reduced environment.** LNCaP cells were pre-treated for 2 hours with 10 mM N-acetyl cysteine (NAC) followed by 8 mM sulforaphane or DMSO control prior to assaying QR activity. The error bars represent the standard deviation of triplicate data points.

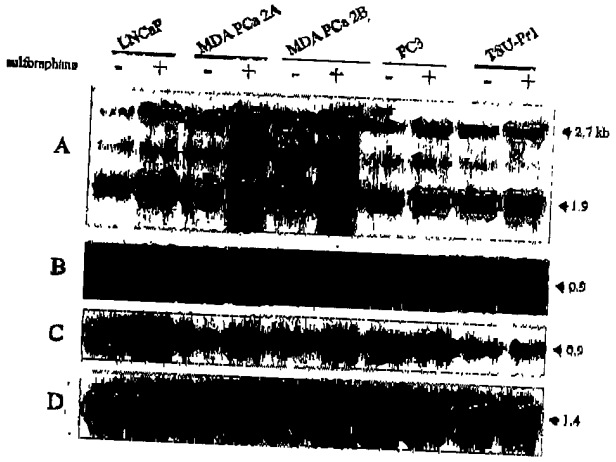

Fig. 1. Transcriptional responses of phase 2 enzymes to sulforaphane in various prostate cancer cell lines. The cell lines LNCaP, MDA PCa 2A, MDA PCa 2B, PC3, and TSU-Pr1 were treated for 8 h with 10 μM sulforaphane or with the DMSO control. Northern blot analyses were performed using (*A*) *NQO-1*, (*B*) *GSTA1*, (*C*) microsomal GST, and (*D*) GAPDH cDNA probes.

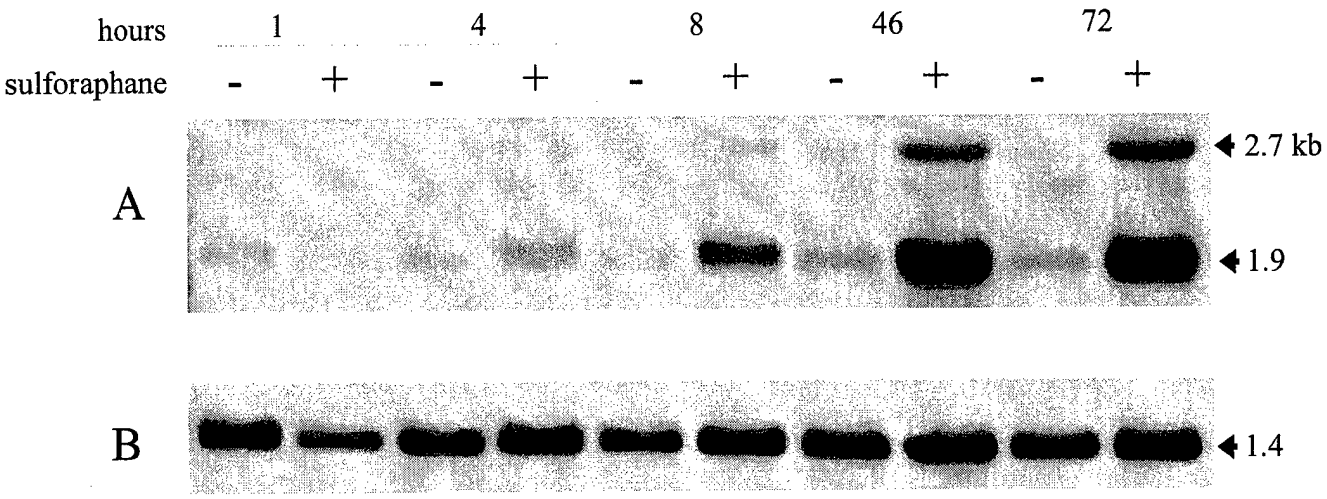

Figure 2

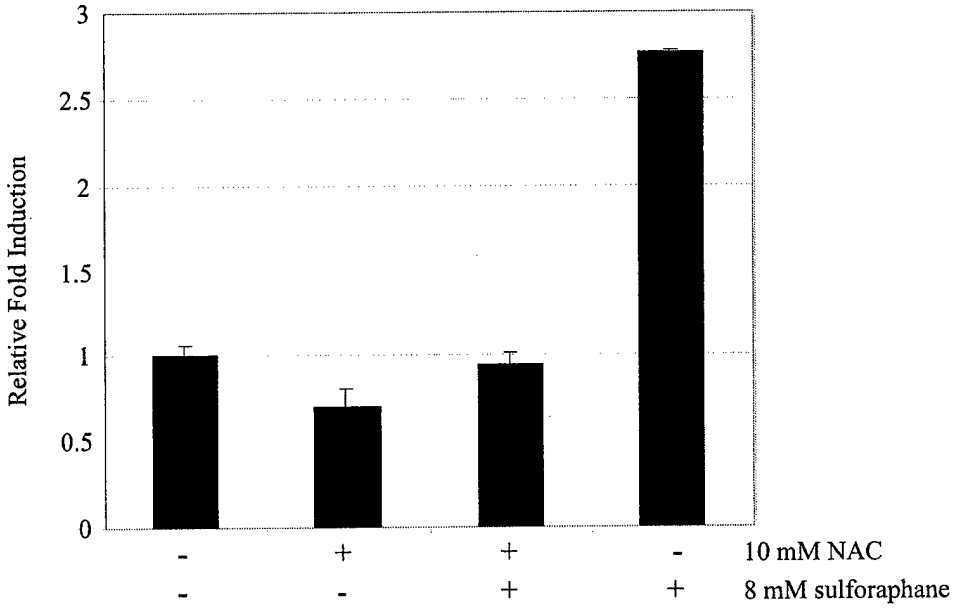
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Figure 3

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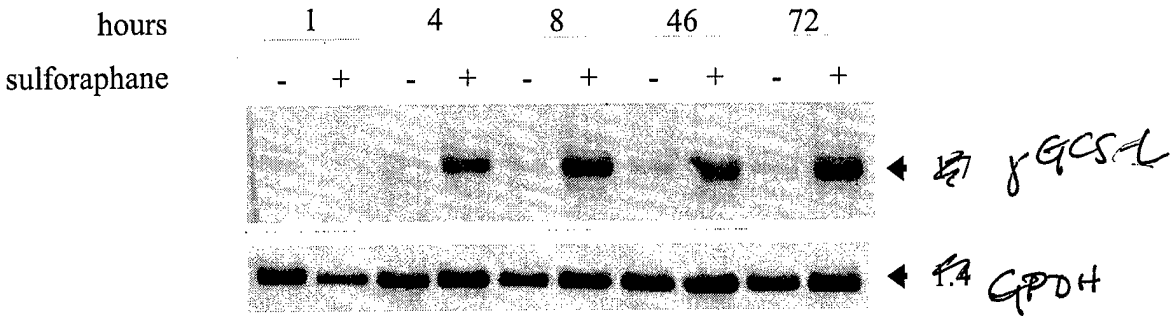
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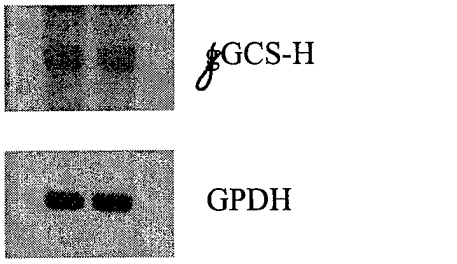
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Figure 4

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PREVENTION OF PROSTATE CANCER

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Samuel E. DePrimo, PhD, Rajesh Shinghal, MD, Genevieve Vidanes, and James D. Brooks, MD

Prostate cancer presents a significant public health challenge, particularly in Western nations. It is the second leading cause of cancer death among men in the United States.[34] Clinical prostate cancer typically manifests late in life, leaving a large window of opportunity for preventive interventions. The incidence of microscopic foci of prostate cancer occurs in greater than 75% of all men by age 70[4]; however, the progression from microscopic foci to clinically manifest cancer of the prostate is significantly more frequent in regions such as North America when compared with Asian countries.[17] This feature of the disease implicates environmental factors as contributors to the progression and morbidity of clinical prostate cancer. Further support for this notion comes from the observation that there is an increased incidence of clinical cancer in Asian men who emigrate to the United States compared with men that remain in Asian countries.[37, 68] Evidence also points to metabolic and genetic risk factors for cancer initiation and progression. Understanding the genetic, metabolic, and environmental factors that contribute to prostate tumorigenesis would sharpen the focus of efforts to develop preventive strategies.

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This article focuses on several potential strategies for prostate cancer prevention. Effective prevention approaches would reduce greatly the public health burden of prostate cancer, as existing therapies for this

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From the Departments of Urology (SED, RS, GV, JDB) and Biochemistry (SED), Stanford University School of Medicine Stanford, California

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disease are limited and can present undesirable side effects. Radical prostatectomy or radiation therapy can be effective against localized tumors; however, advanced or metastatic disease generally is treated with androgen ablation therapy, which invariably results in tumor recurrence during long-term treatment.[58] The existence of prostate cancer biomarkers, such as serum prostate-specific antigen (PSA) levels and prostatic intraepithelial neoplasia (PIN) lesions, allows the design and implementation of clinical prevention trials. In this article, results from epidemiologic studies and preliminary data from clinical trials are emphasized. Much information has been gained from animal studies and in vitro cellular and molecular biology research, and some of these findings are described as well.

HORMONAL APPROACHES TO PREVENTION

Agents That Target Androgen Metabolism

Androgens are necessary for the normal development of the prostate and for prostate carcinogenesis. The role of androgens, particularly dihydrotestosterone (DHT), is evidenced by the observation that prepubertal castration prevents development of benign prostatic hyperplasia (BPH) and prostate cancer.[42] Individuals with a hereditary deficiency of 5-α-reductase enzyme activity, which is necessary for the conversion of testosterone to dihydrotestosterone, fail to develop BPH or prostate cancer.[43] Differences in androgen metabolism have been proposed as a possible contributor to racial and ethnic differences in prostate cancer incidence.[65] Ross et al[63] found that young African-American men had 19% higher levels of serum testosterone than their white counterparts, which correlates with the well-established higher rates of prostate cancer among African-American men. Serum testosterone levels in Japanese men were intermediate between white and black counterparts, despite the lower prostate cancer rates among Japanese men. Japanese men had 25% to 35% lower levels of androstanediol glucoronide, however, an index of 5-α-reductase activity, implying reduced dihydrotestosterone conversion.[64]

Such observations have led to clinical investigation of whether perturbations of the androgen axis might lower prostate cancer incidence. This approach was facilitated by the development of finasteride (Proscar), a competitive inhibitor of 5-α-reductase that reduces levels of dihydrotestosterone and presents fewer side effects than other antiandrogen therapies.[32] A double-blind study of 895 men with BPH showed a decrease in complications related to BPH in patients that received 5 mg of finasteride daily.[33] In an effort to assess the potential of finasteride as a prostate cancer preventive agent, a prospective, randomized Prostate Cancer Prevention Trial (PCPT) involving 18,882 men was initiated in 1994 and has an endpoint of October 2004.[13, 73] All of the men were older than 55 years of age at the start of treatment, had a normal digital rectal

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examination, and serum PSA levels of 3.0 ng/mL or less. After a run-in period, participants were randomized to take either 5 mg/d of finasteride or placebo. All survivors at the end of this 10-year study will undergo a sextant biopsy to assess the period prevalence of prostate cancer.

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Although the PCPT trial should answer whether or not finasteride can prevent prostate cancer, 2 smaller studies have raised questions of finasteride's efficacy. Andriole et al[1] analyzed prostate cancer rates in a randomized, placebo-controlled trial of men with BPH after 4 years of finasteride treatment or placebo. Of the 644 patients who underwent biopsy, 4.7% of men on finasteride and 5.1% of men on placebo were diagnosed with prostate cancer ($P = .7$). The second study evaluated short-term (12 months) finasteride treatment among a small group of men (n = 52) with elevated PSA levels and negative prostate biopsy results. After the 12-month period, prostate cancer was found in 30% of finasteride-treated men and only 4% of untreated men ($P = .025$). Among men with high-grade PIN at the start of the study, 6 of 8 of the finasteride-treated men developed cancer as compared with 0 of 5 of untreated men ($P = .021$).[16] Final results from the PCPT should provide more definitive answers as to whether pharmacologic reduction of 5-α-reductase activity would be a useful preventive strategy. In the future, other, more selective androgen antagonists or partial antagonists may allow effective prevention with acceptably low morbidity.

Insulin-Like Growth Factor-1 and Preventive Strategies

A prospective, nested case-control study within the Physicians' Health Study provided epidemiologic evidence for a role for plasma insulin-like growth factor (IGF-1) levels as a predictor of prostate cancer risk. After comparison of 152 prostate cancer cases and 152 controls, men in the highest quartile of serum IGF-1 levels were shown to have a relative risk of prostate cancer of 4.3 (95% confidence interval [CI], 1.8 to 10.6) compared with men in the lowest quartile.[8] The IGF axis has been implicated in prostate development as well as carcinogenesis and tumor progression. Although much more work is necessary to test whether high serum IGF levels directly contribute to prostate carcinogenesis, the potential exists for using somatostatin analogs or growth hormone–releasing antagonist to suppress partially the growth hormone–IGF-1 axis.[8, 59] Preventive strategies could be designed to include only men with the highest IGF levels.

DIETARY APPROACHES TO PREVENTION

The influence of diet and nutrition on prostate cancer cause is an important and growing area of investigation. Most intriguing leads to date have come from epidemiologic observations and from crude

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assessments of dietary factors that account for geographic disparities in prostate cancer incidence. The much higher incidence in Western nations, where fat intake comprises approximately 40% of total energy intake,[22] compared with Asian countries has led to studies of the role of dietary fat as a risk factor for prostate cancer. As reviewed by Fleshner and Klotz,[24] 11 of 14 case-control studies and 4 of 5 prospective cohort studies confirmed the association between dietary fat intake and higher prostate cancer risk. Animal fat in particular has been linked to higher risk, with red meat consumption having the strongest positive association with advanced prostate cancer (relative risk, [RR] 2.64; 95% CI, 1.21 to 5.77; $P = .02$).[28] Animal studies have shown that xenografts of the human prostate cancer cell line LNCaP grew more slowly in mice fed on a low-fat diet that in their counterparts fed a high-fat diet.[75] Although such results are suggestive, results from more well-controlled, clinical intervention trials are necessary to show fully the efficacy of fat intake reduction as a means of prevention. Specific information on the role of various fatty acids and the effects of changing patterns of fat intake (e.g., saturated versus unsaturated) as well as amount of fat intake is essential.[54]

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Investigation of other dietary components as agents with preventive potential is confounded by the same variables inherent to studies of fat intake and cancer development. Accurate measurement of dietary intake often is lacking in epidemiologic studies, and assignment of long-term preventive function to one or a few nutrients in the face of hundreds or thousands of dietary micronutrients is a difficult proposition. Despite these limitations, patterns have emerged from basic and epidemiologic studies, setting the stage for more focused assessment of specific food components that might confer protective effects against cancer initiation or progression. These components, generally referred to as *micronutrients*, might be useful preventive agents when administered in the form of dietary supplements or in a diet replete with foods rich in these substances.

Many dietary micronutrients are thought to exert some or all of their protective effects because of their ability to act as antioxidants. Oxidative stress resulting from generation of reactive oxygen species can damage DNA, proteins, and lipids and is thought to be an initiating factor in carcinogenesis. Generation of oxidative stress is a potential mechanism by which prostate cancer risk factors, such as androgen levels and dietary fat intake, might increase cancer incidence. Exposure to physiologic levels of androgen has been shown to induce oxidative stress in prostate cancer cell lines,[61, 62] and fatty acid molecules generate reactive oxygen species as they undergo lipid peroxidation.[52] Malins et al[50] sorted normal prostate, BPH, and cancer based purely on the level of oxidized DNA bases in specimens derived from patients.

One genome alteration identified in human prostate cancers also points to oxidative stress as a crucial feature of prostatic carcinogenesis. Virtually all human prostate cancers lose expression of glutathione S-transferase-pi (*GSTP1*).[47] This loss of expression occurs as an early step

in prostatic carcinogenesis as it is found in PIN, a purported prostate cancer precursor lesion.[7] Loss of expression appears secondary to somatically acquired DNA hypermethylation of *CpG islands* in the 5′-regulatory regions of the *GSTP1* gene.[47] *GSTP1* is the primary glutathione transferase expressed in prostate epithelia and is particularly effective at reducing lipid peroxides. Transgenic mice lacking π-class GST activity are more susceptible to carcinogens that act through generation of oxidative DNA damage.[40] Dietary micronutrients that act through attenuating oxidative stress or buttressing cellular defenses may be particularly germane to prostate cancer prevention. It is likely, however, that micronutrients act to prevent initiation and possibly progression through multiple pathways.

DIETARY MICRONUTRIENTS WITH PREVENTIVE POTENTIAL

Vitamin E

Vitamin E is a term that encompasses a group of chemicals that possess antioxidant activity. The form of vitamin E commonly used as a dietary supplement is α-tocopherol. Evidence for a role of vitamin E supplementation in prostate cancer prevention comes from the Finnish Alpha-Tocopherol, Beta-Carotene (ATBC) cancer prevention study.[72] This double-blind, placebo-controlled, randomized clinical trial was designed to measure the effects of β-carotene and α-tocopherol supplementation on the frequency of lung cancer incidence. The study is well known for the surprising result that β-carotene supplementation increased lung cancer incidence. Analysis of secondary endpoints revealed, however, that prostate cancer incidence was decreased 32% and mortality was decreased 41% among men receiving α-tocopherol as compared with controls.[39] Similar to the results for lung cancer, prostate cancer incidence and mortality increased among men taking β-carotene supplements. More recent findings complicate this latter point, however. The randomized Physicians' Health Study trial of β-carotene supplementation suggested a lower risk of prostate carcinoma as well as potential protective effect of β-carotene supplementation among men with the lowest baseline plasma β-carotene levels.[14]

The Health Professional Follow-up Study also showed a protective effect for vitamin E, although that protection was extended only to smokers. Vitamin E supplementation did not reduce prostate cancer risk generally but did show a relative risk of developing metastatic or fatal prostate cancer of 0.44 (95% CI, 0.18 to 1.07) among smokers who were vitamin E users compared with smokers who were not.[19] All men in the ATBC trial were smokers. In a 17-year follow-up study of Swiss men, increased prostate cancer risk was seen only in men who smoked and had the lowest plasma α-tocopherol levels.[19] Together these studies suggest a role for vitamin E in countering the increased oxidative damage

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experienced by smokers, but the argument for vitamin E supplementation in the general population requires further validation.

Lycopene

Several intriguing reports have noted an association between diets rich in tomato products and a reduction in prostate cancer risk. The key component in tomatoes is believed to be lycopene, which is among the most effective oxygen radical quenching agents of all the carotenoids.[18] As with other micronutrients, most of the evidence for a protective effect for lycopene in humans comes from observational studies, although there is evidence that lycopene works in association with α-tocopherol to inhibit prostate cancer cell proliferation in vitro.[56] A review of the published epidemiologic evidence for an inverse association between tomato intake or blood lycopene levels and cancer risk at a defined site claims an inverse association in 57 of 72 studies, 35 of which were statistically significant.[30] One large prospective cohort study, described by Giovannucci et al,[29] included 47,894 men and 812 new cases of prostate cancer and showed an association between lycopene intake and lower risk of prostate cancer (RR, 0.79; 95% CI, 0.64 to 0.99; $P = .04$). Most lycopene was derived from tomatoes, tomato sauce, and pizza. No association was found with consumption of tomato juice, perhaps because the bioavailability of lycopene is enhanced by processes such as heating and mixing with dietary oils or fat.[69]

A study has shown lower prostate cancer risk in men with elevated plasma lycopene levels.[26] In this prospective study, plasma samples were obtained from 1872 men at the initiation of a randomized, placebo-controlled trial of aspirin and β-carotene. After 13 years of follow-up, 578 of these men had developed prostate cancer. Of the antioxidants measured in the plasma samples, only lycopene was found at significantly lower mean levels in cases than in matched controls, particularly among men assigned to placebo. Higher plasma lycopene was associated with reduced risk of prostate cancer. This association was the strongest for aggressive prostate cancers in the highest quintile of plasma lycopene (odds ratio [OR], 0.56; 95% CI, 0.34 to 0.91; $P = .05$). This study is based on a single measurement of plasma lycopene and might not be representative of lycopene levels over the duration of the study. Evidence does exist that *cis*-isomers and *trans*-isomers of lycopene can be concentrated to high levels in the prostate,[11] further suggesting a role in prostate cancer risk reduction. Ultimately, however, these observational studies should be interpreted with caution. Kristal and Cohen[46] emphasized that this work should be considered in light of the discrepancy between the consistent body of evidence linking diets rich in β-carotene to reduced lung cancer risk and the alarming increase in lung cancer in 2 large placebo-controlled trials in patients treated with β-carotene. Protective effects conferred by other components found in carotenoid-rich foods or diets generally rich in vegetables and fruit may confound observational

studies. Clinical dietary intervention trials with lycopene are crucial in further determination of the preventive potential of this compound.

Selenium

Several lines of evidence have implicated selenium, an essential trace element, in cancer prevention. Interest in the cancer preventive property of selenium was sparked initially by an observation that correlated populations with lower cancer mortality rates to geographic regions with high soil selenium content.[67] Several observational studies subsequently suggested an inverse relationship between selenium consumption and later risk of cancer development. The Nutritional Prevention of Cancer study by Clark et al[10] was the first to underscore the role for selenium as a chemopreventive agent for prostate cancer. Although the primary aim of this multicenter, double-blind, randomized, placebo-controlled prevention trial was to determine the effects of selenium supplementation on the incidence of basal or squamous cell skin carcinoma, analysis of the secondary endpoints suggested dramatic reduction in prostate cancer diagnoses in selenium-treated subjects. Participants were treated for an average of 4.5 years and followed for a mean of 6.4 years. Of the patients who developed prostate cancer, 35 received the placebo, and 13 received the 200-μg selenium supplement (RR, 0.37; 95% CI, 0.18 to 0.71; $P = .002$). These results suggest that supplemental selenium beyond the recommended dietary allowance of 70 μg may reduce the risk of prostate cancer diagnosis.

As a follow-up to this intervention trial, Yoshizawa et al[76] examined the association between toenail selenium levels, a reflection of the body selenium pool, and prostate cancer risk in men enrolled in the Health Professions follow-up study. When compared with men in the lowest quintile for toenail selenium levels, there appeared to be a trend for decreased prostate cancer risk in the highest 4 quintiles (95% CI, 0.25 to 0.96; $P = .11$; adjusted for age, smoking, and sampling time relative to diagnosis). When further adjustments were incorporated for diet (lycopene, calcium, and saturated fat) and other prostate cancer risk factors, including family history, body mass index, and vasectomy, the risk for subsequent development of prostate cancer was decreased in the 4 highest quintiles, and the effect did not appear linear (OR, 0.39; 95% CI, 0.18 to 0.84; P for trend .05). Preliminary assessment of serum selenium levels in men enrolled in the Baltimore Longitudinal Study of Aging suggests a similar nonlinear decrease in prostate cancer risk in men with high serum selenium levels.[5]

Although the exact mechanisms behind selenium's preventive effects are largely unknown, in vitro studies suggest that selenium may potentiate antioxidant defenses. Selenoproteins, such as glutathione peroxidase and thioredoxin reductase, play a key role in the antioxidant defense system, and selenium may participate in detecting oxidative stress in the cell.[71] Other work suggests that selenium may act as an

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antineoplastic agent by inhibiting cell growth and DNA synthesis, cell cycle blockage, DNA single-strand breaks, and induction of apoptosis.[27, 41, 44] Further basic investigations as well as ongoing intervention trials would clarify the role of selenium in prostate cancer prevention.

Sulforaphane

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Several population-based studies have linked consumption of vegetables to decreased cancer risk. One report correlated consumption of green vegetables, in particular *Cruciferae,* to decreased prostate cancer risk.[12] Cruciferous vegetables are a rich source of isothiocyanates, the most abundant being sulforaphane. Sulforaphane has been shown to block mouse mammary tumorigenesis and potently induces carcinogen defense systems, most notably the glutathione transferases.[21, 77] Sulforaphane induces these same enzymes in normal prostatic cells in vitro.[76] Because human prostate cancer is characterized by a deficiency of one of the glutathione transferases (GSTP1), sulforaphane may act by compensating for its loss by induction of other glutathione transferases with similar substrate specificity.

Soy/Genistein

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One striking difference between Asian and Western diets is the large disparity in the consumption of soy-based foods. Native Asians consume several-fold higher levels of soy, leading to speculation that soy might have prostate cancer preventive properties. Although scanty, some epidemiologic evidence is suggestive of a protective effect. A prospective study of 12,395 California Seventh-Day Adventist men revealed a 70% reduction of prostate cancer risk among men who consumed soy milk more than once a day.[45] Soy is rich in isoflavones, most notably genistein, which have been reported to exert myriad effects on prostate cancer. Genistein induces growth inhibition of prostate cancer cell lines propagated as tumors in mice and decreases tumor angiogenesis.[79] Genistein may act as a weak phytoestrogen and can alter androgen metabolism through its ability to block 5-α-reductase activity.[20] Genistein also has some capacity to act as an antioxidant.[74] Further work is necessary to assess the relative contributions of these effects in soy and genistein's ability to act as a cancer preventive agent. Results from clinical prevention trials are needed to assess the true efficacy of soy-based foods or derivatives in prostate cancer prevention.

Vitamin D

A growing body of epidemiologic and experimental evidence suggests that vitamin D may play a role in the prevention of prostate cancer. Vitamin D serves as a regulator of calcium and phosphorus absorption

in the small intestine and is a critical mediator of bone metabolism. The active form, 1,25-dihydroxyvitamin D_3, or calcitriol, is synthesized by 3-step process beginning with a UV light–dependent reaction in the skin. Most vitamin D is obtained by this method, although fortified dairy products provide a secondary source.[70] Vitamin D now is recognized as a potent growth and differentiation regulator in many tissues, including the prostate. In vitro experiments show a growth inhibitory effect of vitamin D on an androgen-responsive prostate cancer cell line.[78] Animal models show tumor shrinkage in xenografts. Multiple mechanisms of action, including cell cycle arrest, apoptosis, growth factor modulation, and androgen receptor modulation, have been proposed. Comprehensive summaries of the experimental data are presented by many authors.[1, 23, 51]

The earliest epidemiologic observations were published by Schwartz and Hulka,[66] who hypothesized that vitamin D deficiency was a risk factor for prostate cancer. Further analysis of these data revealed a statistically significant inverse relationship between prostate cancer mortality rates and UV light exposure.[38] Skin pigment, which inhibits the initial conversion to the active form of vitamin D, has been proposed as a possible cause for the increased incidence of prostate cancer in African Americans.

These observations were studied further with retrospective analyses of banked serum samples. Corder et al[15] noted in a study of 181 prostate cancer patients with age-matched and race-matched controls that a lower (1,25) dihydroxyvitamin D_3 level correlated with a statistically significant increase in prostate cancer risk. Other case-control studies have not confirmed this observation, however.[3, 25] The discrepancy between these results could be related to seasonal variability in vitamin D levels as well as the ability to exclude prostate cancer definitively in control populations given the lack of PSA screening at the time of the studies.

Preliminary clinical trials using calcitriol have been reported. In a phase I trial of patients with an early PSA recurrence after definitive local therapy, Gross et al[35] noted that in 6 of 7 patients the rate of rise in serum PSA, or PSA velocity, declined. Further follow-up revealed that this effect has been observed in all 7 patients (D. Feldman: personal communication, 2000). A phase II trial enrolling 13 men with hormone-refractory prostate cancer revealed a decline in PSA values in 2 patients.[53] In both cases, dose-limiting hypercalcemia with the development of renal stones was noted.

Several vitamin D analogs have been developed with more potent antiproliferative effects and fewer calcemic side effects. Such agents are promising in that they avoid the dose-limiting complications of calcitriol therapy and are likely to be employed in future clinical trials as well as in vitro studies.

OTHER PREVENTIVE APPROACHES

Several other dietary and pharmacologic approaches are under investigation as possible means of prostate cancer prevention. Green tea

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and its biologically active component epigallocatechin gallate have been shown to be potent antioxidants and to exert anticarcinogenic effects in several rodent models as well as in prostate cancer cell lines.[36, 55] Differentiation agents such as retinoic acid block tumors of the sex accessory glands in the Lobund-Wistar rat[60] and have been evaluated in phase I clinical trials. The polyamine synthesis inhibitor difluoromethylornithine inhibits prostate cancer cell growth in vitro, although some toxicity in humans has been observed in clinical trials.[49] Modified citrus pectin inhibited metastatic potential in a rat prostate cancer model[57] and has been proposed for use as a chemopreventive agent for several tumor types. The nonsteroidal anti-inflammatory drug sulindac has been shown to inhibit growth and induce apoptosis of prostate cancer cells cultured in vitro and grown as xenografts.[31, 48] Observations such as these set the stage for the development of newer, potentially less toxic agents for distinct and complementary preventive approaches.

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Address reprint requests to
James D. Brooks, MD
Department of Urology/S287
Stanford University School of Medicine
300 Pasteur Drive
Stanford, CA 94305-5118

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