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PRINCIPAL INVESTIGATOR: Bruce J. Trock, Ph.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, Maryland 21205

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Bruce J. Trock, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Johns Hopkins University
Baltimore, Maryland 21205

E-Mail: btrock@jhmi.edu

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The objective of this case-control study is to determine whether oxidative stress, specifically lipid peroxidation, is a risk factor for prostate cancer. Furthermore, the study will investigate with oxidative stress mediates the association between high dietary fat intake and prostate cancer risk. Specifically, the study will compare malondialdehyde (MDA) in serum, as a marker of lipid peroxidation, and deoxyguanosine malondialdehyde (dG-MDA) in peripheral lymphocytes and prostate tumor as a marker of oxidative DNA damage. These associations will be adjusted by dietary measures of fat and antioxidant intake, as well as serum measures of specific fatty acids and antioxidants. Because redox cycling of androgens as recently been shown to be a source of reactive oxygen species, serum androgen levels will also be correlated with MDA, dG-MDA, and risk. If an association between lipid peroxidation and prostate cancer risk is demonstrated, it could lead to new strategies for prevention (especially chemoprevention) and markers that could define men at high risk.
This report covers activities since the grant was moved from Georgetown University to Johns Hopkins School of Medicine in 2001.

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FOREWORD

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NA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI – Signature Bruce Trock Date 6/04/03

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INTRODUCTION

The objective of this case-control study is to determine whether oxidative damage is a risk factor for prostate cancer, and whether this mechanism mediates the association between dietary fat and prostate cancer risk. Specifically, cases and controls will be compared with respect to malondialdehyde (MDA) in serum as a measure of oxidative stress, and deoxyguanosine malondialdehyde (dG-MDA) in peripheral lymphocytes and prostate tumor samples as a measure of oxidative DNA damage. In addition to these measures, dietary intake of fats and specific fatty acids, and of antioxidants will be considered as potential effect modifiers or confounding factors, as will serum antioxidant levels.

This report covers activities since the project transferred from Georgetown University to Johns Hopkins School of Medicine in 2001. There was a long period of inactivity while the study protocol and consent form were being negotiated between the Department of Defense, the Principal Investigator, and Johns Hopkins School of Medicine. The issues were successfully resolved and the study was re-activated in Jan 2003. Since that time, progress has been rapid. We have enrolled 223 prostate cancer cases and 165 controls from Johns Hopkins Hospital and Johns Hopkins Bayview Medical Center. Added to the patients recruited from Georgetown University, the Washington, DC Veterans Administration Medical Center, and Washington Hospital there are a total of 320 cases and 250 controls, of whom 15% are African American. Serum, plasma and lymphocytes are available from 89% of these patients and epidemiology/dietary questionnaire data are available for 85%.

The population enrolled in this case-control study formed the basis for a project in the NCI-funded Johns Hopkins Prostate Specialized Program of Research Excellence (SPORE) award (SPORE Grant #2 P50 CA58236-10, "Project 5: DNA polymorphisms in genes affecting levels of oxidative stress in prostate cells: population studies of association with prostate cancer risk"). That study will build on the existing case-control dataset and analyze a series of single nucleotide polymorphisms (SNPs) associated with generation of or response to oxidative stress, to determine whether any are risk factors for prostate cancer, or modify the risk associated with oxidative stress.

An additional small pilot project has been initiated with Dr. Prakash Rao at the University of South Alabama. This study will measure leptin in the serum of a subset of prostate cancer cases and controls from the parent study. Leptin is a potential risk factor for prostate cancer that may also modify the association of diet with risk. These samples are currently being analyzed.

During the remainder of the funding period we will continue to enroll patients, and begin the analyses of malondialdehyde (MDA), deoxyguanosine malondialdehyde (dGMDA), serum hormones and antioxidants, and dietary data. We expect to complete these analyses in early 2005 and submit manuscripts for publication.

BODY

Study Progress

This section will describe the following:

(a) original study objectives
(b) the change in institutions due to the Principal Investigator's move to another university
(c) delays in re-activating the study at the new institution related primarily to approval of the consent form
(d) progress since the study was re-activated
(e) additional ongoing research that was spun-off of the original grant
(f) plans for concluding the study.

Study Objectives.

Task 1. To complete enrollment of prostate cancer cases scheduled to undergo prostatectomy, and benign urologic surgery controls, from urology clinics at Johns Hopkins University School of Medicine (JHU), Georgetown University (GU), the Veteran's Administration Medical Center (VA) in Washington, DC, and the Washington Hospital Center (WHC). This includes collection of serum, plasma, lymphocytes, epidemiologic and dietary intake data, and (from cases) paraffin-embedded tumor tissue.

Task 2. To measure the following biomarkers:
- Serum malondialdehyde
- Malondialdehyde deoxyguanosine adducts in peripheral blood lymphocytes
- Malondialdehyde deoxyguanosine adducts in prostate tumor tissue
- Complete serum fatty acid profile
- Serum antioxidants including alpha-tocopherol (vitamin E) and carotenoids
- Serum androgens and related hormones or metabolites, including testosterone, dihydrotestosterone, 3α androstanediol glucuronide, and SHBG

Task 3. To conduct a case-control study with the above data to compare the levels of lipid peroxidation biomarkers in cases with controls, to determine the following:
- Whether lipid peroxidation levels modify the association of dietary fat with prostate cancer risk.
- Whether lipid peroxidation biomarkers in serum (MDA) or lymphocytes (dGMDA adducts) provide a good estimate of the extent of oxidative DNA damage in prostate tumors (dGMDA adducts in tumor).
- Whether lipid peroxidation levels are a function of dietary fat and androgens.

Change in Institutions. In April 2001 the Principal Investigator, Dr. Bruce Trock, moved from Georgetown University (GU) to Johns Hopkins University (JHU). The plan at that time was to continue to enroll patients from the original enrollment sites at GU, the Veteran's Administration Medical Center (VA) in Washington, DC, and Washington Hospital Center (WHC), and to also begin enrolling patients from JHU.

Delays in re-activating the study. All research activity and acceptance of new IRB applications at JHU were suspended in July 2001 due to the death of a subject in a research study completely unrelated to Dr. Trock's research. This suspension was lifted in October 2001, and JHU arranged for an outside institutional review board, Western Institutional Review Board (WIRB) to assist in reviewing new applications. Around the same time, in September 2001, Dr. Trock was notified by Dr. Angela Howard (USAMRAA) that the original informed consent form for this study had never been approved, and that the study had not been authorized to enroll patients. Dr. Howard's letter requested that all study activities immediately be suspended. Dr. Trock complied and suspended study activities. The problem had been that Dr. Trock had never received a letter from USAMRAA, dated September 1999 (i.e. the time of the original grant award while at Georgetown) requesting modification to his proposed consent form before the study could be authorized to begin. At that time, a study number was subsequently assigned and the grant funds were released to Georgetown University (GU), so Dr. Trock assumed that the study had been approved to begin, and he began enrolling patients while at GU. Following Dr. Howard's September 2001 letter, a long series of communications took place, during which Dr. Trock provided evidence that no research subjects had been harmed in any way, and the entire grant proposal, study protocol, and consent form were re-reviewed. This process involved communication with Dr. Howard and her successors, including Dr. Adrienne King, Sacelia Heller, Christine Helman, and Shirley Roach, of AMDEX Corporation and the USAMRAA Office of Regulatory Compliance and Quality. Ultimately, everything had been approved except for a clause in the consent form concerning payment for medical care needed in the event that a study subject was injured while participating in the study. Eventually this was resolved to the satisfaction of USAMRAA, Johns Hopkins University, and Dr. Trock. The final consent form and study protocol were approved by WIRB (WIRB Protocol Number 20011642). The study was finally authorized by USAMRAA to begin in January 2003. **This entire Human Subjects Review process was assigned HSRRB Log Number A-09282, and all communications during that process are referenced to that number.**

Progress since re-activation of the study. After moving to JHU, the original intent was to continue to enroll at the three original sites (GU, VA, and WHC), as well as beginning enrollment at JHU. However, because the patient volume for general urology and prostate cancer at JHU far exceeds that of GU and WHC combined, and demographics are similar at all 3 sites, it was decided that enrollment at GU and WHC could be stopped without adversely affecting study

accrual goals or study composition. This step was taken because there was only one Research Nurse funded by the grant, and it was not feasible for her to actively recruit at 4 sites that were over 40 miles apart (e.g. Washington DC to Baltimore). Enrollment at the VA will continue, since 75% of prostate cancer patients there are African American. This will provide the opportunity to examine ethnic differences in risk associated with oxidative stress.

The Brady Urological Institute at JHU is one of the largest clinical urology programs in the world. Annually, approximately 1,000 men undergo radical prostatectomy for prostate cancer, and more than 2000 men are seen for other urological conditions. We initially began recruitment of cases only, to establish the infrastructure and relations with the attending urologists to insure that enrollment would not interfere with clinical practice. The recruitment process requires the Research Nurse to review clinic appointment schedules and surgery schedules up to one month in advance, identify potentially eligible patients based on age, sex, and indication for clinic visit or diagnosis, and when possible, review the patient medical records for eligibility criteria using the JHU Electronic Patient Record. Following implementation of HIPAA, access to this type of information without prior patient assent was no longer permissible so we applied for and received a HIPAA Partial Waiver of Authorization to permit clinicians to provide this information to us. The justification for the Waiver is that identifying and contacting eligible study subjects would not be feasible via other means. We have had no complaints from patients that their information was provided without their prior assent. It is important to note that the Waiver of Authorization is not a waiver of the requirement for consent. It only provides us the means to contact patients, inform them about the study, and then see whether they are interested in learning more about the study and ultimately, going through the informed consent process. The Research Nurse sends each patient a packet with a cover letter explaining the study, a short series of questions to screen for eligibility, the consent form, and the epidemiology and diet questionnaires. When the interval between being notified about a potentially eligible patient and the patient's clinic visit is too short to mail the packet, the Research nurse calls the subject, and if they express willingness to consider the study, she arranges to meet them prior to their appointment to explain the study and obtain informed consent from those willing to participate. Blood samples are obtained at the clinical phlebotomy station for patients who are already scheduled for a blood draw required for their clinic visit, or by the Research Nurse for patients not scheduled for other blood work.

Once this process was going smoothly we began to focus on control recruitment as well. We soon realized that despite the very large patient volumes seen by the Brady Urological Institute, a large fraction of patients being seen for reasons other than prostate cancer (i.e. potential controls) were being seen for other urologic cancers (e.g. bladder, kidney), or had a prior history of other cancers (including non-urologic cancers), and were thus, ineligible. Relatively fewer patients in the target age-range for the study were being seen for general urology problems (e.g. kidney stones, nonmalignant urinary tract obstruction, nephropathies, voiding dysfunction) because such patients are often treated by community urologists rather than going to a large referral center.

The requirement that controls have documented PSA < 2.5 and a normal DRE also reduced the number of eligible patients. Because we realized that a much greater effort was required to screen urology clinic schedules for potentially eligible patients, and the grant did not provide funding to permit us to hire an additional nurse for this task, we temporarily suspended enrollment of cases to focus efforts on control recruitment. We also began to actively recruit controls from the Johns Hopkins Bayview Medical Center, a hospital several miles away from the main Johns Hopkins Hospital. This hospital provides more routine urology care than the urology clinic at the main hospital and is a good source of controls.

We currently enroll two different types of controls. Patients coming for routine urology care unrelated to malignancy and with no history of cancer form the first group ("true controls"). However, despite the fact that these patients will have had a recent (within 2 years) PSA <2.5 ng/ml and a normal DRE, some of these patients will harbor undetected prostate cancer (Thompson 2004). For this reason we also include among the controls men who have had a recent negative prostate biopsy, and are not considered to be at "high risk" of being a false negative (i.e. PSA≤8 ng/ml, no evidence of prostate intraepithelial neoplasia (PIN) or atypia on biopsy, and normal digital rectal exam. These men have about a 20% chance of having prostate cancer missed by the biopsy (false negative). We follow these men for one year to identify any who have a subsequent biopsy that identifies cancer; such men would be switched from the control population to the case population.

The following table summarizes our recruitment of cases and controls to date, showing results from the different clinical sites:

	Cases (n=320)	**Controls (n=250)**
JHU	223	**biopsy negative:** 39, **true control:** 126
GU, VA, WHC	97	**biopsy negative:** 74, **true control:** 11
blood available	279	**biopsy negative:** 105, **true control:** 122
questionnaire(s) available	295	**biopsy negative:** 95, **true control:** 94
white	235	**biopsy negative:** 75, **true control:** 80
black	63	**biopsy negative:** 21, **true control:** 4
asian	3	**biopsy negative:** 1, **true control:** 2
Hispanic	5	**biopsy negative:** 0, **true control:** 0
other	11	**biopsy negative:** 4, **true control:** 3

17% of subjects are African American, with a somewhat higher proportion among cases (20%) than controls (13%). Note that the numbers included in the breakdown of ethnicities do not add up to the total number of subjects – we do not have ethnic data in the database for some individuals for whom we have not yet received their questionnaire. Serum, plasma and lymphocytes are available from 89% of enrolled patients and epidemiology/dietary questionnaire data are available for 85%. We are continuing to contact patients who have not yet returned their questionnaires to obtain additional data. In addition to the cases shown in the table we also have questionnaire data and blood samples from 15 patients with premalignant lesions, i.e. subjects with PIN, HGPIN, and atypia. These may be of interest because, if oxidative damage truly is a risk factor for prostate cancer, these may exhibit levels intermediate between those of cancer cases and controls.

Additional research spun off the parent grant. Two ongoing projects have been based on the existing case-control study and are described below.

1. Johns Hopkins Prostate Cancer SPORE (grant #2 P50 CA58236-10): "Project 5: DNA polymorphisms in genes affecting levels of oxidative stress in prostate cells: population studies of association with prostate cancer risk." This study was funded by the National Cancer Institute in September 2003 as one of five research projects in the Johns Hopkins Prostate Cancer SPORE grant. The study will use the current case-control population. Fifty genes known to be associated with production of reactive oxygen species (ROS), detoxification of ROS, or repair of DNA damage due to ROS will be evaluated. For each of the candidate genes targeted for analysis, we will genotype approximately 8 to 12 single nucleotide polymorphisms (SNPs) per gene in the cases and controls from the parent study. These will be used to identify prostate cancer risk-modifying genes by performing association analyses of genotype frequencies in cases and controls. We will correlate this data with the data on dietary intake, biomarkers of oxidative stress, oxidative damage, and antioxidants from the parent study to determine which genes modify the association of these exposures with risk. Finally, the associations identified in the case-control population will be validated by examining two independent study populations: a cohort study in Washington County, MD (Kathy Helzlsouer, PI), and an African American case-control study population collected at Howard University (Rick Kittles, PI).

2. Serum leptin and prostate cancer risk. This pilot study is a collaboration with Dr. Prakash Rao at the University of South Alabama. Obesity is a potential risk factor for prostate cancer, and is closely correlated with dietary fatty acid intake, a potential source of oxidative stress. Circulating leptin levels are correlated with obesity and androgen activity, and the prostate contains receptors for leptin. Studies *in vitro* have shown that leptin stimulates prostate cancer cell proliferation (Somasundar 2004), but epidemiologic studies have been few and inconsistent (Stattin 2001; Stattin 2003). We decided to examine leptin in a pilot prostate cancer case-control study to determine whether (a) leptin was associated with oxidative stress, and (b)

whether leptin was associated with prostate cancer risk or modified associations with ROS-related exposures. A set of 35 cases was matched to 35 controls on age and race, and were sent to Dr. Rao blinded as to case-control status or any clinical or epidemiological data. Serum leptin levels were assayed using a Human Leptin ELISA kit from Linco Research (Cat.# EZHL-80SK). The assay is a direct sandwich ELISA. The enzyme activity is measured spectrophometrically by the increased absorbency at 450-590nm after acidification of formed products. Because the increase in absorbency is directly proportional to the amount of captured human leptin in the serum,these values can be derived by interpolation from a standard curve generated with reference samples of known concentrations of human leptin. These assays have been completed the week that this report was prepared, and Dr. Rao is sending the data to Dr. Trock to be correlated with case-control status, and ROS-related variables.

Plans for concluding the study. Enrollment has already exceeded the recruitment goals originally defined in the grant application (240 cases and 240 controls). We are beginning to request paraffin-embedded tumor material for analyses of deoxyguanosine malondialdehyde. We have sent dietary data for all subjects enrolled at JHU to Block Dietary Data Systems, Inc., the company that produces the dietary questionnaire and analyzes completed questionnaires for nutrient content. The nutrient data have been quantified and sent back to us for analysis. We are now arranging with out collaborator at University of Toronto, Deparment of Nutrition, Dr. A. V. Rao (not the same Dr. Rao as the one analyzing leptin data) to begin shipping samples for biomarker analysis. Serum will be analyzed for malondialdehyde, fatty acids, and antioxidant profile; lymphocytes and paraffin-embedded tissue will be analyzed for deoxyguanosine malondialdehyde. We expect these analyses, and biostatistical analysis of this data with case-control status, to be completed within 6 months, at which time we will submit papers for publication.

CONCLUSION

This study has surmounted a number of setbacks and has exceeded its accrual goals and formed the basis for additional peer-reviewed funded and unfunded research. The study has assembled a large amount of data and biological specimens, which will be used to evaluate (a) the role of oxidative stress and oxidative damage in prostate cancer, (b) the influence of dietary factors and vitamin supplements on levels of oxidative stress and oxidative damage, (c) the validity of deoxyguanosine malondialdehyde measured in lymphocyte DNA to serve as a dosimeter of levels in the prostate. In addition, we have begun collecting clinical-follow-up data for the prostate cancer patients in the study and will continue to do so, allowing us to analyze in the future the influence of oxidative damage on prostate cancer prognosis.

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APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ELISA	Enzyme-linked immunoabsorbent assay
GU	Georgetown University Medical Center
IRB	Institutional Review Board
MDA	malondialdehyde
dG-MDA	deoxyguanosine malondialdehyde
NCI	National Cancer Institute
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate-specific antigen
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
SPORE	Specialized Program of Research Excellence
VA	Veterans Adminstration Hospital
WHC	Washington Hospital Center
WIRB	Western Institutional Review Board

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Personnel receiving pay from this negotiated effort:

Bruce Trock, Ph.D.
Elizabeth Hopewell, MPH
Patricia Kolmer, RN, BSN
A. Venket Rao, Ph.D. (consultant)