AD_____

Award Number: DAMD17-02-1-0223

TITLE: Dynamic Tissue Culture from Prostate Biopsy Specimens as a Model for Predicting Tumor Radiosensitivity to Ionizing Radiation Treatment

PRINCIPAL INVESTIGATOR: David W. Nyman, M.D.

CONTRACTING ORGANIZATION: University of Arizona
Tucson, Arizona 85722-3308

REPORT DATE: April 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual (1 Apr 2003 - 31 Mar 2004)

4. TITLE AND SUBTITLE
Dynamic Tissue Culture from Prostate Biopsy Specimens as a Model for Predicting Tumor Radiosensitivity to Ionizing Radiation Treatment

5. FUNDING NUMBERS
DAMD17-02-1-0223

6. AUTHOR(S)
David W. Nyman, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Arizona
Tucson, Arizona 85722-3308

E-Mail: DNYMAN@AZCC.ARIZONA.EDU

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Prostate cancer is the most common non-cutaneous malignancy in men. Radiation therapy is a common treatment for this disease however, most patients receive a similar dose of radiation (70-76 Gy) regardless of individual clinical, pathological, or molecular characteristics of the tumor. The hypothesis of this project is that all prostate carcinomas are unique and that by identifying specific tumor markers or other molecular characteristics using our dynamic tissue culture system (Parrish et al, 2002), we can identify those tumors most sensitive to radiation therapy. The specific aims for the first year were to use prostate biopsy tissue, obtained retrospectively, and adapt our organ culture technique to the requirements of prostate biopsy specimens. We have been able to determine the optimal biopsy core size and tissue culture media conditions. We have also demonstrated that basal cells present in the prostate glandular tissue proliferated over the 72 hour time period of organ culture. We have maximized the length of time that tissue remains viable in our dynamic tissue culture system.

We are now ready to begin Aim II of the proposal determining the baseline radiosensitivity of prostate tissue and assessing the roles of p53, bcl-2, and NFKB in the intrinsic radiosensitivity of prostate tissue. We hope to further profile these biomarkers and using them to predict prostate tissue radiosensitivity will aid in the diagnosis and prognosis of this significant cancer.

We have received approval by the IRB at the University of Arizona to enroll up to 30 new patients. Dr. Shona Dougherty, Assoc Professor of Rad. Oncology has agreed to be the new Co-PI. We anticipate enrolling as many as 3-5 patients in the following 6 months. We will be processing these tissues for bio-markers as noted above. We expect to complete the project by April 30, 2005.

14. SUBJECT TERMS
No Subject Terms Provided.

15. NUMBER OF PAGES
26

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction.....4

Body.....5

Key Research Accomplishments.....8

Reportable Outcomes.....9

Conclusions.....10

References.....11

Appendices.....

INTRODUCTION

Prostate cancer is the most common non-cutaneous malignancy in men. Radiation therapy is a common treatment for this disease however, most patients receive a similar dose of radiation (70-76 Gy) regardless of individual clinical, pathological, or molecular characteristics of the tumor. The hypothesis of this project is the concept that all prostate carcinomas are unique and that by identifying specific tumor markers or other molecular characteristics using our dynamic tissue culture system (Parrish et al, 2002), we can identify those tumors most sensitive to radiation therapy. The specific aims for the first year were to adapt our organ culture technique to the requirements of prostate biopsy specimens. We have been able to determine the optimal biopsy core size and tissue culture media conditions. We have also maximized the length of time that tissue remains viable in our dynamic tissue culture system.

We are now ready to begin Aim II of the proposal determining the baseline radiosensitivity of prostate tissue and assessing the roles of p53, bcl-2, and NFKB in the intrinsic radiosensitivity of prostate tissue. We hope to further profile these biomarkers and using them to predict prostate tissue radiosensitivity will aid in the diagnosis and prognosis of this significant cancer.

We have received approval by the IRB at the University of Arizona to enroll up to 30 new patients. Dr. Shona Dougherty, Associate Professor of Radiation Oncology has agreed to be the new Co-PI. We anticipate enrolling as many as 3-5 patients in the following 6 months. We will be processing these tissues for bio-markers as noted above. We expect to complete the project by April 30, 2005. The body, key research accomplishments, conclusions, and references of this report is the same as was submitted to in our previous report of September 2003.

BODY

Introduction

We have previously demonstrated through the production of very thin (275-300 μm) prostate tissue slices, which are highly reproducible, and the use of a dynamic organ culture system in which the tissue is not continuously submerged, that our precision-cut tissue slice system represents an advance over traditional prostate organ culture. Given the retention of stromal-epithelial interactions, the ability to investigate zone-specific features, and the maintenance of cellular viability and function for several days, prostate slices represent a unique *in vitro* model to investigate prostate cellular proliferation and cytotoxicity (Parrish et al, 2002).

Materials and Methods

The dynamic organ culture incubator, titanium rollers, and slice inserts were purchased from Vitron, Inc. (Tucson, AZ). Keratinocyte growth media (K-SFM) and supplements were obtained from Sigma Chemical Co. (St. Louis, MO), as was the lactate dehydrogenase assay system (kit # 340-LD). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY). The ELISA system for the detection of PSA was purchased from Alpha Diagnostics (San Antonio, TX). The proliferation marker MIB-1 (reactive with Ki-67) was obtained from Beckman Coulter (Fullerton, CA). All primary antibodies were detected using biotinylated secondary antibodies followed by a streptavidin-peroxidase conjugate and DAB chromogen with an automated system obtained from Ventana Medical Systems (Tucson, AZ).

Human Prostate Tissue

The University of Arizona Institutional Review Board approved the protocol for the procurement of human tissue, obtained from prostate biopsy. During brachytherapy, the prostate gland was identified using transrectal ultrasound and biopsy was guided by C-arm fluoroscopy. Transperineal biopsy specimens (3-6) were obtained using 18G MaxCore® Disposable Biopsy Units (C.R. Bard, Inc., Murray Hill, N.J.). There were a total of 11 prostates sampled for this study out of a total of 14 patients who signed consent forms.

Biopsy cores

Each biopsy core was approximately 8-10 mm in length with a diameter of 0.0126 mm or 12.6 μm. The cores were placed onto titanium roller inserts that were blotted and loaded into glass scintillation vials containing 1.7 ml of media.

Dynamic Organ Culture System

Keratinocyte serum free media (KSFM) supplemented with (10% v/v) fetal bovine serum (FBS) was the media used in all studies. The media also contained antibiotics, 50 units/ml of Gentamicin and 100 units/ml Penicillin G/ Streptomycin, and 0.625 μg/ml Fungizone an antifungal drug. The scintillation vials were closed with a cap, which has a 2 mm central opening, that allows for gas exchange. Vials were then placed in the dynamic organ culture incubator that was gassed with 95%O_2:5%CO_2 at a flow rate of 1ml/minute. The incubator temperature was a constant 37°C. The media was changed every 24 hours. The dynamic organ culture incubator is unique in that it rotates the scintillation vials allowing the biopsy core on the titanium rollers to be alternately submerged in media and then exposed to the ambient gases (Parrish et al, 1995). This is different from traditional submersion culture techniques used in prostate explant culture and affords much better exchange of nutrients, wastes, and gases.

Tissue Preparation

Three to six punch biopsy specimens were collected during the prostate brachytherapy procedure. All biopsy specimens were immediately placed in chilled, sterile normal saline solution. The first two biopsy specimens were processed *en bloc* for thin section slide preparation. Each specimen was fixed in 10% formalin and then embedded in paraffin. The specimens were sectioned via microtome (5 μm) and then stained. Stains used included H&E for routine pathologic evaluation. Other immunohistochemistry stains include PSA, a measure of normal prostate tissue secretory activity and Ki-67, a nuclear stain for mitotic or proliferative activity. Twenty-four hours after the biopsy, selected specimens were irradiated with various doses of ionizing radiation using a ^{60}Co irradiator. At specific time intervals after irradiation, the specimens were removed from the organ culture incubator and fixed in formalin for subsequent staining. Specimens were stained similarly to the baseline tissues noted above.

The biopsy specimens were labeled as follows: the format will be HPBx-*xy*.

Where HPBx will indicate the Human Prostate Biopsy Study, *x* represents the patient number, *y* represents the sample identifier (total incubation period, whether or not the sample was irradiated, period of incubation after radiotherapy (RT). Note: All irradiation was performed approximately 24 hours after initial biopsy collection. Total incubation time was the incubation period prior to RT plus the incubation period after RT. All Controls will be matched to this total incubation time, so as to eliminate potential changes that may occur simply due to different incubation periods.

Sample Identifier (y)	Meaning (Total incubation period, RT dose, Incubation Period after RT)
1	Control: Incubation for hours (2-4 hrs), No RT
2	Control: Incubation for 24 hours, No RT
3	24 hours, 10 Gy, 1-2 hours after RT
4	Control: 48 hours, no RT
5	48 hours, 10 Gy, incubated for 24 hours after RT
6	Control: 72 hours, No RT
7	72 hours, 10 Gy, incubated for 48 hours after RT

Example: HPBx 2-5 would mean: Human Prostate Biopsy patient number 2, the specimen was incubated for 24 hours prior to RT, received 10 Gy RT, and then was incubated for an additional 24 hours prior to formalin fixation (Total incubation time = 48 hrs)

Glands were graded as negative (glands with PSA expression), luminal (glands with positive PSA staining of luminal cells but negative basal cell expression), or full thickness (abnormal PSA staining, with all cells showing expression). The type of staining for each gland was determined for each slice and expressed as the mean ± standard deviation. The number of Ki-67 immunoreactive nuclei was expressed as the mean value x 100 ± standard error.

Results

Prostate biopsy cores were obtained from a total of 11 patients. 19 patients were consented but specimens were not collected due to technical difficulties-4, withdrew consent-3, and one had previous radiation. These cores were approximately 5 mm in length and the diameter was analogous to the internal diameter of an 18G needle. We attempted to obtain 3 cores from each patient. Then each core could be

cut in half and there would be enough tissue for 6 treatment/time periods. However, not all specimens were adequate due to sampling errors, tissue damage, presence of frank carcinoma, acute and/or chronic prostatitis, to name a few of the difficulties encountered. As a result there was not always enough tissue for each category listed (Table 1).

Histological evaluation.

The initial experiments were performed to investigate the maintenance of organ architecture and cellular heterogeneity of prostate biopsy cores. Histological evaluation of the biopsy cores revealed maintenance of the normal glandular structure of the organ following slicing (Fig. 1-4), suggesting that the slicing process was not associated with dramatic damage. The architecture of the prostate was maintained in cores incubated for up to 72 hours. Although a gradual loss of luminal cells was observed throughout the culture, the basal cell population was not only maintained in the presence of FBS but also proliferated from days 1-3. Ki-67 staining on the other hand, although present, was inconsistent and not comparable to the degree of staining expected with a high degree of proliferation (Fig. 5). There was no evidence of central tissue necrosis usually associated with inadequate penetration of media into the biopsy core.

Key Research Accomplishments

- Determined the optimal prostate biopsy core size for maintaining biopsy specimens in the dynamic tissue culture system. Dimensions of the cores are analogous to the inner diameter of an 18G needle and approximately 3-4 mm in length.
- Determined the optimal tissue culture media conditions. Keratinocyte serum-free media (KSFM) with 10% (vol./vol.) fetal calf serum. The media also contained antibiotics, 50 units/ml of Gentamicin and 100 units/ml Penicillin G/ Streptomycin, and 0.625 µg/ml Fungizone an antifungal drug. Media was changed every 24 hours.
- Determined that prostate biopsy cores can maintain viability up to 72 hours. Tissue architecture was normal, there were no areas of central necrosis, and basal cell proliferation was maximal at 72 hours of incubation.

Reportable Outcomes

The results of this type of work have been previously reported (Parrish et al, 2002).

Conclusions

We have completed the goals outlined in Aim I. We are now ready to submit a new consent form for the purpose of enrolling new patients with prostate cancer. We believe that the goals of Aim II are attainable and we need to begin enrolling patients right away. The original goal of consenting a total of 25 subjects for Aims I and II will need to be revised. The 11 patients from the retrospective group were to expected to provide adequate samples to complete Aim I and then supply further specimens along with another 14 patients to complete Aim II. The total of 25 patients would allow a correlation coefficient of 0.55 with 80% power.

There are no samples left to begin Aim II and we will quickly resubmit our previously approved consent form with the necessary changes. We will be meeting soon with our biostatisticians to assess our new goals as well as to reevaluate the original power calculations. We have a new radiation oncologist who is interested in collaborating with us.

References

Parrish AR, Sallam K, Nyman DW, Orozco J, Cress AE, Dalkin BL, Nagle RB, and Gandolfi AJ. Culturing precision-cut human prostate slices as an in vitro model of prostate pathobiology. Cell Biology and Toxicology. 18:205-219, 2002.

Dynamic Tissue Culture From Prostate Biopsy Specimens as a Model for Predicting Tumor Radiosensitivity to Ionizing Radiation Treatment

Principal Investigator:
David Nyman, D.O.
Department of Pharmacology and Toxicology
University of Arizona
Post Office Box 210207
Tucson, Arizona 85721
Phone: (520) 626-7317

Co-Principal Investigator:
Shona Dougherty, M.B., Ch.B., Ph.D.
Radiation Oncology Department
Post Office Box 245081
Tucson, Arizona 85724
Phone: (520)-626-6724

Johnathan R. Walker, MD
Assistant Professor of Clinical Surgery
PO Box 245071
Tucson, AZ 85724-5071
Phone: (520) 626-6236

Jay Gandolfi, Ph. D.
Department of Pharmacology and Toxicology
University of Arizona
Post Office Box 210207
Tucson, Arizona 85721
Phone: (520) 626-6696

Raymond Nagle, M.D.
Pathology Department
Post Office Box 245043
Tucson, Arizona 85724
Phone: (520) 626-6100

Medical Monitor
Frederick Ahmann, M.D.
Hematology Oncology
Arizona Cancer Center
1515 N. Campbell Avenue
Tucson, Arizona 85724
Phone: (520) 626-8096

Revised: February 16, 2004

Index

1.0 Introduction

2.0 Objectives

3.0 Patients Selection
3.1 Inclusion Criteria
3.2 Exclusion Criteria
3.3 Patient Recruitment

4.0 Pretreatment Evaluation

5.0 Registration Procedure

6.0 Radiation Therapy

7.0 Biopsy Collection
7.1 Retrospective Study Biopsy Collection

8.0 Risks

9.0 Pathology Review

10.0 Patient Assessments

11.0 Data Collection And Storage

12.0 Study Monitoring

13.0 Serious Adverse Event Reporting

14.0 Statistical Considerations

15.0 Study Personnel

16.0 Protocol and Consent Revisions

17.0 Data Analysis

18.0 References

1.0 Introduction

Prostate carcinoma is the most common malignancy in men, reaching an estimated incidence of 179,300 in 1999.[1] Standard therapy for early stage prostate carcinoma includes surgery, external beam radiation therapy, and more recently prostate brachytherapy. Prostate brachytherapy is a single, outpatient surgical procedure that involves the placement of radioactive seeds into the prostate gland under ultrasound guidance. This allows the opportunity for biopsies to be performed in-patient who will undergo a definitive course of radiation therapy for their diagnosis of prostate carcinoma.

Over the past several years, there have been extensive developments in understanding the mechanism of tumorogenesis for prostate carcinomas. In the literature, it has been demonstrated that NFkB, a transcriptional factor that is activated after various cellular stresses, can be activated after ionizing radiation treatment.[2, 3] The end result of NFkB activation results in signals that promote cell survival, in part by blocking radiation induced apoptosis. Similarly, there is clinical data suggesting that the extent of apoptosis may correlate with tumor aggressiveness and treatment outcome for various types of malignancies.[4-8] In prostate carcinoma, there are data suggesting that poorly differentiated tumors, recurrent tumors,[4] and hormone resistant tumors[4, 5, 7] have a lower apoptotic index, and worse treatment outcome. Similarly, there is data suggesting a direct relationship between the level of apoptosis within a tumor and the overall radiosensitivity of the tumor cells.[8] This suggests that the extent of apoptosis may play a role in determining response to treatment, and that a higher apoptotic index may correlate with a more radiosensitive prostate carcinoma.[8-10] However, majority of these studies have been performed in immortalized prostate carcinoma cell lines, and it is uncertain if the same principles can be directly applied to human patients with prostate carcinoma that demonstrate a heterogeneous malignant cell population with various different histological and immunohistochemical features. The biggest limitation in this area has been the lack of direct prostate carcinoma tissue from each patient prior to initiation of radiation therapy. Although every appropriately staged prostate carcinoma patient will have a tissue diagnosis of malignancy through ultrasound guided biopsies of the prostate gland, often these procedures are done in other institutions where the biopsy specimens are no longer available, and additional tissue is difficult to obtain. Similarly, since all of the biopsy specimens are processed, it is not possible to obtain viable tissue for the purposes of doing *in-vitro* studies as described below. The performance of prostate brachytherapy allows an excellent opportunity to collect biopsy specimens from patients during the prostate implant procedure. Since, as a part of the brachytherapy procedure, patients will be under anesthesia, and needles and radioactive seeds will be placed into the prostate gland under ultrasound guidance, the addition of the biopsies should not result in increased morbidity or inconvenience to the patient. These specimens can be collected through ultrasound guided biopsies during the brachytherapy procedure and processed and stained as appropriate. Similarly, a portion of the specimen can be used to determine if information regarding intrinsic tumor radiosensitivity of each patient's individual prostate carcinoma can be obtained from human biopsy specimens.

Preliminary Data

To date, 11 biopsy cores have been obtained from a total of 14 consented patients. These cores were approximately 5 mm in length and the diameter was analogous to the internal diameter of

an 18G needle. We attempted to obtain 3 cores from each patient.

The initial experiments were performed to investigate the maintenance of organ architecture and cellular heterogeneity of prostate biopsy cores maintained in tissue culture media for 24-72 hrs using techniques developed by Dr. Jay Gandolfi and Dr. Ray Nagle at the Arizona Health Sciences Center.[11,12] Histological evaluation (H&E staining) of the biopsy cores revealed maintenance of the normal glandular structure of the organ following biopsy suggesting that the biopsy process was not associated with dramatic damage. The architecture of the prostate was maintained in cores incubated for up to 72 hours. Although a gradual loss of luminal cells was observed throughout the tissue core, the basal cell population was not only maintained in the culture media but also proliferated from days 1 through 3. Ki-67 staining on the other hand, although present, was inconsistent and not comparable to the degree of staining expected with a high degree of proliferation. There was no evidence of central tissue necrosis usually associated with inadequate penetration of media into the biopsy core.

Key research accomplishments in the first year that were reported to the sponsor (Dept. of Defense) were:

- Determined the optimal prostate biopsy core size for maintaining biopsy specimens in the dynamic tissue culture system. Dimensions of the cores are analogous to the inner diameter of an 18G needle and approximately 3-4 mm in length.

- Determined the optimal tissue culture media conditions. Keratinocyte serum-free media (KSFM) with 10% (vol./vol.) fetal calf serum. The media also contained antibiotics, 50 units/ml of Gentamicin and 100 units/ml Penicillin G/ Streptomycin, and 0.625 μg/ml Fungizone an antifungal drug. Media was changed every 24 hours.

- Determined that prostate biopsy cores can maintain viability up to 72 hours. Tissue architecture was normal, there were no areas of central necrosis, and basal cell proliferation was maximal at 72 hours of incubation.

Planned experiments in the next 18-24 months include the enrollment of 30 new patients. After biopsy cores are obtained, the paired specimens (test and control) will be incubated for 48-72 hours and evaluated for radiation-induced apoptosis. Similarly, cells will be stained to determine the subcellular location of NFkB, and the extent of staining will be quantified. Staining of other regulators of apoptosis, such as p53 and bcl-2, will also be determined. These studies will assess the feasibility of evaluating the intracellular levels of various molecular regulators of apoptosis from individual patients prostate biopsy specimens, and correlating these with known pathologic prognostic factors for prostate cancers treated with radiation therapy. If it is determined to be feasible, than further studies with larger patient numbers can be performed to help determine if a consistent molecular event occurs after irradiation in human prostate carcinoma tissue, and whether such an event could be exploited to improve the likelihood of cure after radiation therapy.

2.0 Objectives

a. Evaluating the feasibility of maintaining prostate carcinoma cells obtained from biopsy specimens in tissue culture for several days to assess the extent of radiation

induced apoptosis and the intrinsic radiosensitivity of these cells to ionizing radiation.

b. Assessment of the intracellular levels of various molecular regulators of apoptosis and cell death in biopsy specimens obtained from patients with prostate carcinoma. The predominant focus of this objective will be on the intracellular and intranuclear levels of transcriptional factor nuclear factor κB (NFκB). However, levels of other important regulators of apoptosis such as p53 and bcl-2 will also be evaluated.

c. Correlate the intracellular and intranuclear levels of transcriptional factor NFκB and actual clinical and pathologic prognostic factors (such as PSA, presence of perineural invasion).

d. Correlate the intracellular and intranuclear levels of transcriptional factor NFκB with the extent of radiation induced apoptosis and the intrinsic radiosensitivity of precision-cut prostate carcinoma cells.

The combinations of these studies will serve to evaluate the feasibility of obtaining biopsy specimens from patients undergoing prostate brachytherapy and using the biopsy specimens to obtain information in regards to both intrinsic radiosensitivity as well as the intracellular levels of various molecular regulators of radiation induced apoptosis.

3.0 Patient Selection

3.1 Inclusion Criteria:

Patients will be eligible for this protocol if they are good candidates for prostate seed implant, willing to undergo prostate seed implant and are at least 18 years of age. Patients must have no evidence of metastases. Patients must be in good medical condition to undergo a prostate seed implant, and willing to sign a protocol specific subject consent form.

3.2 Exclusion Criteria:

Women will not be eligible for this study, since prostate carcinoma only occurs in men. Patients who are not good candidates for prostate seed implants will not be eligible for this study. Patients who have a serious underlying medical condition which would impair the ability of the patient to receive protocol treatment, or dementia or significantly altered mental status that would prohibit the understanding and giving of informed consent would also not be eligible for this protocol.

3.3 Patient Recruitment:

Candidates will be identified by the physician who is performing the patient's initial history and physical evaluation. If the patient is felt to be a good candidate for prostate seed implant at that time, the study option will be discussed with them.

4.0 Pretreatment Evaluation

No additional pretreatment evaluation will be required

5.0 Registration Procedure

5.1 Methods

Informed consents will be obtained by physicians in the Department of Radiation Oncology. Typically patients will be seen in Radiation Oncology within one to two months after their initial diagnosis of prostate cancer has been made. Once a decision to proceed with prostate seed implant has been made as the primary treatment for the patients prostate carcinoma, the evaluating physician will discuss this study with the patient. At that time, the goal of the study, benefits, and potential side effects to the patient will be discussed by the physician. The discussion will occur in the Department of Radiation Oncology Clinic area, with the patient, and any additional person they wish to have with them. Subjects will always have the right to withdraw from protocol at any time. If they withdraw prior to biopsy collection, no biopsy will be collected. If they withdraw after biopsy collection, the patient will be notified that the biopsy specimens will be destroyed.

Once a patient is felt to be eligible for prostate brachytherapy, and has agreed to enroll in this protocol and undergo the prostate biopsies, an informed consent will be obtained. The brachytherapy surgery usually occurs within 2-4 weeks after the patient has signed consent. On the day of the prostate brachytherapy procedure, 3 - 6 transperineal punch biopsy specimens will be obtained under ultrasound guidance. These specimens will then be processed as discussed in section 7.0. Otherwise the brachytherapy procedure will be completed as planned. The patient participation (or desire not to participate) will not in any way alter the treatment (in terms of the type of implant, the dose from the implant, whether or not external beam radiation therapy is used in combination with the implant) that they will receive. In participating in this protocol, the patient understands that there will be no financial benefit to them, and they give up all rights to the biopsy specimen that is collected, and how the specimens are used. The only exception to this would be if a patient withdrew consent from study after their biopsy was collected – those biopsy specimens would be destroyed.

6.0 Radiation Therapy

All patients will receive prostate brachytherapy with or with/out external beam radiation therapy. Accepted standard doses for prostate brachytherapy will be used. At present for brachytherapy alone, the patient will receive a total dose of 145 Gy (for I-125 seeds), or 110 Gy (for Pd-103 seeds) to the prostate gland. If the prostate brachytherapy is combined with external beam radiation therapy, then the patient will receiver 45-50 Gy with external beam radiation therapy followed by 110 Gy (I-125 seeds), or 90 Gy (Pd-103 seeds). However, these doses may change as supported by new literature.

7.0 Biopsy Collection

From three to six punch biopsy specimens will be collected during the prostate brachytherapy procedure. All biopsy specimens will be collected and immediately placed in appropriate solution. The first two biopsy specimens will be processed *en bloc* for thin section slide preparation. Each specimen will be fixed in 10% formalin and then embedded in paraffin. The specimens will be sectioned via microtome and then stained. Stains used will include H&E for

routine pathologic evaluation. Other immunohistochemistry stains will include PSA, a measure of normal tissue secretory activity and Ki-67, a nuclear stain for mitotic or proliferative activity. The predominant emphasis will be on transcriptional factor NFkB, both in terms of its intracellular levels, as well as its intranuclear (active form) levels. However, levels of other significant regulators of apoptosis including p53 and bcl-2 will also be assessed. This data will be used for baseline evaluation of each biopsy.

From the third biopsy specimen, precision-cut slices will be generated and maintained in a dynamic organ culture system for up to 72 hours. The biopsies are placed onto titanium roller culture inserts that are blotted and loaded into glass scintillation vials containing 1.7 ml of media: keratinocyte basal media supplemented with (10% v/v) fetal bovine serum (FBS). Media also contains 50 units/ml of Gentamicin, 100 units/ml Penicillin G/ Streptomycin, and 0.625 µg/ml Fungizone. The scintillation vials are closed with a cap, which has a 2 mm central opening, and placed in the dynamic organ culture incubator that is gassed with 95%O_2:5%CO_2 at a flow rate of 1ml/minute. The incubator temperature is a constant 37°C. The media is changed every 24 hours. The dynamic organ culture incubator is unique in that it rotates the scintillation vials causing the biopsies on the titanium rollers to be alternately submerged and then exposed to the ambient gases. This is different from traditional submersion culture techniques used in recent prostate explant culture and affords much better exchange of nutrients, wastes, and gases.

Twenty-four hours after the biopsy, the specimens will be irradiated with various doses of ionizing radiation using a ^{60}Co irradiator. At specific time intervals after irradiation, the specimens will be removed from the organ culture incubator and fixed in formalin for subsequent staining. Specimens will be stained similarly to the baseline tissues noted above. In addition to H&E, PSA, and Ki-67, biopsy sections will be evaluated for radiation-induced apoptosis, the subcellular location of NFκB, and the extent of staining will be quantified. Non irradiated samples will be used as controls to differentiate radiation induced effects from background effects. The independent variable in these ^{60}Co studies is the dose of irradiation. A preliminary treatment protocol is listed in Section 10.

7.1 Retrospective Study Biopsy Collection

Biopsy specimens were obtained during a retrospective study from 11 patients who had signed informed consent and enrolled in the study (HSC # 00-218). The specimens (1-3 biopsies/patient) were maintained in dynamic tissue culture system for 3 days, irradiated at various times during that period, and then returned to the incubator. Subsequently, the specimens were fixed and sectioned, and no further processing has been performed. The purpose of these specimens was to help develop the techniques that will be utilized, while also providing some preliminary data as to how long, and under what conditions the biopsy specimens could be maintained. These slides will be stained for various markers as described in this study. However, using our labeling nomenclature, these slides can be identified by the time of irradiation, and at the time of incubation. The final role for these slides will be determined, once the optimal time and media conditions for the biopsy specimens has been established under Arm 1.

These retrospective samples are part of the 25 required samples. Since enrollment of 19 subjects in the retrospective study resulted in biopsy specimens from 11 patients, approval is being sought to enroll up to 30 more subjects. Thus, upon approval, enrollment will continue until an

additional 14 biopsy specimens are obtained. Enrollment will cease at that time. Therefore, fewer than 25 subjects may be enrolled in this study.

8.0 Risks

There is minimal risk to the patient for the biopsy collection. Possible risks are infection, bleeding, and discomfort. Patients will be observed closely for any possible risks, and treated appropriately. The same precautions that are utilized for patients undergoing prostate brachytherapy alone will be used for patients be treated under this protocol to minimize the risks. There is the possibility of breach of confidentiality; however, steps have been taken to minimize this risk using a coding system for the biopsy specimens collected.

9.0 Pathology Review

One slide from each patient specimen will be stained with H&E and reviewed by a pathologist to confirm the presence of prostate carcinoma. Similarly, other important prognostic factors such as perineural invasion and extracapsular extension will be assessed. Additionally, two to three slides will be stained for the transcriptional factor NFκB. Both the intracellular and the intranuclear levels of NFκB will be assessed and correlated with other pathologic parameters such as the presence of perineural invasion or the presence of lymphovascular invasion. Also the levels of NFκB will be correlated with the radio-responsiveness of the biopsy tissue using the dynamic organ culture system. Additional slides will be stained for various other regulators (p53 and bcl-2), radiation induced apoptosis, and cell survival. These results will be quantified using various molecular biology techniques to help assess the feasibility of the above procedures. This will permit the collection of preliminary data to help develop a model that may allow treatments to be individualized according to each patient's tumor characteristics.

10.0 Patient Assessments

Patient medical records will be reviewed for information relating to their prostate carcinoma. No additional patient assessments will be required with this protocol.

11.0 Data Collection and Storage

Clinical data will be collected and maintained by Dr. Shona Dougherty. Laboratory data will be collected and maintained by Dr. David Nyman. In oral and written presentations of data, patients will not be identified by name or initials. None of the biopsy specimens will be labeled with the patient's name. Since only one prostate brachytherapy procedure is performed per day, there is no potential for mixing up the specimens. The specimens will be labeled using a code as described below. Each specimen will be labeled with a patient number as described below. In a separate computer file, each patient number will be matched with a patient name. This will allow the specimens to be handled while maintaining patient privacy.

Data will be maintained indefinitely in limited access files with no plans for disposal. Original signed Subject Consent forms as well as study data will be kept in the data manager's office in the Department of Radiation Oncology. All biopsy specimens as well as data generated from the biopsy specimens will be kept in Dr. Nagle's laboratory. All clinical data will be kept in the patient's chart. Only authorized personnel involved in the study, or evaluating the study outcome will have access to the data. Similarly, study monitors, as well as all institutional, state

or federal personal reviewing the study will have access to the data. The data will be locked, and kept indefinitely.

All stained slides will be kept until such time as the study has been completed. At that time it will be decided whether the slides should be kept indefinitely or be destroyed.

The biopsy specimens will be labeled as follows: the format will be HPBx-*xy*.

Where HBPX will indicate the Human Prostate Biopsy Study, *x* will represent the patient number, *y* will represent the sample identifier (Total incubation period, whether or not the sample was irradiated, period of incubation after Radiotherapy (RT). Note: All irradiation will occur approximately 24 hours after initial biopsy collection. Total incubation time will be the incubation period prior to RT plus the incubation period after RT. All Controls will be matched to this total incubation time, so as to eliminate potential changes that may occur simply due to different incubation periods.

Sample Identifier (y)	Meaning (Total incubation period, RT dose, Incubation Period after RT)
1	Control: Incubation for hours (2-4 hrs), No RT
2	Control: Incubation for 24 hours, No RT
3	24 hours, 10 Gy, 1-2 hours after RT
4	Control: 48 hours, no RT
5	48 hours, 10 Gy, incubated for 24 hours after RT
6	Control: 72 hours, No RT
7	72 hours, 10 Gy, incubated for 48 hours after RT

Example: HPBx 2-5 would mean: Human Prostate Biopsy patient number 2, the specimen was incubated for 24 hours prior to RT, received 10 Gy RT, and then was incubated for an additional 24 hours prior to formalin fixation (Total incubation time = 48 hrs)

Each fixed tissue block, as well as stained and unstained slides will be marked with this nomenclature. This will allow for identification of the study and the specimen while maintaining patient privacy.

On a computer file, the patient number will be matched with the patient name so that clinical information can be gathered. Also on this file, all of the different time periods at which biopsy samples were treated will be kept, such that a quick overview of what has been done to date will be possible. Since only 3-6 biopsy specimens will be obtained per patient, it will not be possible to perform assays at each of the above possible time points on specimens obtained from each patient.

12.0 Study Monitoring

A medical monitor will be assigned to the study. This individual will be a qualified physician, other than the Principal Investigator, not associated with this particular protocol, able to provide medical care to research subjects for conditions that may arise during the conduct of the study, and who will monitor the subjects during the conduct of the study. The medical monitor will

review all serious and unexpected adverse events (per ICH definitions) associated with the protocol and provide an unbiased written report of the event within 10 calendar days of the initial report. At a minimum, the medical monitor will comment on the outcomes of the adverse event and relationship of the AE to the protocol procedure. The medical monitor will also indicate whether he concurs with the details of the report provided by the study investigator.

13.0 Serious Adverse Event Reporting

Adverse experiences that are both serious and unexpected will be immediately reported by telephone to the USAMRMC Deputy Chief for Regulatory Compliance and Quality (301-619-2165 (non-duty hours call 301-619-2165 and information sent by facsimile to 301-619-7803). A written report will follow the initial telephone call within 3 working days. The written report will be addressed to the U.S. Army Medical Research and Material Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

14.0 Statistical Considerations

It is anticipated that approximately 30 patients will be enrolled in this study, however only 25 patients will be needed to have biopsy collection done. In the previous study (HSC 00-218) 2 patients withdrew their consent prior to performing the biopsies, and three others ~~have~~did not had biopsies collected due to technical difficulties. Only patients who actually undergo biopsies will be counted in data analysis. Enrollment of 30 patients to this study will allow the collection of enough samples and data to allow correlation to be made between laboratory findings (such as intracellular NFκB levels) and characteristic features of each individual patients tumors (such as pretreatment PSA values, presence of perineural invasion, or presence of lymphovascular invasion). Similarly, the correlation between the intracellular levels of various regulators of apoptosis will be correlated with the extent of radiation induced apoptosis that is detected using the precision-cut tissue sections. Twenty-five subjects will allow detection of a correlation coefficient of 0.55 or greater with 80% power (with a two-sided alpha parameter of 0.05).

15.0 Study Personnel

All clinical and IRB reporting responsibilities will be performed by the Principal Investigator for this study, Dr. David Nyman. Dr. Nyman has experience with the dynamic tissue culture system, and will help determine the ideal media conditions and the incubation duration for the biopsy specimens. Similarly, he will perform/supervise all of the immunohistochemical procedures.

Dr. Dougherty, a Radiation Oncologist, is the Co-Investigator she will be involved with recruiting and consenting potential subjects. Similarly, she will supervise the review of medical records.

Dr. Dougherty will also supervise all of the radiation treatments for the biopsy specimens. Dr Johnathan Walker, Assistant Professor Urological Surgery, will be present during biopsy collection.

Drs. Gandolfi and Nagle will be the mentors for Dr. Nyman. Dr. Gandolfi has extensive experience with the development of the dynamic tissue culture system, and will provide guidance

and recommendation for Dr. Nyman for the duration of the grant. Dr. Nagle has extensive research experience in histopatholgy and immunohistochemistry.

Dr. Frederick Ahmann will be the medical monitor for this study and will review all serious and unexpected adverse events (per ICH definitions) associated with the protocol and provide an unbiased written report of the event within 10 calendar days of the initial report. At a minimum, he will comment on the outcomes of the adverse event and relationship of the AE to the protocol procedure. Dr. Ahmann will indicate whether he concurs with the details of the report provided by the study investigator.

16.0 **Protocol and Consent Revision**

All revisions to either the protocol or consent will not be implemented until approval has been received by the Institutional IRB (Human Subjects Review Board) and the Human Subjects Research Review Board.

17.0 **Data Analysis**

Correlation will be made between laboratory findings (such as intracellular NFκB levels) and characteristic features of each individual patient's tumors (such as pretreatment PSA values). Similarly, the correlation between the intracellular levels of various regulators of apoptosis will be correlated with the extent of radiation induced apoptosis that is detected using the precision-cut tissues sections.

18.0 References

1. Landis et al, CA: A J. Clin.8-31, 1999.
2. Wang, C. Y., et al. TNF-and cancer therapy-induced apoptosis: Potentiation by inhibition of NF-kB. Science. 274, 1996.
3. Van Antrep, D. J. et al. Suppression of TNF-alpha-induced apoptosis by NFkB. Science. 274:787-789, 1996.
4. Koivisto P., Visakorpi T., Rantala I., Isola J. Increased cell proliferation activity and decreased cell death are associated with the emergence of hormone-refractory recurrent prostate cancer Journal of Pathology 183:51-56, 1997.
5. Raffo, A. J., et al. Overexpression of bcl-2 protects prostate cancer cells from apoptosis in vitro and confers resistance to androgen depletion in vivo. Cancer Res. 55:4438-4445, 1995.
6. Sheridan, M. T., et al. A high ratio of apoptosis to proliferation correlates with improved survival after radiotherapy for cervical adenocarcinoma. Int. J. Radiol. Biol. Oncol. Phys. 44:507-512, 1999.
7. Colomber, M. et al. Detection of apoptosis-suppressing oncoprotein bcl-2 in hormone-refractory human prostate cancers. Am. J. Path. 143:390-400, 1993.
8. Rupnow, B. A., et al. Direct evidence that apoptosis enhances tumor response to fractionated radiotherapy. Cancer Res. 58:1779-1784, 1998
9. Dewey WC., Ling CC., Meyn RE. Radiation-induced apoptosis: Relevance to radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. 33:781-796, 1995
10. Fisher, D. E. Apoptosis in cancer therapy: Crossing the threshold. Cell. 78:539-542, 1994.
11. Parrish AR., Gandolfi AJ., Brendel K. Precision-cut tissue slices: applications in pharmacology and toxicology. Life Sci. 57:1887-1901, 1995
12. Catania, J. M., et al. Precision-cut tissue slices from transgenic mice as an in-vitro toxicology system. Presented at the Western Pharmocology Meeting in Tucson, AZ, 2000.
13. Condon MS., Kaplan LA., Crivello JF., Horton L., Bosland MC., Multiple pathways of prostate carcinogenesis analyzed by using cultured cells isolated from rats treated with N-methyl-N-nitrosourea and testosterone. Molecular Carcinogenesis. 25 (30): 179-86, 1999 Jul.
14. Magi-Galluzzi C., Murphy M., Cangi MG., Loda M. Proliferation, apoptosis and cell cycle regulation in prostatic carcinogenesis. [Review] [73 refs] Analytical & Quantitative Cytology & Histology 20(5): 343-50, 1998 Oct.
15.MacKay, R. et al. Potential clinical impact of normal-tissue intrinsic radiosensitivity testing [letter] Radiotherapy & Oncology 36 (2):215-6, 1998 Feb.

College of Pharmacy
Department of Pharmacology and Toxicology
P.O. Box 210207

THE UNIVERSITY OF
ARIZONA®
HEALTH SCIENCES CENTER

Tucson, AZ 85721-0207
(520) 626-2823
FAX: (520) 626-2466

March 11, 2004

David Johnson, MD
Human Subjects Committee
1350 N. Vine
PO Box 24-5137
Tucson, AZ 85724

Periodic Review: DYNAMIC TISSUE CULTURE FROM PROSTATE BIOPSY SPECIMENS AS A MODEL FOR PREDICTING TUMOR RADIOSENSITIVITY TO IONIZING RADIATION TREATMENT
(HSC #00-218)

Dear Dr. Johnson:

This report follows a periodic review form sent to IRB on November 13, 2003. This study was closed at that time. No patients have been enrolled since that time. There have been no studies performed nor data generated since that time.

We have completed Aim I of the study and have submitted a new IRB application to open a new study (submitted March 5, 2004). The name of the project is the same. We intend to begin consenting and enrolling new patients for further studies (Aims II and III) in this research project. As noted in the DOD report a total of 25 patients will be required to provide the adequate numbers to achieve a correlation coefficient of 0.55 with 80% power. This may require consenting more than 25 patients (we expect to consent 35 patients) in the next 18 months.

Dr., Shona Dougherty, Assistant Professor of Radiation Oncology, has agreed to serve as Co-PI and will be consenting all patients. She anticipates consenting 25 patients within six months. So there will be adequate patient numbers to fulfill this requirement and these patients will be receiving brachytherapy so there is no increased risk by taking 3 biopsy cores.

No adverse events have been reported since the last update.

A review of the recent literature has not produced any new information regarding risks to patients for this procedure.

Sincerely,

David W. Nyman, D.O., M.S.
Clinical Asst. Professor Pharmaceutical Sciences
Dept. of Pharmacology/Toxicology
University of Arizona

College of Pharmacy
Department of Pharmacology and Toxicology
P.O. Box 210207

THE UNIVERSITY OF
ARIZONA®
HEALTH SCIENCES CENTER

Tucson, AZ 85721-0207
(520) 626-2823
FAX: (520) 626-2466

April 26, 2004

U.S. Army Medical Research and Materiel Command
(MCMR-RMI-S)
504 Scott Street
Fort Detrick, Maryland 21702-5012

Re: Annual Report DAMD17-02-1-0223

Dear Ms. Pawlus:

We are currently awaiting the IRB approval for our latest submission. We have asked for and expect to receive approval to enroll as many as 30 new subjects into our protocol: Dynamic Tissue Culture From Prostate Biopsy Specimens as a Model for Predicting Tumor Radiosensitivity to Ionizing Radiation Treatment.

We have already submitted the project for approval but I have a deadline of April 30, 2004 to contact you. I anticipate sending you the approved report probably the first week of May 2004. We have received approval for the protocol but just have a couple minor areas to smooth out in the consent.

Regards,

David W. Nyman, D.O., M.S.
Clinical Assistant Professor Pharmaceutical Sciences
Dept. of Pharmacology/Toxicology
University of Arizona
1703 E. Mabel
Tucson, AZ 85721-0207