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PRINCIPAL INVESTIGATOR: Alan Pollack, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Texas
M.D. Anderson Cancer Center
Houston, Texas 77030

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13. ABSTRACT (<i>Maximum 200 Words</i>) The investigation pursued here involved the characterization of the molecular changes induced by androgen deprivation (AD) and the potential for these changes to result in a supra-additive interaction with radiation (RT). Molecular markers were measured in human tissue samples, as well as LNCaP cells grown in vitro under different conditions. Another project that was to develop out of this work, but which ended up being accelerated because of promising results, was the radiosensitizing effect of gene therapy (GT) using adenoviral-p53 (Ad5-p53). The results were novel in several ways. First, we established that bcl-2 and bax are independent correlates of biochemical failure for patients treated with radiation alone. These biomarkers show promise for future studies in patients treated with AD+RT. Second, AD was conclusively found not to sensitize hormone sensitive prostate cancer cells to RT, using the LNCaP model. These data have significant clinical implications. Third, the expression of E2F1 and MDM2 in response to AD+RT mirrored the apoptotic response, suggesting that these genes are potential targets for gene therapy strategies. Finally, Ad5-p53 gene therapy caused pronounced sensitization of prostate cancer cells to RT in vitro and in vivo, which lead to a Phase II randomized clinical trial that will be activated in the next two months.

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Phase I Final Progress Report
The Molecular Mechanism of the Supra-additive Apoptotic Response of Prostate Cancer
To Androgen Ablation and Radiotherapy

INTRODUCTION

The most common treatment for prostate cancer is androgen deprivation (AD). Despite the nearly 60 years experience with this approach, how best to use AD and the molecular mechanisms involved are poorly understood. In particular, the combination of AD with radiation (RT) has become standard of care for high risk locoregional prostate cancer, yet, favorable interaction has not clearly been established. Most of the work surrounding the issue of the interaction has come out of our laboratory and because of funding from the Department of Defense there are now data indicating that the interaction is less than previously believed.

There are three main aspects to the work that was done. First, an in vitro model system was developed that paralleled the results observed in vivo using the R3327-G Dunning model (Pollack et al, 1997,1999; Lim Joon et al, 1997). Establishing this system and ensuring that it was representative took longer than anticipated, partly because the results were a departure from what was anticipated. Cultured LNCaP cells when exposed to AD developed a new cell kinetic equilibrium at 3 d wherein cell gain and cell loss were approximately equal and when RT was administered, supra-additive apoptosis was observed. Once these key features of the in vivo model were confirmed, a series of clonogenic assays were done to determine whether AD sensitized cells to RT. This central question was essential to further investigations of the molecular mechanisms surrounding the interaction of AD+RT. After nearly a year of repeating clonogenic assays under various conditions, we concluded that at least in this model system, radiosensitization by AD did not occur Pollack et al (2001, Submitted). Once this was established the second main component of the proposed work, the molecular studies, went forward. Originally, p53, bcl2, and bax were selected as prime candidates for investigation. The biomarkers that were subsequently studied included many more important proteins in the apoptotic pathway. The results from the Western analyses are intriguing in that two, namely MDM2 and E2F1, stood out as correlating with the cell kinetic and apoptotic changes induced by the growth conditions and treatments examined. These data are described below.

The third area that was central to the proposed study, was the measurement of biomarkers in pretreatment, archival, diagnostic tumor material and in tissue exposed to AD for different periods of time. The hypothesis was that such measurements would then be correlated with the in vitro biomarker analyses. Two prostate cancer patient treatment populations were available. Diagnostic material was available from patients treated with RT alone and post-AD tissue was available from patients that had undergone neoadjuvant AD prior to radical prostatectomy. Considerable progress was made in the former group, establishing that Ki-67, bcl-2, and bax were significant and independent correlates of biochemical failure after external beam radiotherapy. The neoadjuvant AD treated prostatectomy group was not completed (Khoo et al, 1999; Cowen et al, 2001).

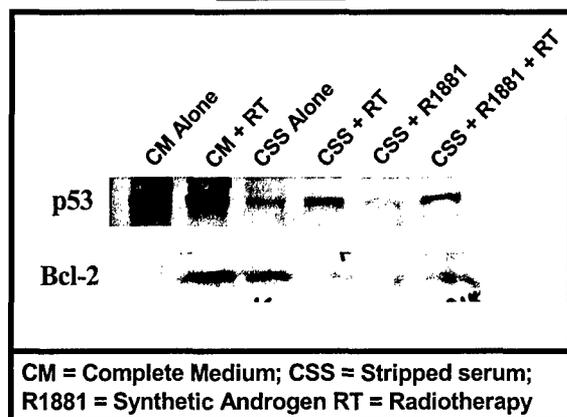
The fourth avenue of research that was pursued with greater enthusiasm than initially predicted was the use of gene therapy to sensitize prostate cancer cells to radiation. The vector that was initially evaluated for radiosensitizing actions was adenoviral-p53 (Ad5-p53). In vitro and in vivo studies demonstrated pronounced radiosensitization by Ad5-p53. As a consequence, the combination of Ad5-p53 plus RT has been applied to the treatment of locally recurrent prostate cancer patients in a clinical trial. Two other adenoviral vectors, namely Ad5-E2F1 and Ad5-C-CAM1, have shown promise as radiosensitizers and are under investigation.

BODY

Task 1. To measure alterations in the expression of p53, bax, and bcl-2 proteins induced by androgen ablation alone.

LNCaP Model: A main objective of the proposal was to use an in vitro model system to determine the molecular mechanisms responsible for the supra-additive apoptotic response observed in Dunning rat prostate tumors grown in vivo with the combination of AD+RT (Pollack et al, 1997, 1999; Lim Joon et al, 1997). The in vitro model involved LNCaP cells cultured in either complete medium (CM), charcoal-

Figure 1



stripped serum (CSS) medium to remove androgens, and CSS+R1881 (synthetic replacement androgen medium). There were inconsistent results for nearly a year with this model, and although radiosensitization was occasionally observed in clonogenic assays, in the majority of experiments radiosensitization was not observed (Pollack et al, 2001, Submitted; See Appendix). The lack of radiosensitization by clonogenic assay was accompanied by a supra-additive apoptotic response, as was described in vivo using the R3327-G Dunning model (Lim Joon et al, 1997; Pollack A et al, 1999.). These findings have clinical implications, in that the main mechanisms behind the improved outcome of patients treated with AD+RT appear to be additive cell killing and/or tumor growth delay that

persists after androgen levels return to normal.

Table 1. Western blot analysis of LNCaP cells grown in vitro. Ratio of band densities.

Group	N	CM+RT/CM	AD+RT/AD	AD+R1881+RT/AD+R1881
p53	4	3.9	2.3	2.9
p21	3	3.0	8.0	7.1
Bcl2	4	0.9	1.1	0.6
Bax	3	0.5	0.8	1.1
MDM2	1	4.3	11.2	5.3
E2F	1	0.8	1.7	0.6
pRb	1	1.0	1.1	1.1

Protein loading on the gels was standardized. N = number of analyses.

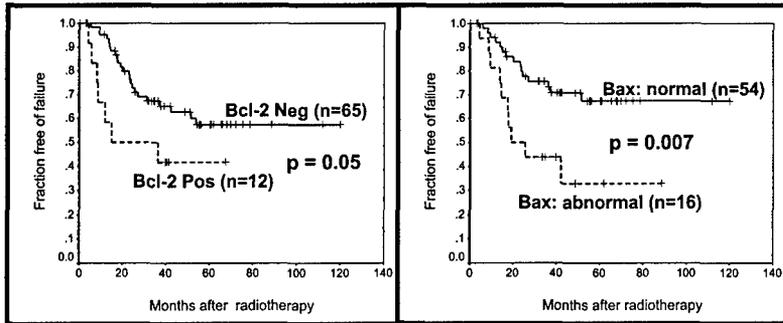
Once the problems with the model had been overcome, Western blot analyses were initiated. The Western blot experiments were structured such that some of the key questions from Tasks 1 and 3 were analyzed together. As shown in Figure 1, the expression of each marker protein was simultaneously analyzed after growth in CM, CM+RT (5 Gy), CSS Alone, CSS+RT, CSS+R1881, and CSS+R1881+RT. The cells were cultured for 3 d in CSS (when done) and analyses were performed 3 hr after RT (when done). Table 1 displays the quantification of band density under these conditions. The results showed that p53, p21 and MDM2 were upregulated in cells treated with RT when grown in CM (**Table 1**). In contrast, Bax and E2F1 expression were depressed and bcl2 and pRb were unchanged. Furthermore, E2F1 and MDM2 levels were substantially altered by AD + RT in an androgen dependent manner; replacement of androgen (AD+R1881+RT) reversed the increase in expression observed with AD+RT. The pattern correlated with the apoptotic response, suggesting that these key proteins may be worth targeting in future studies. The data are still being collected. A minimum of four gels of each marker protein will be analyzed and statistical comparisons generated. If the data on E2F1 and MDM2 hold up and are significant, the effect of antisense treatment on apoptosis and clonogenicity will be investigated.

Human tumor tissue immunohistochemistry.

The other goal of this task was to investigate the prognostic value of pretreatment p53, bcl-2 and bax staining and to compare these levels to those from a cohort of patients that were treated with short course neoadjuvant AD prior to radical prostatectomy. The human tissue findings on the effects of AD on molecular marker levels would then be related to the changes seen in the LNCaP model. Pretreatment prostate biopsy tissue from 106 patients that received external beam radiotherapy at M.D. Anderson Cancer Center between 1987 and 1993 has been analyzed for Ki-67, bcl-2, bax, and bcl-x. One paper (Khoo et al, 1999) compared the predictive value of DNA-ploidy and Ki-67 labeling index, indicating that Ki-67 was more prognostic of patient outcome. The cohort used in this report consisted of 42 patients that underwent transurethral resection

of the prostate prior to treatment, and therefore, had sufficient material for both assays. An extension of this work using the entire group of 106 patients (Cowen et al, 2000) and shows that Ki-67 is independent of

Figure 1. Relationships of bcl-2 and bax to freedom from biochemical failure after RT



pretreatment PSA, Gleason score, and stage as a correlate of biochemical failure. Immunohistochemical analyses of bcl-2, bax, and bclx have been completed and a paper is in preparation. Bcl-2 overexpression, and abnormal bax expression (overexpression or loss of expression) were associated with increased progression after radiotherapy, independent of clinical parameters (Figure 1). The immunohistochemical staining of tissue for bcl-2, bax, and bcl-x from patients that have been treated with androgen ablation prior to radical prostatectomy is now under way. Staining for p53 in both patient cohorts will also be done, although in our experience,

abnormal expression of p53 infrequent in the majority of the clinically localized prostate cancer patients that are treated with radiotherapy.

There were two other clinical projects that are tied to the grant. Patients treated with external beam radiotherapy alone (Pollack et al, 2000a,b) and androgen ablation alone (Kelly et al, 2000) were studied for dose-response and the prognostic value of pretreatment serum testosterone levels, respectively. Since the grant dealt with the development of novel treatment approaches, these projects are relevant. The successful completion of these projects was due in part to the Department of Defense Award and acknowledgement to this effect was given in the publications.

Task 2. To assess whether the mechanism of the supra-additive apoptotic response to androgen ablation plus single fraction radiation and the lack of this response with high single fraction or fractionated doses, is related to changes in p53, bax, or bcl-2 expression.

As described above, Tasks 1 and 2 were addressed simultaneously, in part, in the Western blots in **Table 1**. The experiments examined the molecular changes induced by AD plus single fraction radiation. The results with E2F1 and MDM2 were encouraging and will be pursued further in multifraction experiments.

Task 3. To optimally integrate androgen ablation and radiation based on the molecular marker data obtained and to further enhance apoptosis to this regimen using gene therapy

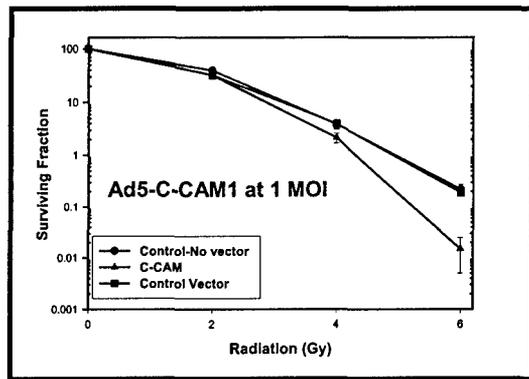
The original intent of Task 3 was to use information from Tasks 1 and 2 to formulate a gene therapy concept. However, during the year when there were inconsistencies in the in vitro LNCaP model (Aim 1), the development of gene therapy strategies to sensitize cells to RT were aggressively pursued ahead of schedule. Colon cancer studies using adenoviral-p53 (Ad5-p53) demonstrated that p53 transgene expression sensitized cells to RT (Spitz et al, 1996). These findings led us to test Ad5-p53 in the p53^{wildtype} LNCaP and p53^{null} PC3 cell lines. In vitro clonogenic survival and apoptosis experiments confirmed that p53 transgene expression sensitized both cell lines to RT (Colletier et al, 2000). In vivo studies were then performed using LNCaP cells grown orthotopically in the prostate and PC3 cells grown s.c. in the leg of nude mice. The results, using serum PSA and tumor volume measurements, established the potent radiosensitization activity of Ad5-p53 in vivo (Cowen et al, 2000). As a consequence, a randomized Phase II clinical trial of RT alone versus Ad5-p53 plus RT for patients with local recurrence after external beam radiotherapy for prostate cancer has been devised (See Appendix). The formulation of this unique clinical trial is a direct result of the funding received from the Department of Defense for preclinical studies. The trial is now under review by the FDA and activation is anticipated in 1-2 months.

There are two other gene therapy projects that have been initiated using adenoviral vectors. The genes under investigation are E2F1, a transcription factor involved in the apoptotic pathway, and C-CAM1, a cell adhesion molecule that appears to have antiangiogenic properties. Ad5-E2F1 was selected because overexpression of E2F1 promotes apoptosis (Hunt et al, 1997) and, as such, may work to preferentially shuttle cells down this pathway after RT, as opposed to the alternate pathway of cell cycle arrest and DNA damage repair. Preliminary in vitro clonogenic and apoptotic data (Salem et al, 1999) demonstrate that Ad5-p53 is as strong a radiosensitizer as Ad5-p53. These findings are now being put together for publication.

The cell adhesion molecule C-CAM1 has been shown by Lin and colleagues (Lin et al, 1999; Luo et al, 1999) to be a potent suppressor of prostate cancer. C-CAM1 expression is regulated by androgen (Hsieh & Lin, 1994) and is downregulated in prostate neoplasms, as compared to normal prostate tissue (Kleinerman et al, 1995). Transfection of C-CAM1 into PC3 prostate tumor cells reduces tumorigenicity and Ad5-C-CAM1 treatment reduces prostate tumor growth.

An Ad5-C-CAM1 vector has been developed and shows similar activity (Lin et al, 1999). The mechanism of the inhibition appears to be related to an effect on host stromal-tumor cell interaction, at least in part to antiangiogenic effects. Preliminary studies of Ad5-C-CAM1 in vitro have shown radiosensitizing activity at high (6 Gy) single fraction doses (Figure 2). The hypothesis is that since C-CAM1 acts through host-stromal-tumor cell interactions, a greater anti-tumor effect will be observed in vivo. In vivo testing has begun.

Figure 2. LNCaP Clonogenic Survival: Ad5-C-CAM1+RT



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In summary, each of the three gene therapy vectors under investigation has shown promise, favorably interacting with RT to promote supra-additive cell killing.

KEY RESEARCH ACCOMPLISHMENTS

1. *Development of the LNCaP in vitro model.* The in vitro LNCaP model mimics other in vivo models in terms of the cell kinetic and apoptotic responses to AD±RT. This development has facilitated our investigation of the molecular changes induced by these treatments. To our knowledge there are no other laboratories actively working on the mechanisms behind the interaction of AD and RT, even though this combination is considered standard of care for high risk locoregional prostate cancer.
2. *Lack of radiosensitization of AD.* A paper has been submitted on these unique findings that the main mechanisms of action of AD+RT are additive cell killing and prolonged tumor growth delay, even after androgen replacement. The often hypothesized radiosensitizing action of AD was shown not to occur in the LNCaP model, which is the most relevant human prostate model available. This study sets the stage for further molecular and antiangiogenesis studies to determine the mechanisms involved.
3. *E2F1 and MDM2 as molecular correlates of the apoptotic response of LNCaP cells to AD+RT.* After testing the changes in expression of a number of key proteins in the apoptotic pathway, the pattern observed in E2F1 and MDM2 levels was parallel to the apoptotic responses under the same conditions. The potential significance is that the manipulation of E2F1 and MDM2 in the setting of AD+RT may further enhance apoptosis and overall cell death. Since we already are working with the Ad5-E2F1 vector, such experiments are underway. A similar approach with MDM2, which may be the more promising of the two, will also be undertaken, funding permitting.
4. *Ki-67, Bcl-2, and Bax as independent biomarkers biochemical failure after RT.* These biomarkers were found to be independent of pretreatment PSA, Gleason score, stage, and RT dose in predicting biochemical failure after RT. These findings have led to further immunohistochemical analyses in a cohort of patients treated in RTOG protocol 86-10 (Pilepich et al, 1995) with RT±AD. The staining of Ki-67 and bcl-2

have been completed and a preliminary data analysis has been performed on Ki-67. The goal is to use biomarkers such as these to stratify patients in future national randomized protocols.

5. *Radiosensitization by Ad5-p53, Ad5-E2F1, and Ad5-C-CAM1.* The results with Ad5-p53 have led to a Phase II randomized trial that is 1-2 months from activation. The findings with E2F1 are at least as significant. The Ad5-C-CAM1 vector tests a novel concept for radiosensitization. These translational studies set the stage for future clinical trials that will define the role of GT+RT for prostate cancer.

REPORTABLE OUTCOMES

Manuscripts and Abstracts

- 1) Khoo VS, Pollack A, Cowen D, Lim Joon D, Patel N, Terry NHA, Zagars GK, von Eschenbach AC, Meistrich ML, Troncoso P. Relationship of Ki-67 labeling index to DNA-ploidy, S-phase fraction, and outcome in prostate cancer treated with radiotherapy. *Prostate* 41:166-172, 1999.
- 2) Pollack A, Ashoori F, Sikes C, Lim Joon D, von Eschenbach AC, Zagars GK, Meistrich ML: The early supra-additive apoptotic response of R3327-G prostate tumors to androgen ablation plus radiation is not sustained with multiple fractions. *Int J Radiat Oncol Biol Phys* 46:153-158, 1999.
- 3) Cowen D, Salem N, Ashoori F, Meyn R, Meistrich ML, Pollack A. Prostate cancer radiosensitization in vivo with adenovirus-mediated p53 gene therapy. *Int. J. Radiat. Oncol. Biol. Phys.* 45(Suppl 3):184-185, 1999.
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- 7) Pollack A, Smith LG, von Eschenbach AC. External beam radiotherapy dose-response characteristics of 1127 men with prostate cancer treated in the PSA era. *Int. J. Radiat. Oncol. Biol. Phys.* 48:507-512, 2000.
- 8) Pollack A, Zagars GK, Smith LG, Lee JJ, von Eschenbach A, Antolak J, Starkschall G, Rosen I. Preliminary results of a randomized radiotherapy dose escalation study comparing 70 Gy to 78 Gy for prostate cancer. *J. Clin. Oncol.* 18:3904-3911, 2000.
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- 10) Pollack A, Salem N, Ashoori F, Hachem P, Sangha M, von Eschenbach AC, Meistrich ML. Lack of prostate cancer radiosensitization with androgen deprivation. (Submitted, 2001).

Clinical Translational Research

Principal Investigator: A. Pollack

Title: "A phase II randomized study of adenovirus-p53 plus radioactive seed implant versus seed implant alone for PSA relapse after external beam radiotherapy". This protocol is a direct extension of the laboratory data and has been approved at M.D. Anderson Cancer Center.

CONCLUSIONS

Two treatment strategies with the goal of sensitizing prostate cancer cells to radiation were investigated. One strategy, combining AD with RT, proved not to result in overall cell killing that was supra-additive, while the other strategy, combining GT with RT, proved to be strongly supra-additive. Despite the lack of radiosensitization from AD, there appear to be important additive effects, which may work to prolong prostate cancer patient survival. Molecular studies of the expression of a number of key proteins in the apoptotic pathway revealed that the pattern of E2F1 and MDM2 expression mirrored the apoptotic response

of LNCaP cells to AD alone, RT alone, AD+RT, and reversal of AD using the synthetic androgen R1881. These findings suggest that the E2F1 and MDM2 genes are potential targets for enhancing the apoptotic response of androgen sensitive prostate cancer cells to RT. Indeed, Ad5-E2F1 is a potent radiosensitizer.

The significance of the results lies in two areas. First, the mechanisms governing the response of prostate cancer cells to AD+RT are much better understood, although much further work in this area is needed. We are unaware of any other group devoting attention to this issue. Second, the GT+RT laboratory data has translated into a clinical trial that has the potential to dramatically alter salvage therapy of locally recurrent prostate cancer. We are clearly at the cusp of applying GT in the clinic, and the Ad5-p53, Ad5-E2F1, and Ad5-C-CAM1 vectors show promise. The ultimate reward is to see this effort applied clinically and the Ad5-p53 + RT trial will be open in the next two weeks.

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APPENDICES

Relationship of Ki-67 Labeling Index to DNA-Ploidy, S-Phase Fraction, and Outcome in Prostate Cancer Treated With Radiotherapy

Vincent S. Khoo,¹ Alan Pollack,^{1*} Didier Cowen,¹ Daryl Lim Joon,²
Nalini Patel,² Nicholas H.A. Terry,² Gunar K. Zagars,¹
Andrew C. von Eschenbach,³ Marvin L. Meistrich,² and Patricia Troncoso⁴

¹Department of Clinical Radiation Oncology, U.T. M.D. Anderson Cancer Center,
Houston, Texas

²Department of Experimental Oncology, U.T. M.D. Anderson Cancer Center,
Houston, Texas

³Department of Urology, U.T. M.D. Anderson Cancer Center, Houston, Texas

⁴Department of Pathology, U.T. M.D. Anderson Cancer Center, Houston, Texas

BACKGROUND. Our purpose was to evaluate the relationship of Ki-67 labeling index (Ki67-LI) to deoxyribonucleic acid (DNA) ploidy, S phase fraction (SPF), other clinical prognostic factors, and clinical outcome for patients with prostate cancer treated by external beam radiotherapy.

METHODS. Tissue was retrieved from 42 patients who underwent transurethral resection of the prostate before treatment with external beam radiotherapy between 1987-1993. DNA histogram profiles were classified as diploid (diploid + near-diploid) and nondiploid (tetraploid + aneuploid). Immunohistochemical staining of Ki-67 by the MIB-1 monoclonal antibody was used to calculate Ki67-LI. Median patient follow-up was 62 months. Treatment failure was defined as two consecutive rises in serum prostate-specific antigen (PSA) or clinical evidence of disease recurrence.

RESULTS. The mean and median Ki67-LIs were 3.1 and 2.4, respectively (range, 0-12.4). Mean Ki67-LI values were significantly associated with higher stage, Gleason score, and pretreatment PSA. Nondiploid tumors had significantly higher Ki67-LIs, as did patients who failed radiotherapy over the follow-up period. SPF was not significantly correlated with Ki67-LI. As a categorical variable, the most significant relationships were seen when Ki67-LI was subdivided into thirds around the median (Ki67-LI \leq 1.5%, Ki67-LI >1.5-3.5%, and Ki67-

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Current address of Vincent S. Khoo is the Academic Unit of Radiotherapy, Royal Marsden Hospital, Downs Road, Sutton, Surrey SM25PT, UK.

Current address of Daryl Lim Joon is the Peter MacCallum Cancer Institute, St. Andrews Place, East Melbourne, Victoria 3002, Australia.

*Correspondence to: Alan Pollack, M.D., Ph.D., Department of Radiation Oncology (Box 97), U.T. M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

E-mail: APollack@notes.mdacc.tmc.edu

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LI >3.5%). This trichotomous variable correlated significantly with pretreatment PSA ($P = 0.0008$), tumor stage ($P = 0.016$), Gleason score ($P = 0.024$), and treatment failure ($P = 0.0015$), but not with DNA-ploidy ($P = 0.15$). In actuarial univariate analyses, Ki67-LI appeared to be a more significant predictor of patient outcome ($P = 0.003$) than DNA-ploidy ($P = 0.035$).

CONCLUSIONS. The Ki67-LI correlated with known prognostic factors such as pretreatment PSA, tumor stage, and Gleason score, and was also weakly related to DNA-ploidy. In comparison to DNA-ploidy, Ki67 LI seems to be a better correlate of treatment outcome. *Prostate* 41:166–172, 1999. © 1999 Wiley-Liss, Inc.

KEY WORDS: DNA-ploidy; Ki-67; MIB-1; prostate cancer; prostate-specific antigen; radiotherapy

INTRODUCTION

The deoxyribonucleic acid (DNA) content of prostate tumors has repeatedly been shown to be predictive of disease outcome [1–15]. In our experience [16–18], DNA-ploidy is an independent correlate of biochemical and/or clinical failure after radiotherapy for clinically localized prostate cancer. The potential of DNA-ploidy for enhancing the prognostic classification of prostate cancer is evident from these studies; however, the application of such measurements clinically has been limited. Flow cytometry and image analysis, the two most common methods for quantifying DNA content, are technically demanding methods. The classification of histograms into diploid, tetraploid, and aneuploid is highly variable between investigators and not entirely objective. Some improvement in the resolution of overlapping cell populations is obtained by analyzing DNA in combination with other parameters, such as nuclear protein [17], but this adds complexity to an assay already difficult to standardize.

Immunohistochemical staining of the proliferation marker, Ki-67, has been shown to reasonably approximate the growth fraction in prostate cancers and other malignancies [19–22]. In contrast, DNA content histograms are strictly a freeze-frame of the proportion of cells distributed about the cell cycle phases. Although such histograms provide an approximation of the fraction of cells in S phase (SPF), the Ki-67 labeling index (Ki67-LI) is a more functional estimate of proliferation. The relationships between Ki67-LI and the DNA content parameters of DNA-ploidy and SPF are poorly documented for prostate cancer [23–25]. In addition, a number of reports indicate that Ki67-LI is significantly related to prostate cancer patient outcome after radical prostatectomy or androgen ablation therapy [26–32]. Preliminary results in radiotherapy-treated patients are also encouraging [33]. The purpose of this report was to explore the correlation of DNA content parameters and Ki67-LI, and to determine the relative predictive value of these factors for the outcome of patients treated with radiotherapy.

MATERIALS AND METHODS

Patient Characteristics

Sections from transurethral resection of the prostate (TURP) specimens were used for this study because the tissue requirements for MIB-1 immunohistochemical staining and DNA-ploidy by flow cytometry were beyond those of most needle biopsy specimens. There were 151 patients with prostate cancer diagnosed from TURP who were referred to the M.D. Anderson Cancer Center (MDACC) between 1987–1993. Paraffin-embedded prostatic sections were obtained from 42 of these patients. All patients were treated with definitive radiotherapy only; no patient received neoadjuvant or adjuvant androgen ablation, or underwent radical prostate surgery or surgical lymph node dissection. The workup of patients treated with radiotherapy at MDACC was described previously [34].

Pretreatment serum prostate-specific antigen (PSA) levels were determined in all patients. The median and mean pretreatment PSAs were 4.1 and 8.8 ng/ml, respectively (range, 0.3–92 ng/ml). The median and mean age was 68 years (range, 56–79 years). The median follow-up was 62 months (range, 19–121 months). The clinical stages for the study population were: stage T1 in 27 patients (64%); stage T2 in 8 (19%); stage T3 in 6 (14%); and stage T4 in 1 (2%). The Gleason scores were: 5 in 6 patients (14%); 6 in 15 (36%); 7 in 13 (31%); 8 in 3 (7%); 9 in 4 (10%); and 10 in 1 (2%).

The median external beam radiotherapy dose was 64 Gy, with a mean of 65 Gy and a range of 60–78 Gy. Radiotherapy was delivered via a four-field box with 18 MV photons, using a shrinking field technique in all but one patient, who received a conformal six-field boost after 46 Gy to a total dose of 78 Gy [34]. The dose was specified to the isocenter at 2 Gy per day. After the completion of radiotherapy, patients were followed at 3–6-month intervals with history, clinical examination, and repeat serum PSA for 2 years and then every 6–12 months thereafter.

Biochemical failure (a rising PSA profile) was defined as two or more consecutive rising PSA values

TABLE I. Percent Ki-67 Staining by Various Potential Prognostic Factors

Grouping	N	% Ki-67		P*
		Mean	± SE	
All patients	42	3.1	± 0.4	
Stage				
T1/T2	35	2.4	± 0.3	0.003
T3/T4	7	6.7	± 1.4	
Gleason score				
2-6	21	2.0	± 0.3	0.017
7-10	21	4.2	± 0.7	
Pretreatment PSA				
≤10	33	2.2	± 0.3	0.0003
>10	9	6.4	± 1.0	
DNA-ploidy				
Diploid	27	2.5	± 0.4	0.04
Nondiploid	11	4.7	± 1.1	
Percent S-phase				
≤2.5	14	2.9	± 0.8	0.53
>2.5	15	3.2	± 0.7	
Treatment failure				
No	29	2.3	± 0.4	0.0009
Yes	13	4.9	± 0.7	

*Mann-Whitney test; SE, standard error.

higher mean Ki67-LI values were seen with stage T3/T4 disease, Gleason score ≥ 7 , pretreatment PSA >10 , or nondiploidy. No association was seen between SPF (stratified by the median value) and Ki67-LI. Treatment failure correlated with higher Ki67-LIs.

As a categorical dichotomous variable, stratified around the median value, Ki67-LI was a correlate of palpable stage, Gleason score, and pretreatment PSA (Table II). A weaker, borderline-significant association was found with DNA-ploidy. With the exception of DNA-ploidy, these correlations were more significant when the patients were divided into thirds, based on Ki67-LI as a trichotomous variable (Table III). High Ki67-LIs above 3.5% were seen in significantly more patients with T3/T4 disease, Gleason scores ≥ 7 , and pretreatment PSAs >10 . Of the patients who failed biochemically, 62% had a Ki67-LI $>3.5\%$.

The relationship of Ki67-LI with actuarial biochemical failure is shown in Figure 1. Ki67-LI predicted failure when used as either a dichotomous or trichotomous variable. The most significant correlation was seen with the latter (Table IV), in which no failures were evident at 4 years if the Ki67-LI was $\leq 1.5\%$, and 67% failed if the Ki67-LI was $>3.5\%$. Only pretreatment PSA was a more significant determinant of failure. When Cox proportional hazards regression was performed, the only independent correlate of failure was pretreatment PSA.

TABLE II. Distribution of Patients by Ki-67 Staining as a Dichotomous Variable

Grouping	Percent (n) patients by Ki-67 ^a		P*
	≤2.4	>2.4	
Stage			
T1/T2	54 (19)	46 (16)	0.05
T3/T4	14 (1)	86 (6)	
Gleason score			
2-6	67 (14)	33 (7)	0.01
7-10	29 (6)	71 (15)	
Pretreatment PSA			
≤10	61 (20)	39 (13)	0.001
>10	0 (0)	100 (9)	
DNA-ploidy			
Diploid	59 (16)	41 (11)	0.07
Nondiploid	27 (3)	73 (8)	
Percent S-phase			
≤2.5	57 (8)	43 (6)	0.57
>2.5	47 (7)	53 (8)	
Treatment failure			
No	62 (18)	38 (11)	0.005
Yes	15 (2)	85 (11)	

^aPatients were stratified by %Ki-67 staining of $\leq 2.4\%$ and $>2.4\%$.

^bChi-square test.

TABLE III. Distribution of Patients Stratified by Ki-67 Staining as a Trichotomous Variable

Grouping	Percent (n) patients by Ki-67 ^a			P*
	≤1.5	>1.5-3.5	>3.5	
All				
Stage				
T1/T2	37 (13)	43 (15)	20 (7)	0.016
T3/T4	0 (0)	29 (2)	71 (5)	
Gleason score				
2-6	38 (8)	52 (11)	10 (2)	0.024
7-10	24 (5)	29 (6)	48 (10)	
Pretreatment PSA				
≤10	40 (13)	46 (15)	15 (5)	0.0008
>10	0 (0)	22 (2)	78 (7)	
DNA-ploidy				
Diploid	41 (11)	37 (10)	22 (6)	0.15
Nondiploid	9 (1)	46 (5)	46 (5)	
Percent S-phase				
≤2.5	36 (5)	36 (5)	29 (4)	0.87
>2.5	27 (4)	40 (6)	33 (5)	
Treatment failure				
No	45 (13)	41 (12)	14 (4)	0.0015
Yes	0 (0)	39 (5)	62 (8)	

^aPatients were stratified by %Ki-67 staining of $\leq 1.5\%$, $>1.5-3.5\%$, and $>3.5\%$.

*Trended chi-square test.

also reported associations between Ki67-LIs and DNA-ploidy. These results indicate that the parameters of Ki67-LI and DNA-ploidy are significantly, albeit weakly, related.

Several studies have examined the prognostic importance of Ki67-LI in patients with prostate cancer. In nearly every report, Ki-67-LI has been predictive of patient outcome in actuarial univariate analyses. While the majority have confirmed the independence of Ki67-LI as a correlate of patient outcome in multivariate analysis [26,27,28,29,32,33], others have not [24,43]. The number of patients in our study ($n = 42$) was inadequate to accurately assess the independence of Ki67-LI as a predictor of freedom from failure. Pre-treatment PSA was the only correlate by Cox proportional regression in this series. Prior studies with larger numbers of patients have established that Gleason score, clinical stage, and DNA-ploidy are also independent correlates [16,17,34].

CONCLUSIONS

The Ki67-LI is significantly related to other prognostic factors, such as pretreatment PSA, Gleason score, and stage, and is a predictor of patient outcome. The data suggest that Ki67-LI is a stronger correlate of prostate cancer patient outcome following radiotherapy than DNA-ploidy or SPF. A Ki67-LI $>3.5\%$ was associated with a particularly poor prognosis. Prospective evaluation of pretreatment prostate tumor biopsy Ki67-LI will help to clarify the role of this potentially useful cell kinetic marker.

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BIOLOGY CONTRIBUTION

THE EARLY SUPRA-ADDITIVE APOPTOTIC RESPONSE OF R3327-G PROSTATE TUMORS TO ANDROGEN ABLATION AND RADIATION IS NOT SUSTAINED WITH MULTIPLE FRACTIONS

ALAN POLLACK, M.D., PH.D.,* FARAMARZ ASHOORI, M.D.,[†] CHARLES SIKES, B.S.,[†]
DARYL LIM JOON, M.D.,[†] ANDREW C. VON ESCHENBACH, M.D.,[‡] GUNAR K. ZAGARS, M.D.,* AND
MARVIN L. MEISTRICH, PH.D.[†]

Departments of *Radiation Oncology and [†]Experimental Radiation Oncology and [‡]Urology, University of Texas, M.D. Anderson Cancer Center, Houston, Texas

Purpose: The treatment of R3327-G tumor-bearing rats with androgen ablation (AA) via castration results in a supra-additive increase in apoptosis when 2–8 Gy γ -irradiation (RT) is given as a single dose 3–14 days afterwards. We report here the dose response and effect of multiple fractions on this supra-additive apoptotic response.

Materials and Methods: Dunning R3327-G tumors were grown in the flanks of Copenhagen rats and the experiments were initiated at a tumor volume of 1.0–1.5 cc. Androgen ablation was achieved by castration 3 days prior to γ -irradiation. Apoptosis was measured with a terminal deoxynucleotidyl transferase dUTP-biotin nick end-labeling assay 6-h after RT, unless otherwise specified.

Results: The dose response of the supra-additive apoptotic response was assessed by irradiating castrated animals with single doses of 2, 4, 8, or 16 Gy ($n = 5$ per group); tumor cell apoptosis at 6-h following irradiation was $2.4\% \pm 0.7\%$ (\pm SEM), $4.2\% \pm 0.8\%$, $6.5\% \pm 1.4\%$, and $1.6\% \pm 0.3\%$, respectively. The RT only and AA only controls had $< 1\%$ apoptosis. The effect of fractionated RT on apoptosis was investigated to determine if the supra-additive apoptotic response was sustained with repeated 2–8 Gy fractions. When tumor-bearing animals were treated with repeated daily 2-Gy fractions, there was a reduction in the level of the supra-additive apoptotic response. After five 2-Gy fractions at 24-h intervals, apoptosis in the combined treated tumors was at levels seen in the AA controls. This raised the possibility that more than 24 h are required for recovery of the high supra-additive apoptotic levels seen after one fraction. When the interfraction interval was extended to 96 h, there was no significant increase in apoptosis over the additive effect of AA and RT. Although there was a decline in supra-additive apoptosis with repeated fractions, a dose response for tumor growth delay was evident for RT alone using 2.5-Gy fractions. Moreover, the combination of AA + fractionated RT resulted in a supra-additive enhancement in tumor growth delay to 5 cc.

Conclusion: The early supra-additive apoptotic response from AA and single fraction radiation is not seen at high single fraction doses and is not sustained with repeated fractions. Therefore, the classical apoptotic response that occurs within 24 h of irradiation is not likely to be the main mechanism responsible for any clinical benefit seen with this combination. © 2000 Elsevier Science Inc.

Prostate cancer, Apoptosis, Androgen ablation, Radiation.

INTRODUCTION

Clinical and laboratory data suggest that androgen ablation (AA) plus radiation (RT) results in improved prostate cancer control rates. Several randomized trials have documented a highly significant increase in freedom from biochemical failure for AA plus RT over radiation alone (1–4). A survival benefit was seen in two of the trials. The lack of an AA alone arm in these studies compromises interpreta-

tion because a survival benefit has been described for early AA, as compared with deferred AA (5). A key question has been whether the combination of AA + RT results in more cell killing than either treatment given individually and, if so, whether apoptosis is the principal mechanism of this interaction.

Laboratory studies using animal and human prostate cancer models have shown that cell killing is enhanced with AA + RT over either treatment given individually using a

Reprint requests to: Alan Pollack, M.D., Ph.D., Department of Radiation Oncology (97), University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.
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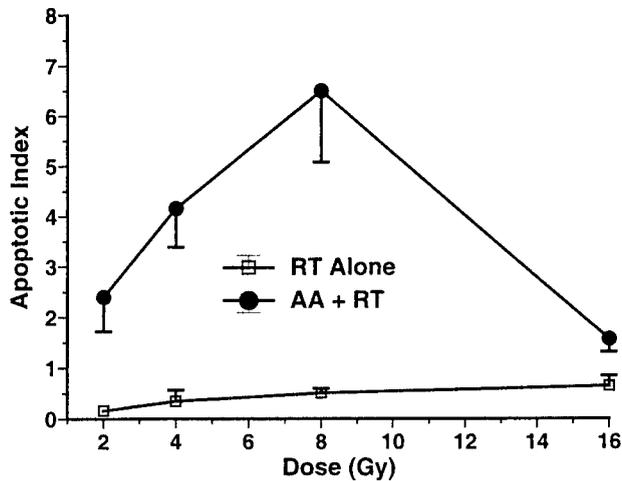


Fig. 1. Supra-additive apoptotic dose-response for R3327-G tumors grown in castrated rats for 3 days, irradiated, and then removed for TUNEL staining 6-h later (solid circles). The intact irradiated RT alone controls are shown (open squares).

group control for 16 Gy (assayed at 6 h) was 0.93% in Fig. 1 and 1.94% in Fig. 2. These values are much lower than for AA plus 8 Gy as shown in Fig. 1 (6.0%) and described previously (9).

The effect of fractionated RT on apoptosis was investigated to determine if the supra-additive apoptotic response was sustained with repeated 2–8 Gy fractions. When tumor-bearing animals were treated with repeated daily 2-Gy fractions beginning 3 days after AA, and apoptosis measured 6-h after the administration of the last fraction, there was a reduction in the level of the supra-additive apoptotic response relative to that seen after a single 2-Gy fraction. Figure 3 illustrates that with as few as three daily fractions in the AA + RT treated tumors the supra-additive apoptotic response is lost. After five daily 2-Gy fractions, apoptosis in the combined treated tumors was at the level of the castrated control.

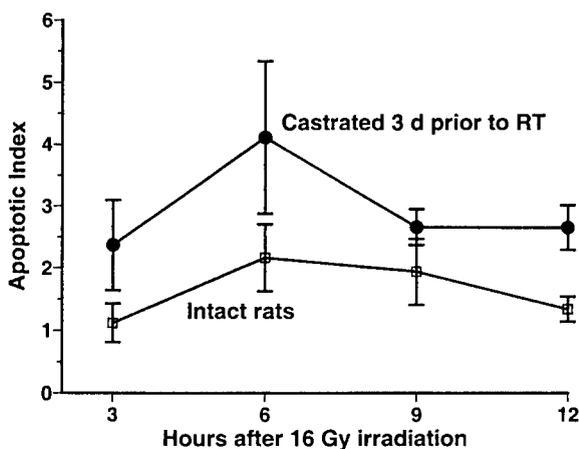


Fig. 2. Effect of varying the time after AA plus 16 Gy irradiation on measurable apoptosis. Tumor-bearing rats were castrated, irradiated 3 days later with 16 Gy, and examined for apoptosis at different times thereafter (solid circles).

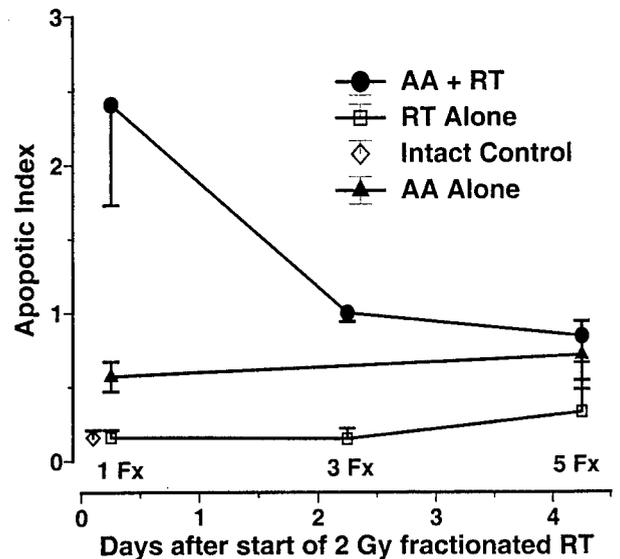


Fig. 3. R3327-G tumor apoptotic response to 1, 3, or 5 daily 2 Gy fractions. Tumor-bearing rats were castrated, irradiated with the first fraction 3 days later, and removed 6-h after the last fraction (solid circles). The following controls are shown: RT alone = open squares; intact unirradiated control = open diamonds; AA alone = solid triangles.

Other investigators have described that maximal recovery of the apoptotic response after single fraction radiation requires more than 24 h (13, 14). This raised the possibility that more than 24 h are required for recovery of the supra-additive apoptotic levels seen after one fraction. Tumor-bearing animals were castrated and, 3 days later, were irradiated with 2 Gy; followed 24-, 48-, or 96-h later with a second fraction of the same dose. The tumors were removed and prepared for the TUNEL assay 6-h after the second

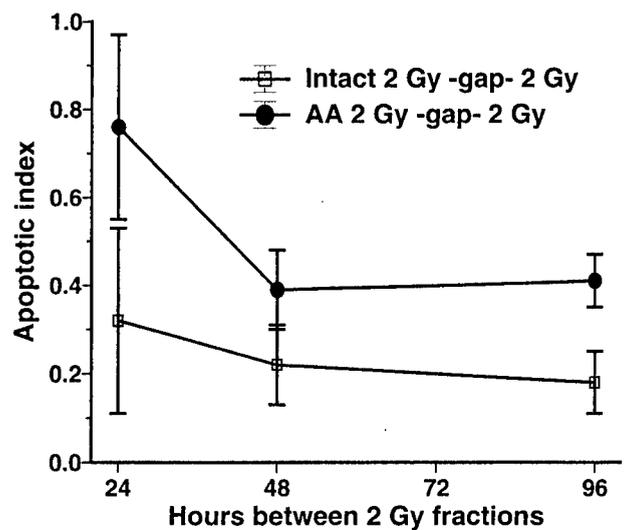


Fig. 4. Effect of varying the interval between two fractions of 2 Gy on the apoptotic response of R3327-G tumors grown for 3 days in castrated rats (solid circles). Apoptosis was measured 6-h after the second fraction. The intact controls are also shown (open squares).

tate cancers to radiation is also very low (23, 24) and under the best circumstances probably only contributes about 5% to overall cell killing. Prostate cancer is not unique in this regard, as for most tumors tested, apoptosis accounts for a fraction of the loss of reproductive integrity (25).

The finding that AA and single fraction RT of 2–8 Gy caused supra-additive apoptosis (9), when applied in a specific sequence to prostate tumors *in vivo*, suggested that this cell death mechanism might take precedence, leading to increased overall cell killing. Confirmation of this tenet seemed apparent with the observation that enhanced apoptosis to AA plus single fraction RT was associated with supra-additive tumor growth delay. Implicit in these data is potential for considerably greater tumor eradication with fractionated radiotherapy, if supra-additive apoptosis occurred with each fraction.

Meyn and colleagues (13, 14) found that ovarian carcinoma (Oca-I) cells grown *in vitro* exhibited a significant, albeit reduced, apoptotic response when a second fraction was applied 24-h after the first. They also observed about a twofold further recovery of the apoptotic response when the interfraction interval was lengthened to 5 days. A similar pattern was established by Mirkovic *et al.* (14) using lymphoma cells grown *in vivo*.

The data described here using the Dunning R3327-G rat prostate model reveal that supra-additive apoptosis to AA + RT is not repeated with additional fractions of γ -radiation. Nor was a supra-additive apoptotic response evident when the interfraction interval between two fractions of 2 or 8 Gy was extended to 96 h (Figs. 4 and 5). No recovery of the supra-additive apoptotic response was noted within the limits of the study. Thus, the reduction in apoptosis under these conditions appears not to be consistent with changes in the distribution of cells in the cell cycle caused by split-dose irradiation (26, 27). We chose not to go beyond 96 h because such extended times would be impractical in designing a clinical regimen that optimized the apoptotic response. The level of cell killing evidenced by apoptosis after multiple fractions was not concordant with the tumor

growth delay results. Figure 7, and the resulting calculations of the enhancement factor, demonstrate that the combination of AA with multiple RT fractions causes a greater delay in tumor growth than the addition of the individual effects of AA and RT. Supra-additive tumor growth inhibition with AA + RT is probably related to an increase in overall cell death; however, an alternative mechanism is that tumor proliferation was suppressed. The former explanation, that AA + RT causes increased overall cell killing via necrosis, is supported by the reduction in TCD50 observed under these conditions in this cell line (6).

The single fraction dose-response experiment shown in Fig. 1 provides additional evidence that apoptosis levels after AA + RT appear to be contrary to the expected dose response achieved with measures of overall cell killing, such as tumor growth delay (15). Irradiation with AA plus 16 Gy resulted in a lower apoptotic index than AA plus 8 Gy. In contrast, tumors treated with RT alone had consistently higher levels of apoptosis when the dose was increased from 8 to 16 Gy, which is the dose-response pattern that others have reported (13, 14).

Apoptosis is clearly a secondary mechanism of cell killing from irradiation (25, 28). Our data show that apoptosis is suppressed with the administration of multiple fractions in the setting of androgen deprivation. Likewise, the apoptotic index was lower to AA plus 16 Gy given in a single dose, as compared with AA plus 8 Gy. The administration of AA + RT alters the relationship of apoptosis to overall cell killing, suggesting that AA affects the expression of key proteins in the apoptotic pathway or that the impact of known proteins is diminished by changes in the expression of less characterized downstream factors. Along these lines, Kyprianou *et al.* (29) have observed that bcl-2 overexpression delays radiation-induced apoptosis without affecting clonogenic survival. From these results, and those described here with AA plus fractionated RT, it would appear that under certain conditions the level of apoptosis is not reflective of overall cell death.

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Prostate Cancer Radiosensitization *in Vivo* with Adenovirus-mediated *p53* Gene Therapy¹

Didier Cowen,² Naji Salem,² Faramarz Ashoori, Ray Meyn, Marvin L. Meistrich, Jack A. Roth, and Alan Pollack³

Departments of Experimental Radiation Oncology [D. C., N. S., F. A., R. M., M. L. M.], Thoracic and Cardiovascular Surgery [J. A. R.], and Radiation Oncology [A. P.], University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

An adenovirus 5 vector containing wild-type *p53* cDNA (Ad5-*p53*) and a cytomegalovirus promoter was used to generate *p53* transgene expression. Control vector (Ad5-*pA*) contained the poly-adenosine sequence. PC3 cells (2×10^6) were injected s.c. into the legs of nude mice. Treatment with Ad5-*p53* was initiated at a tumor volume of 200 mm³. Three intratumoral injections (days 1, 4, and 7) were given with 3×10^8 plaque-forming units, followed by 5 Gy pelvic irradiation (day 8) in one fraction using a cobalt-60 source. Tumor volume measurements were obtained every 2 days. LNCaP cells (2×10^6) were injected orthotopically into the prostates of nude mice, and tumor weight was approximated using serum prostate-specific antigen (PSA) obtained from weekly tail vein bleedings. The target PSA for the start of the studies was 5 ng/ml. The intraprostatic injections of Ad5-*p53* were done twice (days 1 and 2) and followed by 5 Gy pelvic irradiation on day 3.

The PC3 tumor volume growth curves were log transformed and fitted using linear regression. The times (in days) for the tumors to reach 500 mm³ were calculated as 10.7 ± 0.7 (\pm SE) for the saline control (no virus), 9.8 ± 2.1 for Ad5-*pA*, 15.6 ± 1.6 for Ad5-*p53*, 14.6 ± 1.5 radiation therapy (RT; 5 Gy), 14.6 ± 1.5 for Ad5-*pA* plus RT, and 31.4 ± 5.3 for Ad5-*p53* plus RT. The Ad5-*p53* plus RT times were significantly different from the other groups. An en-

hancement factor of 3.4 was calculated, indicating supra-additivity.

LNCaP tumor growth was determined via weekly serum PSA measurements. Treatment failure was determined using two PSA-based methods; a serum PSA of >1.5 ng/ml or two rises in PSA during 6 weeks posttreatment. The results were similar using either end point. Treatment with Ad5-*p53* plus 5 Gy resulted in significantly fewer PSA failures (<30%), as compared with Ad5-*p53* alone (64–73%) and the other controls (~80–100%) These results are also consistent with a supra-additive inhibition of tumor growth. Tumor growth *in vivo* was inhibited supra-additively when *p53*^{null} and *p53*^{wildtype} prostate tumors were treated with Ad5-*p53* and 5 Gy radiation.

INTRODUCTION

Patients at high risk of PSA⁴ relapse after external beam radiotherapy may be identified using the pretreatment clinical parameters of PSA, Gleason score, and stage (1, 2). The question then, is how best to treat this group. External beam radiotherapy to conventional doses is inadequate, and the main mechanism appears to be failure to completely eradicate the disease locally. Local persistence is evident in most patients that exhibit a rising PSA in this setting, because prostate biopsies are positive in the majority of those that are investigated. Although dose escalation results from a number of institutions indicate modest reductions in biochemical failure rates for high-risk patients (3–5), dose-related improvements in outcome have been modest and are still wanting. One approach that holds promise is radiosensitization.

Recent clinical (6–8) and animal (9–11) studies have described improved results when androgen ablation is combined with radiation. The results suggest a supra-additive interaction between these treatments. The clinical gains from the combination have been encouraging to a limited degree but have been associated with significant long-term side effects. Clearly, a radiosensitization strategy that has fewer systemic side effects is desirable. The potential for radiosensitization using gene therapy is relatively untapped. Our approach has been to alter the intracellular molecular milieu such that cell death via apoptosis is favored over cell cycle delay and repair in response to radiation. This concept was manifest from *in vitro* experiments (12) involving two prostate cancer cell lines using a replication defective adenovirus 5 vector containing a *p53*^{wildtype} cDNA construct (Ad5-*p53*). A key question was whether Ad5-*p53* would sensitize prostate cancer cells that did not express *p53* (PC3 line), as well as those that expressed *p53*^{wildtype} (LNCaP

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² Present address: Institut Paoli Calmettes, 232 Boulevard Sainte Marguerite, Marseille 13009, France.

³ To whom requests for reprints should be addressed, at Department of Radiation Oncology, 1515 Holcombe Boulevard (Box 97), Houston, TX 77030. Phone: (713) 792-0781; Fax: (713) 794-5573; E-mail: Apollack@notes.mdacc.tmc.edu.

⁴ The abbreviations used are: PSA, prostate-specific antigen; Luc, luciferase; Ad5, adenovirus 5; pfu, plaque-forming units; PBS, phosphate-buffered saline; NHS, normal horse serum.

line). The results showed that clonogenic survival was reduced and apoptosis enhanced supra-additively in both cell lines when Ad5-p53 was combined with radiation. Thus, p53 gene replacement was not the only mechanism responsible for the radiosensitization observed.

In the present study, the effect of Ad5-p53 on the *in vivo* tumor growth response of PC3 and LNCaP cells to radiation was investigated. Whereas the *in vitro* data demonstrate radiosensitization by this vector under ideal conditions, these experiments are necessary to verify that p53 gene delivery plus radiation is effective *in vivo*.

MATERIALS AND METHODS

Cell Lines. The PC3 and LNCaP cell lines were obtained from the American Tissue Type Collection and were maintained in cell culture, using liquid nitrogen for long-term storage. Cells were cultured for a period of ~2 months, before taking a new aliquot from liquid nitrogen storage. Both PC3 and LNCaP cells were cultured in a 5% CO₂ incubator at 37°C in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 IU/ml Pen-Strep solution.

***In Vivo* Ad5-p53 Vector Treatment.** An adenovirus 5 vector containing wild-type p53 cDNA (Ad5-p53) and a cytomegalovirus promoter was used to generate p53 transgene expression (13). The main control vector used contained the polyadenosine sequence (Ad5-pA); however, an adenoviral-Luc vector (Ad5-Luc) containing the cDNA for luciferase was also used as a control in some studies. We have used these control vectors interchangeably and have not seen a difference in clonogenicity or apoptosis (12). PC3 cells (2×10^6) were injected s.c. into the legs of nude mice. Treatment with Ad5-p53 was initiated at a tumor volume of 200 mm³. Three intratumoral injections (days 1, 4, and 7) were given with 3×10^8 plaque-forming units, followed by 5 Gy irradiation in one fraction using a cobalt-60 source. Tumor volume measurements were obtained every 2 days.

LNCaP cells (2×10^6 in 24 μ l) were injected orthotopically into the prostates of nude mice. Tumor weight was approximated using serum PSA obtained from weekly tail vein bleedings. There is a linear relationship between tumor (plus prostate) weight and serum PSA; linear regression results revealed that tumor weights of 0.15, 0.3, and 0.6 g correlated with PSAs of 1.1, 11.1, and 31.1 ng/ml. The target PSA for the studies was 5 ng/ml, which correlated with a tumor weight of 0.208 g, which was found at a median of 6 weeks after orthotopic injection. The animals were then anesthetized via s.c. injection of 100 μ l of a 0.02 mg/ μ l solution of Ketamine in 0.9% saline, the prostate was surgically exposed, and 4.5×10^8 pfu injected in 24 μ l. The intraprostatic injections were done twice (days 1 and 2), and 5 Gy pelvic irradiation using a cobalt-60 source was administered 24 h later (day 3).

Calculation of Enhancement Factor. As a determination of supra-additivity in PC3 tumor volume growth delay from the combination of Ad5-p53 + 5 Gy, an enhancement factor was calculated (9). The tumor volume curves for each tumor-bearing animal were first log-transformed, and the absolute delay in tumor growth to 500 mm³ relative to the saline control was calculated. These values were used to calculate the en-

hancement factor [Abs delay (Ad5-p53 + RT - Ad5-p53)/Abs delay (PBS + RT alone)], which measures the relative increase of the combined treatment (taking into consideration the effects of the Ad5-p53 vector) over radiation alone. The Ad5-pA controls were not included because significant delays over the saline controls were not observed. An enhancement factor of >1.0 is indicative of supra-additivity between Ad5-p53 and radiation.

Measurement of Serum PSA. Human PSA was measured in the serum obtained from tail vein bleedings. From each blood draw, 30 μ l of serum were diluted 1:5 in PSA diluent (Abbott Labs, Abbott Park, IL) and analyzed for PSA concentration on an IMX analyzer (Abbott Labs). The results are expressed in ng/ml.

Apoptosis and p53 Staining. A terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was used to quantify apoptosis in tissue sections from PC3 and LNCaP tumors injected *in vivo* with Ad5-p53 as described above. The tumors were removed and fixed in 10% neutral formalin overnight and embedded in paraffin. Sections were then mounted on silane-coated slides as described previously (9, 11). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining of apoptotic cells was accomplished using the ApopTag (Oncor, Gaithersburg, MD) kit. The cells were counterstained with hematoxylin. Positive controls were included with each group of samples stained.

The immunohistochemical staining of p53 was performed as outlined previously (14). Briefly, paraffin-embedded tissue sections mounted on slides were deparaffinized, hydrated, and treated for 30 min with 0.3% H₂O₂. Antigen retrieval was accomplished with three high power microwave treatments of 5 min each. Nonspecific staining was blocked by incubating 15 min with 2% NHS in PBS (NHS-PBS). Primary Ab6 anti-p53 antibody (Calbiochem-Novabiochem Corp., San Diego, CA) was used at a 1:100 dilution in NHS-PBS, incubating on the slide overnight at room temperature. After rinsing the slide four times in PBS, biotinylated second antibody (1:200 in NHS-PBS) was added for 30 min. The biotinylated second antibody and other reagents for peroxidase staining were supplied in a kit from Vecta Laboratories (Vectastain ABC kit; Vecta Labs, Burlington, CA). After rinsing off the second antibody, the Vectastain Elite ABC reagent was added for 30 min, the slides were washed, peroxidase substrate solution was added for 20 min, and the cells were counterstained with Mayer's hematoxylin.

RESULTS

The experiments with the PC3 line were designed to determine the ability of intratumoral Ad5-p53 plus radiation to enhance tumor volume growth delay over Ad5-p53 alone. The hypothesis was that the administration of Ad5-p53 would replace p53 function in PC3 cells, which are p53^{null}. The replacement of p53 function would maximize the chance for apoptosis in response to radiation. Injection of Ad5-p53 into PC3 tumors resulted in increased p53 expression and apoptosis in portions of the tumor 24 h later (Fig. 1), as compared with Ad5-Luc control vector. The data indicate that Ad5-p53 treatment resulted in functional p53 expression *in vivo*.

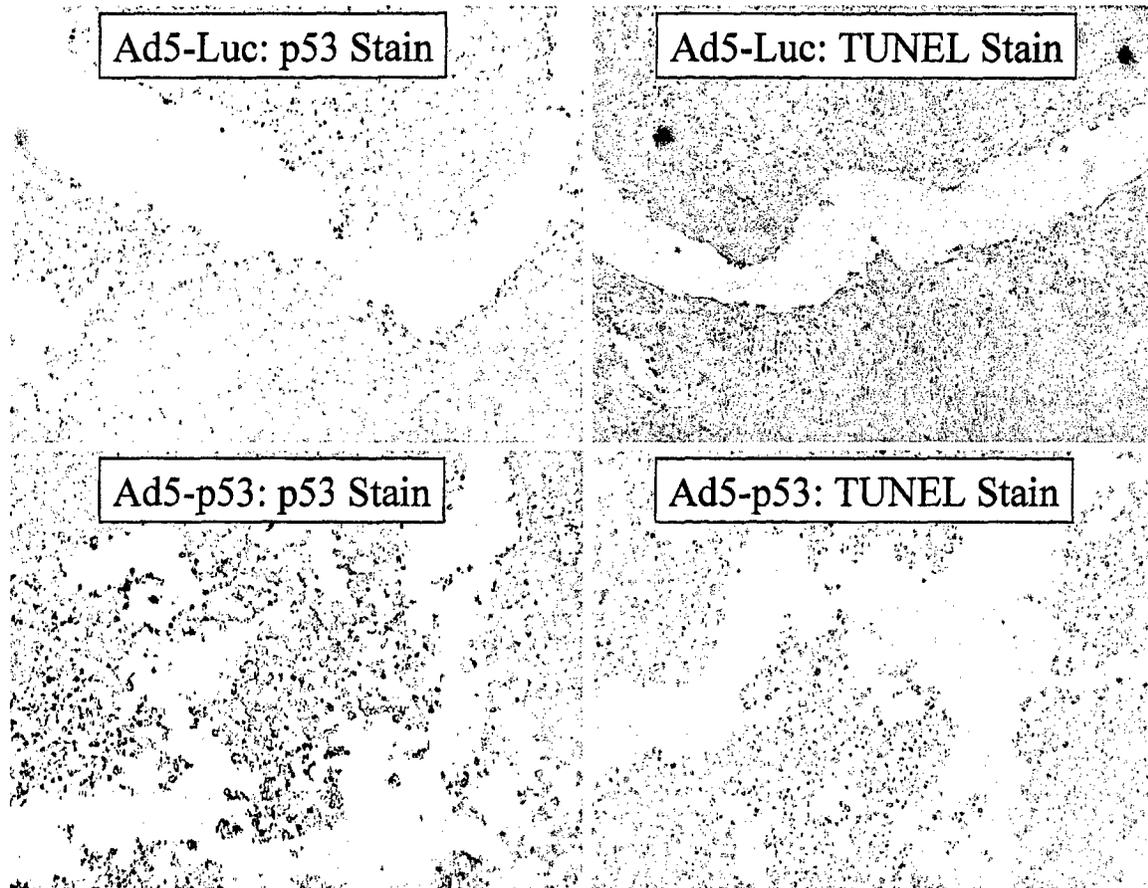


Fig. 1 Replacement of p53 and induction of apoptosis in PC3 tumors treated with Ad5-p53 *in vivo*. A single intratumoral injection of Ad5-p53 at 4.5×10^8 pfu in 24 μ l resulted in the expression p53 (bottom left) and apoptosis (bottom right). Injection of Ad5-Luc at 4.5×10^8 pfu did not cause detectable p53 expression (top left) or enhanced apoptosis (top right).

Fig. 2 shows the tumor volume growth delay response of PC3 cells grown in the legs of nude mice to three Ad5-p53 intratumoral injections, with or without single-fraction 5 Gy of γ -irradiation. There were a number of controls, including injection of PBS alone, PBS + 5 Gy, Ad5-pA control vector alone, and Ad5-pA + 5 Gy. The Ad5-p53 vector was administered alone and in combination with 5 Gy. The results illustrate a substantial tumor volume growth delay for Ad5-p53 plus radiation, as compared with the other treatment groups, including Ad5-p53 alone. Table 1 summarizes the absolute time to reach 500 mm³, which was calculated from the log-transformed tumor volume growth curves from each animal. The absolute delay was about three times that seen for the PBS alone and Ad5-pA alone controls and was about two times that for the PBS + 5 Gy, Ad5-pA + 5 Gy, and Ad5-p53 alone groups. One-way ANOVA (Scheffe test) showed that absolute tumor growth delay from Ad5-p53 + 5 Gy was significantly greater than from all of the other treatments. The enhancement factor was calculated to be 3.4, indicating a supra-additive affect on tumor growth.

LNCaP cells are p53^{wildtype}, leaving to question the mechanism for potentiation of the tumor growth inhibitory action of radiation by Ad5-p53 on such cells. *In vitro* data (9) suggested that p53 overexpression as a consequence of treatment with

Ad5-p53 enhanced the apoptotic response and reduced cell survival of LNCaP cells exposed to radiation. The *in vivo* experiments performed here were designed to test whether LNCaP tumors grown in the prostates of nude mice, and therefore under the influence of stromal-epithelial interactions, would be inhibited supra-additively to Ad5-p53 plus radiation. Because LNCaP cells produce PSA, the orthotopic system closely parallels human prostate cancer. Serum PSA obtained through tail vein bleeding is a surrogate for tumor weight and/or volume. This is illustrated in Fig. 3, where a highly significant relationship was found between serum PSA and tumor (plus prostate) weight. Thus, serum PSA after treatment was used to determine the failure rates for the various treatments tested.

The two methods used to assess biochemical failure are similar to those used in patients with prostate cancer. In one, a 6-week posttreatment serum PSA value of >1.5 ng/ml (threshold PSA method) was considered evidence of failure, and in the other a rising PSA on two consecutive weekly bleedings or a single rise of >1.5 ng/ml (rising PSA method) over the 6-week posttreatment period was considered evidence of failure. The pretreatment and 6-week posttreatment PSA results are summarized in Table 2. The average pretreatment PSA was 4.86 ng/ml. There were no statistically significant differences between the

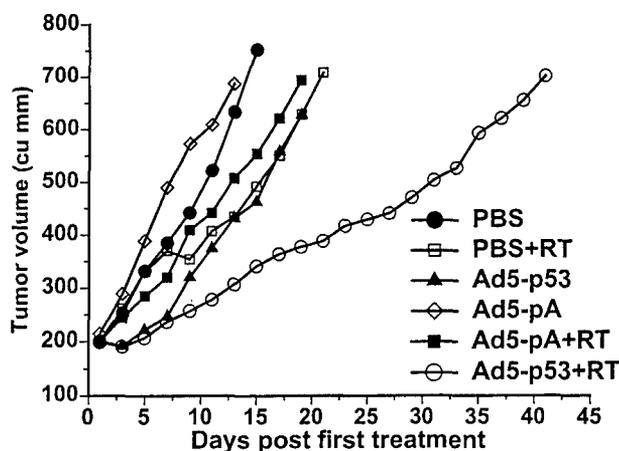


Fig. 2 Effect of Ad5-p53 + 5 Gy radiation on PC3 tumor volume growth. The groups shown include PBS control (●), PBS + 5 Gy RT (□), Ad5-pA vector alone (◇), Ad5-pA + 5 Gy RT (■), Ad5-p53 alone (▲), Ad5-p53 + 5 Gy RT (○). The number of animals per group is shown in Table 1. This is a representative experiment of two.

Table 1 Delay in PC3 tumor growth to a volume of 500 mm³ induced by Ad5-p53 and/or 5 Gy Radiation

Group	Absolute delay (n)	Delay from saline control
PBS	10.7 ± 0.7 (4)	
Ad5-pA	9.8 ± 2.1 (3)	0
Ad5-p53	15.6 ± 1.6 (5)	4.9
PBS + 5 Gy	15.4 ± 2.2 (5)	4.7
Ad5-pA + 5 Gy	14.6 ± 1.5 (5)	3.9
Ad5-p53 + 5 Gy	31.4 ± 5.3 (5) ^a	20.7 ^b

^a Significantly different ($P < 0.05$) from other groups by one-way ANOVA (Scheffe test).

^b Enhancement factor, 3.4.

treatment groups in terms of pretreatment PSA. Table 2 also displays the 6-week posttreatment PSAs. Although Ad5-p53 + 5 Gy resulted in the lowest mean posttreatment PSA, the only statistically significant difference between this group and the others was with the PBS-only group. Mean posttreatment PSAs are not an accurate reflection of response because once biochemical failure is established, PSA rises quickly. Table 2 illustrates this variability in posttreatment PSAs, showing that the Ad5-p53 + RT group had the lowest median posttreatment PSA (0.5 ng/ml), and yet in one animal that failed, the PSA rose to over 56 ng/ml. The more meaningful end points of the threshold and rising PSA methods are reflective of clinical biochemical end points that have been widely adopted clinically.

Table 3 shows that both methods resulted in similar estimates of treatment failure. Treatment with Ad5-p53 + 5 Gy resulted in significantly fewer PSA failures (<30%), as compared with Ad5-p53 alone (64–73%) and the other controls (~80–100%). This trend was highly statistically significant. The determining component was the distinctive reduction in biochemical failure from Ad5-p53 plus radiation over Ad5-p53 alone and the other controls. In univariate analysis (χ^2), the Ad5-p53 plus radiation failure rates by both the threshold and

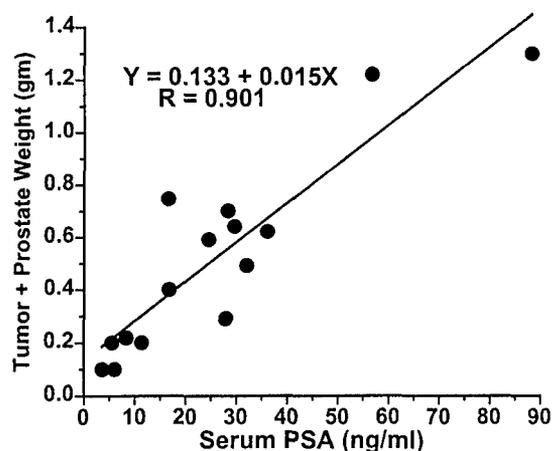


Fig. 3 Relationship of LNCaP tumor plus prostate weight to serum PSA in untreated LNCaP-bearing nude mice. LNCaP cells were injected into the prostates of nude mice, and at various times thereafter serum PSA and tumor plus prostate weights were assayed.

rising PSA methods were significantly lower than all of the other groups, including the Ad5-p53 alone group. There was a clear-cut advantage to the Ad5-p53 plus radiation combination. The reduction in biochemical failure from this combination appeared to be greater than the additive effects of the individual treatments.

DISCUSSION

The eradication of locally advanced or high-risk prostate cancer with radiation has proven more difficult than believed previously. The clinical application of PSA as an end point has been the principal factor leading to this realization. Although modern series document improved outcome with higher radiation doses, the gains have been modest and not without side effects (5, 15, 16). The need for novel methods of radiosensitization is apparent. Androgen ablation has shown promise as a radiation sensitizer of androgen-sensitive cancer cells (9–11); however, the morbidity from prolonged androgen ablation in men with prostate cancer is significant. Novel approaches to radiosensitization with reduced systemic effects are more desirable, and gene therapy offers promise in this regard.

The *p53* gene product has been shown to be a key factor in the radiation response pathways governing cell cycle arrest and repair and apoptosis (17–19). A number of studies have indicated that *p53* replacement in tumor cell lines with altered *p53* expression reduces tumorigenicity and promotes apoptosis (20–24) and sensitizes tumor cells to radiation (25–28). These effects are less conclusive in cases of *p53* transgene overexpression in *p53*^{wildtype} tumors. For *p53*^{wildtype} tumors treated with *p53* gene therapy, the inhibition of tumorigenesis and promotion of apoptosis have ranged from significant (12) to nearly absent (29, 30). Likewise, the action of *p53* gene transfer plus radiation on tumor cell lines with *p53*^{wildtype} expression has been variable; some reports have described radiosensitization of *p53*^{wildtype} tumors (29, 31), and others have not (30). In our *in vitro* experience (12), apoptosis was induced in the absence of radiation by *p53* transgene expression in the *p53*^{wildtype} LNCaP line

Table 2 Pretreatment and posttreatment PSAs in nude mice bearing orthotopic LNCaP tumors

Group	n	Serum PSA in ng/ml		
		Pretreatment ^a Mean ± SE	At 6 wk ^b Mean ± SE	At 6 Wk Median (range)
PBS alone	5	2.82 ± 0.52	44.74 ± 8.44	42.2 (22.1–69.9)
PBS + 5 Gy	10	4.36 ± 0.91	10.06 ± 2.26	11.7 (0.0–21.7)
Ad5-pA alone	10	5.52 ± 0.69	25.83 ± 6.78	23.1 (2.9–64.0)
Ad5-pA + 5 Gy	5	6.40 ± 1.19	15.13 ± 4.69	17.9 (1.4–27.3)
Ad5-p53 alone	11	4.62 ± 1.10	12.18 ± 4.24	5.7 (0.0–46.4)
Ad5-p53 + 5 Gy	14	5.12 ± 1.01	6.83 ± 4.17	0.5 (0.1–56.1)

^a No significant differences between groups by one-way ANOVA (Scheffe test).

^b The Ad5-p53 + 5 Gy group was significantly different ($P < 0.05$) from the PBS alone group by one-way ANOVA (Scheffe test). If the PSA rose rapidly, the mice were sacrificed before 6 weeks and the PSA value used was <6 weeks. At 6 wk, PSA 6 weeks after treatment; Ad5-pA, adenoviral control vector with polyadenylated sequence; Ad5-p53, adenoviral p53 vector.

Table 3 Treatment failure using biochemical criteria in nude mice bearing orthotopic LNCaP tumors

Group ^a	Threshold PSA ^b		Rising PSA ^c	
	Failure free	Failure	Failure free	Failure
PBS	0% (0)	100% (5)	0% (0)	100% (5)
PBS + 5 Gy	20% (2)	80% (8)	30% (3)	70% (7)
Ad5-pA	0% (0)	100% (10)	10% (1)	90% (9)
Ad5-pA + 5 Gy	20% (1)	80% (4)	20% (1)	80% (4)
Ad5-p53	37% (3)	73% (8)	36% (4)	64% (7)
Ad5-p53 + 5 Gy	71% (10)	29% (4) ^d	79% (11)	21% (3) ^d

^a Ad5-pA, adenoviral control vector with polyadenylated sequence; Ad5-p53, adenoviral p53 vector.

^b Treatment failure was defined PSA above 1.5 ng/ml at 6 weeks after treatment. The failure rates were significantly different overall by trended χ^2 ($P \leq 0.001$).

^c Treatment failure was defined as two consecutive rises in PSA or a single rise of >1.5 ng/ml within 6 weeks of treatment. The failure rates were significantly different overall by trended χ^2 ($P \leq 0.001$).

^d The rate of failure was significantly lower in the Ad5-p53 plus 5 Gy group, as compared with each of the other groups (χ^2 ; $P < 0.05$).

to about the same degree as for the p53^{null} PC3 line; adenoviral-mediated p53 radiosensitization using a clonogenic survival assay was also observed in these lines.

The prostate is amenable to direct intraprostatic injection of gene therapy vectors (32). A foremost concern with such a strategy is whether sufficient radiosensitization can be accomplished with relatively few supplemental gene therapy treatments during radiotherapy. The efficacy of intraprostatic gene therapy should be established with two to three intraprostatic injections during a radiation course because of cost, convenience, and potential morbidity issues with more than three injections. The current investigation establishes that two to three intratumoral injections results in substantial sensitization in both p53^{null} and p53^{wildtype} prostate cancer lines. The enhancement in PC3 tumor growth inhibition by three daily intratumoral injections of Ad5-p53, followed a day later by a single 5 Gy radiation, was calculated to be >3-fold, relative to the controls. A similar effect was observed for p53^{wildtype} LNCaP cells using serum PSA as a measure of failure to control tumor growth. The rising PSA profile is the earliest and most sensitive end point in the documentation of treatment failure in patients with prostate cancer and is highly correlated with eventual clinical disease relapse. The orthotopic LNCaP model used here is decidedly representative of human prostate cancer, from the dependence on stromal growth factors for tumorigenicity (33), to the secretion of PSA in proportion to tumor weight (Fig. 3), as well as the

sensitivity to radiation. With two intratumoral injections of Ad5-p53 plus single-fraction radiation, PSA response was sustained for >6 weeks in close to 80% by the rising PSA method. Freedom from a rising PSA was seen in 36% of Ad5-p53 alone control group and 20–30% of the control irradiated groups (Table 3). Thus, the freedom from failure rate in the Ad5-p53 + 5 Gy group was greater than the additive effect of the controls.

In conclusion, our results confirm the feasibility of sensitizing prostate cancer cells to radiation *in vivo* using adenoviral-mediated p53 gene therapy. By our estimation, based on prior *in vitro* (12) and *in vivo* data, the radiosensitization achieved in prostate cancer patients treated with Ad5-p53 and fractionated radiotherapy should be substantial. The data described here represent the minimum expected gain from combining Ad5-p53 and radiation, because all of the intratumoral injections were given before radiotherapy and only a single radiation fraction was used. The strategy currently being instituted in patients involves three injections of Ad5-p53 into the prostate at 2-week intervals during fractionated or low-dose-rate radiotherapy. Because transgene p53 expression lasts at least 5–7 days depending on cell type (34, 35), sensitization could occur for 35–45% of the daily radiation treatments, which typically ranges from 34 to 42 fractions over 6.8–8.5 weeks. Using intensity modulated radiotherapy and hypofractionation (36), it may be possible to shorten overall treatment time without increasing side effects; this would facilitate sensitization by Ad5-p53 for >50% of the

radiation fractions administered. Treatment of LNCaP cells *in vitro* (9) resulted in about a 2.5-fold reduction (0.187–0.072) in the surviving fraction at 2 Gy. If radiosensitization of this magnitude were sustained for even just 35–45% of the radiation fractions, tumor control probability would be expected to increase substantially (37). Radiotherapy dose-escalation studies (3–5, 38) have established that most radiation failures are attributable to local persistence of disease and that more aggressive local therapy is justified. Gene therapy is an ideal approach in this setting.

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Serum testosterone is not a correlate of prostate cancer lymph node involvement, but does predict biochemical failure for lymph node positive disease

Jason F. Kelly, M.D., Alan Pollack, M.D., Ph.D.*, Gunar K. Zagars, M.D.

Department of Radiation Oncology, The University of Texas, M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

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Abstract

Previously we found that serum testosterone (serum-T) correlated with the development of distant metastasis in patients with clinically localized prostate cancer treated with radiotherapy. In this report, the relationship of serum-T to lymph node positivity and to patient outcome for patients with regional lymph node involvement treated with androgen ablation alone was investigated. Serum-T was available in 514 of 854 men with clinically localized prostate cancer who underwent pelvic lymphadenectomy at M.D. Anderson Cancer Center between 1984 and 1993. Pretreatment prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) were assayed in 98% and 95% of patients, respectively. Androgen ablation was achieved via orchiectomy or a luteinizing hormone releasing hormone agonist. Median follow-up was 66 months for the node positive subgroup ($n = 92$). Serum-T did not correlate with palpable stage, Gleason score, pretreatment PSA, or lymph node involvement. Age ≤ 60 years and pretreatment PAP > 0.8 mU/ml correlated significantly with higher serum-T. In lymph node positive patients treated with androgen ablation, higher serum-T levels corresponded to both pretreatment PSA > 10 ng/ml and PAP > 0.8 mU/ml. Serum-T predicted for biochemical failure, but not metastatic relapse or overall survival. Actuarial 5-year biochemical failure rate was 73% for serum T > 500 ng/dl and 57% for serum-T ≤ 500 ($p = 0.009$). Multivariate analysis showed serum-T to be an independent correlate of rising PSA, both as a continuous ($p = 0.001$) or categorical ($p = 0.037$) variable. Serum-T did not significantly correlate with lymph node positivity, and therefore is not a marker for regional disease spread. However, serum-T was significantly associated with biochemical failure in node-positive patients treated with androgen ablation alone. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Testosterone; Prostate cancer; Lymphadenectomy; Androgen ablation

Testosterone has long been known to influence the development, growth, and progression of prostate cancer. Permanent androgen deprivation early in life correlates with an essential absence of risk for prostatic carcinoma, and androgen blockade after tumor formation usually results in regression. The precise relationship between serum testosterone (serum-T) values and clinical outcome, however, is unclear and appears to be influenced by disease extent. In patients with metastatic prostate carcinoma treated with androgen ablation, elevated serum-T has been reported to be prognostic for improved outcome [1–5], but in men treated with radiation therapy for localized prostate cancer, high pretreatment serum-T (> 500 ng/dl) was recently correlated with metastatic relapse [6].

The relationship between pretreatment serum-T and regional lymph node spread is poorly understood [7]. More-

over, the association of serum-T with outcome in regional lymph node positive (Stage D1) patients treated with androgen ablation is unknown. These relationships were investigated in a large patient cohort that underwent pelvic lymphadenectomy as part of the initial evaluation for clinically localized prostate cancer.

1. Methods and materials

Between 1984 and 1993, 854 men with clinically localized prostate cancer underwent pelvic lymphadenectomy at the University of Texas, M.D. Anderson Cancer Center (MDACC) as a prelude to radical prostatectomy or radiation therapy. The surgical technique has been described previously [8]. Of the 854 patients, 514 also had pretreatment serum-T determination as part of their routine evaluation unrelated to their clinicopathologic profile; these patients form the parent cohort for this analysis. Total serum-T was measured with the Equate radioimmunoassay (Binax, Portland, Maine) with a reported normal range of 260 to 1,250 ng/dl.

* Corresponding author. Tel.: +001-713-792-3400; fax: +001-713-792-3642.

Most of the patients had pretreatment evaluation that included serum prostatic acid phosphatase (PAP) and prostate specific antigen (PSA) assays. PAP levels were measured using the enzymatic Roy method (upper normal limit 0.8 mU/ml) [9] in 504 patients (98%). The mean PAP was 0.36 ± 0.22 mU/ml (range 0.10–2.5, median 0.30). PSA values assayed prior to 1993 used the immunoenzymatic Hybritech test (lower limit 0.3 ng/ml), which was then replaced with the TOSOH assay (lower limit 0.1 ng/ml). Pretreatment PSA results were available in 488 patients (95%). Mean pretreatment PSA was 16.5 ± 22.0 ng/ml (range 0.3–247.3, median 9.7). The men ranged in age from 35 to 77 years (mean 64, median 64). Clinical palpatory T-categories [10] were: T1, 115 (22%); T2, 216 (42%); and T3, 183 (36%). Gleason score (equal to the sum of two patterns) distribution was: Gleason 2, 3 (<1%); Gleason 3, 33 (6%); Gleason 4, 73 (14%); Gleason 5, 100 (20%); Gleason 6, 113 (22%); Gleason 7, 115 (22%); Gleason 8, 57 (11%); Gleason 9, 16 (3%); and Gleason 10, 4 (<1%).

Within this parent cohort, 92 men with pathologically positive regional lymph nodes were identified as receiving androgen ablation as their sole initial therapy. Androgen ablation was either surgical via bilateral orchiectomy or medical using a luteinizing hormone releasing hormone (LHRH) agonist most often without sustained peripheral androgen blockade. Follow-up duration for the lymph node positive patients was a mean of 65 months (range 19–115, median 66) with patients being seen approximately every 6 months with interval history, physical examination, serum PSA evaluation, and other testing as indicated. Biochemical failure was assessed in the node positive patients treated with androgen ablation and was defined as two or more successive rises in the PSA above the nadir value. Distant metastases were verified by imaging studies.

The nonparametric Mann-Whitney test was performed to evaluate differences between means [11]. The Spearman

and Kendall tests were used where appropriate [11]. Actuarial curves were calculated using the Kaplan-Meier and Berkson-Gage methods, and the log-rank statistic was applied to assess the significance of differences between the curves [12]. A proportional hazards model with Cox's log-linear hazard function was used for multivariate analysis [12].

2. Results

Mean serum testosterone for the parent cohort was 446 ± 173 ng/dl (range 22–1,600, median 423). Serum-T levels did not correlate with palpable stage, Gleason score, pretreatment PSA, or lymph node involvement (Table 1). The only factors that correlated with serum-T were age and pretreatment PAP. The significance of the latter relationship was driven by the 10 patients with elevated PAP levels >0.8 mU/ml, in whom serum-T was higher (542 ± 127 ng/dl).

When serum-T was analyzed as a dichotomous variable ($T \leq 500$ vs. >500 ng/dl), as described previously [2], only pretreatment PAP showed a trend toward significance (Table 2). No relationship was observed between serum-T as a categorical variable and age, palpable stage, Gleason score, PSA, or lymph node status.

For the lymph node positive patients treated with androgen ablation the mean serum-T was 450 ± 168 ng/dl (range 93–945, median 420). In this subgroup a significant relationship between serum-T and both pretreatment PSA and PAP levels was found (Table 3). Pretreatment PSAs >10 were significantly associated with higher serum-T values. A positive correlation was also seen for pretreatment PAP >0.8mU/ml. This same association was again found for pretreatment PAP, but not PSA, when serum-T was analyzed as a dichotomous variable (≤ 500 vs. >500 ng/dl) in this lymph node positive subset (Table 4).

In terms of patient outcome, there were a total of 42 biochemical failures, 16 distant failures, and 19 deaths among

Table 1
Pretreatment serum testosterone for all patients by potential prognostic factors

Grouping		Number	Serum-T (ng/dl; mean \pm SD)	P-value*
Age	≤ 60	142	470 ± 175	0.030
	> 60	372	437 ± 171	
Palpable stage	T1/T2	331	453 ± 178	0.25
	T3	183	434 ± 161	
Gleason score	2–6	322	444 ± 167	0.95
	7–10	192	449 ± 181	
Pretreatment PSA	≤ 10	252	451 ± 185	0.73
	> 10	236	450 ± 162	
Pretreatment PAP	≤ 0.4	393	448 ± 181	0.037**
	$> 0.4 \leq 0.8$	101	424 ± 140	
	> 0.8	10	542 ± 127	
Lymph node positive	No	383	449 ± 172	0.37
	Yes	131	438 ± 173	

* Mann-Whitney nonparametric test.

** Kendall-Spearman correlation.

PSA, prostate specific antigen; PAP, prostatic acid phosphatase.

Table 2
Distribution of all patients by pretreatment serum testosterone (ng/dl) as a dichotomous variable

Grouping		Patient % (number)		P-value*
		Serum-T \leq 500	Serum-T > 500	
Age	\leq 60	26 (93)	32 (49)	0.16
	>60	74 (267)	68 (105)	
Palpable stage	T1/T2	64 (229)	66 (102)	0.57
	T3	36 (131)	34 (52)	
Gleason score	2-6	62 (224)	64 (98)	0.76
	7-10	38 (136)	36 (56)	
Pretreatment PSA	\leq 10	53 (177)	49 (75)	0.49
	>10	47 (159)	51 (77)	
Pretreatment PAP	\leq 0.4	76 (270)	82 (123)	0.065
	>0.4 \leq 0.8	22 (79)	15 (22)	
	>0.8	1 (5)	3 (5)	
Lymph node positive	No	74 (265)	77 (118)	0.47
	Yes	26 (95)	23 (36)	

* Chi-square.

PSA, prostate specific antigen; PAP, prostatic acid phosphatase.

the men with involved lymph nodes. With respect to the crude failure rates, mean serum-T only correlated with biochemical failure (Table 3); this association was not evident when serum-T was categorized (Table 4). However, serum-T as a categorical variable correlated with actuarial biochemical failure, which was 73% at 5 years for serum-T > 500 ng/dl ($n = 26$) and 57% for serum-T \leq 500 ($n = 62$) (Figure 1). Cox proportional multivariate analysis showed serum-T to be an independent correlate of rising PSA, both as a continuous ($P = 0.001$) or categorical ($P = 0.037$) variable. Other significant covariates were Gleason score ($P = 0.047$) when serum-T was used as a continuous variable and pretreatment PSA ($P = 0.077$) when serum-T was used as a

categorical variable. No correlation of serum-T to actuarial distant metastases was observed (Figure 2).

3. Discussion

The prognostic usefulness of pretreatment serum testosterone levels in patients with prostate cancer is unclear. For patients with distant metastatic disease treated with androgen ablation, high pre-ablation serum-T has been shown to correlate with improved response and survival [1-5]. In contrast, we reported previously that high pretreatment serum-T predicted the earlier development of distant metasta-

Table 3
Pretreatment serum testosterone for lymph node positive patients treated with androgen ablation alone by potential prognostic factors

Grouping		Number	Serum-T	P-value*
			(ng/dl; mean \pm SD)	
Age	\leq 60	17	486 \pm 153	0.25
	>60	75	442 \pm 171	
Palpable stage	T1/T2	20	427 \pm 151	0.43
	T3	72	457 \pm 173	
Gleason score	2-6	38	480 \pm 170	0.15
	7-10	54	429 \pm 164	
Pretreatment PSA	\leq 10	19	391 \pm 145	0.019
	>10	60	490 \pm 174	
Pretreatment PAP	\leq 0.4	56	422 \pm 161	0.037**
	>0.4 \leq 0.8	29	470 \pm 176	
	>0.8	7	590 \pm 118	
Rising PSA	No	50	412 \pm 153	0.023
	Yes	42	496 \pm 175	
Distant relapse	No	76	451 \pm 171	0.83
	Yes	16	448 \pm 159	
Alive	No	19	428 \pm 166	0.49
	Yes	73	456 \pm 169	

* Mann-Whitney nonparametric test.

** Kendall-Spearman correlation.

PSA, prostate specific antigen; PAP, prostatic acid phosphatase.

Table 4
Distribution of patients with positive lymph nodes by pretreatment serum testosterone (ng/dl) and potential prognostic factors

Grouping		Patient % (number)		P-value*
		Serum-T ≤ 500	Serum-T > 500	
Age	≤60	16 (10)	25 (7)	0.29
	>60	84 (54)	75 (21)	
Palpable stage	T1/T2	23 (15)	18 (5)	0.55
	T3	77 (49)	82 (23)	
Gleason score	2-6	42 (27)	39 (11)	0.79
	7-10	58 (37)	61 (17)	
Pretreatment PSA	≤10	29 (15)	14 (4)	0.13
	>10	71 (36)	86 (24)	
Pretreatment PAP	≤0.4	66 (42)	50 (14)	0.042
	>0.4 ≤ 0.8	31 (20)	32 (9)	
	>0.8	3 (2)	18 (5)	
Rising PSA	No	59 (38)	43 (12)	0.14
	Yes	41 (26)	57 (16)	
Distant relapse	No	83 (53)	82 (23)	0.94
	Yes	17 (11)	18 (5)	
Alive	No	22 (14)	18 (5)	0.66
	Yes	78 (50)	82 (23)	

* Chi-square.

PSA, prostate specific antigen; PAP, prostatic acid phosphatase.

sis in clinically localized (T1-3, NX or N0, M0) adenocarcinoma of the prostate treated with radiotherapy [6]. In the current study of patients with regional lymph node metastasis treated with androgen ablation, serum-T was a strong

correlate of biochemical failure, but not distant metastasis. Although serum-T has consistently been found to predict outcome for patients with prostate cancer, extent of disease influences the nature of this relationship. The following dis-

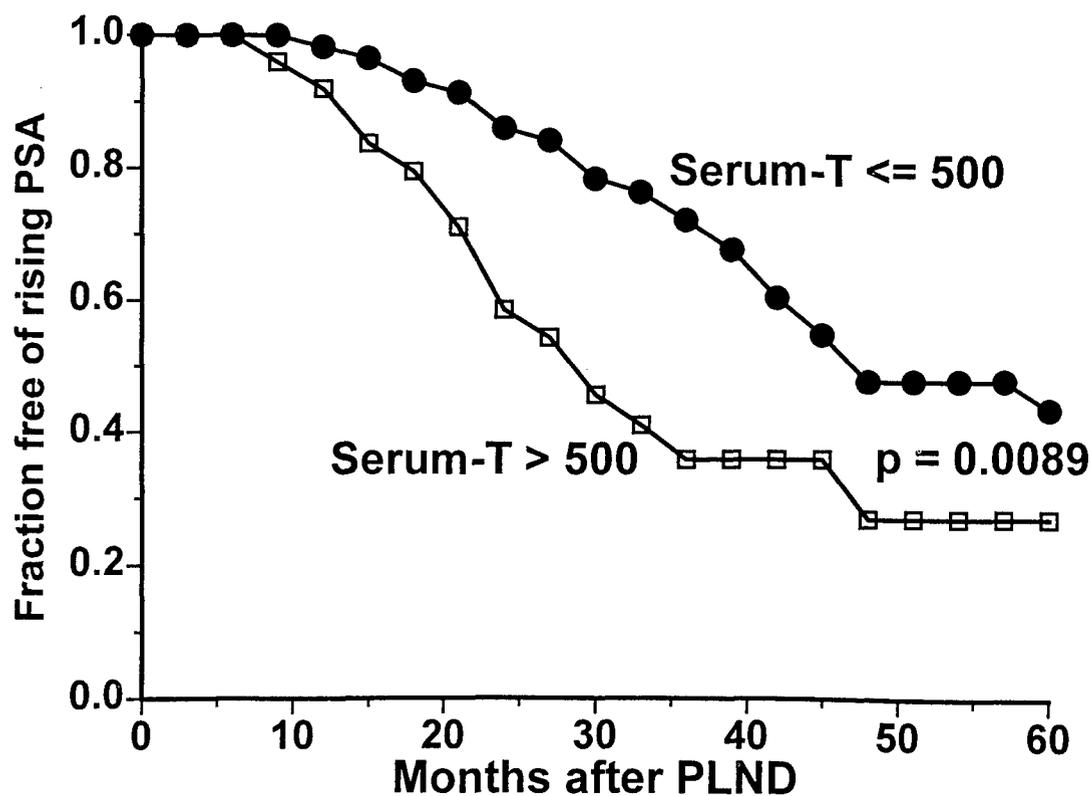


Fig. 1. Actuarial freedom from biochemical failure by serum testosterone (Serum-T) for lymph node positive patients treated with androgen ablation. PSA, prostate specific antigen, PLND, positive lymph node disease.

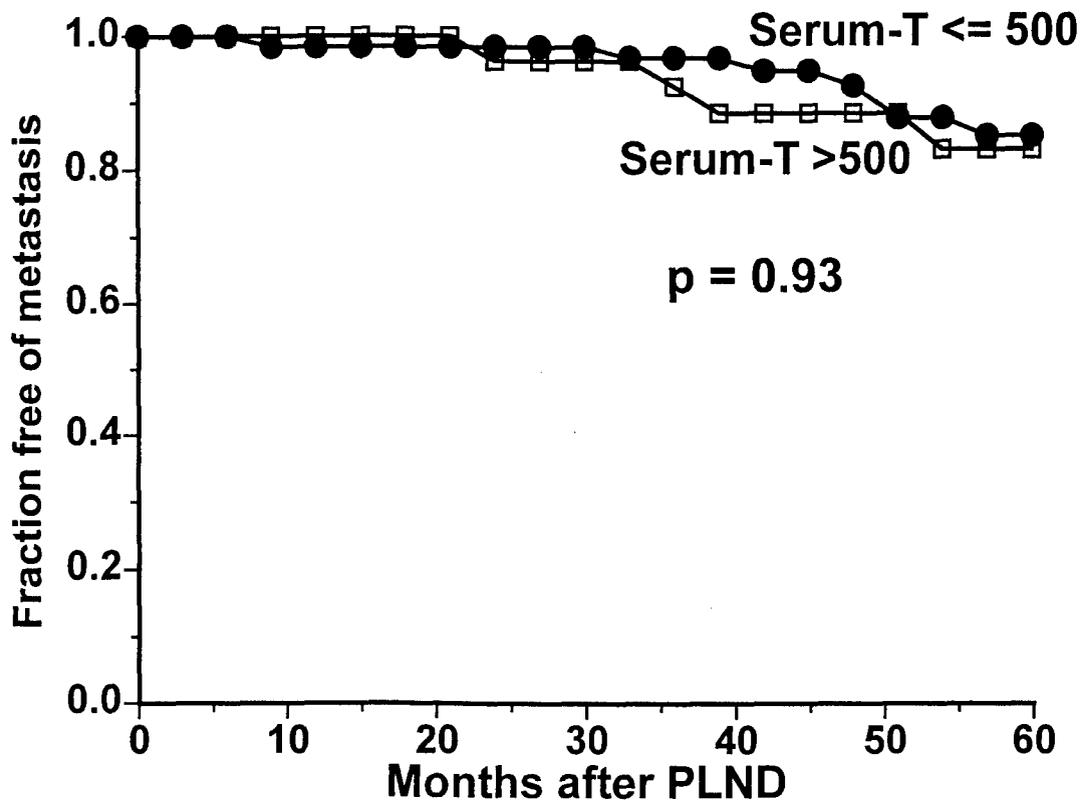


Fig. 2. Actuarial freedom from distant metastases by serum testosterone (Serum-T) for lymph node positive patients treated with androgen ablation. PLND, positive lymph node disease.

cussion examines the possible reasons for these differences and the potential value of serum-T measurements.

3.1. Serum-T as a correlate of distant metastasis in clinically localized prostate cancer treated with radiotherapy

In our prior study [6], pretreatment serum-T was related only to distant metastasis; no association with biochemical failure was observed. One explanation for these findings is that in the setting of clinically localized prostate cancer treated in the PSA era with radiotherapy, a rising PSA represents local persistence/progression of disease much more often than distant metastasis [13,14]. Although there are patients whose disease metastasizes distantly soon after the completion of treatment in whom a rising PSA represents distant failure, these are a small subset of the total. High serum-T levels would presumably be maintained during and after radiotherapy and therefore, could promote a more rapid progression of local failures to distant failures [15,16]. One would then expect that the correlation of pretreatment serum-T to distant metastasis would be sustained. In this setting, pretreatment serum-T should eventually be associ-

ated with biochemical failure, because biochemical failure would correlate more strongly with distant metastasis over time.

3.2. Serum-T as a correlate of biochemical failure in lymph node positive prostate cancer treated with androgen ablation

The data described herein extend our previous observations, indicating that pretreatment serum-T is indeed a determinant of patient outcome. However, in lymph node positive patients treated with androgen ablation, high pretreatment serum-T was related to biochemical failure and not distant metastasis. In this population, serum-T levels are reduced during androgen ablation, and the principle effect on disease progression would probably be the result of altering response to androgen ablation. At face value, a high pretreatment serum-T could be interpreted as causing a diminished response to androgen ablation. The problem with this potential mechanism is that a reduced response to androgen ablation is incongruous with several studies of patients with distant metastatic disease. In patients with distant metastases treated with androgen ablation, a high serum-T:

was related to a more favorable outcome [1–5]. Another possibility is that high pretreatment serum-T predisposes to micrometastases not visualized on bone scan. This mechanism would be consistent with the findings in clinically localized patients that suggest that the principle effect of serum-T is an alteration in the rate of progression to distant metastasis. Once distant metastases develop, a sustained high serum-T leads to a better response to androgen ablation; patients with distant metastasis in the setting of a low serum-T metastasize as a consequence of other factors and have a poorer response to androgen ablation.

The reason that serum-T was associated with biochemical failure in lymph node positive patients treated with androgen ablation and distant metastasis in patients with clinically localized disease treated with radiotherapy is probably due, at least in part, to the contrasting relationship of biochemical failure to distant metastasis. As described above, several lines of evidence [13,14] indicate that biochemical failure is most representative of persistent/progressive disease in the prostate in patients with clinically localized prostate cancer. This relationship is much less clear for lymph node positive patients.

Distant metastases comprise approximately 50% of all failures at 5 years [17] in lymph node positive patients treated with androgen ablation versus approximately 5% for clinically localized prostate cancer [18]. The regional metastases present in lymph node positive (stage D1) patients predispose to already having subclinical distant metastases at diagnosis and a more rapid onset of progression to distant metastases. In these patients, a rising PSA signifies hormone refractory disease and it is certain that the majority have at least microscopic distant metastases. Lankford et al. [19] reported that prostate radiotherapy rarely results in sustained freedom from biochemical failure in patients with local progression following androgen ablation. Thus, high pretreatment serum-T could predispose to micrometastatic disease, and subsequent biochemical failure may herald progression at these distant sites prior to documentation by imaging studies. The finding that serum-T did not correlate with distant failure in lymph node positive patients could be related to type 2 error, due to the small absolute number of patients failing distantly ($n = 16$). In fact, tumor grade, which is a strong correlate of distant spread in most prostate cancer populations including those with positive lymph nodes treated with androgen ablation [17], was not a significant correlate in this cohort. More events are needed to evaluate the relationship of serum-T to distant metastasis. This relationship may yet become evident with further follow-up.

It is important to note that, although serum-T was related to biochemical progression, there was no significant association with lymph node positivity rates (Table 1). Monda et al. [7] also found that serum-T was not associated with lymph node positivity in a smaller group of patients that underwent pelvic lymph node dissection before planned radical prostatectomy. High levels of serum-T do not appear to influence regional-nodal spread.

4. Summary and conclusions

High pretreatment serum-T levels have been shown to be independently related to patient outcome in two prostate cancer cohorts analyzed at MDACC. In the cohort described herein, in lymph node positive patients treated with androgen ablation, high serum-T was associated with increased biochemical failure. However, a number of studies have shown that high serum-T correlates with a more favorable prognosis [1–5]. We hypothesized that high pretreatment serum-T promotes the more rapid evolution of distant metastasis and that the increased biochemical failure in the lymph node positive patients was an early marker of the clinical manifestation of this process. Although this hypothesis ties the findings of these two groups together, a number of variables that act on the development of androgen insensitivity, such as androgen receptor mutations [20], have not been considered. These results must be confirmed by other groups.

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BIOLOGY CONTRIBUTION

ADENOVIRAL-MEDIATED P53 TRANSGENE EXPRESSION SENSITIZES BOTH WILD-TYPE AND NULL P53 PROSTATE CANCER CELLS *IN VITRO* TO RADIATION

PHILIP J. COLLETIER, M.D.,*¹ FARAMARZ ASHOORI, M.D.,[†]
DIDIER COWEN, M.D.,^{†2} RAYMOND E. MEYN, PH.D.,[†] PHILIP TOFILON, PH.D.,[†]
MARVIN E. MEISTRICH, PH.D.,[†] AND ALAN POLLACK, M.D., PH.D.*

Departments of *Radiation Oncology and [†]Experimental Radiation Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

Purpose/Objective: The effect of adenoviral-mediated p53 transgene expression on the radiation response of two human prostate cancer cell lines, the p53^{wild-type} LNCaP and p53^{null} PC3 lines, was examined. The objective was to determine if this vector sensitizes cells to radiation independently of their p53 status.

Methods and Materials: A recombinant adenovirus-5 vector (RPR/INGN 201, Introgen Therapeutics, Houston, TX) containing a CMV promoter and wild-type p53-cDNA (Ad5-p53) was used to facilitate p53 transgene expression. A multiplicity of infection (MOI) of 10–40 viral particles per cell was used, based on Ad5/CMV/lacZ infection and staining for the β -galactosidase reporter gene product. Clonogenic assays were performed to evaluate the degree of sensitization to radiation of viral-transduced cells compared with irradiated nontransduced controls. The relative efficacy of these treatments to induce apoptotic cell death was determined using the TUNEL assay.

Results: The delivery of Ad5-p53 (10 MOI) reduced control plating efficiency from 36.5% to 0.86% in the LNCaP cell line and from 75.1% to 4.1% in the PC3 cell line. After correcting for the effect of Ad5-p53 on plating efficiency, the surviving fraction after 2 Gy (SF2) of gamma-irradiation was reduced over 2.5-fold, from 0.187 to 0.072, with transgene p53 expression in the LNCaP cell line. Surviving fraction after 4 Gy (SF4) was reduced over 4.5-fold, from 0.014 to 0.003, after Ad5-p53 treatment. In the PC3 cell line, Ad5-p53 (40 MOI) reduced SF2 over 1.9-fold from 0.708 to 0.367, and SF4 over 6-fold from 0.335 to 0.056. In both the LNCaP and PC3 cell lines, the combination of Ad5-p53 plus radiation (2 Gy) resulted in supra-additive apoptosis (~20% for LNCaP and ~15% for PC3 at 50 MOI), above that seen from the addition of the controls; control vector Ad5-pA plus RT (0.15% for LNCaP and 1.44% for PC3), Ad5-p53 alone (28.6% for LNCaP and 21.7% for PC3), RT alone (0% for LNCaP and 0.23% for PC3), or Ad5-pA alone (0.1% for LNCaP and 0.29% for PC3).

Conclusion: The clonogenic survival and apoptosis data demonstrate that p53 transgene expression sensitizes human prostate adenocarcinoma cells *in vitro* to irradiation. As this effect was observed in both the p53^{wild-type} LNCaP and p53^{null} PC3 lines, radiosensitization was independent of p53 status. © 2000 Elsevier Science Inc.

Prostate cancer, Gene therapy, p53, Radiotherapy, Apoptosis.

INTRODUCTION

The process by which irradiated cells die has been shown to involve two principal mechanisms: mitotic and apoptotic cell death. The importance of apoptosis as a mechanism of radiation-induced cell death has been found to vary greatly according to cell type, being most prevalent in lymphomas and essentially absent in sarcomas (1). Apoptosis is also

present as a cause of cell death in irradiated normal tissues. In prostate cancer, previous work from our laboratories has shown that early apoptosis at 3–24 h after radiation does not appear to be the dominant mechanism of cell death (2, 3). Although current data suggest that apoptosis is a secondary mechanism of cell death from radiation, alterations in the expression of proteins that regulate this pathway have been associated with resistance to treatment and the development

Reprint requests to: Alan Pollack, M.D., Ph.D., Department of Radiation Oncology, 1515 Holcombe Boulevard (Box 97), Houston, TX 77030. Tel: (713) 792-0781; Fax: (713) 794-5573; E-mail: Apollack@notes.mdacc.tmc.edu

¹ Current address: Therapeutic Radiology Associates, PC, 411 Laurel St, #C-100, Des Moines, IA 50314.

² Current address: Institut Paoli Calmettes, 232 Blvd Sainte Marguerite, Marseille 13009, France.

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of hormone refractory disease (4, 5). The hypothesis that formed the basis for the studies described here was that prostate cancer cell death could be enhanced overall by manipulating the intracellular molecular processes that govern apoptosis in response to radiation.

Apoptosis propensity has been found to be variably dependent on the presence of the tumor suppresser gene p53 (6–10). The response of cells with wild-type p53 status to ionizing radiation is characterized by a rise in the level of p53 protein within hours of treatment (8). This increase in p53 precedes G1 arrest and apoptosis, and p53 has been found to have a central role in these responses. Transfection of p53 mutant cells with wild-type p53 plasmids has induced both G1 arrest and apoptosis in the absence of other stressors such as chemotherapy and radiation (11, 12). Enhancement of p53 expression through gene therapy has been shown to induce apoptosis in several cell lines, including prostatic carcinomas. These findings have naturally led to intensive investigation as to whether replacement of p53^{wild-type} status, and attendant control of the cell cycle, might restore apoptosis and enhance radiation response via this mechanism of cell death. In this report, we investigate whether p53 transgene expression, resulting from a gene therapy approach, is effective at sensitizing p53^{wild-type} and p53^{null} human prostate cell lines, and whether apoptosis is a major cell death mechanism under these conditions.

METHODS AND MATERIALS

Cell culture

The p53^{wild-type} LNCaP and p53^{null} PC3 prostate cancer cell lines were chosen to study the effects of transgene p53 expression on radiation response. These cell lines were obtained from the American Type Culture Collection. All cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum, 1% 200 mM L-glutamine, 1% 10,000 IU/mL Pen-Strep solution, and incubated in a 5% CO₂ incubator at 37°C.

Adenoviral vector

The Ad5-p53 adenoviral vector (RPR/INGN 201, Houston, TX) used in this experiment has been described previously (13). The p53 expression cassette consists of a genome 35.4 kb in size. The replication defective vector includes a human cytomegalovirus (CMV) promoter, human wild-type p53 cDNA, simian virus 40 early polyadenylation signal, two cDNA-specific primers, and two viral genome-specific primers. This replaces the E1 region of the Ad5 genome. Before transduction, purified virus was aliquoted so that all virus had been subjected to the same number of freeze-thaw cycles. Infection of cell lines was accomplished by dilution of viral stock to the multiplicity of infection (MOI) value of 10–40 viral particles per cell, based on infection with Ad5/CMV/lacZ and staining for the β -galactosidase reporter gene product (14). This Ad5- β gal vector was utilized in some experiments as a control vector.

We also used a polyadenylation sequence-only vector (Ad5-pA) as a control in some experiments.

Gene transduction and cell line irradiation for clonogenic survival

A total of 5×10^5 cells were plated into sterile T25 flasks (Falcon Plastics, Lincoln Park, NH) and, typically, 2×10^6 cells were available for transduction in each flask after 48 h. Virus was diluted in serum-free DMEM/F12 until ready for transduction. The cells in each flask were washed in phosphate-buffered solution to remove any residual serum that might bind with the virus and decrease the MOI. The viral solution (1 mL) was then gently placed onto the monolayer. The flasks were returned to the incubator for a total of 1 h. At 10-min intervals, the flasks were gently rocked to ensure even mechanical distribution of the viral solution over the cells. Control flasks, with and without control vector, were exposed to identical manipulations during this process. After 1 h in the incubator, 4 mL of complete medium with serum was added to each flask. This effectively ended the transduction process. The flasks were then returned to a dedicated incubator. Forty-eight hours after viral exposure, flasks were removed from the incubator and placed on ice for 20 min. Flasks were then irradiated with a high dose-rate cesium unit (4 Gy/min). Immediately after irradiation, flasks were trypsinized, serial dilutions performed, and known numbers of cells replated into 100-mm dishes. The plates were incubated for approximately 12 days for macroscopic colony formation. The colonies were stained with gentian violet, and counted.

The surviving fraction relative to the unirradiated cells was calculated. Triplicate determinations of each radiation dose and dilution were performed in every experiment and an intraexperiment average calculated. The points shown on the clonogenic survival curves are the interexperiment averages calculated from the intraexperiment averages.

TUNEL staining

Apoptosis was measured using a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay. The procedure involved culturing, fixing, and staining the cells directly on glass slides. The slides were prepared for cell culture by marking a 1.5–2.0-cm circle with a hydrophobic slide marker (Research Products International, Mount Prospect, IL), cleaning with 70% ethanol for 30 min, and sterilizing by UV lamp exposure (30 W) for 30 min at a 20-cm distance. The sterile slides were placed in a 100-mm cell culture dish and 150 μ l of culture medium containing cells (3×10^4 for PC3 and 7×10^4 for LNCaP) was placed into the center of each slide. The cells were cultured for 24 h to allow attachment and then an additional 15 mL was added to the plates. The plates were incubated another 2–4 days, until an even distribution of nonconfluent cells was present. The cells on the slide monolayers were counted using an inverted microscope with a grid and then treated by first rinsing in 15 mL of PBS and adding 100 μ l of viral suspension at the desired MOI in

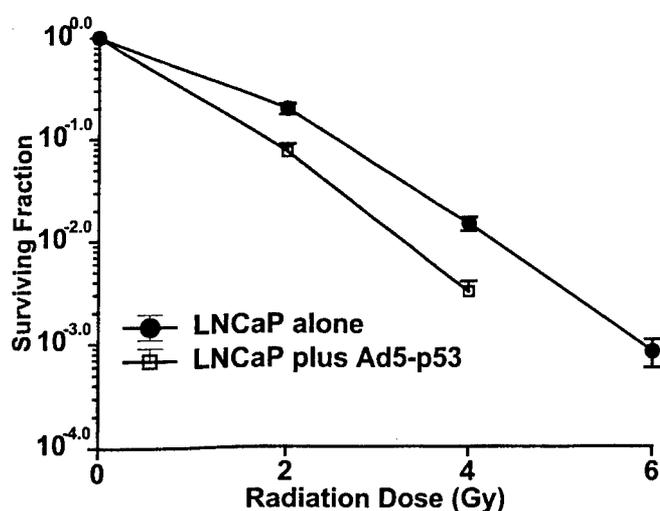


Fig. 1. Clonogenic surviving fraction of LNCaP cells without (solid circles) and with Ad5-p53 (open squares). The error bars represent one standard deviation above and below the mean.

serum-free medium. The cells were incubated for 1 h at 37°C, and 100 μ L of complete medium with serum added. The cells were further incubated another 24 h before irradiation, with 2 or 8 Gy given in a single fraction. After irradiation, the cells were incubated for 6 h at 37°C, the medium removed, and the cells fixed in 4% formaldehyde for 30 min. The formaldehyde was then removed and the slides placed in 70% ethanol. The TUNEL staining was done within a few days of fixation.

The TUNEL staining of apoptotic cells was accomplished using the ApopTag (Oncor, Gaithersburg, MD) kit (15). The apoptotic index was calculated by dividing the number of apoptotic cells by the total number of cells (16). The cells were counterstained with hematoxylin. Positive controls were included with each group of samples stained.

RESULTS

Radiosensitization of LNCaP cells using Ad5-p53

Eleven control experiments were performed to accurately characterize the clonogenic response of LNCaP cells to irradiation without the presence of viral vector. Four experiments were then performed with Ad5-p53 plus radiation. An MOI of 10 was selected based on preliminary data that demonstrated approximately 50% transduction efficiency. Treatment with Ad5-p53 reduced control plating efficiency from 36.5% to 0.86% in the LNCaP cell line. After normalizing data to correct for the plating efficiencies, the surviving fraction after 2 Gy of γ -irradiation (SF2) was 0.187 without Ad5-p53 compared to 0.072 with the vector (Fig. 1). Similarly, the surviving fraction after 4 Gy (SF4) was reduced from 0.0143 to 0.0031 with Ad5-p53 exposure. These differences were statistically significant ($p < 0.05$, Student's *t*-test).

To demonstrate that the radiosensitization was due to the presence of p53 in the viral vector, rather than nonspecific

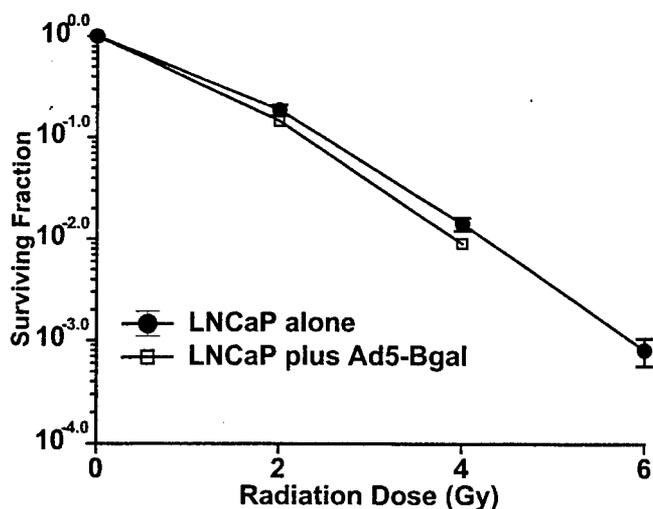


Fig. 2. Clonogenic surviving fraction of LNCaP cells without (solid circles) and with Ad5- β gal (open squares). The error bars represent one standard deviation above and below the mean.

effects of the vector itself, experiments were performed using Ad5- β gal. Significant radiosensitization of LNCaP cells with Ad5- β gal was not observed (Fig. 2). The plating efficiency of LNCaP cells exposed to Ad5- β gal at 10 MOI was the same as without any virus.

Radiosensitization of PC3 cells using Ad5-p53

Very reproducible clonogenic cell survival results were observed in PC3 cells irradiated in the absence of virus. The PC3 cell line was quite susceptible to Ad5-p53 alone, exhibiting a reduction in control plating efficiency from 75.1% to 4.1% (Fig. 3). The SF2 after adjusting for plating efficiency was 0.708 without vector and 0.367 with the addition of Ad5-p53 at 40 MOI. The SF4 decreased from 0.335 without virus to 0.056 with transduction. These differences were statistically significant ($p < 0.05$, Student's *t*-test).

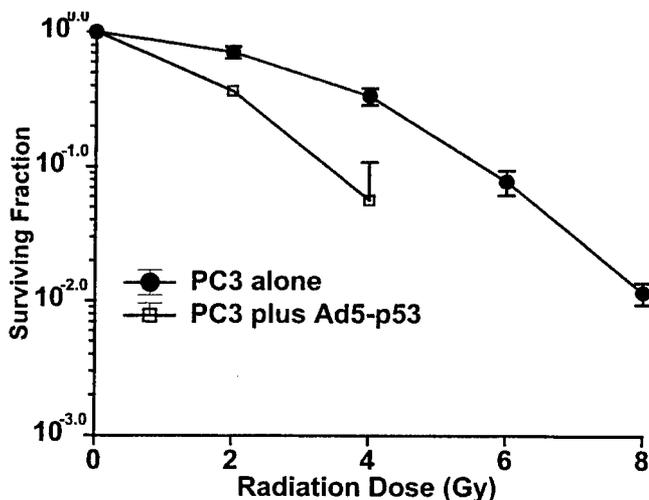


Fig. 3. Clonogenic surviving fraction of PC3 cells without (solid circles) and with Ad5-p53 (open squares). The error bars represent one standard deviation above and below the mean.

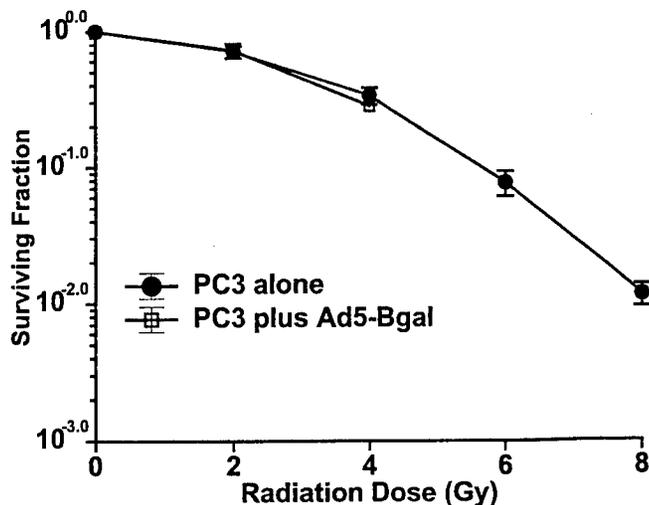


Fig. 4. Clonogenic surviving fraction of PC3 cells without (solid circles) and with Ad5- β gal (open squares). The error bars represent one standard deviation above and below the mean.

Figure 4 demonstrates that Ad5- β gal did not significantly radiosensitize PC3 cells. The plating efficiency of PC3 cells exposed to Ad5- β gal at 40 MOI was 24%, as compared to without virus, and 457%, as compared to Ad5-p53. The plating efficiency of PC3 cells exposed to Ad5-p53 was 5.25%, as compared to without virus. Thus, a reduction on clonogenic survival from Ad5- β gal was seen at MOIs of 40%, but this reduction was much less than that seen with Ad5-p53.

Apoptotic response to Ad5-p53 plus 2-Gy radiation

The *in vitro* apoptotic response of the LNCaP and PC3 cell lines to Ad5-p53 plus radiation is shown in Table 1. The control virus reported in these studies was Ad5-pA; the results with Ad5- β gal and Ad5-pA were similar in clonogenic and apoptosis experiments. The results revealed a

Table 1. Apoptotic response of LNCaP and PC3 cells to Ad5-p53 plus 2-Gy radiation using the TUNEL assay

Vector	2-Gy RT	Apoptotic Index*	
		LNCaP	PC3
No	No	0	0.0067 \pm 0.0067
No	Yes	0	0.23 \pm 0.21
Ad5-p53 25 MOI	No	1.24 \pm 0.50	0.63 \pm 0.42
Ad5-p53 25 MOI	Yes	21.43 \pm 9.19	6.69 \pm 5.74 [†]
Ad5-p53 50 MOI	No	28.63 \pm 12.97	21.70 \pm 1.59
Ad5-p53 50 MOI	Yes	48.62 \pm 16.73	37.93 \pm 0.66 [†]
Ad5-pA 50 MOI	No	0.10 \pm 0.07	0.29 \pm 0.20
Ad5-pA 50 MOI	Yes	0.15 \pm 0.15	1.44 \pm 1.32

* Mean (\pm SEM) of 3 or more values from separate experiments; 2,000 cells counted per group. The radiation was administered 24 h after viral exposure and the cells fixed for scoring of apoptosis 6 h after irradiation.

[†] $p < 0.05$ by one-way ANOVA using least significant difference test, as compared to above group without radiation.

MOI = multiplicity of infection.

significant supra-additive increase in apoptosis in both cell lines when this treatment combination was used, as compared to the controls. These findings suggest that apoptosis may be a significant mechanism of cell death when prostate cancer cells are treated with Ad5-p53 plus irradiation.

DISCUSSION

Radiotherapy is the most common treatment for high-risk prostate cancer. However, through the use of PSA to monitor the efficacy of treatment, it has become apparent that few high-risk patients are cured with radiotherapy alone (17, 18); the results with radical prostatectomy are probably worse (19, 20). While a proportion of such high-risk patients fail distantly early after the onset of a rising PSA, there is evidence to suggest that the main site of initial failure is local (21). Consequently, novel techniques for radiosensitization hold promise for improving the cure fraction. One strategy is to combine androgen ablation with radiation. Preliminary evidence indicate radiosensitization occurs when androgen ablation and radiation are applied in a particular sequence (2). However, the clinical trials that have been published to date (22, 23) do not sort out the advantage of androgen ablation plus radiation over androgen ablation alone, making conclusions of real benefit of the combination unclear. Even if androgen ablation does sensitize prostate cancer cells to radiation, there is a need to develop new methods of radiosensitization based on molecular mechanisms. Our approach has been to alter the intracellular milieu on a molecular level such that cell death via apoptosis is the favored pathway following exposure to radiation.

The p53 gene product is prototypical of a gene therapy target that is integral to radiation response. Gene therapy strategies based on p53 have been shown to radiosensitize a number of different types of human tumor cells. This includes cancers cells of the colon (14), head and neck (24, 25), ovary (26), and brain (27, 28). The intent of the present study was to examine whether this gene therapy approach could be extended to cancer of the prostate and, because most regionally localized prostate cancers express wild-type p53, to determine if prostate lines with differing p53 status would have similar responses to this treatment.

The data presented here indicate that Ad5-p53 transduction into cultured human prostate adenocarcinoma cells results in reduced clonogenicity from the vector alone, as well as sensitization to irradiation. The exposure of p53^{wild-type} LNCaP cells to Ad5-p53 at 10 MOI reduced clonogenicity by over 40-fold. When these cells were treated with radiation plus Ad5-p53 there was a further 2.5–4.5-fold reduction in surviving fraction after correcting for the effects of Ad5-p53 alone, as compared to radiation alone. Similar, if slightly more enhanced, effects were observed for p53^{null} PC3 cells. The data indicate a substantial cell killing action from the vector that is compounded by single fraction radiation. Moreover, there is potential for radiosensitization to be manifest in each repeated radiation

fraction for the duration of transgene expression, which is estimated to be 5–7 days (29, 30). That these effects were observed both in the p53^{wild-type} LNCaP and p53^{null} PC-3 cell lines indicates the independence on p53 status and the broad applicability of this strategy in prostate cancers with divergent molecular phenotypes.

The results suggest that the mechanism of Ad5-p53 toxicity is not exclusively due to the restoration of "normal" p53 function, because LNCaP cells express wild-type p53. We have also observed that p53 expression in LNCaP cells is enhanced within hours of irradiation (data not shown), typical of other cell lines with functional p53. The data presented here demonstrate that even in the presence of wild-type p53, the induction of p53 transgene overexpression by Ad5-p53 promotes apoptosis as the preferred response after irradiation. The greatest radiosensitization by Ad5-p53, however, was seen with p53 replacement in p53^{null} PC3 cells. PC3 cells were radiosensitized to a slightly greater degree than p53^{wild-type} LNCaP cells, as determined on the basis of dose modification factors (DMF) calculated at the 10% survival levels from Figs. 1 and 3. The DMF for PC3 was 1.67 versus 1.42 for the LNCaP line. The observation of supra-additive toxicity in both cell lines

attests to the potential application of Ad5-p53 for the sensitization of prostate cancer cells to radiation, independent of pretreatment p53 status.

In conclusion, the use of p53 gene therapy has made it possible to preferentially induce cell death to irradiation rather than transient G1 arrest and unwanted repair. The data presented here show that Ad5-p53 causes significant prostate cancer cell killing, as well as radiosensitization. Localized high-risk prostate cancer has many potential advantages as a model for the testing of gene therapy (31). Prostate cancer has a long natural history, which ameliorates some of the concerns of an inefficient delivery system by virtue of the fact that repeated applications are possible and anatomically the prostate is relatively easy to access via transperineal injection of gene vector delivery systems. With the advent of PSA as both a screening and follow-up endpoint, it has become clear that the eradication of prostate cancer is more difficult than was previously believed. Using the pretreatment prognostic factors of PSA, Gleason score, and palpatory stage, patients at high risk of failing radiotherapy alone may be identified and targeted using gene therapy techniques.

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3D-CRT

EXTERNAL BEAM RADIOTHERAPY DOSE RESPONSE CHARACTERISTICS OF 1127 MEN WITH PROSTATE CANCER TREATED IN THE PSA ERA

ALAN POLLACK, M.D., PH.D., LEWIS G. SMITH, M.D., AND ANDREW C. VON ESCHENBACH, M.D.

Departments of Radiation Oncology, and Urology, The University of Texas, M.D. Anderson Cancer Center, Houston, TX

Purpose: To characterize the relationship of radiotherapy dose to prostate cancer patient outcome, with an emphasis on the influence of pretreatment prognostic variables.

Methods and Materials: The 1127 Stage T1-T4 prostate cancer patients examined were treated consecutively with definitive external beam radiotherapy at the University of Texas-M.D. Anderson Cancer Center from 1987 to 1997. All had a pretreatment prostate-specific antigen (PSA) level. Treatment failure was defined as two consecutive PSA elevations on follow-up. There were 994 patients treated with a four-field box throughout to 60-70 Gy after a small reduction at 46 Gy and 161 treated with a six-field conformal boost after 46 Gy to 74-78 Gy. No patient received neoadjuvant or adjuvant androgen ablation. Median follow-up was 51.8 months.

Results: Patients were divided into three radiotherapy dose groups consisting of ≤ 67 Gy ($n = 500$), $> 67-77$ Gy ($n = 495$), and > 77 Gy ($n = 132$). Relative to other prognostic factors, there were fewer patients treated to the highest dose level with a pretreatment PSA (PSAB) ≤ 4 or > 20 ng/ml, Stage T3/T4 disease, or a Gleason score of 2-6. Actuarial 4-year freedom from biochemical failure (bNED) rates for the entire cohort were 54%, 71%, and 77% ($p < 0.0001$) for the low-, intermediate-, and high-dose groups. PSAB, palpable stage, and Gleason score were also highly significant. In Cox proportional hazards regression, dose ($p < 0.0001$ as a continuous or categorical variable) was an independent predictor of bNED, as were the other prognostic factors. Pairwise univariate comparisons showed that an increase in dose from ≤ 67 Gy to $> 67-77$ Gy was associated with improved bNED rates for all PSAB (≤ 10 and > 10), stage (T1/T2 and T3/T4), and Gleason score (2-6 and 7-10) subgroups tested. In contrast, the only prognostic group that benefited from raising dose from $> 67-77$ Gy to > 77 Gy was patients with a PSAB > 10 ng/ml; although trends were noted for Stage T1/T2 and Gleason 2-6 patients. Patients with the combined features of a PSAB > 10 ng/ml and Stage T1/T2 disease had 4-year bNED rates of 61% and 93% at the intermediate- and high-dose levels. A strongly significant linear association between dose (60-78 Gy) and 4-year actuarial bNED was demonstrated for patients with these intermediate-risk features.

Conclusion: Prostate cancer dose response to external beam radiotherapy should be considered in the context of pretreatment prognostic factors. Our data indicate that, for favorable patients with a PSAB of ≤ 10 ng/ml, intermediate doses of $> 67-77$ Gy provide the same rate of control as higher doses. However, longer follow-up may reveal a benefit to dose escalation > 77 Gy, even in this favorable subset. Substantial and clinically relevant enhancements in bNED were seen at all dose levels for moderate-risk patients, such as those having a PSAB > 10 ng/ml and Stage T1/T2 disease. Sustained bNED was not realized for high-risk patients, even using 78 Gy; these patients may be best treated with higher doses, whole pelvic irradiation, and/or androgen ablation plus radiation.
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Radiotherapy, Dose, Prostate-specific antigen.

INTRODUCTION

A number of studies in the prostate-specific antigen (PSA) era have indicated that prostate cancer patient outcome, particularly freedom from a rising PSA (biochemical, no evidence of disease, bNED), may be improved by increasing the dose delivered using external beam radiotherapy (1-4). However, doses of > 70 Gy may not be necessary for some patients and others may require additional treatment, such as still higher doses or

adjuvant androgen ablation. While attempts have been made to establish radiotherapy dose (RT dose) requirements based on prognostic factors, the results are far from conclusive. In this report, the impact of dose escalation was examined in the context of the well-established pretreatment prognostic variables of PSA, Gleason score, and palpable stage. The patients that most clearly benefited from treatment to > 77 Gy were Stage T1/T2 patients with a PSA > 10 ng/mL.

Reprint requests to: Alan Pollack, M.D., Ph.D., Department of Radiation Oncology (Box 97), M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. E-mail: Alan_Pollack@radonc.mdacc.tmc.edu

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Table 1. Distribution of patients by dose and pretreatment prognostic factors

Factor	Group	% Patients (n)			p*
		≤67 Gy	>67–77 Gy	>77 Gy	
Pre-TX PSA	≤ 4	23 (115)	12 (59)	8 (11)	<0.0001
	>4–10	32 (162)	48 (239)	54 (72)	
	>10–20	25 (124)	30 (148)	33 (43)	
	> 20	20 (99)	10 (49)	5 (6)	
Stage	T1/T2	71 (354)	72 (356)	77 (101)	0.25
	T3/T4	29 (146)	28 (139)	23 (31)	
Gleason score	2–6	74 (365)	51 (251)	49 (65)	<0.0001
	7	18 (87)	33 (162)	31 (41)	
	8–10	8 (39)	16 (78)	20 (26)	

Abbreviations: Pre-Tx PSA = pretreatment prostate specific antigen; RT = radiotherapy.

* Chi-square.

METHODS AND MATERIALS

Patient characteristics

Radiotherapy was the sole primary treatment for prostate cancer in 1127 men treated in the PSA era between 1987 and 1997. All had a pretreatment PSA. No patient had evidence of regional nodal or distant metastasis, although 98 patients did have a negative staging pelvic lymphadenectomy. Median follow-up from the end of radiotherapy for those alive at the time of analysis ($n = 991$) was 51.8 mo. Median follow-up for the three dose groups (3) was 77 mo for ≤ 67 Gy ($n = 401$ alive at last contact), 41 mo for >67 –77 Gy ($n = 466$), and 34 mo for >77 Gy ($n = 124$). Patient age ranged from 46–84, with a median of 69 yr.

The distribution of patients by palpable T-category was 2% in T1A, 7% in T1B, 23% in T1C, 15% in T2A, 18% in T2B, 7% in T2C, 9% in T3A, <1% in T3B, 18% in T3C, and <1% in T4B. Transrectal ultrasound findings (5) and extent of biopsy involvement (6) were not considered in staging. Transurethral resection (TURP) <6 months prior to radiotherapy was done in 163 patients, and of these, 27 had Stage T3/T4 disease. Gleason score was available in 1114 patients and was 1% Gleason 2, 4% Gleason 3, 13% Gleason 4, 16% Gleason 5, 28% Gleason 6, 26% Gleason 7, 10% Gleason 8, 2% Gleason 9, and <1% Gleason 10.

Median pretreatment PSA (PSAB) was 8.4 ng/mL, with a mean of 12.0 ± 0.4 ng/mL and range of 0.3–150 ng/mL. The TOSOH Medics (San Francisco, CA) Assay (lower limit 0.1 ng/mL) has been used since 1993 and the Hybritech assay (lower limit 0.3 ng/mL) prior to that time. There were 12 patients, of 949 measured, who had an elevated pretreatment prostatic acid phosphatase (PAPB, upper limit 0.8 mU/mL) by the enzymatic assay (7).

Radiotherapy was administered using a conventional four-field approach throughout in 982 patients. After 46 Gy at 2 Gy per fraction to the isocenter, a small reduction was made and treatment continued to 60–70 Gy using 18-MV photons. Conformal radiotherapy via 3-dimensional treatment planning (3D-CRT) was used as a boost in 145. The conventional and 3D-CRT boost techniques have been de-

tailed previously (8); the isocenter dose range was 74–78 Gy. A six-field arrangement consisting of laterals and four obliques at 30–40 degrees above and below the true laterals was used for 3D-CRT.

Follow-up PSAs were scheduled at 3-month intervals for the first 2 years and every 6 months thereafter. A rising PSA was considered evidence of prostate cancer relapse, and was defined as three or more consecutive rises on follow-up. There were a total of 9439 PSA values for the 1127 patients, an average of 8.38 per patient. Median follow-up was 51.8 months, with a minimum potential follow-up of 1.4 years.

Actuarial freedom from a rising PSA (bNED) curves were calculated using the life table and Kaplan-Meier methods (9). The onset of a rising PSA was defined as the average time between the date of the PSA obtained prior to the first risen value and the date of the first risen value. The log rank test was used to determine statistically significant differences (9). Multivariate analysis was performed using Cox proportional hazards regression (10). Statistical corrections for multiple comparisons were not made.

RESULTS

The patients were divided into three dose groups, representative of patients treated to low ≤ 67 Gy ($n = 500$; range 60–66 Gy, median 66 Gy), intermediate >67 –77 Gy ($n = 495$; range 68–76 Gy, median 70 Gy), and high isocenter doses of >77 Gy ($n = 132$; 78 Gy in all). Table 1 shows the distribution of patients by dose and other prognostic factors. The high-dose group was comprised of proportionally fewer patients with a PSA of ≤ 4 or >20 ng/mL, Stage T3/T4 disease, and Gleason score 2–6. The relationships with PSAB and Gleason score were significant, while stage was not. There were fewer patients treated to the higher dose with a PSAB >20 ng/mL and/or T3/T4 disease because the majority in more recent years were treated with androgen ablation plus radiation, and were not included in this analysis. The

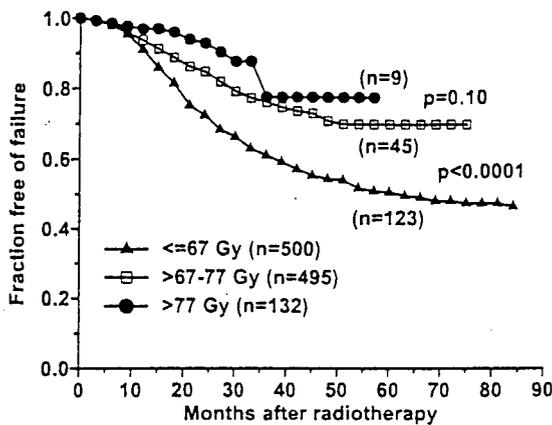


Fig. 1. Actuarial bNED for all patients by dose group (≤ 67 Gy, >67 – 77 Gy, and >77 Gy). The numbers next to the symbol legends are the total number of patients in each group. The numbers next to the curves are the numbers of patients at risk at 5 years when possible (the >77 Gy curve is out to 57 mo). The p -values for the pairwise comparisons of ≤ 67 Gy vs. >67 – 77 Gy and >67 – 77 Gy vs. >77 Gy are shown.

patient features for those treated with >67 – 77 Gy were generally intermediate between those treated to ≤ 67 Gy and >77 Gy.

The association of dose to bNED for the entire cohort is shown in Fig. 1. The pairwise comparisons demonstrate a highly significant difference in bNED rates between the low- and intermediate-dose groups, and a borderline difference between the intermediate- and high-dose groups. Table 2 summarizes the 4- and 7-year actuarial bNED rates for RT dose, as well as the other factors significant in univariate analysis—PSAB, palpable stage, and Gleason score. Multivariate analysis by Cox proportional hazards regression demonstrated that all of these factors correlated independently with bNED (Table 3). The two analyses depicted show that similar results were obtained by including PSAB and RT dose as continuous or categorical variables.

The data presented indicate that elevating dose from ≤ 67 Gy to >67 – 77 Gy results in a dramatic improvement in bNED rates across all patient prognostic groups. Table 4 validates this affirmation; the pairwise comparisons of ≤ 67

Table 3. Multivariate results by Cox proportional hazards regression of biochemical failure: all patients*

Factor	Grouping	Chi-square	p
PSAB and RT dose as continuous variables			
PSAB	Continuous	79	<0.0001
Stage	T1/T2 vs. T3/T4	49	<0.0001
Gleason score	2–6 vs. 7–10	26	<0.0001
RT Dose	Continuous	42	<0.0001
PSAB and RT dose as categorical variables			
PSAB	≤ 10 vs. >10 ng/ml	88	<0.0001
Stage	T1/T2 vs. T3/T4	42	<0.0001
Gleason score	2–6 vs. 7–10	35	<0.0001
RT Dose	≤ 67 vs. >67 – 77 vs. >77 Gy	60	<0.0001

* There were 1104 patients available for these analyses.

PSAB = pretreatment (baseline) prostate-specific antigen; RT = radiotherapy.

Gy to >67 – 77 Gy were significantly different for each prognostic subgroup tested. The strength of these findings is grounded in the large patient numbers and long follow-up available for the low- and intermediate-dose groups.

Of the three RT dose groups, the high-dose group had the fewest patients and shortest follow-up, which may have contributed to the borderline increase in bNED over the intermediate-dose group when all patients were analyzed (Fig. 1). A Cox proportional hazards analysis for bNED was performed with patients who received >67 Gy, to further assess the significance of escalating dose beyond this level. Dose was a significant independent covariate when PSAB and RT dose were included as continuous variables, and was of borderline significance when included as categorical variables (Table 5). While high RT dose was documented to independently enhance bNED rates, this enhancement was most pronounced for patients with specific prognostic features. The pairwise comparisons in Table 4 show that a PSAB >10 ng/mL was significantly associated with greater bNED when the dose was escalated above 77 Gy. Figure 2 illustrates this effect and shows that increasing the dose beyond 77 Gy did not affect bNED for patients with a PSAB of ≤ 10 ng/mL. Trends for improved outcome with doses >77 Gy were seen for Stage

Table 2. Factors correlating with actuarial bNED in univariate analysis

Factor	Group	n	4-yr % bNED (nr)	7-yr % bNED (nr)	p
Pre-Tx PSA	≤ 10 ng/mL	658	79 (223)	71 (43)	<0.0001
	>10 ng/mL	469	41 (95)	34 (11)	
Stage	T1/T2	811	71 (239)	66 (38)	<0.0001
	T3/T4	316	45 (79)	34 (16)	
Gleason score	2–6	681	69 (228)	62 (49)	<0.0001
	7–10	433	54 (87)	41 (5)	
RT Dose	≤ 67 Gy	500	54 (182)	47 (54)	<0.0001
	>67 – 77 Gy	495	71 (113)	—	
	>77 Gy	132	77 (23)	—	

Abbreviations: n = overall patient number in group; nr = number at risk at time indicated; — = not enough patients at risk to provide a meaningful estimate of bNED.

Table 4. Actuarial univariate 4-year results of prognostic factors by dose

Factor	Group	4 Year %bNED (n)			<i>p</i> [§]
		≤67 Gy	>67–77 Gy	>77 Gy	
Pre-Tx PSA	≤10 ng/ml	73 (277)	85 (298)*	84 (83)	0.0026
	>10 ng/ml	31 (223)	51 (197)*	68 (49)*	<0.0001
Stage	T1/T2	62 (354)	80 (357)*	90 (101) [†]	<0.0001
	T3/T4	37 (146)	51 (139)*	56 (31)	0.0013
Gleason score	2–6	61 (365)	77 (251)*	94 (65) [‡]	<0.0001
	7–10	36 (126)	66 (240)*	62 (67)	<0.0001

* Pairwise *p* < 0.05, as compared to next lowest dose level.

[†] Pairwise *p* = 0.14, as compared to next lowest dose level.

[‡] Pairwise *p* = 0.07, as compared to next lowest dose level.

[§] Overall *p* value, log rank.

T1/T2 and Gleason 2–6 patients, but not for Stage T3/T4 or Gleason 7–10 patients (Table 4).

Patients at intermediate risk (PSAB >10 ng/ml and Stage T1/T2 disease or PSAB >10 ng/ml and Gleason 2–6 disease) showed the most pronounced improvements in bNED. Figure 3 displays the striking results for Stage T1/T2 patients with a PSAB >10 ng/mL. Actuarial 4-year bNED was 61% for the intermediate-dose group versus 93% for the high-dose group (*p* = 0.0148). The results were similar for patients with a PSAB >10 ng/mL and Gleason score 2–6 (not shown), although there were only 23 patients treated to >77 Gy and the significance of the difference from the intermediate-dose group was borderline (*p* = 0.07). There were not enough patients with a PSA ≤10 ng/mL and Stage T3/T4 disease to evaluate the consequence increasing dose from >67–77 Gy (*n* = 58) versus >77 Gy (*n* = 17).

The dose–response relationship for patients with a PSAB >10 ng/mL and Stage T1/T2 disease was expanded by examining the 4-year actuarial bNED rates for five dose levels. The dose levels were ≤65 Gy (*n* = 74), >65–67

Gy (*n* = 61), >67–69 Gy (*n* = 24), >69–77 Gy (*n* = 92), and >77 Gy (*n* = 35). Figure 4 shows that for this patient population there was a highly significant linear relationship between mean dose at each level and 4-year bNED. A distinct dose–response was not observed for patients with PSAB ≤10 ng/mL, even when Stage T1/T2 and T3/T4 patients were analyzed separately. Likewise, no dose–response was found for all Stage T3/T4 patients or when grouped by PSAB >10 ng/ml; however, the numbers of patients who received >77 Gy in these groups were small.

DISCUSSION

The decision tree for prostate cancer patients considering definitive treatment is expansive and confusing. The simple choice of external beam versus surgery is no more. The radiotherapy options range from permanent seed implant monotherapy to various combinations of external beam plus implants. With the ability to more precisely target the prostate using 3D-CRT or intensity-modulated radiotherapy (IMRT), dose escalation using external beam as a single modality has been brought to the forefront. Which patients are in need of higher doses above the standard of 70 Gy, be it through the use of an implant boost or external beam alone, remains to be defined. Hanks *et al.* (1) have reported that the greatest improvement in bNED using external beam alone is for the patient with a PSAB >10 ng/ml. However, they recently described that patients with PSABs <10 ng/ml also benefited (11). Zelefsky *et al.* (2) summarized the results of the Memorial Sloan-Kettering sequential dose escalation trial, finding that intermediate- and high-risk patients had significantly better bNED rates when the dose was above 75.6 Gy. They did not find a significant improvement when low-risk patients were treated with these doses.

In our earlier analysis, dose affected outcome for those with PSABs >4–10 ng/ml, as well as >10 ng/ml (3). The findings described herein more firmly establish a dose response for patients with PSABs ≤10 ng/ml, mainly for doses ≤77 Gy. One constant in the analyses was the inferior results for patients treated to ≤67 Gy. The prac-

Table 5. Multivariate results by Cox proportional hazards regression of biochemical failure: patients who received >67 Gy*

Factor	Grouping	Chi-square	<i>p</i>
<u>PSAB and RT dose as continuous variables</u>			
Pre-Tx PSA	Continuous	42	<0.0001
Stage	T1/T2 vs. T3/T4	13	0.0003
Gleason score	2–6 vs. 7–10	14	0.0002
RT dose	Continuous	5	0.0279
<u>PSAB and RT dose as categorical variables</u>			
Pre-Tx PSA	≤10 vs. >10 ng/ml	30	<0.0001
Stage	T1/T2 vs. T3/T4	20	<0.0001
Gleason score	2–6 vs. 7–10	18	<0.0001
RT dose	>67–77 vs. >77 Gy	3	0.0830

* There were 616 patients available for this analysis.

Pre-Tx PSA = pretreatment prostate specific antigen; RT = radiotherapy.

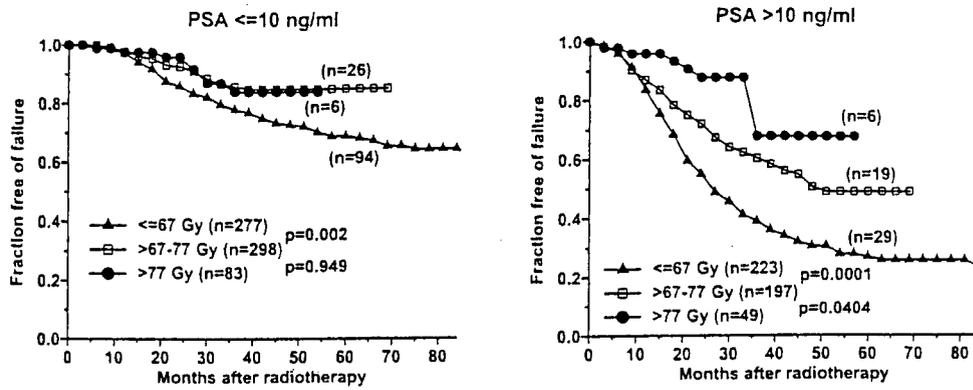


Fig. 2. Actuarial bNED dose response for patients with PSAB ≤ 10 (left) and PSA > 10 ng/ml (right). The numbers next to the curves are the numbers of patients at risk at 5 years when possible (the > 77 Gy curves are out to 54–57 mo). The overall p -values for each graph were < 0.0001 . The p -values for the pairwise comparisons of ≤ 67 Gy vs. $> 67-77$ Gy and $> 67-77$ Gy vs. > 77 Gy are shown.

tice of delivering 66 Gy to the isocenter, as is still done in many centers in the United States and abroad, must be abandoned. The improvement in 4-year bNED rates observed by increasing the dose from ≤ 67 Gy to $> 67-77$ Gy was 16% for PSAB < 10 ng/ml, 29% for T1/T2 disease, and 26% for Gleason score 2–6 (Table 4). These highly significant and independent gains were sustained to 7 years (not shown), lending credence to the assertion that the minimum isocenter dose should be 70 Gy in all cases. No benefit in actuarial bNED was observed using doses above 77 Gy in these favorable patients, although follow-up for the high-dose group was relatively short.

An enhancement in bNED attributable to raising dose from the intermediate- to the high-dose levels was also documented in some patients. A borderline significant difference was seen in univariate (Fig. 1) and multivariate (Table 5) analyses. The strength of this relationship was dampened by the lack of dose response in some prognostic subgroups, which ranged from the more favorable (PSAB < 10 ng/mL) to the generally unfavorable (T3/T4, Gleason

7–10). In fact, the PSAB > 10 ng/mL group was fairly unfavorable, with a 4-year bNED rate of 51% at the intermediate dose. Of those with a PSAB > 10 ng/mL, it was the intermediate-risk Stage T1/T2 patients who profited the most from escalating dose to > 77 Gy (Fig. 3). These patients displayed an exceptional linear dose–response relationship between 60 and 78 Gy (Fig. 4) that was not seen for other patient groups. Possibly with longer follow-up and greater numbers of patients treated to > 77 Gy, other groups, such as those with a PSAB ≤ 10 ng/ml and Stage T3/T4 disease might exhibit a dose response.

SUMMARY AND RECOMMENDATIONS

There is ample evidence from this report and others that dose is a significant determinant of treatment outcome for clinically localized adenocarcinoma of the prostate. Dose thresholds, above which further escalation results in little apparent improvement, were evident for certain prognostic groups. A pretreatment PSA ≤ 10 ng/mL was associated with a dose response from ≤ 67 Gy to $> 67-77$ Gy, but not

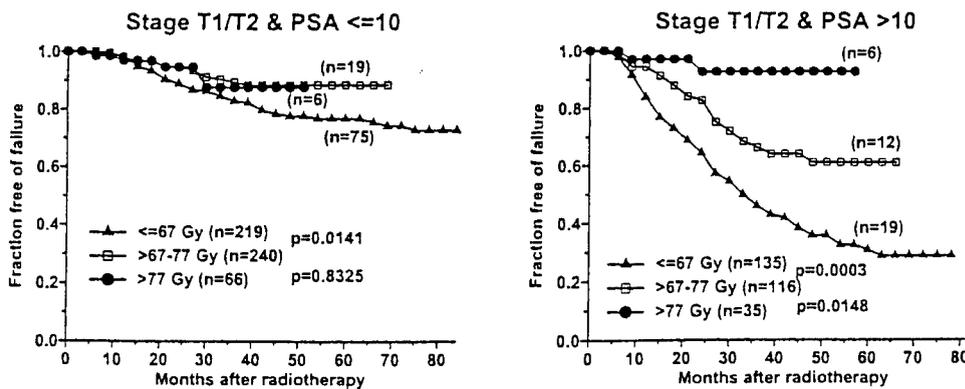


Fig. 3. Actuarial bNED dose response for patients with Stage T1/T2 disease subdivided by whether PSAB was ≤ 10 (left) or > 10 ng/mL (right). The numbers next to the curves are the numbers of patients at risk at 5 years when possible (the > 77 Gy curves are out to 51–57 mo). The overall p -values were 0.028 (left) and < 0.0001 (right). The p -values for the pairwise comparisons of ≤ 67 Gy vs. $> 67-77$ Gy and $> 67-77$ Gy vs. > 77 Gy are shown.

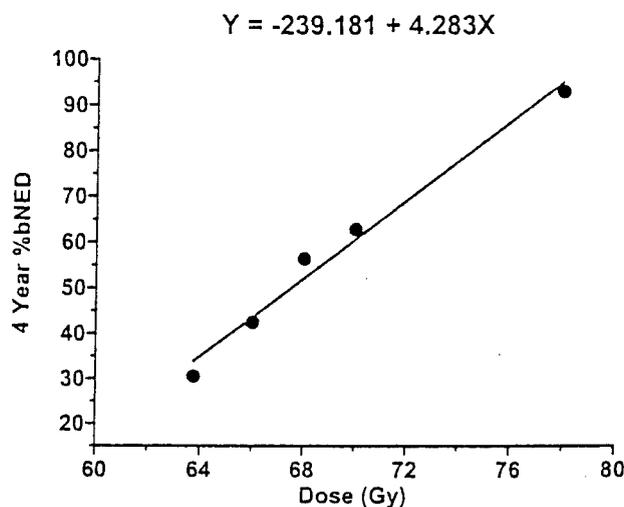


Fig. 4. Actuarial 4-year bNED rates for the mean doses of 5 dose levels are plotted and fitted using linear regression. The formula for the curve is displayed. The relationship was significant at $p < 0.0001$.

to >77 Gy. Since the majority of patients in the intermediate-dose group received 68–70 Gy, it appears that 70 Gy is sufficient. An advantage to giving >70 Gy in these favorable patients would be difficult to prove, but, the possibility remains that with longer follow-up, even this group may benefit from doses beyond this level. Intermediate-risk patients, such as those with a PSA >10 ng/ml and T1/T2 disease, and/or Gleason 2–6, plainly displayed a highly significant dose response and should receive >70 Gy using 3D-CRT or IMRT. Another relatively intermediate-risk group, those with a PSA ≤ 10 ng/ml and Stage T3/T4 disease, might manifest improved bNED with >77 Gy; however, patient numbers were insufficient for this determination. Moreover, there were not enough patients with pretreatment PSAs >20 ng/mL, a traditionally high-risk group, to evaluate the impact of dose >77 Gy. Most likely, high-risk patients with T3/T4 and/or a PSA >20 ng/mL will necessitate more than dose escalation above 77 Gy for adequate control and the recommendation for now is androgen ablation plus radiotherapy (12, 13).

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Preliminary Results of a Randomized Radiotherapy Dose-Escalation Study Comparing 70 Gy With 78 Gy for Prostate Cancer

By Alan Pollack, Gunar K. Zagars, Lewis G. Smith, J. Jack Lee, Andrew C. von Eschenbach, John A. Antolak, George Starkschall, and Isaac Rosen

Purpose: To determine the effect of radiotherapy dose on prostate cancer patient outcome and biopsy positivity in a phase III trial.

Patients and Methods: A total of 305 stage T1 through T3 patients were randomized to receive 70 Gy or 78 Gy of external-beam radiotherapy between 1993 and 1998. Of these, 301 were assessable; stratification was based on pretreatment prostate-specific antigen level (PSA). Dose was prescribed to the isocenter at 2 Gy per fraction. All patients underwent planning pelvic computed tomography scan to confirm prostate position. Treatment failure was defined as an increasing PSA on three consecutive follow-up visits or the initiation of salvage treatment. Median follow-up was 40 months.

Results: One hundred fifty patients were randomized to the 70-Gy arm and 151 to the 78-Gy arm. The difference in freedom from biochemical and/or disease failure (FFF) rates of 69% and 79% for the 70-Gy and 78-Gy groups, respectively, at 5 years was marginally

significant (log-rank $P = .058$). Multiple-covariate Cox proportional hazards regression showed that the study randomization was an independent correlate of FFF, along with pretreatment PSA, Gleason score, and stage. The patients who benefited most from the 8-Gy dose escalation were those with a pretreatment PSA of more than 10 ng/mL; 5-year FFF rates were 48% and 75% ($P = .011$) for the 70-Gy and 78-Gy arms, respectively. There was no difference between the arms (~80% 5-year FFF) when the pretreatment PSA was ≤ 10 ng/mL.

Conclusion: A modest dose increase of 8 Gy using conformal radiotherapy resulted in a substantial improvement in prostate cancer FFF rates for patients with a pretreatment PSA of more than 10 ng/mL. These findings document that local persistence of prostate cancer in intermediate- to high-risk patients is a major problem when doses of 70 Gy or less are used.

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DOSE ESCALATION WITH limited morbidity is now possible with techniques such as three-dimensional conformal radiotherapy (3DCRT) and intensity-modulated radiotherapy (IMRT) that more precisely target the prostate with greater sparing of the surrounding normal tissues. A randomized trial performed in the pre-prostate-specific antigen (PSA) era using protons to escalate dose showed that higher doses reduced treatment failure for patients with Gleason scores of 8 to 10.¹ The patients in that trial had relatively advanced disease and the findings may not

be representative of contemporary prostate cancer patients treated to higher doses with photons. A number of PSA-era retrospective studies have examined the sequential increase in dose over time and in most,²⁻⁷ but not all,⁸ dose has been found to reduce biochemical failure rates. The potential fault in such sequential comparisons is that there may have been an uneven distribution of unaccountable prognostic factors during the study period. Indeed, an unprecedented stage migration has occurred over the last 10 years as a consequence of refinements in the application of PSA and ultrasound to screening.^{9,10} Only a contemporary, prospective, randomized trial would eliminate this bias. Such a trial was performed at M.D. Anderson from 1993 through 1998, and the preliminary analysis is reported here.

From the Departments of Radiation Oncology, Biostatistics, Urology, and Radiation Physics, The University of Texas M.D. Anderson Cancer Center, Houston, TX.

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Address reprint requests to Alan Pollack, MD, PhD, Department of Radiation Oncology (Box 97), M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030; email apollack@notes.mdacc.tmc.edu.

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PATIENTS AND METHODS

Protocol Entry Criteria and Goals

The protocol was approved and activated by the Institutional Review Board at M.D. Anderson Cancer Center, and the first patient was randomized in March 1993. Patients with stage T1 through T3, Nx/N0, and M0 prostate cancer were eligible. All patients were required to have a pretreatment serum PSA analysis. Prior history of pelvic radiotherapy, radical prostatectomy, or androgen ablation was not permitted. Moreover, planned neoadjuvant or adjuvant androgen ablation was not permitted. Bone scan was performed if the PSA level was

more than 8 ng/mL. A pelvic computed tomography (CT) scan performed if the pretreatment PSA level was more than 20 ng/mL. There were only 16 patients who had a pretreatment PSA level of more than 20 ng/mL, because soon after opening the trial we instituted a treatment policy of combined androgen ablation plus radiotherapy for these high-risk patients. Only two patients underwent pelvic lymph node dissection before enrollment and both were lymph node-negative. No patient had evidence of metastatic disease.

The hypothesis of the protocol was that the higher radiation dose would result in a 15% long-term improvement in freedom from relapse or an increasing PSA level. Assuming direct causality from differences in local tumor control, an estimated 150 patients per arm would be required.¹¹ Between March 1993 and June 1998, 305 patients who met the eligibility criteria were enrolled in the study. Of these, 301 were assessable and are the subject of this report. The four nonassessable patients included two who withdrew before radiotherapy was administered; one chose surveillance and one underwent radical prostatectomy. Prostate cancer was not confirmed pathologically at M.D. Anderson after enrollment in one patient, and one patient withdrew consent and stopped radiotherapy after 3 weeks of treatment. There were also four assessable patients who were classified as protocol violations. Two patients received androgen ablation (one in each arm) after completing radiotherapy. Two patients who were randomized to receive 78 Gy of radiation received 70 Gy; one withdrew consent during radiotherapy and 3DCRT planning was compromised in one patient secondary to obesity. The analyses described were performed for the assessable patients by intent-to-treat as they were randomized. Of the 301 assessable patients, 150 were randomized to the 70-Gy arm and 151 were randomized to the 78-Gy arm.

Patient Characteristics

The mean and median pretreatment PSA values were 9.4 and 7.8 ng/mL, 9.1 and 7.5 ng/mL, and 9.8 and 7.8 ng/mL, respectively, for the entire cohort, the 70-Gy group, and the 78-Gy group. Median follow-up values for the entire cohort, the 70-Gy group, and the 78-Gy group were 40, 39, and 42 months, respectively.

Patients were staged using the American Joint Committee on Cancer 1992 palpable staging system. Transrectal ultrasound findings, extent of biopsy involvement, and number and position of positive biopsies were not considered in staging.^{12,13} The distribution of patients by palpable T category for the entire study population was 1% in T1b, 28% in T1c, 23% in T2a, 18% in T2b, 10% in T2c, 8% in T3a, and 12% in T3c. Transurethral resection of the prostate was performed less than 6 months before radiotherapy in 13 patients; of these patients, two had stage T3/T4 disease. Gleason score was available in 300 of the 301 assessable patients: 2% had Gleason 4 disease, 7% had Gleason 5, 40% had Gleason 6, 33% had Gleason 7, 14% had Gleason 8, 3% had Gleason 9, and less than 1% had Gleason 10.

Radiotherapy Techniques

A conventional four-field box was used for the initial 46 Gy in all patients. Dose was specified to the isocenter and was delivered at 2 Gy per fraction per day. The anterior-posterior fields were typically 11 × 11 cm and the laterals were 11 × 9 cm. A corner block was placed over the bladder and the rectum was split on the lateral fields. As described previously,¹⁴ the 70-Gy patients were planned using contrast in the rectum and bladder, although within 1 week of starting treatment, a pelvic CT scan was performed to confirm that the prostate was within the field. Minor adjustments in the field based on pelvic CT scan were made in less than 5% of the 70-Gy patients. After 46 Gy, a conven-

tional four-field boost was delivered to approximately 9 × 9 cm fields, continuing at 2 Gy per fraction to a total dose of 70 Gy to the isocenter. The 78-Gy patients were planned without contrast from the planning pelvic CT scan. The first 46 Gy was delivered using the same conventional four-field box arrangement as was used for the 70-Gy patients.¹⁴ After 46 Gy, a six-field 3DCRT boost was used to bring the total isocenter dose to 78 Gy. The clinical target volume was the prostate and seminal vesicles. The 3DCRT boost margins were 0.75 cm to 1.0 cm from the clinical target volume to the block edge in the posterior and superior dimensions and 1.25 to 1.5 cm in the anterior and inferior dimensions. Using these margins, the proportion of rectum that received ≥ 60 Gy was similar for the two treatment methods.¹⁴

End Points and Statistics

The main end point of the study was the survival analysis of freedom from biochemical and/or disease failure (FFF), which was defined as time from completion of treatment to an increasing PSA level and/or clinical-radiographic relapse. An increasing PSA profile was evidence of biochemical failure and was defined as three or more increases on follow-up visits per the American Society of Therapeutic Radiation Oncology consensus guidelines.¹⁵ The onset of an increasing PSA level was defined as the average time between the date of the PSA level obtained before the first increasing value and the date of the first increasing value. One patient was considered to have experienced treatment failure without evidence of an increasing PSA level because salvage prostatectomy was performed when frank carcinoma was found on a prostate biopsy at 2 years. The χ^2 test was used to test for differences in proportions. Survival curves were calculated from the completion of radiotherapy using the Kaplan-Meier and Berkson-Gage methods.¹⁶ The log-rank test was used to compare the survival curves.¹⁶ Multiple-covariate analysis was performed using Cox proportional hazards regression.¹⁷

Secondary study end points were freedom from distant metastasis, overall survival, and prostate biopsy positivity at 2 years after completion of treatment for patients who were free of failure. The prostate biopsies were classified into the four categories of negative, atypical/suspicious but not diagnostic of carcinoma, carcinoma with treatment effect, and frank carcinoma without treatment effect.

RESULTS

Three hundred one patients were randomized in the trial, with 150 in the 70-Gy arm and 151 in the 78-Gy arm. Stratification was based on pretreatment PSA level, and there were no statistically significant differences between the treatment arms in terms of the distribution of patients by pretreatment PSA level, palpable stage, or Gleason score (Table 1). Only two patients with category T3 disease had undergone transurethral resection of the prostate within 6 months of radiotherapy.

There were 35 patients (23%) in the 70-Gy arm and 22 patients (15%) in the 78-Gy arm who had evidence of treatment failure at the time of this analysis. Figure 1 shows the Kaplan-Meier curves based on FFF rates for the two treatment arms. The difference in FFF rates was marginally significant ($P = .058$), with rates of 69% for patients in the 70-Gy group and 79% for patients in the 78-Gy group. The 5-year FFF rates for the potential prognostic factors of

Table 1. Distribution of Patients by Treatment Arm and Pretreatment Prognostic Factors

Factor	70-Gy Arm		78-Gy Arm		P*
	No. of Patients	%	No. of Patients	%	
Pre-Tx PSA					.94
≤ 4 ng/mL	16	11	18	12	
> 4-10 ng/mL	81	54	80	53	
> 10 ng/mL	53	35	53	35	
Stage					.26
T1/T2	124	83	117	78	
T3	26	17	34	23	
Gleason score					.93
2-6	72	48	76	50	
7	51	34	49	33	
8-10	26	17	26	17	

Abbreviation: Pre-Tx, pretreatment.

* χ^2 test.

pretreatment PSA level, Gleason score, and clinical stage are listed in Table 2. All of these factors were highly significant in single-covariate analysis. Multiple-covariate survival analysis of FFF by Cox proportional hazards regression revealed that the study randomization of 70 Gy versus 78 Gy was significant and independent of the other key prognostic factors of pretreatment PSA level, palpable disease stage, and Gleason score (Table 3).

An analysis was then performed to determine which patients benefited most from the 8-Gy dose increment. Figure 2 shows that when the pretreatment PSA level was ≤ 10 ng/mL, the 5-year FFF rates were similar at approximately 80%. In contrast, a pretreatment PSA level of more than 10 ng/mL was associated with a significantly higher 5-year FFF rate of 75% for the patients who received 78 Gy

Table 2. Single-Covariate Survival Analysis of FFF

Factor	No. of Patients	% 5-Year FFF	No. at Risk*	P†
All patients	301	74	31	
Pre-Tx PSA				.0008
≤ 10 ng/mL	195	81	18	
> 10 ng/mL	106	61	13	
Stage				< .0001
T1/T2	241	82	26	
T3	60	50	5	
Gleason score				.0006
2-6	148	84	16	
7-10	152	66	15	
Randomization				.058
70 Gy	150	69	16	
78 Gy	151	79	15	

*Number at risk at 5 years.

†Log-rank test.

versus 48% for the patients who received 70 Gy. Table 4 lists these findings and shows that 78 Gy was associated with improved FFF rates in the setting of T3 and Gleason 2 through 6 disease.

The FFF gain from the higher dose for patients with a pretreatment PSA level of more than 10 ng/mL was attributable mainly to those with intermediate risk features. Figure 3 displays the FFF curves for patients with category T1/T2 disease and PSA level of more than 10 ng/mL. The 5-year FFF rates were 90% for the 78-Gy patients and 60% for the 70-Gy patients. Comparable results were obtained for those with Gleason score of 2 through 6 and PSA level of more than 10 ng/mL (91% v 56% FFF at 4 years for 78 and 70 Gy) and those with category T3 disease and PSA level ≤ 10 ng/mL (75% v 44% FFF at 3 years for 78 and 70 Gy); however, patient numbers were small and the differences were not significant.

The other secondary end points examined in the protocol were freedom from distant metastasis, overall survival, and

Months	0	10	20	30	40	50	60
70 Gy	150	137	106	71	49	32	16
78 Gy	151	144	106	77	54	31	15

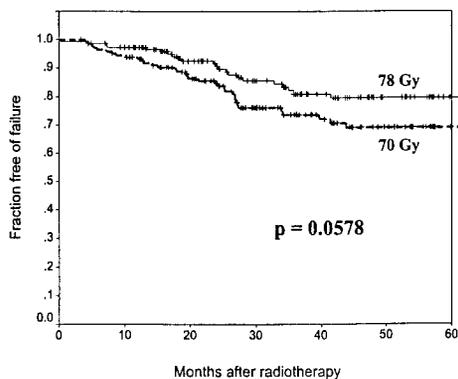


Fig 1. Kaplan-Meier FFF curves for all patients by dose randomization (70 Gy v 78 Gy). The numbers of patients at risk at 10-month intervals are shown above the graph.

Table 3. Multiple-Covariate Survival Analysis of FFF by Cox Proportional Hazards Regression

Factor	RR	CI	P
Pretreatment PSA as a categorical variable			
Pre-Tx PSA, ≤ 10 versus > 10 ng/mL	2.06	1.19-3.57	.01
Stage, T1/T2 versus T3	2.41	1.37-4.25	.003
Gleason score, 2-6 versus 7-10	2.29	1.25-4.18	.005
Randomization, 70 Gy versus 78 Gy	0.55	0.32-0.94	.028
Pretreatment PSA as a continuous variable			
Pre-Tx PSA, continuous	1.06	1.03-1.09	.0001
Stage, T1/T2 versus T3	2.18	1.22-3.90	.011
Gleason score, 2-6 versus 7-10	2.26	1.24-4.13	.005
Randomization, 70 Gy versus 78 Gy	0.50	0.28-0.86	.011

NOTE. There were 299 patients available for these analyses.

Abbreviations: RR, relative risk; CI, 95% RR confidence intervals.

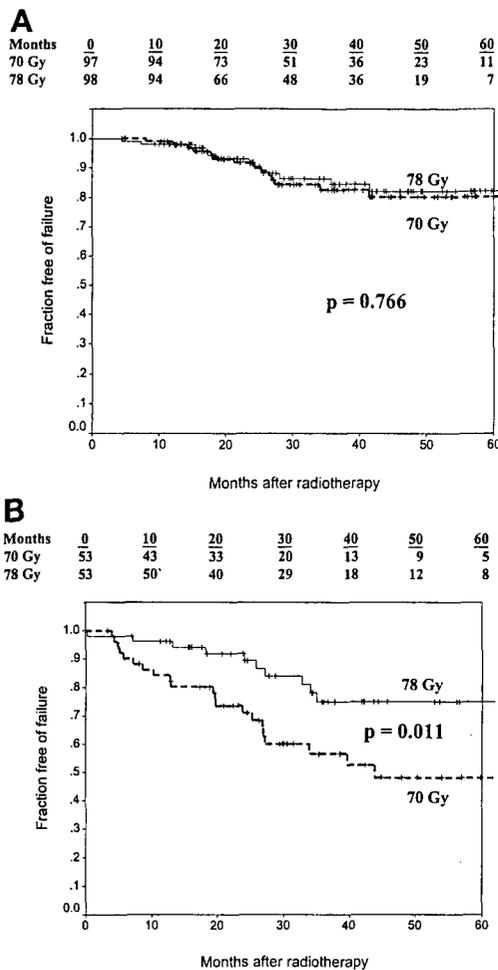


Fig 2. Kaplan-Meier FFF curves for patients with (A) pretreatment PSA levels of ≤ 10 ng/mL and (B) PSA levels of more than 10 ng/mL by dose randomization (70 Gy v 78 Gy). The numbers of patients at risk at 10-month intervals are shown above the graphs.

2-year prostate biopsy positivity rates. Distant metastases were documented in six patients in the 70-Gy arm and two patients in the 78-Gy arm. Of these, one patient had hormone-refractory disease and the others were started on androgen ablation when distant metastasis was observed. The overall 5-year freedom-from-distant-metastasis rates were similar for the two treatment groups at 95% to 98%. Likewise, equivalent rates of 98% to 100% were seen when the pretreatment PSA level was ≤ 10 ng/mL. In contrast, for those with a pretreatment PSA level of more than 10 ng/mL, the 5-year freedom-from-distant-metastasis rate for the 78-Gy patients was higher (98% v 87%; $P = .054$), as shown in Fig 4. There were 18 deaths in the study group over the follow-up period, with eight occurring in the 70-Gy arm and 10 occurring in the 78-Gy arm. Overall survival at

Table 4. Single-Covariate Survival Analysis of Prognostic Factors by Dose

Factor	Randomization						Pt
	70 Gy			78 Gy			
	% 5-Year FFF	No. of Patients	No. at Risk†	% 5-Year FFF	No. of Patients	No. at Risk†	
Pre-Tx PSA							
≤ 10 ng/mL	80	97	11	82	98	7	.77
> 10 ng/mL	48	53	5	75	53	8	.011
Stage							
T1/T2	77	124	15	87	117	11	.11
T3	36	26	1*	61	34	4*	.047
Gleason score							
2-6	76	72	8	93	76	8	.05
7-10	64	77	8	68	75	7	.39

*4-year results.

†Log-rank test.

‡Number at risk at 5 years unless otherwise indicated.

5 years was the same for the two groups at 90% to 91%. Moreover, there was no difference between the groups in overall survival when the pretreatment PSA level was ≤ 10 ng/mL (94% to 95% at 5 years) or more than 10 ng/mL (84% to 86% at 5 years).

Consent for sextant transrectal ultrasound-guided prostate biopsies at 2 years after the completion of radiotherapy was acquired at protocol enrollment. Additional biopsies were obtained from any hypoechoic areas that were suggestive of abnormality on ultrasound. At the time of this analysis, 168 patients had undergone prostate biopsy. There were 18 patients who had evidence of an increasing PSA level at biopsy, with 11 in the 70-Gy arm and seven in the 78-Gy arm. A total of 150 patients were free from biochemical and/or disease failure at the time of the 2-year end point prostate biopsy, and these are the subject of the following analysis. The goal was to determine whether the 2-year biopsy findings were a surrogate for eventual biochemical failure. The pathologic classification of the material fell into four categories: (1) completely negative, (2) atypical cells that were suggestive of abnormality but not diagnostic of carcinoma, (3) carcinoma with treatment effect, and (4) frank carcinoma without treatment effect. Table 5 shows that there was an uneven distribution of patients between the four pathologic classification groups. Proportionally fewer patients had no tumor and more had cells that were atypical or suggestive of abnormality in the biopsy material in the 70-Gy arm, whereas more patients had carcinoma with treatment effect and fewer had frank carcinoma in the 78-Gy arm. Pooling those who did not have evidence of carcinoma in the biopsy specimen (the no-tumor and atypical/suggestive of abnormality groups [biopsy-negative]) and comparing them with patients with evidence of carcinoma (the carcinoma with treatment effect and frank carcinoma)

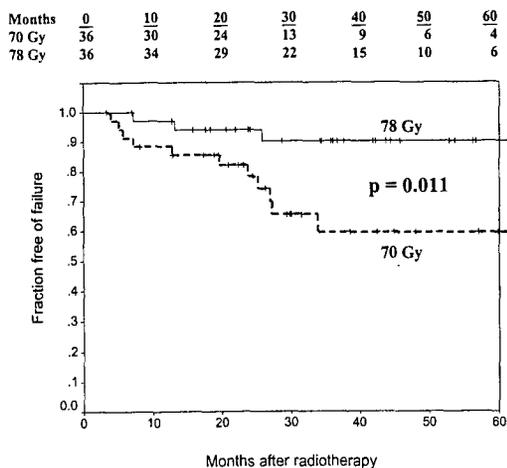


Fig 3. Kaplan-Meier FFF curves for patients with pretreatment PSA levels of more than 10 ng/mL and stage T1/T2 disease by dose randomization (70 Gy v 78 Gy). The numbers of patients at risk at 10-month intervals are shown above the graph.

noma groups [biopsy-positive]) did not reveal a significant difference in the distribution of patients by treatment arm (Table 5). The overall 2-year biopsy positivity rate at 2 years was 31%, with 28% in the 70-Gy arm and 35% in the 78-Gy arm ($P = .33$). Figure 5 shows that the biopsy-positive and biopsy-negative patients segregated together in terms of Kaplan-Meier FFF estimates. The 5-year FFF rate was 88% for biopsy-negative patients and 67% for biopsy-positive patients ($P = .0004$). The randomization between 70 Gy and 78 Gy did not have a significant effect on biopsy-negative or biopsy-positive FFF rates.

DISCUSSION

The development of more accurate methods for delivering radiation to the target tumor site has considerably altered the practice of radiation oncology. Three-dimensional treatment planning and conformal radiotherapy have rapidly gained acceptance and are being widely applied in both academic and community practices. IMRT, which has the capability of taking dose delivery precision to another level, is also being used with increasing frequency for the treatment of patients with prostate cancer.^{18,19} The quandary is that these technological advances have outpaced the establishment of suitable criteria for application such that improved patient outcome with low morbidity is ensured.

Retrospective single-institution prostate cancer dose-escalation studies²⁻⁸ have been at the forefront of the employment of 3DCRT and IMRT. The relationship between higher doses and enhanced tumor control is encouraging; however, this is weakened by the sequential nature of these observations. The M.D. Anderson retrospective data²⁻⁴ il-

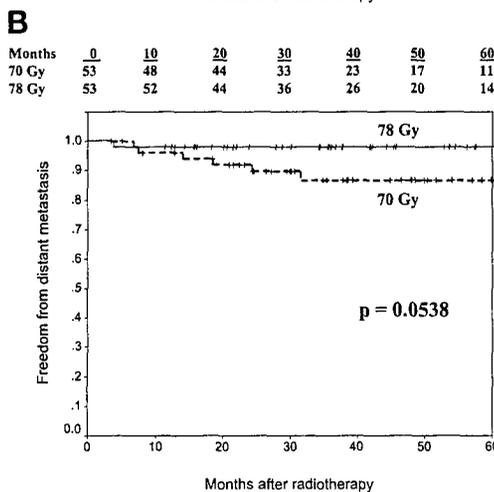
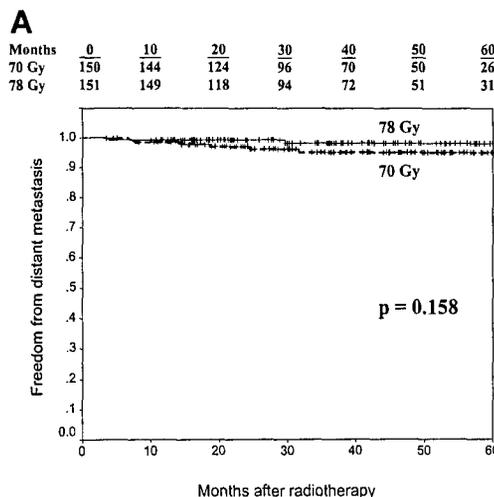


Fig 4. Kaplan-Meier freedom from distant metastasis for (A) all patients and (B) those with PSA levels of more than 10 ng/mL by dose randomization (70 Gy v 78 Gy). The numbers of patients at risk at 10-month intervals are shown above the graphs.

lustrate this point. For PSA-era patients treated between 1987 and 1995, median radiotherapy doses increased from 64 Gy (range, 60 to 68 Gy) in 1987 through 1989 to 70 Gy (range, 66 to 78 Gy) in 1994 through 1995.² Doses were incrementally increased as it became apparent, through the recognition of posttherapy increasing PSA level as a valid end point, that standard doses were not as effective as previously believed. In a recent update of our retrospective experience,⁴ increasing the dose from ≤ 67 Gy to more than 67 to 77 Gy resulted in improved FFF rates for all patient prognostic categories, including favorable patients with pretreatment PSA levels of ≤ 10 ng/mL. The main advantage of increasing the dose from more than 67 to 77 Gy to 78 Gy was observed for patients with intermediate- to high-risk clinical features. Other investigators have reported analogous patterns.

Table 5. Distribution of Patients by 2-Year Prostate Biopsy Pathologic Features and Treatment Arm

Group*	Biopsy-Negative*				Biopsy-Positive*				P
	No Tumor		Atypical/Susp Cells		Carcinoma + Tx Effect		Frank Carcinoma		
	No. of Biopsies	%	No. of Biopsies	%	No. of Biopsies	%	No. of Biopsies	%	
70-Gy arm	29	37	28	35	13	17	9	11	.029†/.33‡
78-Gy arm	34	48	12	17	20	28	5	7	

*Patients without evidence of biochemical and/or clinical relapse are analyzed. Four pathologic groups are shown: no tumor; atypical cells suggestive of abnormality but not diagnostic of carcinoma; carcinoma with treatment effect; and frank carcinoma without treatment effect.

† χ^2 test for all four groups.

‡ χ^2 test for biopsy-positive versus biopsy-negative.

Hanks et al⁵ have demonstrated higher 5-year FFF rates for intermediate-risk patients (pretreatment PSA ≥ 10 to < 20 ng/mL), going from 29% at less than 71.5 Gy to 57% at 71.5 to 75.7 Gy and finally to 73% at more than 75.7 Gy. These investigators also described an increase in 5-year FFF rates for high-risk patients (pretreatment PSA level of > 20 ng/mL) from 8% at less than 71.5 Gy to 28% to 30% at 71.5 Gy and above. Zelefsky et al⁶ found significantly improved FFF rates as a function of dose for intermediate- and high-risk patients in their sequential dose-escalation trial. The 4- to 5-year FFF rates for doses of 64.8 to 70.2 Gy and 75.6 to 81.0 Gy were approximately 53% and approximately 79%, respectively, for intermediate-risk patients and approximately 20% and approximately 53%, respectively, for high-risk patients. The Cleveland Clinic Group also recently noted a lower 5-year FFF rate when less than 72 Gy was used (54%), as compared with ≥ 72 Gy (85%) in a cohort of 738 men treated with radiotherapy. Dose was an

independent predictor of FFF and was significant for patients with favorable and unfavorable prognostic attributes. A drawback of studying the effects of dose on patients treated serially over time is that these policies were accompanied by sweeping refinements in the ability to diagnose prostate cancer earlier on the basis of PSA level and prostate imaging.^{9,10} Resultant stage migration has further complicated such nonrandomized comparisons and strengthened the need for confirmation in the setting of randomized trials.

Shipley et al¹ performed a randomized dose-escalation trial of 67.2 Gy versus 75.6 Gy in 202 patients with locally advanced prostate cancer. The delivery of the higher dose was accomplished using a proton boost. In this pre-PSA study, there was no overall difference in the rate of disease freedom or survival based on the increased dose. However, local control at 8 years was significantly enhanced from 19% to 84% ($P = .0014$) in a dose-dependent manner. Because this trial involved patients with locally advanced disease who were treated in the pre-PSA era with protons, the findings may not be representative of contemporary prostate cancer patients treated with photons. Although a number of randomized dose-escalation trials are underway around the world, the results described here are the first in the PSA era, to our knowledge, to document the benefit of dose.

The findings presented here verify the retrospective analyses. An 8-Gy increase in dose was responsible for a borderline significant improvement in FFF rates from 69% to 79%. This improvement was mainly attributable to the gains seen for intermediate- to high-risk patients, particularly those with pretreatment PSA levels of more than 10 ng/mL. Intermediate-risk patients, such as those with T1/T2 disease and pretreatment PSA level of more than 10 ng/mL, exhibited a 50% increase in FFF rate at the higher dose. The data also suggested that other intermediate-risk patients may benefit from dose escalation, although there were not enough patients in other intermediate-risk subgroups to make any meaningful comparisons. Future randomized trials should target these patients. Moreover, the distant

Months	0	10	20	30	40	50	60
Negative	63	63	63	53	42	24	13
Atyp/Susp	40	40	39	33	22	13	6
Tx Effect	33	33	33	18	11	8	4
Frank Ca	14	14	13	9	5	4	2

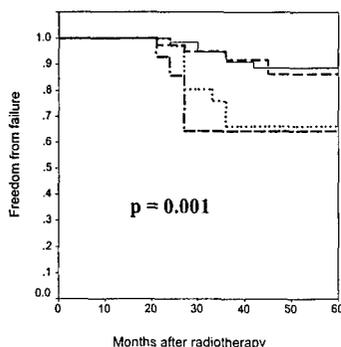


Fig 5. Kaplan-Meier FFF curves for the 150 failure-free patients who underwent prostate biopsy 2 years after radiotherapy, divided by biopsy classification. The numbers of patients at risk at 10-month intervals are shown above the graph. Biopsy results: —, frank carcinoma; ···, cancer with treatment effect; - - -, cells that are atypical or suggestive of abnormality; —, negative. Abbreviations: Atyp/Susp, cells that are atypical or suggestive of abnormality; Tx, treatment; Ca, carcinoma.

metastasis rate was reduced by the administration of 78 Gy in patients with intermediate risk features (Fig 4B).

A number of retrospective analyses have indicated that local failure is highly associated with distant metastasis²⁰⁻²² for patients with prostate cancer who are treated with radiotherapy. Although a marginally significant reduction in distant metastasis as a consequence of dose escalation was observed in the study presented here, the absolute difference was slight. Longer follow-up would make the results more convincing. Confirmatory evidence that dose affects distant failure has been described by Hanks et al.²³ They performed a matched pair analysis of 714 patients, one half of whom were treated to less than 74 Gy versus ≥ 74 Gy. Freedom from biochemical failure, freedom from distant metastasis, and survival were all significantly higher for the patients treated to the ≥ 74 -Gy dose level.

The other end point examined in our randomized trial was prostate biopsy positivity at 2 years after the completion of radiotherapy. Biopsy positivity was seen in 31% of patients, and there was no difference between the two dose groups. There were, however, more patients in the 78-Gy group who had carcinoma with treatment effect. A number of investigators have reported that many such cases convert to biopsy-negative status over time,^{24,25} with some patients converting to biopsy-negative status beyond 2 years. Yet our time-to-event data (Fig 5) indicate that biochemical failure rates are identical for those with biopsies showing carcinoma with treatment effect and those with frank carcinoma. Zelefsky et al⁶ have quantified biopsy positivity at ≥ 2.5 years posttreatment and have documented a

relationship between higher doses and significantly lower biopsy positivity rates. Their results parallel ours to the degree that there was a greater frequency of biopsy negativity and treatment effect as dose was increased. Longer follow-up will reveal the percentage of those patients having carcinoma with treatment effect in the biopsy who convert to biopsy-negative status and the prognostic value of this conversion in terms of FFF rate. The patients in our series that demonstrated carcinoma with treatment effect are undergoing repeat biopsy yearly to determine the conversion rate to biopsy-negative status. The final analysis of the trial is projected to be in the latter half of 2001, when minimum patient follow-up will be at least 3 years from treatment completion.

In summary, this preliminary analysis shows that significant gains in FFF and freedom from distant metastasis are realized with a modest radiotherapy dose increment of 8 Gy for patients with pretreatment PSA levels of more than 10 ng/mL. Because there were only 16 patients with pretreatment PSA levels of more than 20 ng/mL, most were intermediate in risk. The greatest impact of dose was observed for those with a balance of prognostic features between favorable and intermediate, such as stage T1/T2 disease and pretreatment PSA level of more than 10 ng/mL. These patients should be targeted for future dose-escalation trials.

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**Ki-67 Staining Is An Independent Correlate Of Biochemical Failure In Prostate Cancer
Treated With Radiotherapy**

**Didier Cowen,^{1,2} Patricia Troncoso,³ Vincent S. Khoo,^{1,4} Gunar K. Zagars,⁵
Andrew C. von Eschenbach,⁶ Marvin L. Meistrich¹ and Alan Pollack,^{5,*}**

¹Department of Experimental Radiation Oncology, U.T. M.D. Anderson Cancer Center

³Department of Pathology, U.T. M.D. Anderson Cancer Center

⁵Department of Radiation Oncology, U.T. M.D. Anderson Cancer Center

⁶Department of Urology, U.T. M.D. Anderson Cancer Center

² Current Address: Institut Paoli Calmettes, 232 Bvd Sainte Marguerite, Marseille 13009, France

⁴ Current address: Academic Unit of Radiotherapy, Royal Marsden Hospital, Downs Road, Sutton,
Surrey SM25PT, UK.

*Correspondence: Alan Pollack, M.D., Ph.D.
Department of Radiation Oncology (Box 97)
1515 Holcombe Boulevard, Houston, Texas 77030
Tel: (713) 792-0781; Fax: (713) 794-5573
e-mail: APollack@notes.mdacc.tmc.edu

ABSTRACT

PURPOSE. To determine the significance of Ki-67/MIB1 staining as a marker of patient outcome for prostate cancer patients treated with radiotherapy.

MATERIALS AND METHODS. Pretreatment archival prostate biopsy tumor tissue was available from 106 stage T1-T4 prostate cancer patients treated with external beam radiotherapy between 1987 and 1993 at M.D. Anderson Cancer Center. Diagnosis was made from prostate needle biopsy in 64 cases and from TURP in 42 cases. All patients had a pretreatment PSA and no patient had evidence of metastasis. Immunohistochemical staining for MIB1 was used to determine the percentage of Ki-67 positive tumor cells, the Ki-67 labeling index (Ki67-LI). Biochemical failure after radiotherapy was defined as 3 consecutive rises in PSA on follow-up. No patient failed clinically without evidence of biochemical failure. Median follow-up was 62 months.

RESULTS. The mean and median Ki67-LI for the entire cohort was 3.2 and 2.3 (range 0 – 13.8). The mean and median Ki67-LIs for those diagnosed by needle biopsy were 3.2 and 2.3 (range 0.1 – 13.8), and by TURP were 3.1 and 2.4 (range 0 – 12.4). For all patients, mean Ki67-LI levels were significantly higher with Stage T3/T4 disease, Gleason 7-10 disease, and in those that developed treatment failure. Similar relationships were observed when the Ki67-LI was dichotomized into low ($\leq 3.5\%$) and high ($> 3.5\%$) groups. Actuarial freedom from biochemical failure (bNED) when Ki67-LI was low and high was 76 and 33% at 5 years ($p < 0.0001$, log rank). Similar statistically significant differences were observed when the TURP and needle biopsy groups were analyzed separately. Cox proportional hazards regression showed that dichotomized Ki67-LI was an independent correlate of bNED, along with pretreatment PSA, Gleason score, and clinical stage.

CONCLUSIONS. The Ki67-LI obtained from pretreatment prostate cancer tissue is a strong independent predictor of failure after radiotherapy using biochemical criteria. This prognostic factor was equally valuable for patients diagnosed by TURP or needle biopsy.

Running Title: Prostate cancer Ki-67 labeling index

KEY WORDS: Ki-67, MIB-1, Prostate cancer, Prostate specific antigen, Radiotherapy.

INTRODUCTION

The clinical progression of prostate cancer is slow and this is reflected in various proliferation markers. Pretreatment serum prostate specific antigen (PSA) doubling is usually greater than 12 months (1, 2), as is tumor doubling time based on cell kinetic parameters (3). Likewise, the in situ nucleoside analogue labeling index is usually less than 6% (4, 5, 6) and potential doubling time is most often >24 hr (6). Flow cytometric, as well as image analysis, have shown that most tumors are DNA-diploid (7) and are comprised of less than 5% S phase cells (8, 9). The Ki-67 labeling index (Ki67-LI) is another proliferation marker that is determined via a rapid, simple immunohistochemical method. The Ki67-LI, measured using MIB-1 antibody, provides an accurate estimate of growth fraction (10-13) and in many studies has been found to be a predictor of outcome for patients treated with radical prostatectomy (14-22). Preliminary data indicate that this static approximation of growth fraction is more strongly associated with patient outcome than DNA ploidy (23). However, few studies have investigated whether Ki67-LI is an independent predictor of prostate cancer patient outcome after treatment with radiotherapy in the PSA era (21). In this report, the Ki67-LI from pretreatment biopsy specimens in 106 prostate cancer patients treated with radiotherapy in the PSA era was established to be a strong independent correlate of biochemical failure.

MATERIALS AND METHODS

Patient Characteristics

Archival paraffin-embedded pretreatment prostate cancer tissue was available in 106 patients treated with external beam radiotherapy from 1987-1993. Every patient had a pretreatment PSA. No patient received androgen ablation neoadjuvantly or adjuvantly, underwent lymph node dissection for staging, or underwent radical prostatectomy. The diagnosis of prostate cancer was made by transurethral resection of the prostate (TURP) in 42 (40%) and by ultrasound-guided transrectal prostate needle biopsies in 64 (60%).

Median and mean pretreatment PSA levels were 7.6 and 11.8 ng/ml for all patients, 9.8 and 13.7 ng/ml for the needle biopsy group, and 4.1 and 8.8 ng/ml for the TURP group. Median follow-up was 62 months for the entire group, 62 months for the needle biopsy group, and 61 months for the TURP group. There were 74 patients with stage T1/T2 disease and 32 with T3/T4 (only one had T4) disease.

Immunohistochemical Staining of Ki-67/MIB1

The monoclonal antibody, MIB-1 (Immunotech, SA MAC Inc., Germany), was used to determine the proportion of tumor cells staining positive for Ki-67, the Ki-67 labeling index (Ki67-LI). As described previously (23), slide-mounted paraffin-embedded prostatic tissue sections were deparaffinized in xylene, rehydrated sequentially in ethanol (100%, 90%, 70%) and placed into a 1% phosphate buffered solution (PBS, pH 7.4). The sections were then heated in a conventional 600 W. microwave oven at maximum power for 3 x 5 minutes. The sections were left at room temperature for 40 minutes and 2% normal horse serum added to block non-specific protein binding. The sections were incubated with MIB-1 antibody (1:50 dilution) overnight at 4°C in a

humidified chamber. Detection of the bound MIB-1 antibody involved applying the VECTASTAIN Elite ABC reagents (Vector Laboratories Inc, Burlingame, CA) using Avidin DH: biotinylated horseradish peroxidase H complex with 3,3'-diaminobenzidine (Polysciences Inc., Warrington, PA) and Mayer's hematoxylin (Fisher Scientific, Fair Lawn, NJ). Appropriate positive controls (HeLa cells) were included in each immunohistochemical run to verify the specificity of MIB-1 and negative controls were produced by substituting the primary antibody with PBS in duplicate sections.

Tissue Specimens and Ki67-LI

Needle biopsy and TURP sections were reviewed by the study pathologist (P.T.) and graded according to the Gleason system. Sections representative of the tumor with the highest grade were selected for immunohistochemical analysis. When possible, 2000 tumor cells were counted for the determination of Ki67-LI. Any nuclear staining, regardless of intensity, was considered positive for MIB-1. The Ki67-LI was expressed as a percentage of immunoreactive tumor cells to the total counted tumor cells. Two of the investigators (V.K. and D.C.) scored the slides without any prior knowledge of the patient data or treatment related outcomes. The mean (\pm SEM) Ki67-LIs for the two counts were $2.5 \pm 0.3\%$ (\pm SEM) and $3.8 \pm 0.3\%$, and were statistically different (student's t-test and Wilcoxon signed ranks test, for paired samples). The averages of these independent counts were used for the analyses.

Statistics

The chi-square test was used to assess the significance of differences between proportions (24). Non-parametric comparisons between independent groups were performed using the Mann-

Whitney test. Kaplan-Meier curves were calculated from the completion of radiotherapy, with tests of statistical significance based on the log-rank statistic (25). Biochemical failure was defined as three PSA rises on follow-up (26). The onset of a rising PSA was defined as the average time between the date of the PSA obtained prior to the first risen value and the date of the first risen value.

RESULTS

The mean Ki67-LI was 3.2% and there was no statistically significant difference between the mean Ki67-LIs from the TURP and needle biopsy specimens (Table 1). For the entire group, as well as for those diagnosed by TURP, mean Ki67-LI was significantly higher in the presence of T3/T4 and Gleason score 7-10 disease, as well as when there was evidence of biochemical failure. Also, no correlation was seen between pretreatment PSA and Ki67-LI for the entire cohort; however, a significant relationship was observed for those diagnosed from TURP. For patients diagnosed by needle biopsy, no associations between Ki67-LI and the other pretreatment prognostic factors were discerned. The reason for the differences in the relationships of Ki67-LI in the patients diagnosed by needle biopsy and TURP is uncertain, but could be based on inherent biologic divergence or technical factors, such as the amount of tissue available for analysis. The one correlation that was evident for patients diagnosed by needle biopsy and TURP, as well as for the entire group was between Ki67-LI and treatment failure. The mean Ki67-LI was significantly higher for those manifesting a rising PSA after radiotherapy. Radiotherapy dose has been shown to be a determinant of outcome (27) and so was investigated here. There was no relationship between dose and mean Ki67-LI.

Previously we found (23) that, for patients diagnosed with prostate cancer by TURP, a Ki67-LI >3.5% was associated with a poor prognosis. Table 2 shows that the correlations between dichotomized Ki67-LI and stage, Gleason score, pretreatment PSA, radiotherapy dose, and treatment failure were the same as for mean Ki67-LI in Table 1.

Figure 1 shows the Kaplan-Meier freedom from biochemical failure (bNED) survival analysis for the entire cohort, the patients diagnosed by TURP and those diagnosed by needle

biopsy. There was no difference statistically between the two diagnostic groups. The univariate 5 year bNED survival results for the factors associated with patient outcome are shown in Table 3. Stage T3/T4, Gleason score 7-10, pretreatment PSA >10 ng/ml and Ki67-LI >3.5% predicted strongly for reduced bNED rates. Radiotherapy dose did not correlate with bNED in this cohort. Figure 2 displays the bNED survival curves for dichotomized Ki67-LI, subdivided by diagnostic group. A high Ki67-LI consistently was associated with a lower bNED rate, independent of whether diagnosis was based on TURP or needle biopsy. The results of Cox proportional hazards regression for bNED are shown in Table 4. Ki67-LI was a highly significant correlate of bNED, along with pretreatment PSA, stage, and Gleason score.

The relationship of Ki67-LI to freedom from distant metastasis was also examined to determine if the reduction in bNED associated with a high Ki67-LI was due to metastasis. The absolute percentage of patients with distant metastasis and nodal metastasis identified in the follow-up period was 4%(n=4) and 3%(n=3), respectively. Figure 3 shows that 98% and 94% were free of distant metastasis by Kaplan-Meier analysis when the Ki67-LI was low and high, respectively. This difference was not significant. Of the 37 patients that had a rising PSA, 20 were investigated by prostate biopsy and imaging, and 13 were found to have local disease persistence, one with concurrent nodal and distant spread. Therefore, the initial rise in PSA appears to be due to incomplete eradication of local disease in most cases.

DISCUSSION

The Ki67-LI by immunohistochemical staining provides a noninvasive, relatively rapid, determination of growth fraction, which has prognostic value. In the vast majority of reports (14-16,18-21) Ki67-LI has been observed to be a correlate of biochemical and/or disease outcome for patients with prostate cancer. Table 5 summarizes a number of contemporary series, showing that in the majority Ki-67 immunostaining was also independent of other prognostic factors in multivariate analysis.

The predictive usefulness of immunohistochemical Ki-67 staining for patients treated with radiotherapy has only been reported by one other group (21). Scalzo et al (21) classified Ki-67 staining into low and high groups based on the number positive cells per high powered microscopic field. Even though the classification of Ki-67 staining by the number per high powered field is less exacting than the quantification of labeling index, they found a correlation of Ki-67 staining with biochemical failure in univariate and multivariate analyses. Our data in 106 cases also demonstrated that Ki-67 is a significant predictor of biochemical relapse in patients with clinically localized prostate cancer treated with radiotherapy. The Ki67-LI cut-point of 3.5% that we used was taken from a prior analysis of the patients diagnosed by TURP. In the analysis described here, this Ki67-LI cut-point was also strongly associated with biochemical failure in patients diagnosed from needle biopsy tissue. The pooled TURP and needle biopsy Ki67-LI analysis was of sufficient power to document that Ki67-LI is independent of pretreatment PSA, Gleason score, and stage as a correlate of a rising PSA after radiotherapy.

The series' displayed in Table 5 included patients followed for progression after observation (deferred treatment) or androgen ablation (18, 19), radical prostatectomy (14-17, 20, 22), or radiotherapy (21). The predictive merit of Ki-67 immunohistochemical staining appears to be

unaffected by the treatment used. Concerning factors in the application of Ki67-LI clinically are interobserver variability in the estimation of Ki67-LI and the way the data are categorized. The two investigators that quantified the staining in our study had slightly, but statistically, different estimates of Ki-67-LI on a case-by-case basis. Disparity in data categorization is also evident; Scalzo et al (21) and Kallakury (22) used the number of positive cells per high powered field, whereas the quantification of Ki67-LI is the more typical and reproducible method. Moreover, there has been considerable disparity in the Ki67-LI cut-points used to assess failure risk. Ki67-LI cut-points have ranged from 1% (17) to 25% (15) and it is clear that this discrepancy is reflective of median Ki67-LI differences. Since inconsistency in Ki67-LI cut-points is apparent among patients treated by radical prostatectomy, the median differences do not appear to be solely a consequence of inherent attributes of the patient populations examined. A number of other technical variables might contribute to this diversity in absolute Ki67-LI levels, such as loss of Ki-67 antigen staining with storage (28), antigen retrieval, the monoclonal antibody, and the classification of positive staining (interobserver variation). Stricter standardization of the method needs to be established before widespread clinical application is feasible.

CONCLUSION

The Ki67-LI is strongly associated with biochemical relapse after radiotherapy. A high Ki67-LI, which was defined here as >3.5%, resulted in a bNED rate of only 33%, versus 76% for those with a low Ki67-LI. Our data suggest that local disease persistence most commonly accounts for a rising PSA after radiotherapy. Since prostate cancer proliferation rates are exceptionally low when compared to those of other tumor sites, even in the high Ki67-LI group, it is unlikely that accelerated tumor repopulation is responsible for the presumed resistance to radiotherapy. The more

probable mechanism is that a high Ki67-LI is associated with tumor aggressiveness and radioresistance.

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Table 1. Percent Ki-67 staining by various potential prognostic factors.

Grouping		TURP %Ki-67	Biopsy %Ki-67	All %Ki-67
		Mean + SE(n)	Mean ± SE(n)	Mean ± SE(n)
All Patients		3.1 ± 0.4 (42)	3.2 ± 0.4(64)	3.2 ± 0.3(106)
Stage	T1/T2	2.4 ± 0.3 (35)	3.0 ± 0.5(39)	2.7 ± 0.3(74)
	T3/T4	6.7 ± 1.4 (7)*	3.4 ± 0.6(25)	4.2 ± 0.6(32)*
Gleason Score	2-6	2.0 ± 0.3 (21)	2.3 ± 0.5(17)	2.1 ± 0.3(38)
	7-10	4.2 ± 0.7 (21)*	3.6 ± 0.5(46)	3.8 ± 0.4(67)*
Pretreatment PSA	≤ 10 ng/ml	2.2 ± 0.3 (33)	3.3 ± 0.5(34)	2.8 ± 0.3(67)
	> 10 ng/ml	6.4 ± 1.0 (9)*	3.1 ± 0.6(30)	3.8 ± 0.6(39)
Radiotherapy Dose	≤ 67 Gy	2.9 ± 0.4(34)	2.9 ± 0.5(35)	2.9 ± 0.3(69)
	> 67 Gy	4.1 ± 1.3(8)	3.6 ± 0.6(29)	3.7 ± 0.5(37)
Treatment Failure	No	2.3 ± 0.4 (29)	2.5 ± 0.4(40)	2.4 ± 0.3(69)
	Yes	4.9 ± 0.7 (13)*	4.3 ± 0.7(24)*	4.5 ± 0.5(37)*

*p<0.05 by both Student's t-test and Mann – Whitney test; SE = standard error.

Table 2. Distribution of patients by Ki-67 staining as a dichotomous variable.

Group		%Patients(n)					
		TURP %Ki-67		Biopsy %Ki-67		All %Ki-67	
		≤3.5	>3.5	≤3.5	>3.5	≤3.5	>3.5
Stage	T1/T2	80(28)	20(7)	67(26)	33(13)	73(54)	27(20)
	T3/T4	29(2)	71(5)*	60(15)	40(10)	53(17)	47(15)*
Gleason Score	2-6	91(19)	9(6)	71(12)	29(5)	82(31)	18(7)
	7-10	52(11)	48(10)*	61(28)	39(18)	58(39)	42(28)*
Pretreatment PSA	≤ 10 ng/ml	85(28)	15(5)	62(21)	38(13)	73(49)	27(18)
	> 10 ng/ml	22(2)	78(7)*	67(20)	33(10)	56(22)	44(17)
Radiotherapy Dose	≤ 67 Gy	74(25)	26(9)	63(22)	37(13)	68(47)	32(22)
	> 67 Gy	63(5)	37(3)	66(19)	35(10)	65(24)	35(13)
Treatment Failure	No	86(25)	14(4)	78(31)	22(9)	81(56)	19(13)
	Yes	39(5)	61(8)*	42(10)	58(14)*	41(15)	59(22)*

*p<0.05, Chi-square

Table 3. Univariate analysis of correlates of 5 year bNED.

Grouping		N	%5 yr bNED	P*
Stage	T1/T2	74	74	<0.0001
	T3/T4	32	31	
Gleason Score	2-6	38	84	<0.0001
	7-10	67	45	
Pretreatment PSA	≤ 10 ng/ml	67	76	<0.0001
	> 10 ng/ml	39	32	
RT Dose	≤ 67 Gy	69	59	0.89
	> 67 Gy	37	62	
Ki67-LI	≤ 3.5%	71	76	<0.0001
	> 3.5%	35	33	

*log-rank test.

Table 4. Cox proportional hazards multivariate analysis of factors predictive of bNED.

Variable	Grouping	Chi-square	RR(95%CI)	p
Ki87-LI	≤ 3.5% vs >3.5%	8.8	2.8(1.4-5.4)	0.003
Pretreatment PSA	≤ 10 vs >10 ng/ml	8.2	2.7(1.3-5.5)	0.004
Stage	T1/T2 vs T3/T4	7.6	2.6(1.3-5.0)	0.006
Gleason Score	2-6 vs 7-10	7.1	3.4(1.3-9.2)	0.008

RR = relative risk; CI = confidence intervals.

Table 5. Contemporary series investigating the association of Ki-67 with prostate cancer patient outcome.

<u>Author(Ref)</u>	<u>Year</u>	<u>N</u>	<u>Tx</u>	<u>F/U</u>	<u>%Ki67-LI(n)</u>	<u>Failure</u>	<u>Univar</u>	<u>Multivar</u>
Bubendorf(14)	1996	137	Prostx	5 yr	<7.5%	20*	Sig	Sig
Bettencourt(15)	1996	180	Prostx	4 yr	≥7.5%	35*		
					<1%(18)	17*		
					1-25%(90)	31*		
Stapleton(16)	1997	47	Prostx	5 yr	≥26%(72)	56*	Sig	Sig
Coetzee(17)	1997	244	Prostx	2 yr	Median 2.4%	----	Sig	NS
					<1%	----	NS	NS
					≥1%	----		
Stattin(18)	1997	125	Obs/AA	6 yr	≤3%(99)	18†		
					>3%(26)	63†	Sig	Sig
Borre(19)	1998	221	Obs/AA	>5 yr	≤10%	37		
					>10%	62	Sig	Sig
Keshgegian(20)	1998	208	Prostx	4 yr	≤6.4%(106)	8*		
					>6.4%(102)	20*	Sig	Sig
Scalzo(21)	1998	42	XRT	-----	≤5Nuc/HPF	----		
					>5Nuc/HPF	----	Sig	Sig
Kallakury(22)	1999	132	Prostx	4 yr	1-3Nuc/HPF	----		
					4-6Nuc/HPF	----		
					>7Nuc/HPF	----	NS	NS
Current Report	2000	106	XRT	5 yr	≤3.5%(71)	24*		
					>3.5%(35)	67*	Sig	Sig

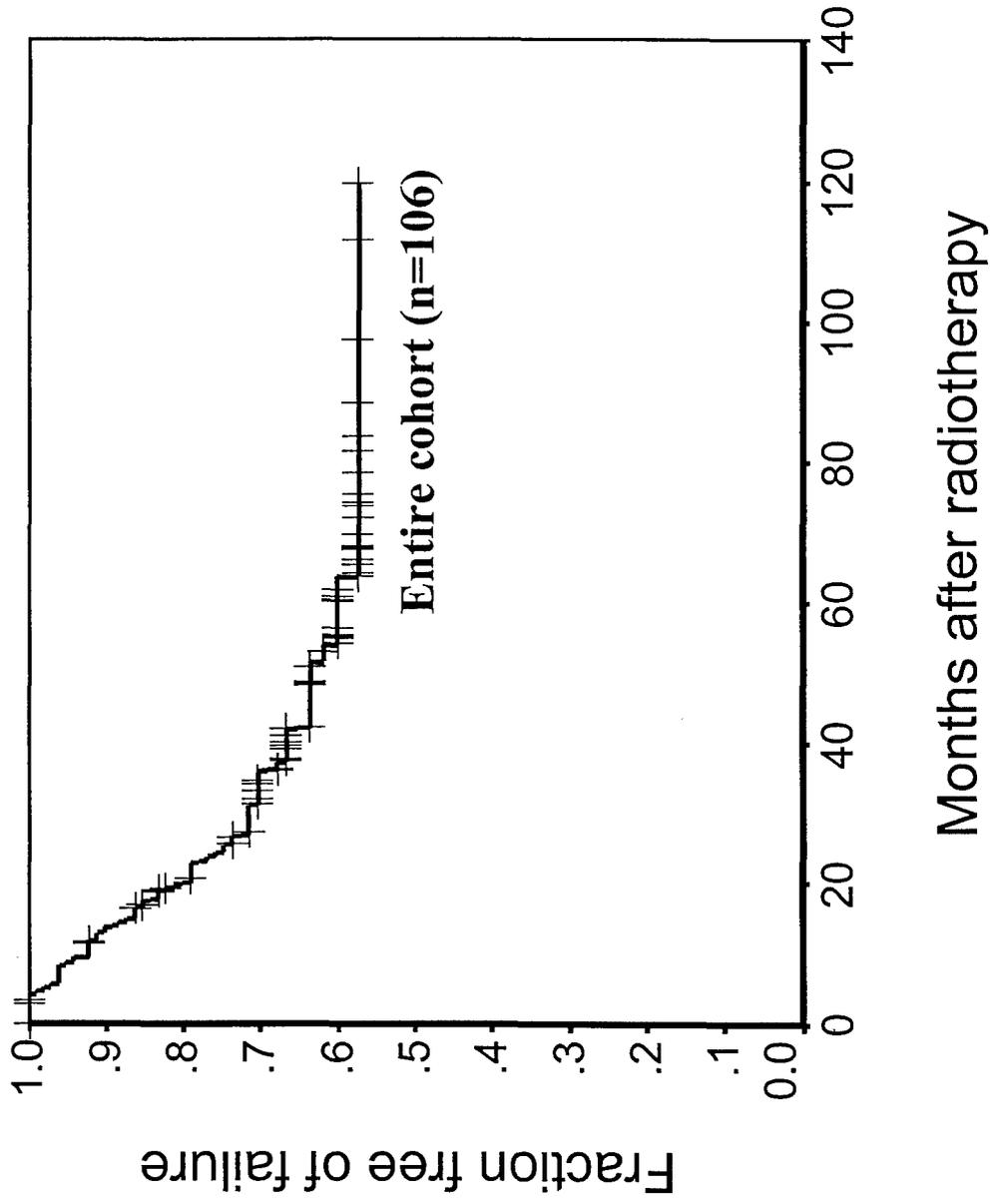
* Failure (biochemical and/or disease); † Cancer specific death.

F/U = followup; Prostx = prostatectomy; TURP = transurethral resection of the prostate; Obs = observed; AA = androgen ablation; XRT = radiotherapy; Sig = significant; NS = not significant.

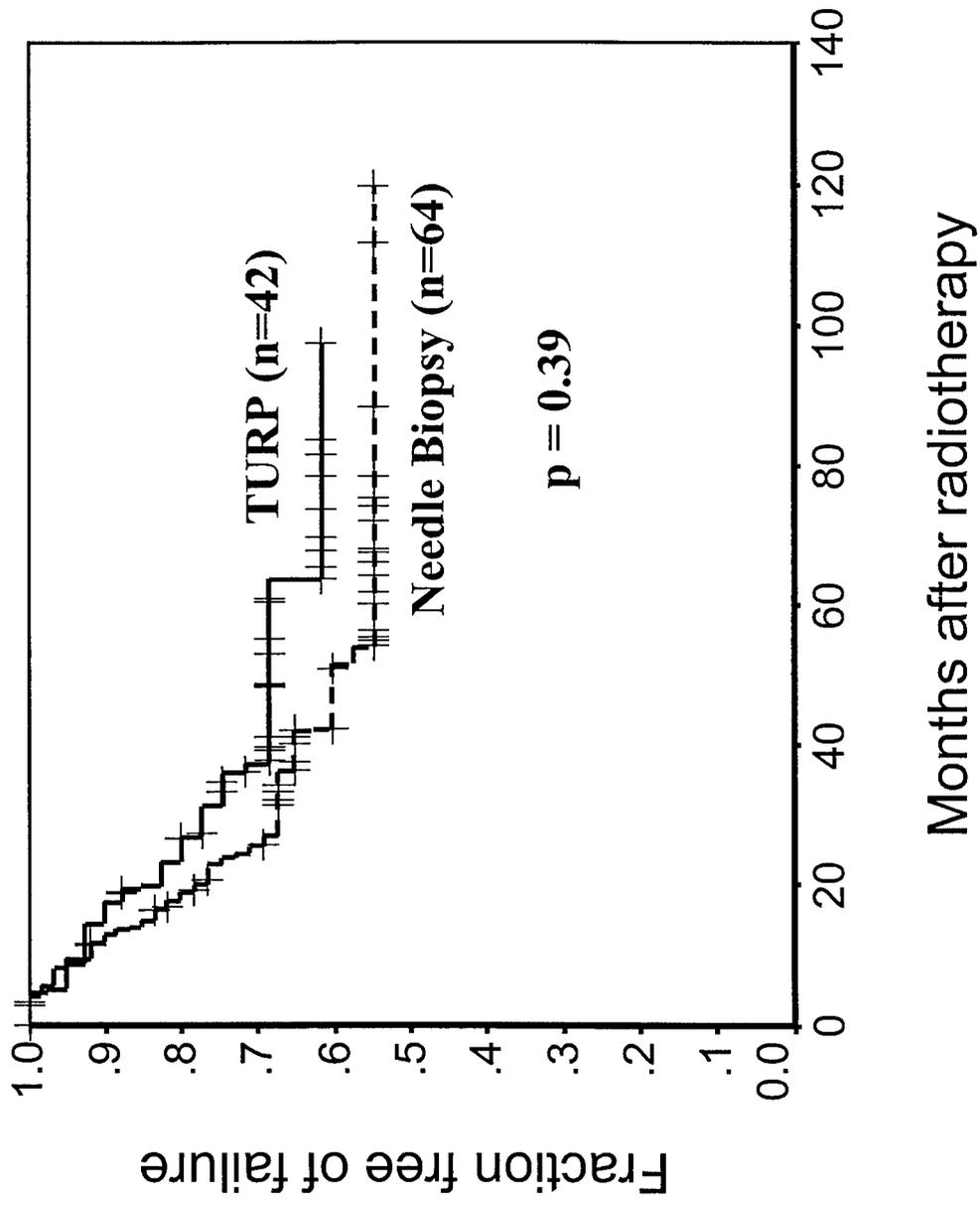
FIGURE LEGENDS

- Figure 1. Kaplan-Meier freedom from biochemical failure analysis for the entire cohort (left) and those diagnosed with prostate cancer based on TURP or needle biopsy (right).
- Figure 2. Kaplan-Meier freedom from biochemical failure analysis based on Ki67-LI ($\leq 3.5\%$ vs $> 3.5\%$) for patients diagnosed by TURP (left), patients diagnosed by needle biopsy (middle), and all patients (right). The solid line and dashed line curves are for Ki67-LI $\leq 3.5\%$ and $> 3.5\%$, respectively.
- Figure 3. Kaplan-Meier freedom from distant metastasis analysis based on Ki67-LI ($\leq 3.5\%$ vs $> 3.5\%$). The solid line and dashed line curves are for Ki67-LI $\leq 3.5\%$ and $> 3.5\%$, respectively.

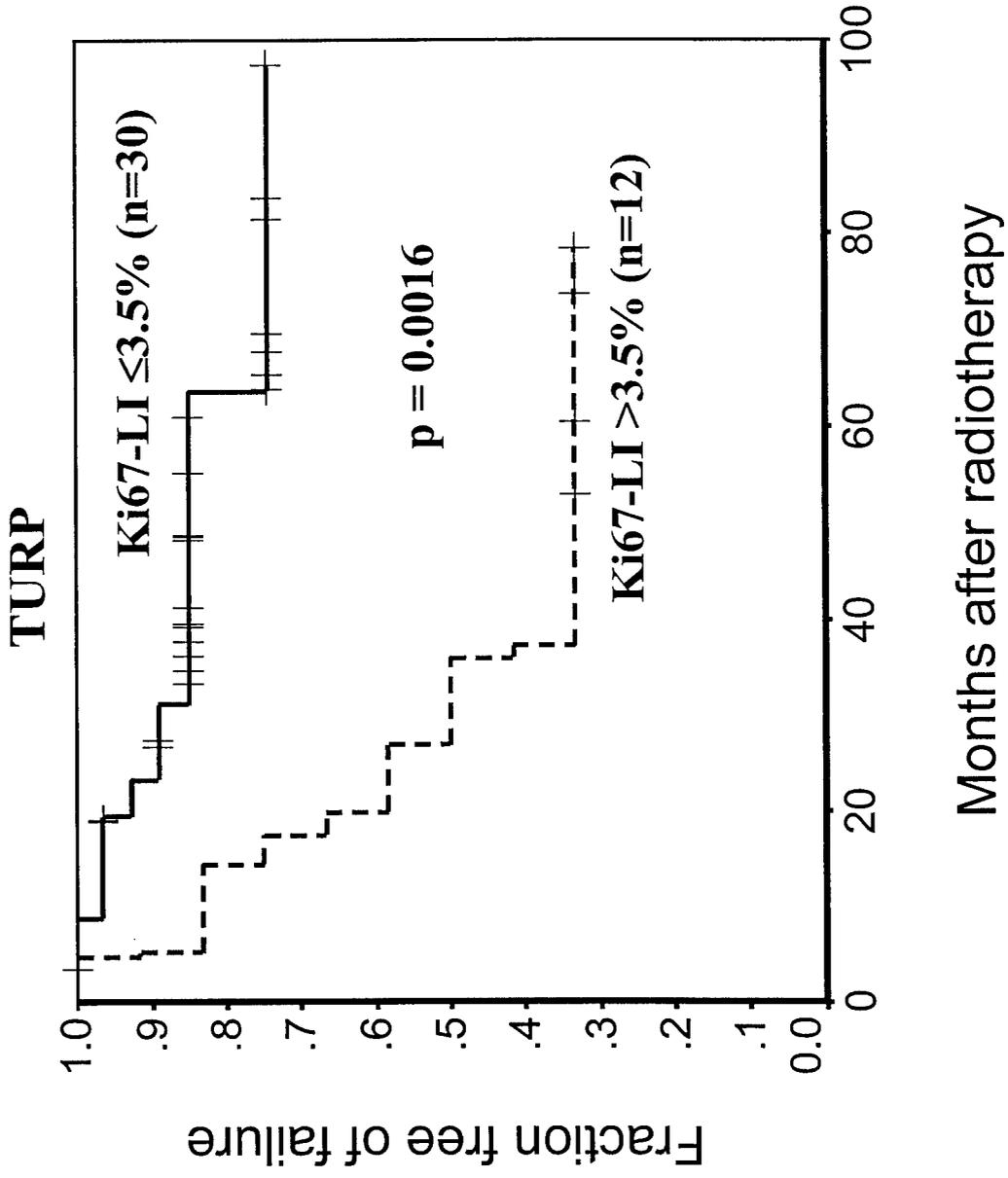
Cowen et al, Fig 1 (left)



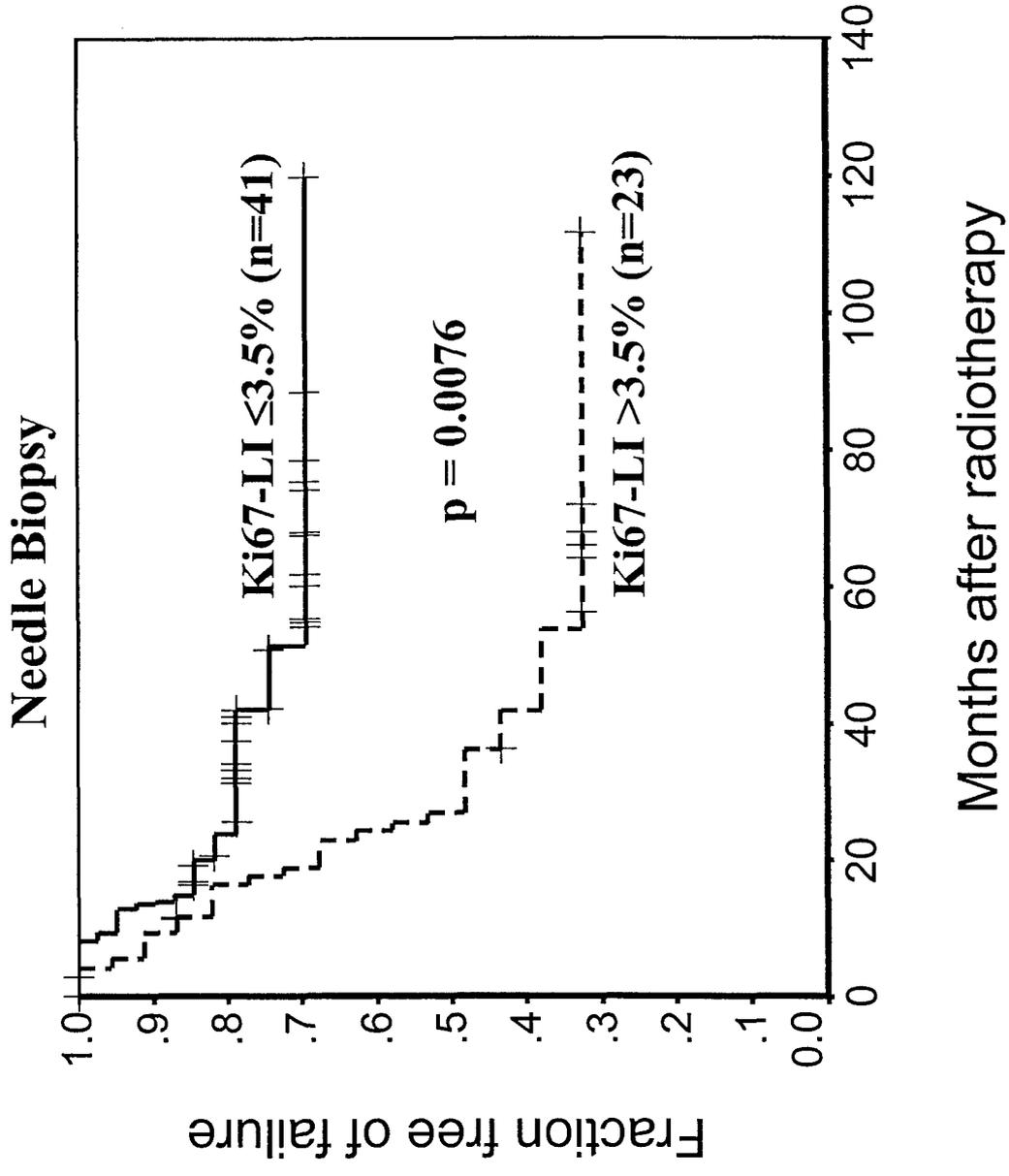
Cowen et al, Fig 1 (right)



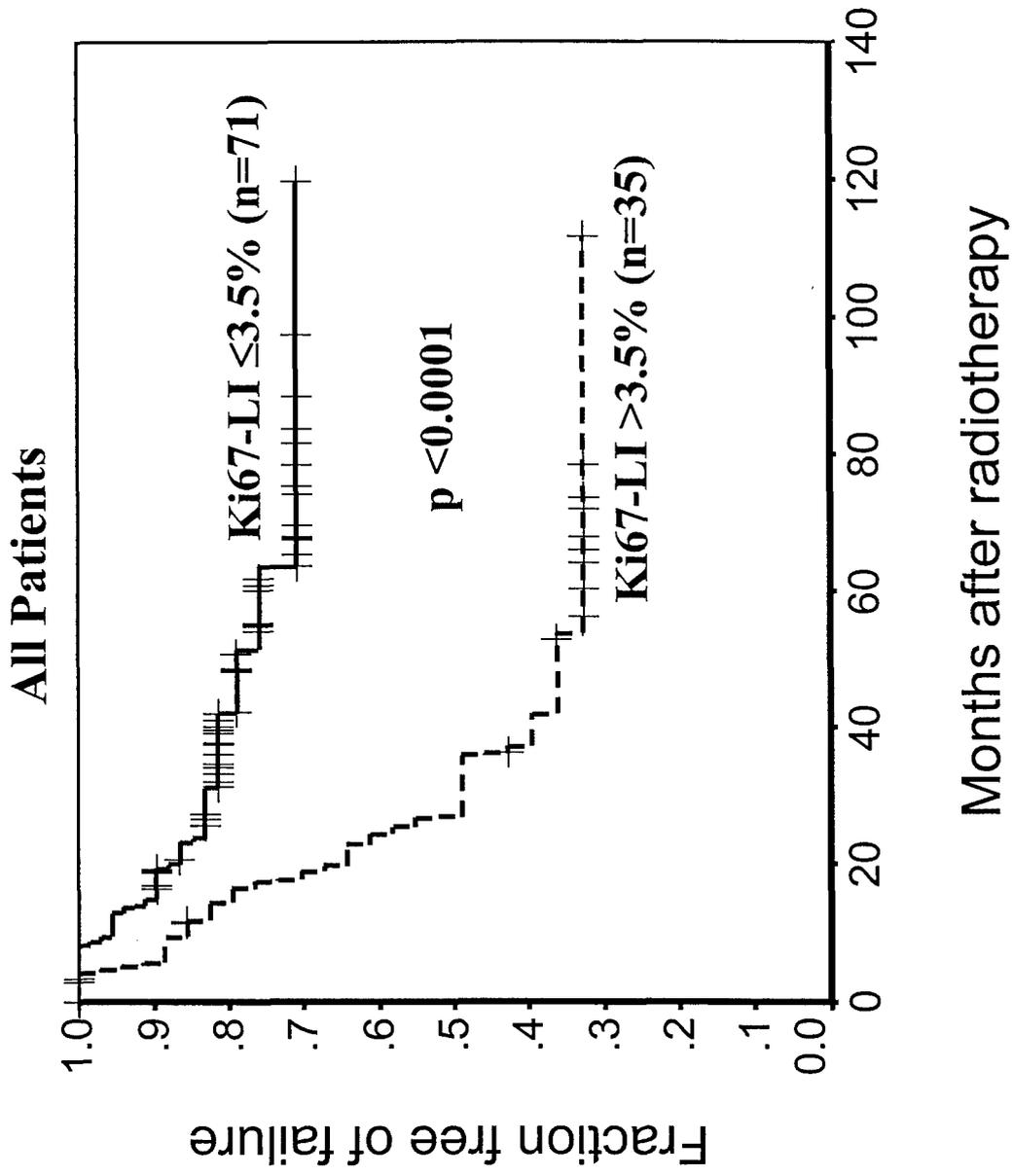
Cowen et al, Fig 2(left)



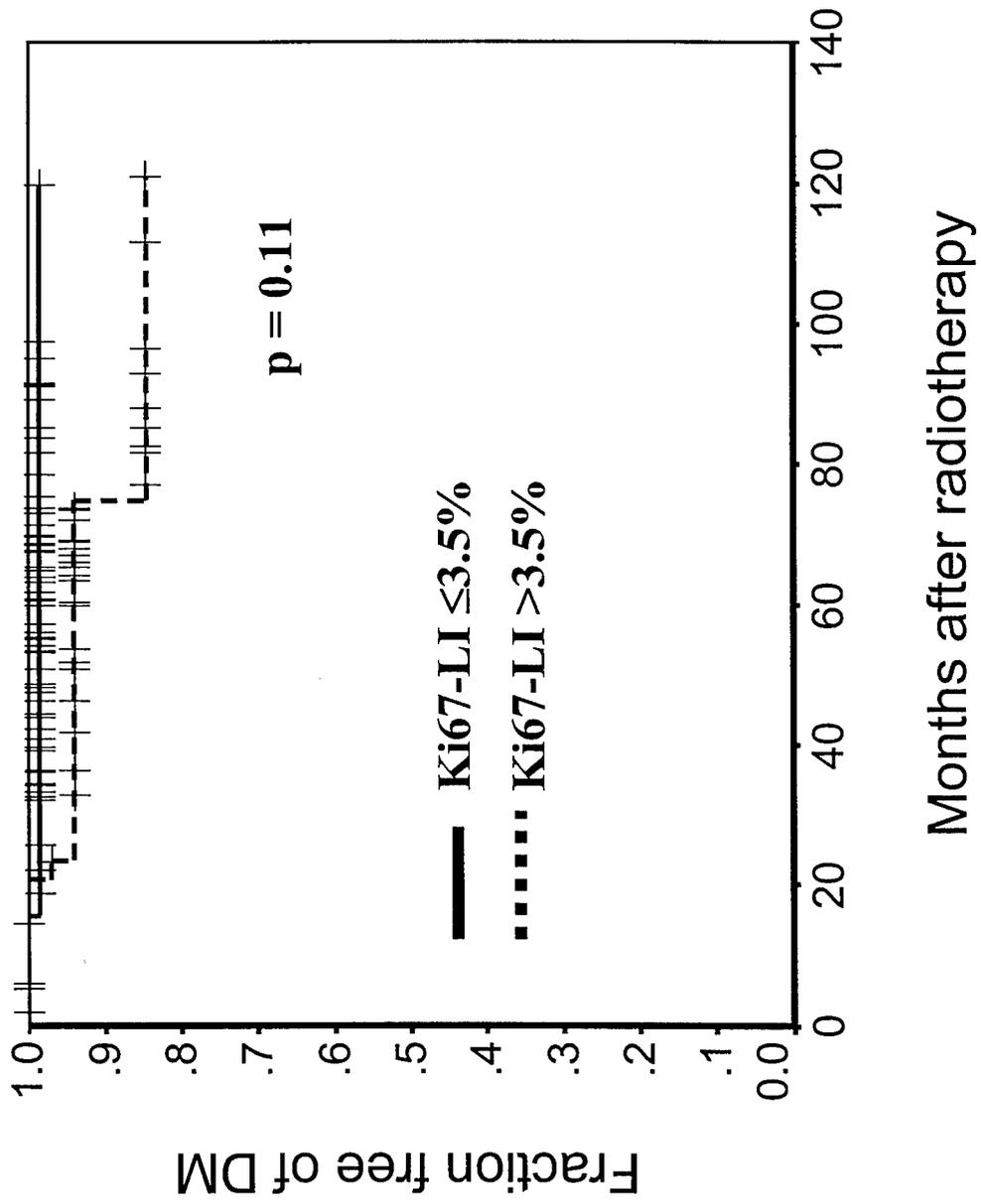
Cowen et al, Fig 2(middle)



Cowen et al, Fig 2(right)



Cowen et al, Fig 3



**LACK OF PROSTATE CANCER RADIOSENSITIZATION BY
ANDROGEN DEPRIVATION**

Alan Pollack, M.D., Ph.D*, Naji Salem, M.D.**¹, Faramarz Ashoori, M.D.** , Paul Hachem,
B.S.** , Mamta Sangha, M.D.** , Andrew C. von Eschenbach, M.D.[#] ,
Marvin L. Meistrich, Ph.D.**

Departments of Radiation Oncology* , Experimental Radiation Oncology** , and Urology[#] ,
University of Texas M.D. Anderson Cancer Center, Houston, TX 77030

Running Title: Lack of radiosensitization from androgen deprivation

Key Words: Prostate cancer, apoptosis, androgen deprivation, radiation

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Correspondence: Alan Pollack, M.D., Ph.D., Department of Radiation Oncology (97),
University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas
77030, Telephone, (713) 792-3400, Fax, (713) 792-3642.

¹ Current Address: Naji Salem, M.D., Institut Paoli-Calmettes, 13273 Marseille, France.

ABSTRACT

Purpose: The majority of clinical trials have shown that high grade prostate cancer patients treated with androgen deprivation (AD) plus radiation (RT) have a survival advantage over those treated with RT alone. One possible mechanism for such a favorable interaction is that AD sensitizes cells to radiation. Animal model studies have provided suggestive evidence that AD sensitizes cells to radiation, but this mechanism is difficult to confirm conclusively *in vivo*. This question was investigated in LNCaP cells grown *in vitro*.

Materials & Methods: LNCaP cells were cultured *in vitro* in DMEM-F12 medium, containing 10% fetal bovine serum (complete medium or CM). AD was achieved by culture in charcoal stripped serum (SS) containing medium. Replacement of androgen was done by adding the synthetic androgen R1881 at 1×10^{-10} M to SS. Apoptosis was measured with a terminal deoxynucleotidyl transferase dUTP-biotin nick end-labeling (TUNEL) assay. Clonogenic survival was used to determine overall cell death and the results were corrected for differences in plating efficiency from the various growth conditions.

Results: LNCaP cells were grown in CM, SS, or SS+R1881 medium, and cell counts obtained at 3, 4, and 5 days. Cell number increased exponentially in CM, whereas no increase in cell number was observed in SS medium. Cell counts from growth in SS+R1881 were intermediate between these extremes. Apoptosis was measured to determine if the combination of AD+RT *in vitro* resulted in supra-additive cell death, as has been previously described in an *in vivo* model system. The cells were cultured for 3 days before RT and apoptosis quantified 24 hr after RT. There was a consistent supra-additive increase in apoptosis in cells exposed to AD+RT (2 or 8 Gy), as compared to either treatment given individually. In contrast, significant radiosensitization

by AD was not observed by clonogenic survival even when the conditions of AD were varied. No radiosensitization was observed upon incubation in SS medium for 3, 4, or 5 days before RT, or extending AD after RT for 6 hr before plating or 24 hr after plating.

Conclusion: The results show that in LNCaP prostate tumor cells supra-additive apoptosis does not translate into radiosensitization by clonogenic survival. Since clonogenic survival is a measure of overall cell death, either the level of apoptosis is too small a component of overall cell death or the increases in apoptosis occurred in a subpopulation that would have been killed by other mechanisms. Although the findings indicate that AD does not act by sensitizing prostate cancer cells to RT, the additive cell death and growth inhibitory effects of AD+RT are clinically meaningful.

INTRODUCTION

Although Huggins and Hodges (1) first described the clinical response of patients with prostate cancer to hormone therapy in the 1940's, remarkably little progress has been made in defining how best to use androgen deprivation (AD) in the treatment of patients with locally advanced and metastatic prostate cancer. The effects of AD on prostatic carcinoma are dramatic, particularly for clinically localized disease. Androgen deprivation results in cytologic changes and a reduction in the proportion of tumor cells due to a shift to quiescence and apoptosis (2,3). While AD is quite effective at causing dormancy and reducing tumor burden, complete eradication of prostate cancer rarely occurs. Biochemical progression in high risk prostate cancer patients receiving AD as monotherapy is usually evidenced in 5 years (4). The systemic side effects from AD aside, the selective action of AD on clinically localized prostate cancer provides a means for altering the molecular milieu in cells of prostate origin, without such effects on the surrounding normal tissue cells. These characteristics have lead to the tactic that AD used in combination with RT would increase tumor control through either an additive effect on cell killing (both treatments cause apoptosis) or possibly a supra-additive interaction.

At least four randomized clinical trials have documented a survival advantage for AD+RT over RT alone in prostate cancer patients with high risk (particularly high grade) features (5-8). However, none of these trials included an AD alone arm. The early administration of AD alone has been shown to result in a survival advantage over delayed AD (9), leaving to question whether the addition of RT to AD is beneficial. The best data in support of the use of AD+RT come from tumor model studies (10-15). While these reports suggest an interaction between AD and RT, none specifically address the question of whether AD sensitizes prostate

cancer cells to radiation when all mechanisms of cell death are considered. The investigation described here focuses on the global cell killing effects of AD+RT, as defined by clonogenicity.

MATERIALS AND METHODS

LNCaP cell culture system. LNCaP cells were grown in vitro in DMEM-F12 medium, containing 10% fetal bovine serum, 1% L-Glutamine, and 1% Penicillin-Streptomycin (complete medium or CM), as described previously (16). Approximately 5×10^5 cells were plated and cultured in 10 ml of medium in 100 mm dishes in a 5% CO₂ incubator at 37 °C. The cells were typically cultured for 24 hr in complete medium before the culture conditions were altered. Androgen deprivation was achieved by culture in charcoal stripped serum (SS) containing medium. The SS medium was prepared by adding dextran coated charcoal (Sigma c-6197, St. Louis, MO) at 10% weight per volume of FBS and incubating for 45 minutes at room temperature on a rocker platform. The charcoal was removed by centrifuging at 5000 rpm for 10 minutes at 4°C and filtering the supernatant with a 0.22 micron low protein binding filter (#430769, Corning Incorporated, Corning, NY). Replacement of androgen was done by adding the synthetic androgen R1881 (NEN Life Science Products, Boston, MA) at 1×10^{-10} M to SS medium.

Radiation Treatment and Clonogenic Assay. The culture flasks were irradiated in a high dose rate cesium unit (3.6 Gy/min). In most experiments the cells were immediately trypsinized after irradiation in preparation for clonogenic assay, although in some experiments there was a delay of 6 hr. The trypsinized cells were then serially diluted and known numbers replated into 100 mm dishes. Clonogenic survival was determined after incubation in CM, SS, or SS+R1881, with or without single dose γ -irradiation. The clonogenic survival results were corrected for

differences in plating efficiency from the various growth conditions. The dilutions for clonogenic assay were done in triplicate the results were averaged together (intra-experiment averages). The data points shown in the clonogenic survival graphs represent the average from multiple experiments (inter-experiment average). The number of experiments performed is described in the figure legends.

Measurement of Apoptosis.

A terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to measure apoptosis, as described previously (13,14,16). The assay was performed on cells that were cultured directly on glass slides (16). After growth under the specified conditions, the cells were fixed onto the slides with 4% formaldehyde for 30 min, the formaldehyde rinsed free using 70% ethanol, and the slides stored in 70% ethanol. TUNEL staining was done within a few days using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). A positive control, consisting of irradiated mouse intestinal crypts, was included with each staining run. The cells were counterstained with hematoxylin. The apoptotic index was determined by dividing the number of apoptotic tumor cells by the total number of tumor cells, multiplied by 100.

RESULTS

Figure 1 shows that the growth of LNCaP cells is slowed considerably when cultured in medium containing serum stripped of androgen using charcoal (SS medium). The addition of the synthetic androgen R1881 at 1×10^{-10} M to SS medium (SS+R1881) accelerated growth, although it never reached the rate of cells grown in complete medium (CM). The R1881 concentration was derived from titration experiments, which demonstrated that $1 - 5 \times 10^{-10}$ M was optimal and that higher concentrations led to less growth promotion (not shown). The administration of 2 or 8 Gy RT at 3 days after starting AD resulted in an increase in apoptosis for the AD+RT group (*Table 1*), above that observed of the AD and RT controls. The level of apoptosis observed is consistent with that described in prior studies in R3327-G tumors grown in vivo. These data demonstrate supra-additive cell death in the form of apoptosis.

A number of clonogenic survival experiments were performed in an attempt to determine whether AD plus RT causes overall supra-additive cell killing (radiosensitization) in LNCaP cells grown in vitro. The assays were performed under various conditions including incubating for 3 d (Figure 2), 4 d (Figure 3), and 5 d (Figure 4) in SS (androgen deprived) medium before radiation. The curves shown are corrected for differences in plating efficiency. The plating efficiencies for cells grown for 3 days in CM medium ($n = 14$) were $24.5\% \pm 3.1\%$ (\pm SEM) for 0 Gy, $4.8 \pm 0.9\%$ for 2 Gy, and $0.8 \pm 0.3\%$ for 4 Gy. While there were statistically significant differences in plating efficiencies between the dose groups, no differences were observed as a result of growth in the different medium conditions (CM, SS, or SS+R1881). Similar plating efficiency results were observed for LNCaP cells cultured 4 and 5 days before plating for the clonogenic assay, although plating efficiencies were lower. For example, the plating efficiency

for cells grown for 4 ($n = 4$) and 5 days ($n = 7$) in CM medium and not irradiated were $14.5\% \pm 4.3\%$ and $18.8\% \pm 3.1\%$. Figures 2, 3, and 4 indicate that the overall effects of AD+RT on cell death are not supra-additive.

One possible explanation for the lack of overall radiosensitization by AD in vitro is that the cells were immediately plated in CM after RT for the survival assay. Extending AD beyond the time of radiation may be critical to demonstrating radiosensitization. Figure 5 shows that continuing to incubate the cells for 6 hr after irradiation did not alter the clonogenic response. Furthermore, plating SS medium grown cells for the clonogenic assay in SS medium and incubating for another 24 hr before adding unabsorbed serum did not result in radiosensitization (Figure 6).

DISCUSSION

Fundamental to the appropriate development of clinical strategies involving AD plus RT is understanding the basic principles governing the possible interaction. Prior clinical studies have established that the combination of AD+RT is superior to RT alone (4-8). Most of the randomized trials have established a survival benefit from AD+RT, at least in high grade subsets (5-8). The data, however, do not directly address the concern that prolonged AD, administered as sole treatment, results in a survival benefit in locally-advanced patients (9). A key question, therefore, is whether AD sensitizes cells to radiation, and if an interaction does exist, the extent to which this occurs.

The data published to date suggest an interaction between AD+RT; yet, all can be explained by alternative mechanisms when considered with findings described here that AD does not sensitize androgen sensitive LNCaP prostate cancer cells to RT (Figures 3-6). Zietman and colleagues (10-12) described a reduction in the radiation dose needed to control 50% (TCD50) of animals of androgen sensitive R3327-G and Shionogi tumors. Timing was critical, with the largest reduction in dose seen when AD was started before RT and when tumor shrinkage was at its peak. These data can be explained by an additive effect from AD+RT, although an interaction is possible. Previously we found that, in response to AD, R3327-G tumors grown in vivo have a pronounced shift to quiescence, which reaches steady state in 3 days (3). The new cell kinetic equilibrium at 3 days was associated with a supra-additive increase in apoptosis that was sequence-specific and time-limited (13). Supra-additive apoptosis was seen only when AD preceded RT and gradually diminished over the ensuing 2 – 3 weeks. More recently, we reported that supra-additive apoptosis was not sustained with multiple radiation fractions (14). The lack of apoptosis with repeated fractions was seen despite a consistent supra-additive increase in tumor

volume growth delay. The results suggested that apoptosis was not the major cell death mechanism responsible for the combined effects of AD plus RT or, alternatively, that tumor growth delay was not related to cell death and was due to a slowing of tumor growth.

The data presented herein confirm and extend the *in vivo* experiments described previously. The LNCaP *in vitro* system reflects the *in vivo* R3327-G system in several respects. Androgen deprivation *in vitro* was accomplished by growing the cells in charcoal stripped serum-containing medium. The response of LNCaP cells to AD was pronounced; net cell numbers did not increase significantly between 3 and 5 days (Figure 1) and supra-additive apoptosis was documented at 3 days after single fraction radiation of 2 Gy (Table 1). Thus, the system is ideal for testing the hypothesis that AD sensitizes cells to radiation.

Clonogenic survival experiments were conducted under various conditions to determine whether overall cell killing from AD+RT was supra-additive. The results showed no evidence for an interaction between AD and RT, when cell death is measured globally via clonogenic assay. That apoptosis was supra-additively enhanced to a small degree, confirms the suspicion from other reports that this mode of cell death has a minor role (14).

What are the clinical implications of the inability of AD to sensitize prostate cancer cells to RT? The answer lies in considering all of the tumor model data together. Beyond doubt, there is an increased inhibition of prostate cancer growth from the combination of AD+RT. Ruling out radiosensitization as an explanation of the reduction in TCD50 observed by Zietman et al (10-12), leaves additive cell killing as the principal mechanism for their findings. In contrast, the tumor regrowth delay results that we described in the past (13,14) were indicative of supra-additivity. Differences in tumor regrowth delay may occur via differences in cell killing, or may be explained by slower growth after treatment concludes. Granfors et al (15) recently described

that the growth rate of R3327-PAP tumors in vivo remained low after AD+RT, even after AD was withdrawn by testosterone supplementation. Their data support the hypothesis that AD+RT alters the kinetics of prostate cancer growth in a supra-additive fashion. Therefore the two mechanisms that account for all of the animal and clinical data on the effects of AD+RT are 1) additive cell killing and 2) reduced prostate cancer cell proliferation, even after testosterone levels return. These mechanisms would account for the significant improvements in survival observed, despite a lack of overall radiosensitization by AD. Moreover, early AD administration could affect the course of micrometastatic disease.

Androgen deprivation remains a critical component in the armamentarium of methods that have been, and will continue to be, used against high risk prostate cancer. The caution is that tumor growth delay may be a major component of the clinical survival results observed to date with AD+RT. Although younger men treated with AD+RT have improved survival over RT alone, the cause specific survival benefit may not be as great as anticipated if the contribution of tumor growth delay over additive cell killing is significant. As a consequence, cause specific survival curves for AD+RT and RT alone may eventually come closer together. Long follow-up in clinical studies of AD+RT is necessary to determine the extent to which additive cell killing and tumor growth delay contribute to cause specific survival.

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Table 1. Apoptosis from androgen deprivation and radiation.

Serum Group	Mean Apoptotic Index \pm SEM(n)		
	0 Gy	2 Gy	8 Gy
Complete Medium	0(8)	0.1 \pm 0.1(8)	0.3 \pm 0.1(3)
Charcoal Stripped	8.0 \pm 2.1(8)*	21.2 \pm 5.1(8)* [‡]	15.7 \pm 4.9(3)*
Charcoal Stripped + R1881	2.8 \pm 1.0(8)*	1.4 \pm 0.8(8)*	2.6 \pm 1.9(3) [†]

One way ANOVA by least significant difference and Scheffe tests were used:

* $p < 0.05$ compared to above group;

[†] $p < 0.05$ (0.06 by Scheffe) compared to above group.

[‡] $p < 0.05$ (0.07 by Scheffe) compared to dose group to left.

FIGURE LEGENDS

- Figure 1. Effect of growth in complete medium (CM), charcoal stripped serum containing medium (SS), or SS plus synthetic androgen (SS+R1881) on LNCaP in vitro cell number over time. There were 5×10^5 cells plated on day 0. Representative experiment of three.
- Figure 2. Clonogenic survival of LNCaP cells grown for 3 days in CM, SS, or SS+R1881 medium before irradiation and plating. Each point in the CM and SS curves is an average of 17 experiments. Each point in the SS+R1881 curve is an average of 14 experiments. The bars represent the standard error of the mean (SEM).
- Figure 3. Clonogenic survival of LNCaP cells grown for 4 days in CM, SS, or SS+R1881 medium before irradiation and plating. Each point in the CM, SS, and SS+R1881 curves is an average of 4 experiments. The bars represent the standard error of the mean (SEM).
- Figure 4. Clonogenic survival of LNCaP cells grown for 5 days in CM, SS, or SS+R1881 medium before irradiation and plating. Each point in the CM, SS, and SS+R1881 curves is an average of 7 experiments. The bars represent the standard error of the mean (SEM).
- Figure 5. Clonogenic survival of LNCaP cells grown for 3 days in CM, SS, or SS+R1881 medium before irradiation, and incubated another 6 hr in the same medium before plating. Each point in the CM, SS, and SS+R1881 curves is an average of 5 experiments. The bars represent the standard error of the mean (SEM).

Figure 6. Clonogenic survival of LNCaP cells grown for 3 days in CM, SS, or SS+R1881 medium before irradiation, plated for clonogenic assay in the same medium, and after 24 hr unabsorbed fetal bovine serum (1 ml) was carefully added. Each point in the CM, SS, and SS+R1881 curves is an average of 3 experiments. The bars represent the standard error of the mean (SEM).

Figure 1.

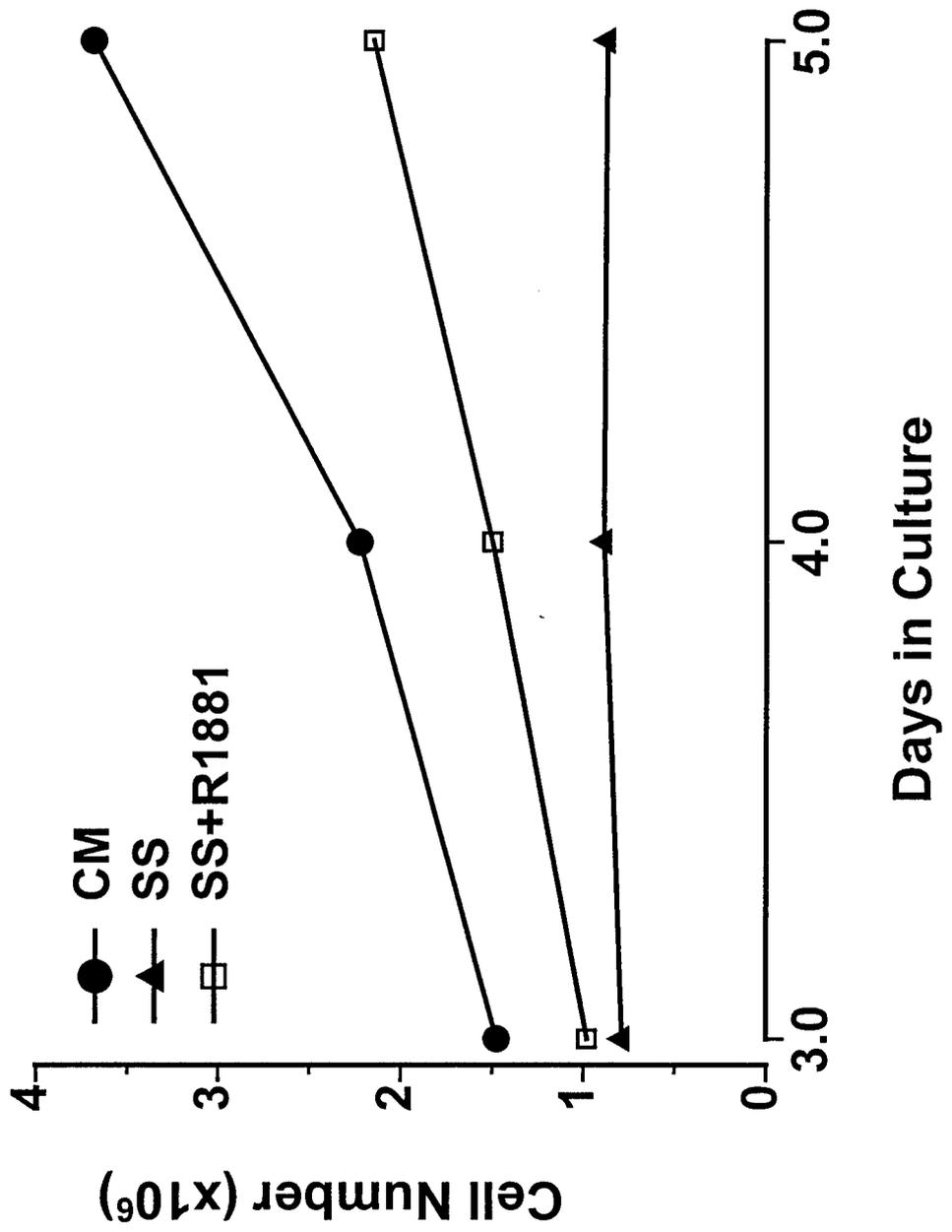


Figure 2

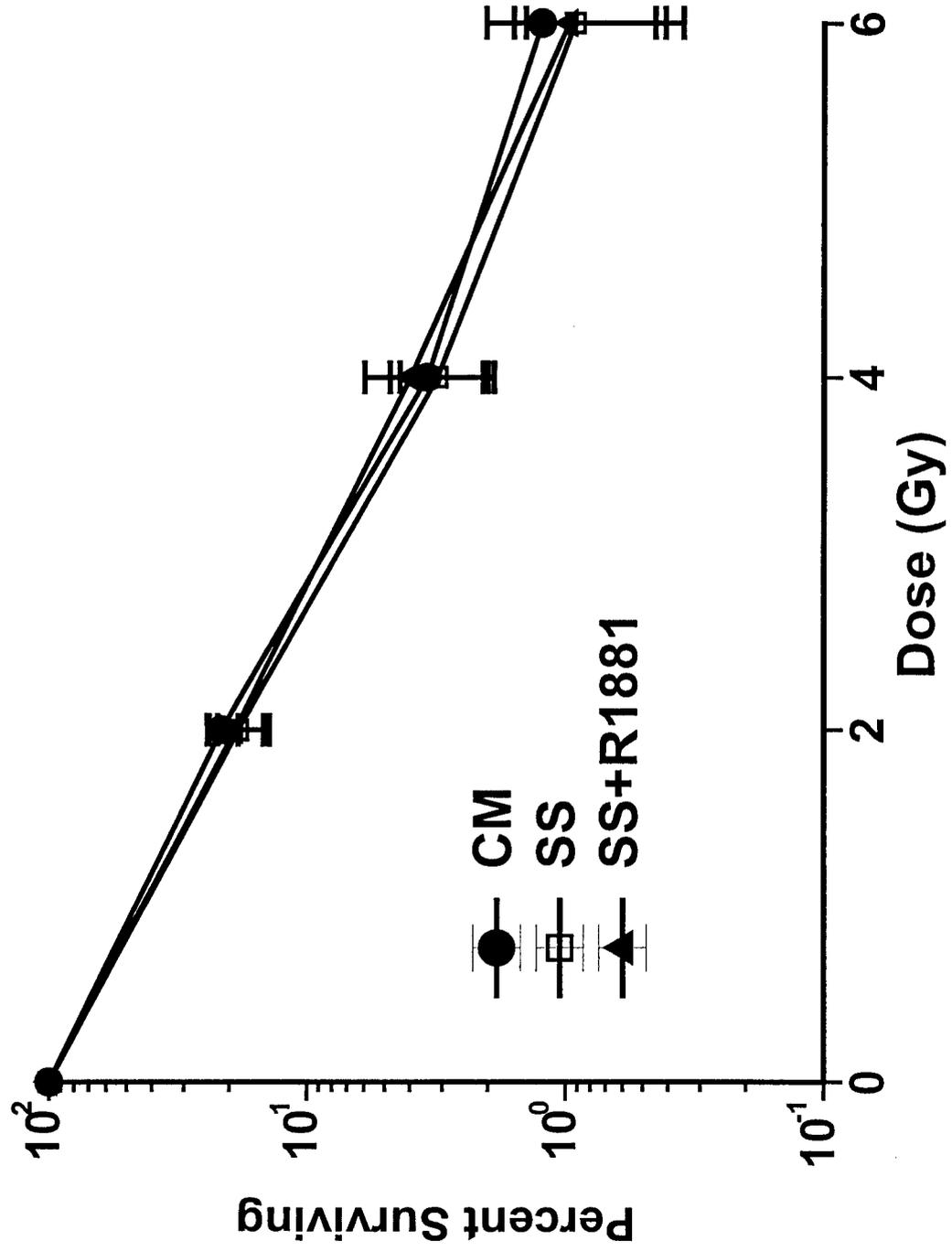


Figure 3.

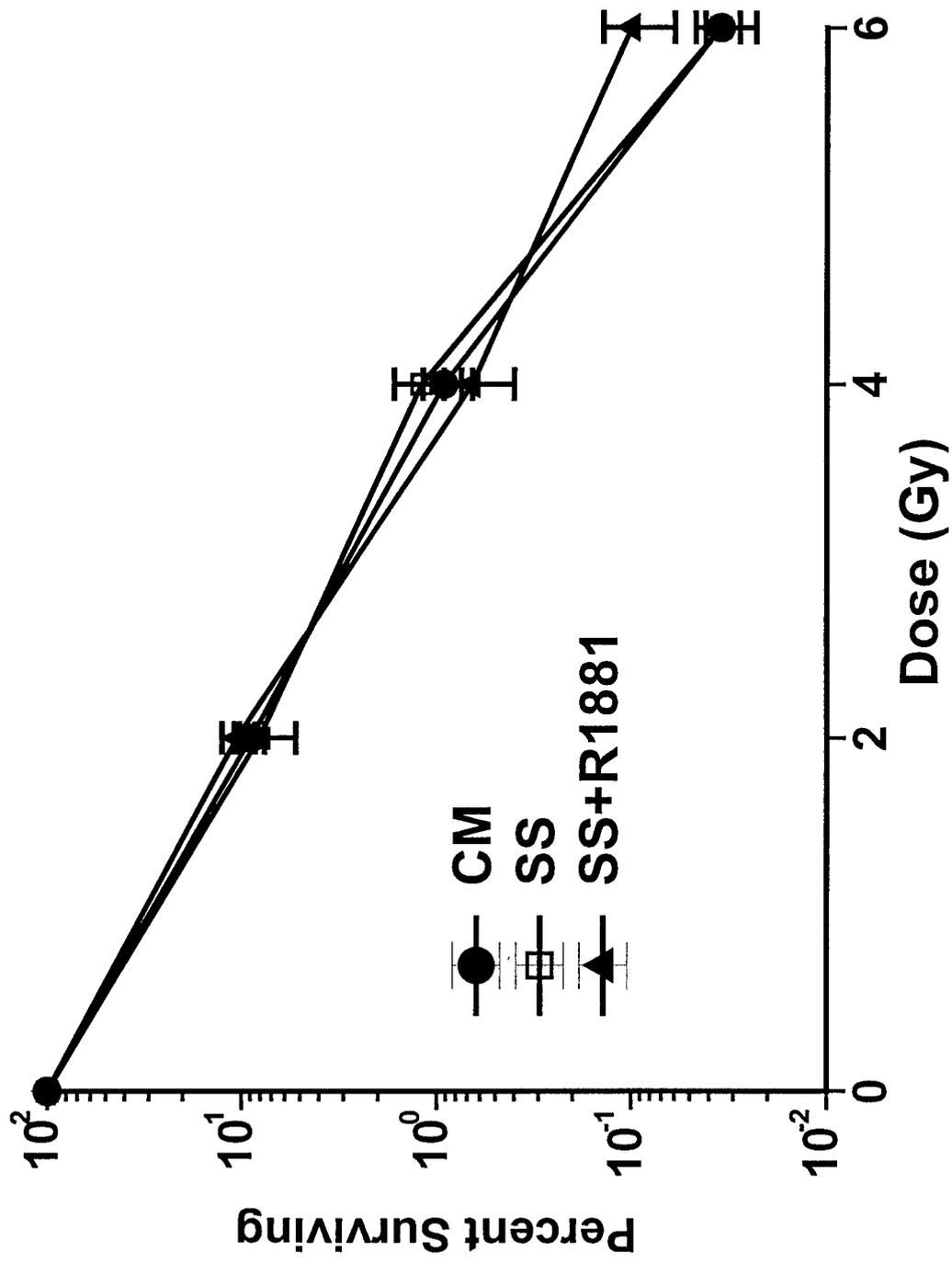


Figure 4.

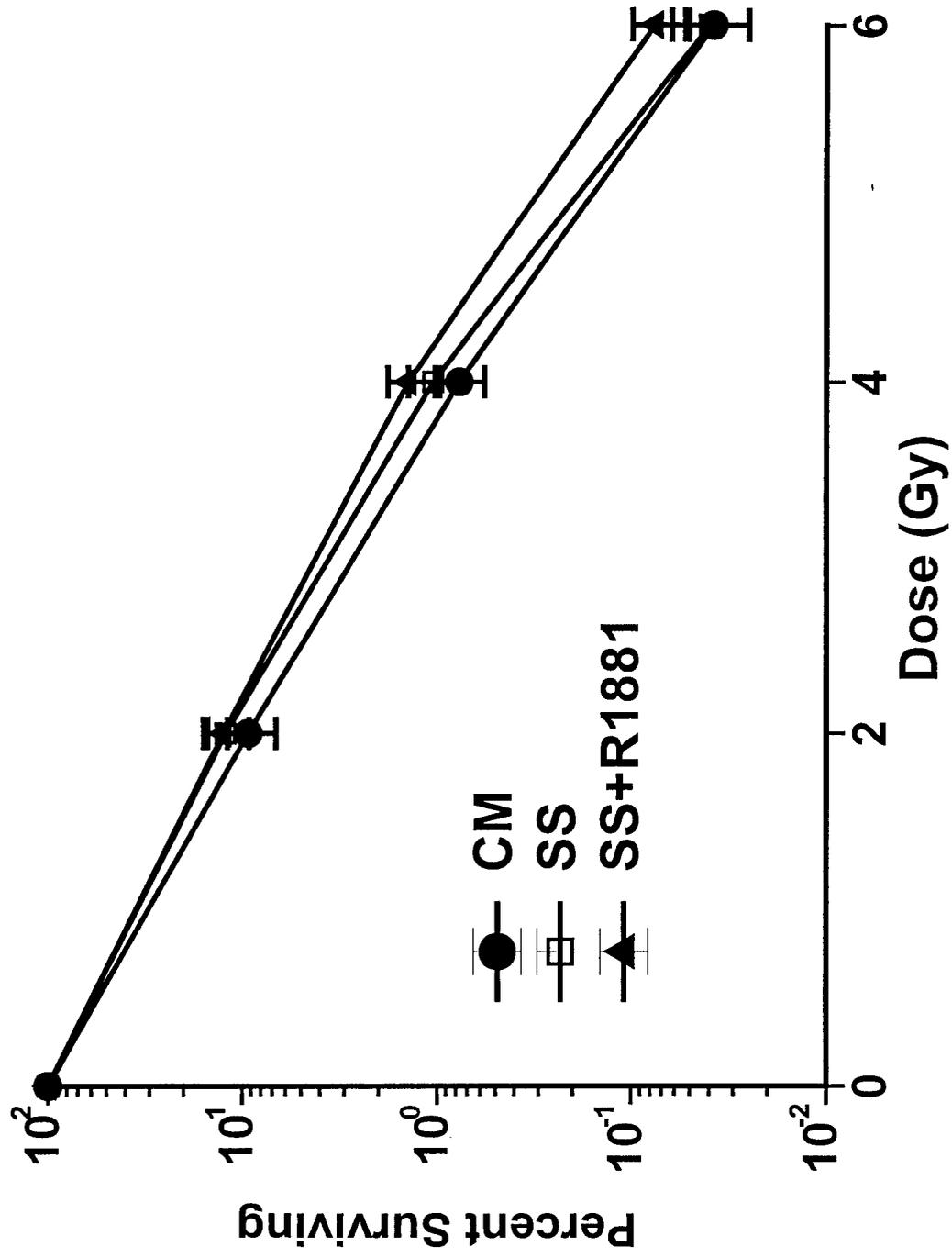


Figure 5.

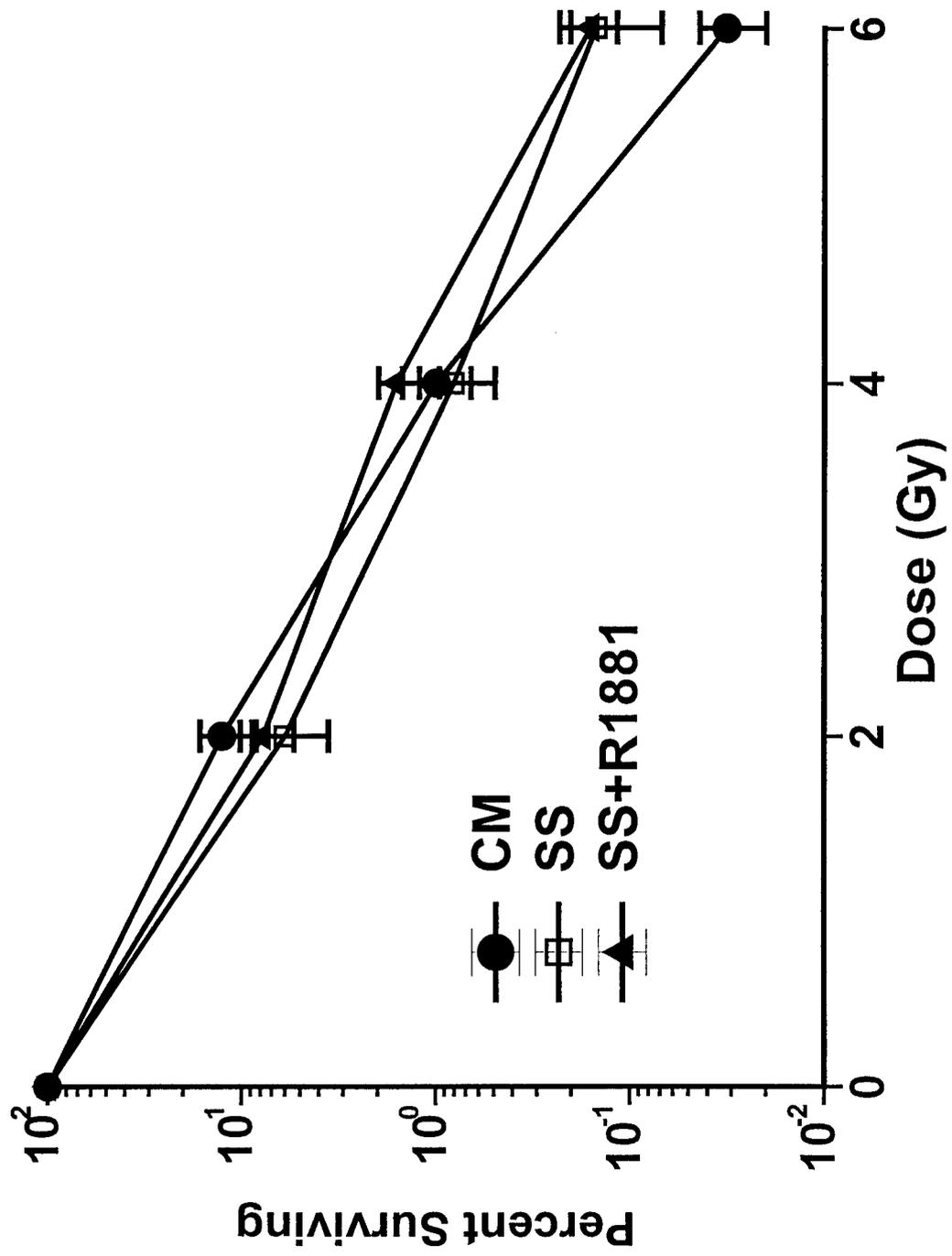
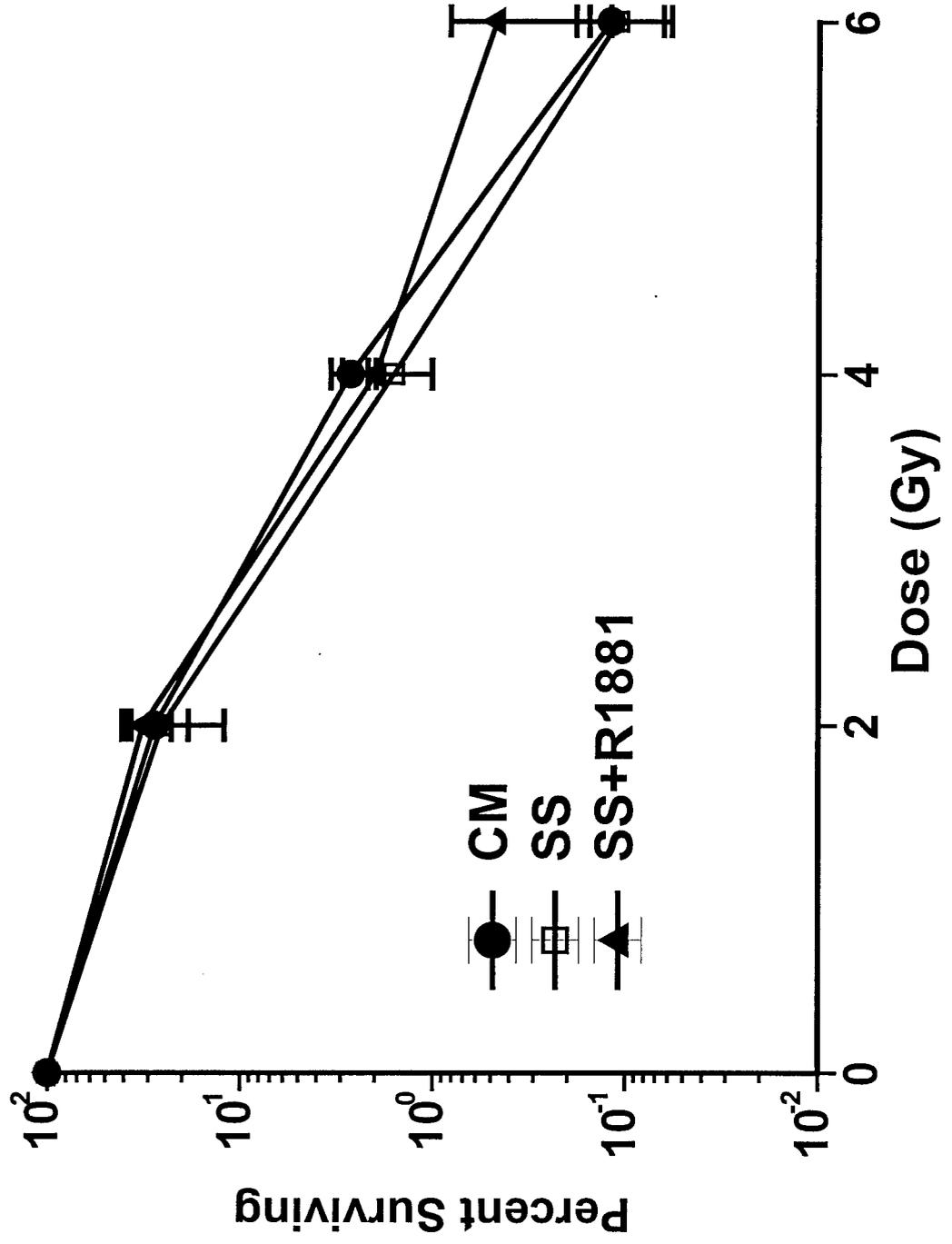
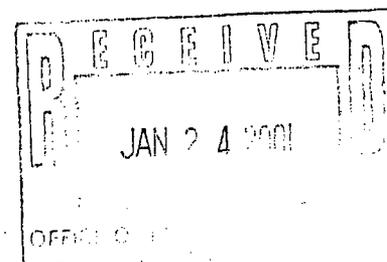


Figure 6.



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CANCER CENTER



Department of Radiation Oncology - 97
713-792-3400
FAX 713-794-5573

MEMORANDUM

TO: Leonard A. Zwelling, M.D., M.B.A.
Associate Vice President for Research Administration

FROM: Dr. Alan Pollack *AP*
Professor
Department of Radiation Oncology- Box 97

DATE: January 16, 2001

SUBJECT: Protocol ID 99-205 entitled "A Randomized Phase II Study of Adenovirus-p53 Plus Radioactive Seed Implant Versus Seed Implant Alone for PSA Relapse After External Beam Radiotherapy."

The above protocol has been revised for resubmission and details of the changes made are attached. I have also enclosed a tagged/highlighted copy of the previous protocol and a copy of the revised current protocol.

Please note that there has been a delay in activating this trial. The protocol with these revisions will be sent to the FDA and once the protocol is approved, activation will be requested.

ZL/ sc

Enc.

Cc: Criselda Molina

The University of Texas M.D. ANDERSON CANCER CENTER	
PROTOCOL NUMBER:	<u>1099-205</u>
REVISION DATE(S):	<u>1-16-01</u>
APPROVED BY:	<u><i>AM</i></u> AMAN U. BUZDAR, M.D. IRB Chairman of designee
APPROVAL DATE:	<u>1-25-01</u>
ACTIVATION DATE:	<u>Upon Activation</u>

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A Comprehensive Cancer Center designated by the National Cancer Institute
located in the Texas Medical Center

ID99-205 Revisions:

Protocol Abstract Page:

Objectives: Add as follows:

4. To determine the morbidity of treatment through prospective assessment of urinary and bowel radiation reactions, and through the administration of quality of life questionnaires at 1 month, 3 months, 6 months, 1 year and 2 years after treatment.

Eligibility Criteria: page 2 – Change as follows:

2nd sentence: change as follows:

Evidence of a rising PSA (3 consecutive rises on follow-up).

3rd sentence – change as follows:

PSA doubling time should be greater than 1 year.

Treatment Plan: 5th line – change as follows:

will be administered in three intraprostatic injections at 3×10^{12} viral particles per injection.

Patient Evaluation: Add as follows:

Quality of life questionnaires will be administered before treatment and at 1 month, 3 months, 6 months, 1 year and 2 years.

All PC-spes – change to : PC-Spes

1.0 Objectives: Add as follows:

- 1.4 To determine the morbidity of treatment through prospective assessment of urinary and bowel radiation reactions, and through the administration of quality of life questionnaires at 1 month, 3 months, 6 months, 1 year and 2 years after treatment.

5.4 I-125 Seed Implant:

5.4.1 2nd line – change as follows:

protocol enrollment to confirm that the prostate is ≤ 65 cc in size.

6.0 Pretreatment

Add as follows: 6.9.3 Administration of pretreatment questionnaires including the UCLA Prostate Cancer Index (Litwin, et al 2000), FACT-P QOL, AUA Symptom Index and Late Effects Questionnaires.

7.0 Post-Treatment Evaluation: Change as follows:

7.1 4th line – years, and every 6 months thereafter. Rectal exam will not be done until 3 months after the last injection.

7.2 The UCLA Prostate Cancer Index (Litwin, et al, 2000) and the FACT-P quality of life (FACT-P QOL: Esper et al, 1997) questionnaires Appendix B , will provide the determination of QOL from the patient's perspective. The AUA Symptom Index (Appendix B, Section 4) will also be obtained. These questionnaires will be administered prior to the implant and at 1 month, 3 months, 6 months, 1 year and 2 years after completion of treatment. In addition, a more specific questionnaire designed to assess radiotherapy sexual, urinary and bowel late side effects (Appendix B, Sections

2 & 3) will be administered. This questionnaire will be administered prior to the implant and 2 years after completion of therapy. The completed questionnaires should be mailed to Joy Phillips, RN, Department of Radiation Oncology (Box 97) within 7 days of completion.

Appendix A = Examinations, Test To Be Done and Schedules: Delete QOL Questionnaires and change as follows:

	Prior to Imp	F/U visits
QOL Questionnaires:		
UCLA Prostate Cancer Index	x	x ^c
FACT-P QOL	x	x ^c
AUA Symptom Index	x	x ^c
Late Effects Questionnaire	x	x ^d

c Administer questionnaires at 1 month, 3 months, 6 months, 1 year and 2 years after completion of treatment.

d Administer questionnaire at 2 years after completion of treatment.

Appendix B (Section 1 of 4) change to Appendix B (Section 1 of 5)
 Appendix B (Section 2 of 4) change to Appendix B (Section 2 of 5)
 Appendix B (Section 3 of 4) change to Appendix B (Section 3 of 5)
 Appendix B (Section 4 of 4) change to Appendix B (Section 4 of 5)
 UCLA Prostate Cancer Index will be Appendix B (Section 5 of 5)

Add the following to References:

Litwin MS, Pasta DJ, Yu J, Stoddard ML, Flanders SC. Urinary function and bother after radical prostatectomy or radiation for prostate cancer: a longitudinal, multivariate quality of life analysis from the cancer of the prostate strategic urologic research endeavor. J Urol 2000; 164:1973-7.

Change Reference as follows:

Pollack A, Zagars GK, Smith LG, Antolak JA, Rosen II. (2000) Preliminary results of a randomized dose escalation study comparing 70 Gy to 78 Gy for the treatment of prostate cancer. J Clin.Oncol Vol 18, No 23 (2000):pp 3904 - 3911

Revisions to ID99-205:

All Ad5-p53 = change to Ad5CMV-p53

All INGN 201 = change to RPR/INGN 201

3.0 Drug Information: p53 Adenoviral Vector (INGN 201) = change to :
Ad5CMV-p53 Adenoviral Vector (RPR/INGN 201)

6.0 Pretreatment and Treatment Evaluation

Delete as follows: 6.5.5 Plasma Sample....

6.7 Pretreatment mucosal/sputum samples

6.7.1 Rectal Swab

6.7.2 Sputum/Saliva sample

6.8 Change to: 6.7 Pretreatment Imaging

Appendix A = change as follows:

History & Physical & concomitant meds

Hepatitis B and C screen

Delete HIV, Rectal swab & sputum

Add EKG (prior to implant)

Abstract Page = reformat entirely into MDACC format

Abstract page: Eligibility: #8 change to : Prostate volume \leq 65 cc.

4.0 Patient Eligibility – change as follows:

4.8 Transrectal prostate volume \leq 65 cc, determined <1 month prior to enrollment.

4.9 Androgen ablation is permitted if it was for \leq 6 months and was stopped >1 year prior protocol enrollment. This includes natural hormones, such as PC-Spes. Patient agrees not to initiate hormone therapy within 2 years of completion of treatment, except under advisement of protocol physicians.

6.0 Pretreatment and treatment Evaluation – change as follows:

Replace 6.5.4 HIV Serology with Hepatitis B and C Screen

Delete 6.5.5

Delete 6.7, 6.7.1 and 6.7.2

6.8.1. change to : 6.7.1 Prostate transrectal ultrasound volume study within 3 months prior to treatment.

6.8.2 change to : 6.7.2 Chest x-ray within 1 month prior to treatment.

6.8.3 Change to : 6.7.3 Bone scan \leq 3 months prior to treatment.

6.8.4 Change to: 6.7.4 CT-scan pelvis \leq 3 months prior to treatment.

6.9 Change to: 6.8 EKG within 1 month prior to treatment.

6.12 Change to: 6.11 A pelvic CT-scan will be performed within 24hr after the implant to determine seed placement.

6.13.3 Change to: 6.12.3 Day 15 (\pm 1 day): prior to second vector administration

7.0 Post Treatment Evaluation

7.5 Add as follows: Prostate biopsies in absence of a rising PSA : see section 8.3.1

Additional Revisions to ID 99-205:

Appendices to be added-

Appendix I: Assessment/ Screening Forms

Appendix J: Study Medication Administration Form

Appendix K: Follow-up Form

Appendix to be changed-

Appendix H: Day 16 through Day 39 was added and dates (spaces) were added to the Medical Record form.

*Deborah Kuban, MD was added as a collaborator to the title/signature/collaborator page.

*The entire protocol was repageanated to reflect the additions to the body.

THE UNIVERSITY OF TEXAS M.D. ANDERSON CANCER CENTER
DEPARTMENT OF RADIOTHERAPY

A RANDOMIZED PHASE II STUDY OF ADENOVIRUS-p53 PLUS RADIOACTIVE
SEED IMPLANT VERSUS SEED IMPLANT ALONE FOR PSA RELAPSE AFTER
EXTERNAL BEAM RADIOTHERAPY

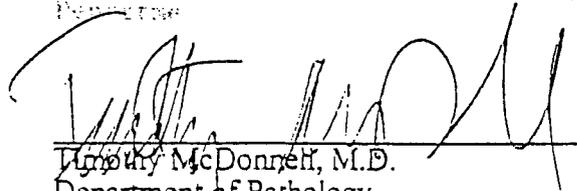
- Abstract
- Eligibility Checklist
- 1.0 Objectives
- 2.0 Background
- 3.0 Drug Information
- 4.0 Patient Eligibility
- 5.0 Treatment Plan
- 6.0 Pretreatment and Treatment Evaluations
- 7.0 Post-treatment Evaluation
- 8.0 Data Collection
- 9.0 Reporting of Adverse Reactions
- 10.0 Statistical Considerations
- 11.0 References

- Appendix A: Tests and Schedules
- Appendix B: QOL Questionnaires
- Appendix C: Zubrod Performance Status Scale
- Appendix D: Clinical Staging
- Appendix E: Acute Toxicity Grading
- Appendix F: Late Toxicity Grading
- Appendix G: Reporting of Adverse Events
- Appendix H: Temperature Log
- Appendix I: Assessment Screening Forms
- Appendix J: Study Medication Administration Form
- Appendix K: Follow-up Form
- Informed Consent

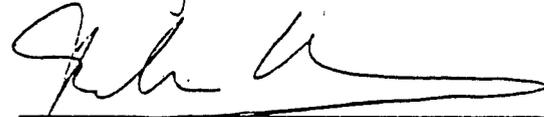
PRINCIPAL INVESTIGATOR:



Alan Pollack, M.D., Ph.D.
Department of Radiation Oncology



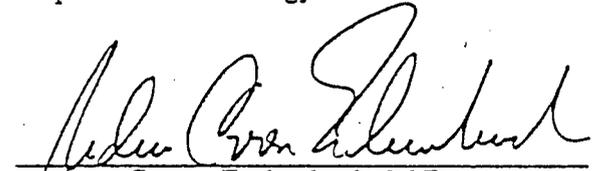
Timothy McDonnell, M.D.
Department of Pathology



Patricia Troncoso, M.D.
Department of Pathology



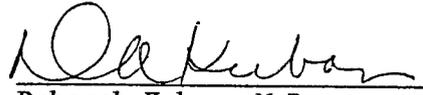
Curtis Pettaway, M.D.
Department of Urology



Andrew C. von Eschenbach, M.D.
Department of Urology



Kim-Anh Do, Ph.D.
Department of Biomathematics



Deborah Kuban, M.D.
Department of Radiation Oncology

COLLABORATORS:

Christopher Logothetis, M.D.
Department of GU Medical Oncology

Lewis Smith, M.D.
Department of Radiation Oncology

Louis Pisters, M.D.
Department of Urology

Timothy McDonnell, M.D.
Department of Pathology

Patricia Troncoso, M.D.
Department of Pathology

Curtis Pettaway, M.D.
Department of Urology

Andrew C. von Eschenbach, M.D.
Department of Urology

Kim-Anh Do, Ph.D.
Department of Biomathematics

Deborah A. Kuban, M.D.
Department of Radiation Oncology

THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER

Protocol Abstract Page

Study Chairman: Alan Pollack, M.D., Ph.D.

Department: Radiation Oncology

Phone: 713-792-0781

Box: 97

Full Title: "A Randomized Phase II Study of Adenovirus-p53 Plus Radioactive Seed Implant Versus Seed Implant Alone For PSA Relapse After External Beam Radiotherapy"

Objectives:

1. To determine the feasibility and toxicity of administering wild type p53 in an adenovirus (Ad5CMV-p53) vector plus salvage I-125 seed implantation, as compared to I-125 seed implant alone, for patients with biochemical failure after external beam radiotherapy.
2. To examine the differences in molecular response in the two treatment arms in terms of pathological (necrosis by H&E staining and apoptosis by TUNEL assay), proliferation (Ki-67/MIB-1), and molecular (p53 and bcl-2) parameters.
3. To assess response to the treatments via prostate biopsy at 1 and 2 years (pathologic response), nadir PSA level with 2 years minimum follow-up (biochemical response), and clinical response (palpable response). The main response parameter will be prostate biopsy at 1 year.
4. To determine the morbidity of treatment through prospective assessment of urinary and bowel radiation reactions, and through the administration of quality of life questionnaires at 1 month, 3 months, 6 months, 1 year and 2 years after treatment.

Rationale: (Be concise as possible)

A rising PSA after external beam radiotherapy for patients with clinically localized prostate cancer is usually representative of local persistence of disease. Those with a negative metastatic work-up are left with the main options of observation, androgen ablation alone, salvage prostatectomy, or salvage brachytherapy. The first two options are palliative. Salvage prostatectomy is associated with considerable morbidity, although 30-50% may be salvaged. Salvage brachytherapy appears to be associated with less morbidity and salvage rates of about 35%. The rationale is to sensitize the prostate cancer cells to radiation by injecting Ad5CMV-p53 directly into the prostate, allowing for enhanced cure rates using lower seed implant radiation doses. This strategy should reduce morbidity and enhance efficacy over salvage seed implant monotherapy.

Eligibility Criteria: (List Major Criteria)**Inclusion:**

Prior external beam radiotherapy for clinical stage T1-T3 (Nx, M0) adenocarcinoma of the prostate.

Evidence of a rising PSA (3 consecutive rises on follow-up.)

PSA doubling time should be greater than 1 year.

A positive post-external beam radiotherapy prostate biopsy for adenocarcinoma.

No evidence of metastases by bone scan and CT-scan of the pelvis.

PSA \leq 10 ng/ml.

Prostate volume \leq 65 cc.

Androgen ablation is permitted if it was for \leq 6 mo and was stopped over 1 year prior to enrollment.

Zubrod Performance Status \leq 2.

No history of grade 3 radiation reaction to external beam radiotherapy

No history of HIV positivity or chronic hepatitis B or C infections. No history of steroid medications of greater than two months duration. (Such patients are considered to have been treated with hormonal therapy).

Exclusion:

N/A

Treatment Plan:

Patients with a rising PSA post-external beam radiotherapy will be randomized between Ad5CMV-p53 gene therapy using INGN 201 plus I-125 seed implant and I-125 seed implant alone. Stratification will be based on whether androgen ablation was given prior to the implant and whether the pre-implant PSA was \leq ng/ml or above. The I-125 seed implant dose will be 110 Gy specified to a planning target volume of 2 – 5 mm around the prostate. Ad5CMV-p53 gene therapy will be administered in three intraprostatic injections at 3×10^{12} viral particles per injection. The first injection of INGN 201 will be at the time of the I-125 implant and the second and third injections at two and four weeks thereafter.

Does your research involve the use of Recombinant DNA technology? Yes No N/A

If Yes, appropriate forms are obtainable through the Office of Research.

Statistical Considerations:

A total of 74 patients will be entered; 37 per arm. The primary objective of this study is to assess efficacy in terms of pathologic response by prostate biopsy at 1 year and toxicity in terms of bladder and rectal late morbidity \geq grade 3 for Ad5CMV-p53 gene therapy plus radioactive I-125 seed implant versus seed implant alone in patients refractory to external beam radiotherapy. From the sample of 37 patients for each arm, if (a) there are 14 or fewer responses for the combined treatment arm, then the treatment is rejected due to inadequate response; (b) if there are 9 or more adverse events then the treatment is rejected due to excessive toxicities; (c) if there are more than 14 responses and fewer than 10 adverse events then the treatment is recommended for further consideration. With the above rules for the combined treatment arm, the overall type I errors are 2% (poor response and excessive toxicity), 10% (poor response and acceptable toxicity), 14% (good response and excessive toxicity), and a type II error will be 14%. Interim analysis for possible early trial termination will be performed after 15 patients in each arm have been enrolled. The purpose of the randomization is to provide unbiased estimators of the effects of adding gene therapy on the variables of interest.

Patient Evaluation: (Pretreatment and Interim Testing)

All patients must have a post-external beam radiotherapy prostate biopsy, pre-seed implant prostate specific antigen (PSA), complete blood count with differential and platelets, PT, PTT, prostate ultrasound volume, chest x-ray, bone scan, and pelvic CT-scan before study enrollment. Prostate biopsies will be obtained at 1 day and again at 2 years after the first p53 injection. The patient will undergo history and physical examination post-implant at 2 weeks, one month, every 6 months for two years, and every 12 months thereafter. Serum PSA will be drawn post-implant at 2 weeks, one month, every 3 months for two years, and every 6 months thereafter.

Quality of life questionnaires will be administered before treatment and at 1 month, 3 months, 6 months, 1 year and 2 years.

Estimated Accrual: 74

It is estimated that accrual will be 2 participants per month.

Site of Study:

This protocol is performed on an: Inpatient Outpatient Both

Length of Stay: (What is the length and frequency of hospitalization?)

N/A

Return Visits: (How often must participants come to M. D. Anderson?)

2 weeks, 1 month, every 6 months for 2 years and then annually.

Home Care: (Specify what [if any] treatment may be given at home)

N/A

Where will the Study be Conducted:

- A) Only at MDACC
- B) MDACC + Community Programs (CCOP/Network)
- C) Independent Multicenter Arrangements

Competing Protocols: (List the Protocol Number[s])

N/A

Name of Research Nurse/Data Manager:

Mary Jane Oswald, Data Manager
Joy Phillips, R.N., Research Nurse

If your protocol has a diagnostic step requiring informed consent and registration on the protocol (e.g., a blood test or biopsy) that will determine whether or not the patient will subsequently receive or not receive experimental therapy; please check the appropriate box(es) so that the appropriate registration process may be established.

Applicable? Yes N/A

Blood Test: Yes No Biopsy: Yes No Other: Yes No

Public Display of Protocol on the Office of Protocol Research Web Site:

The Office of Protocol Research maintains a website (www.clinicaltrials.org) listing protocols actively accruing patients. No information is given about drug dose or schedule. Would you like this protocol listed on this website?

Yes No

If this protocol has a corporate sponsor, we also need to get the sponsor's written approval to post the trial on the website. Would you like OPR to send a letter requesting this permission to the sponsor?

Yes No N/A

Name of Sponsor/Funding Source:

Sponsor:

Sponsor Contact:

Company Address:

City:

State:

Country:

Zip Code:

Telephone:

Fax Number:

Eligibility Checklist

- ____(Y) Prior external beam radiotherapy of the prostate for clinical Stage T1-T3 (Nx, M0) adenocarcinoma of the prostate.
- ____(Y) No prior radical prostate surgery.
- ____(Y) Evidence of a rising PSA (3 consecutive rises on follow-up) after external beam treatment.
- ____(Y) PSA doubling time of >1 year. Calculated PSA doubling time: _____ months.
- ____(Y) No evidence of metastases by bone scan within 1 mo of enrollment.
- ____(Y) No enlarged adenopathy on CT-scan of the pelvis within 1 month of enrollment.
- ____(Y) No palpable evidence of palpable extraprostatic tumor extension on rectal exam.
- ____(Y) PSA \leq 10. Current PSA: _____ ng/ml done within two weeks of enrollment.
- ____(Y) Prostate volume by ultrasound is \leq 65 cm³. Current prostate volume by ultrasound: _____ done within 1 month of enrollment.
- ____(N) Androgen ablation, except for below.
____(Y) May be have been used for \leq 6 months, but must have been stopped for >1 year prior to study enrollment. This includes PC-Spes.
- ____(Y) Zubrod Performance Status \leq 2.
- ____(Y) No history of grade 3 late radiation reaction from external beam radiotherapy.
- ____(Y) No history of HIV positivity or chronic hepatitis B or C infections.
- ____(Y) No history of steroid medications of greater than two months duration. (Such patients are considered to have been treated with hormonal therapy).

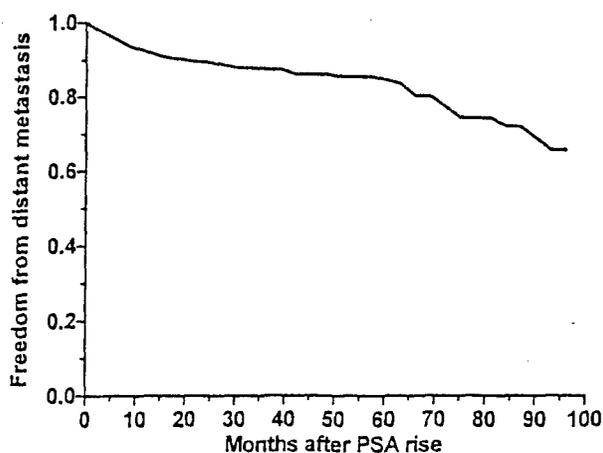
1.0 OBJECTIVES

- 1.1 To determine the feasibility and toxicity of administering wild type p53 in an adenovirus (Ad5CMV-p53) vector plus salvage I-125 seed implantation, as compared to I-125 seed implant alone, for patients with biochemical failure after external beam radiotherapy.
- 1.2 To examine the differences in molecular response in the two treatment arms in terms of pathological (necrosis by H&E staining and apoptosis by TUNEL assay), proliferation (Ki-67/MIB-1), and molecular (p53 and bcl-2) parameters.
- 1.3 To assess response to the treatments via prostate biopsy at 1 and 2 years (pathologic response), nadir PSA levels with 2 years minimum follow-up (biochemical response), and clinical response (palpable response). The main response parameter will be prostate biopsy at 1 year.
- 1.4 To determine the morbidity of treatment through prospective assessment of urinary and bowel reactions, and through the administration of quality of life questionnaires at 1 month, 3 months, 6 months, 1 year and 2 years.

2.0 BACKGROUND

- 2.1 Failure patterns after external beam radiotherapy. The rising PSA profile after external beam radiotherapy or surgery is a harbinger of disease relapse. Biochemical failure precedes clinically detectable disease relapse by 3-5 years on average (Pollack et al, 1994) and a number of studies show that it is a correlate of distant metastasis. From our database of 1160 men with stage T1-T4, Nx/N0, M0 prostate cancer treated solely with external beam radiotherapy, every case of distant relapse was preceded by biochemical relapse (Smith et al, in press). In the majority of cases with isolated biochemical failure, local tumor persistence is documented on prostate biopsy as the only initial evidence of disease. For the 341 men with evidence of a rising PSA, the distant metastasis rate was 34% at 8 yr (calculated from the time of a rising PSA; Figure 1). The PSA doubling time after treatment, be it external beam radiotherapy (Smith et al, In press) or surgery (Pound et al, 1999), correlates with the development of distant metastasis. A PSA doubling time of ≤ 1

Figure 1. Freedom from distant metastasis in patients with a rising PSA after external beam radiotherapy



year was associated with an 8 yr actuarial rate of distant metastasis of 53%, as compared to 17% when the doubling time was >1 yr. The inherently long lag-time between biochemical and distant failure, local persistence as the main site of failure, and ability to select patients with a low risk of developing distant metastasis based on PSA doubling time suggests that many of these patients might be salvaged with aggressive local treatment.

2.2 Salvage therapy. External beam radiotherapy is a common treatment for prostate cancer and has proven to be efficacious. However, local recurrence, documented by prostate biopsy, is seen in over 30% and represents the most common site of first failure. The salvage of patients with local recurrence after external beam radiotherapy (EBR) has in general been poor. In selected patients with delayed recurrence >2 yr after EBR and in whom the PSA is ≤ 10 ng/ml, salvage radical prostatectomy appears to control about 45% for 5 yr (Amling et al, 1999; Gheiler et al, 1998; Bochner et al, 1998; Roger et al, 1995; Pontes et al, 1993). However, the morbidity of this approach is quite high, particularly for men >70 years old. An alternative that is gaining favor is salvage implant monotherapy using Iodine-125 or palladium-103 (Grado et al, 1999; Butler et al, 1997; Dattoli et al, 1997; Cumes et al, 1981; Goffinet et al, 1980). The problem with re-irradiating the prostate with brachytherapy is that the dose used is often limited for fear of sequelae. The more recent ultrasound-based studies (Grado et al, 1999; Butler et al, 1997; Dattoli et al, 1997) have demonstrated reasonable complication rates with biochemical control (freedom from a rising PSA) seen in about 35%. *The key question posed is whether radiosensitization using p53 gene therapy is feasible and of low morbidity, and whether the tumor is completely eradicated from the prostate using this strategy.* This key question will be addressed via a randomized Phase II trial comparing radioactive I-125 seed implant monotherapy with Ad5CMV-p53 gene therapy and seed implant.

There are a small number of studies with limited patient numbers (Grado et al, 1999; Butler et al, 1997; Dattoli et al, 1997; Cumes et al, 1981; Goffinet et al, 1980) in which brachytherapy has been explored as salvage for local disease persistence after external beam radiotherapy. Salvage brachytherapy is an old concept (Cumes et al, 1981; Goffinet et al, 1980) that has recently been improved upon through the use of ultrasound guidance of seed placement. There are now a number of groups with experience, even though much of the available data is immature and has not been formally published. Drs Stock and Stone (1998) have a large experience in the treatment of prostate cancer with brachytherapy and have treated over 100 patients using Palladium-103 for external beam failures (personal communication). Using a prescribed dose of 90 Gy they have noted few cases of incontinence and no rectal complications. Drs. Beyer and Priestly (1997) have used Iodine-125 for salvage in over 50 patients with local progression after external beam radiotherapy to a prescribed dose of 112 Gy. They found a 25% incontinence rate (personal communication). The largest published experience in the ultrasound era is by Grado et al (1999) wherein 49 patients were treated with full dose Palladium-103 (120 Gy) or Iodine-125 (160 Gy). Actuarial 3 and 5 year disease-specific survival was 89% and 79%, and biochemical control was 48% and 34%. No serious complications were observed.

2.3 Gene therapy studies in model systems. Ad5CMV-p53 is the prototypal gene therapy vector. p53 transgene expression has been shown to radiosensitize colon cancer cells in vitro and in vivo (Spitz et al, 1997), and has been used in lung cancer patients on protocol as a radiosensitizer (personal communication, Jack Roth, M.D.). Our in vitro and in vivo

experiments have confirmed that Ad5CMV-p53 sensitizes using human prostate cancer cells to radiation. The model systems used were the p53^{wildtype} LNCaP and p53^{null} PC3 lines. The exposure of either of these cell lines in vitro with Ad5CMV-p53 (multiplicity of infection of 40-70) 24 hr prior to irradiation at single fraction doses of 2-6 Gy caused significantly reduced clonogenicity, as compared to control vectors (Ad5-βGal or Ad5-polyadenylation sequence [Ad5-pA]) plus radiation or radiation alone (Collitier et al, 1998). The enhanced cell killing from Ad5CMV-p53 plus radiation was clearly supra-additive (Figure 2).

Figure 2A: Effect of Ad5CMV-p53 + RT on PC3

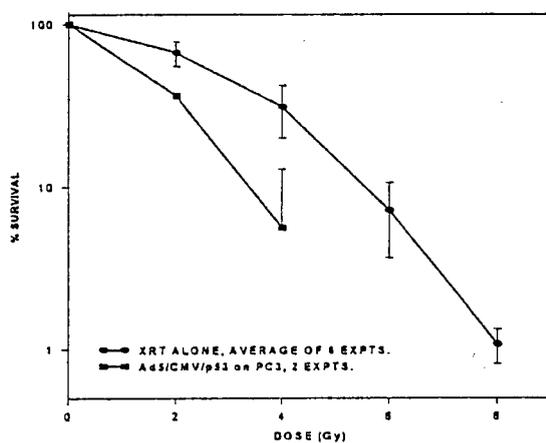
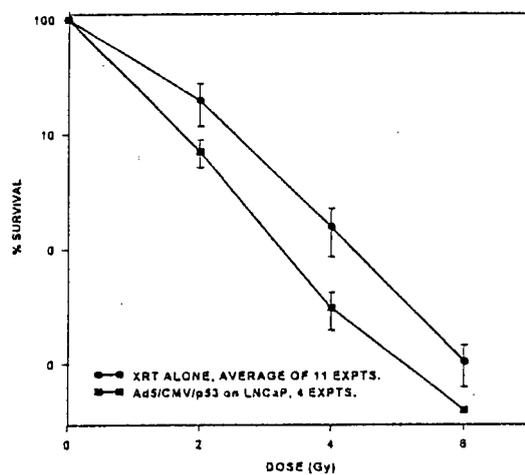


Figure 2B: Effect of Ad5CMV-p53 + RT on LNCaP



In vivo experiments in nude mice using PC3 involved the implantation of 2×10^6 cells subcutaneously in a hind leg and beginning treatment at a tumor volume of 200 mm^3 . Three intratumoral injections (days 1, 4, and 7) were given with 3×10^8 plaque forming units, followed by 5 Gy pelvic irradiation in one fraction using a cesium source. Tumor volume measurements were obtained three days per week. The PC3 tumor volume growth curves were log-transformed, and fitted using linear regression. The times (in days) for the tumors to reach 500 mm^3 were calculated as 10.7 ± 0.7 for the saline control (no virus), 9.8 ± 2.1 for Ad5-pA (virus control), 15.6 ± 1.6 for Ad5CMV-p53, 14.6 ± 1.5 RT (5 Gy), 14.6 ± 1.5 for Ad5-pA plus RT, and 31.3 ± 5.3 for Ad5CMV-p53 plus RT. The latter group times were significantly different at $p < 0.05$ (one way ANOVA, Scheffe test) from all of the other groups. The absolute delay in tumor growth to 500 mm^3 relative to the saline control was used to calculate the enhancement factor [Abs delay(Ad5CMV-p53+RT - Ad5CMV-p53)/RT], which was 4.0. The Ad5-pA controls were not included in the calculation because no significant delays were seen.

LNCaP cells (2×10^6 in 24 μl) were injected orthotopically into the prostates of nude mice and tumor weight approximated using serum PSA obtained from weekly tail vein bleedings. There is a linear relationship between tumor weight and serum PSA; linear regression results revealed that tumor weights of 7, 10, and 20 mg correlated with PSAs of 1.4, 3.0, and 8.5 ng/ml. The target PSA for the studies was 5 ng/ml. The animals were then anesthetized, the prostate surgically exposed, and 4.5×10^8 pfu injected in 24 μl . The intraprostatic injections were done twice (days 1 and 4), and 5 Gy pelvic irradiation was administered 24 hr later (Day 5). LNCaP tumor growth was determined via weekly serum PSA measurements, and a serum PSA of >1 ng/ml six weeks after treatment was considered evidence of regrowth. There were five animals per group. The number of animals with evidence of regrowth were 5 for the saline control, 5 for Ad5-pA, 4 for Ad5CMV-p53, 4 for RT alone (5 Gy), 4 for Ad5-pA + RT, and 1 for Ad5CMV-p53 + RT. These results are also consistent with a supra-additive inhibition of tumor growth.

2.4 Ad5CMV-p53 gene therapy studies in prostate cancer patients. There is substantial evidence that Ad5CMV-p53 sensitizes both $p53^{\text{wildtype}}$ and $p53^{\text{null}}$ prostate cancer cells. The technique of intraprostatic injection has already been devised and the effect of p53 transgene expression has been documented. A protocol examining the effects of injecting Ad5CMV-p53 prior to radical prostatectomy has recently been completed. There were 30 patients that were enrolled and 26 received escalating doses of Ad5CMV-p53 from 3×10^{10} to 3×10^{12} viral particles per injection for 3 injections. The preliminary data show an increase in p53 expression and apoptosis 24 hr following injection. Low grade fever was the most common side effect. No significant and measurable increase in surgical or other toxicity was encountered at the highest dose level (Tables 1). This is the dose level that will be used in the current trial.

Table 1: Ad5CMV-p53 Plus Prostatectomy Side Effect Events

Event	Number of Patients with			
	Grade 1	Grade 2	Grade 3	Grade 4
Perineal Pain	12	10	0	0
Fever	11	19	0	0
Chills	6	5	0	0
Headache	2	2	0	0
Hematospermia	0	3	0	0
Hematuria	1	1	0	0
Scrotal Edema	1	2	0	0

Preliminary evidence for antitumor activity existed by many parameters: Regression of prostate cancer by 25% occurred within six weeks in 7/26 available for analysis at this time. In addition, the serum PSA (Fig 3) concentration declined in the majority of patients (data not shown) The decline in the serum PSA concentration occurred in most patients despite repeated puncture of the prostate which frequently results in a substantial rise in the PSA concentration. Biopsies at 24 hr after p53 injection showed enhanced apoptosis (Table 3). The pathological findings from radical prostatectomy at revealed that the patients had high grade cancer which was regionally advanced, which suggests that p53 therapy alone is insufficient for patient in this category and that patients with poor prognostic features were reliably selected.

Figure 3. Changes in tumor volume from preoperative Ad5CMV-p53.

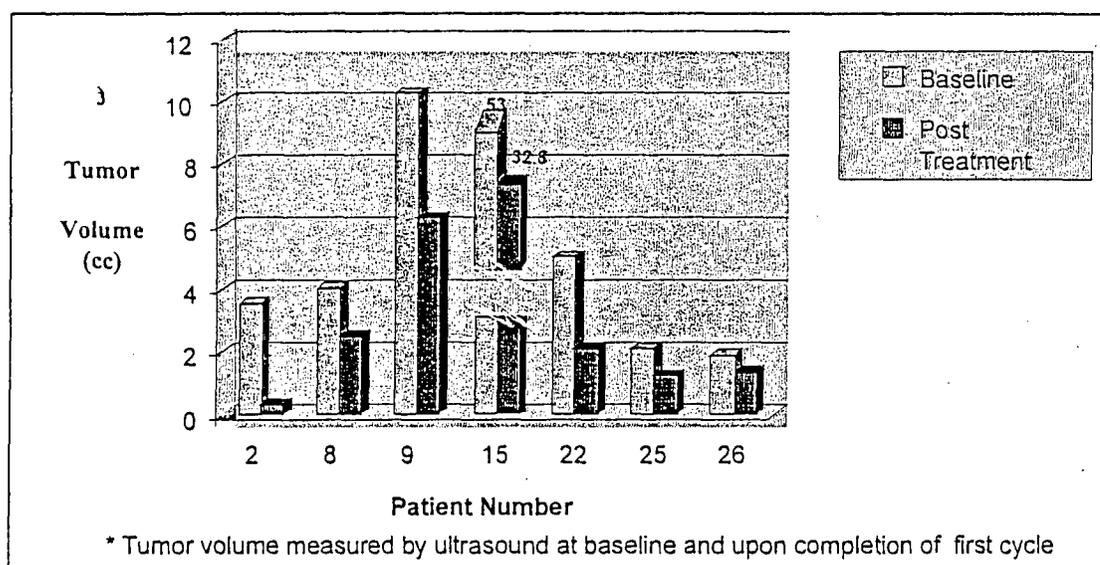


Table 3. The induction of apoptosis 24 hr after Ad5CMV-p53 treatment.

Pt #	p 53		A p o p t o t i c I n d e x	
	Baseline	Day 1	Baseline	Day 1
14	-	+	0.4 %	0.8 %
17	-	+	0.6 %	2.75 %
18	-	+	0.6 %	0.5 %
19	-	+	0.5 %	1.6 %
20	-	-/+	0.3 %	2.7 %
21	-	++	0.8 %	4.6 %
23	+	-/+	1.13 %	0.6 %
24	-	-/+	0.4 %	1.1 %

These are the first clinical data demonstrating that p53 transgene expression results in increased programmed cell death in human prostate cancer.

- 2.5 Quality of life, Prostate Cancer Late Side Effect, and AUA symptom score questionnaires. These questionnaires (Appendix B) will be given to the patients to assess the effects of the proposed treatments on these measures. The purpose of the questionnaires is to provide descriptive information which will form the basis for larger trials to be performed in the future. The quality of life questionnaire will be the FACT-P (Esper et al, 1997), a validated prostate cancer therapy instrument. The radiotherapy late side effect questionnaire has been used by our group in the past (Ngyuen et al, 1997) and others (Jonler et al, 1994; Crook et al, 1996). The AUA symptom score (Barry et al, 1992) is routinely used and has proven valuable in assessing patients undergoing prostate implants (Lee et al, In press).
- 2.6 Molecular markers as response parameters. There are a number of studies at MDACC and elsewhere which have correlated alterations in the expression of certain biomarkers, such as Ki-67/Mib-1, p53, and bcl-2 with prognosis (McDonnell et al, 1992; Columbel et al, 1993; Bubendorf; et al, 1996; Moul et al, 1996; Grignon et al, 1997; Byrne et al, 1997; Uzoaru et al, 1998). The changes in these biomarkers appear to be associated with progression to androgen independence. In addition, the apoptotic index will be measured using the TUNEL assay (Chyle et al, 1996). These biomarkers will be measured on the pretreatment (pre-EBR), post-EBR (pre-implant), and day 1 post-implant biopsy specimens. Every attempt will be made to obtain biopsy material obtained at other institutions. The goal is to quantify the alterations in the expression of these biomarkers that is induced by the treatments and ultimately to determine if these changes reflect response.
- 3.0 **DRUG INFORMATION: Ad5CMV-p53 Adenoviral Vector (RPR/INGN 201)**
- 3.1 Labeling
RPR/INGN 201 will be labeled as follows:
Ad5CMV-p53 1.0mL MM/DD/YY
P/N XX-XXXXXX B/N XXXXXXXXXX
NTROGEN Therapeutics, Inc.
Store: $\leq -60^{\circ}\text{C}$ Caution: New Drug for Investigational Use Only
Nominal Concentration: XEXX vp/mL or XEXX pfu/mL
- 3.2 Shipping/Receiving
- 3.2.1 RPR/INGN 201 will be supplied by Introgen to the study site. Study material will be shipped on dry ice by overnight courier following packaging procedures in accordance with applicable local, state, and federal guidelines. Study Material Accountability records will be maintained at the study site.
- 3.2.2 All unused study material must be returned to Introgen. Spent vials may be discarded by the study site after they have been inventoried by an Introgen representative in accordance with Institutional Biosafety Committee policy and procedures.
- 3.2.3 A detailed procedure for ordering and shipping clinical materials will be maintained in the Study File.

3.3 Packaging

RPR/INGN 201 will be provided in single use 1 mL Nunc vials as a frozen viral suspension in Dulbecco's phosphate-buffered saline with 10% (v/v) glycerol.

3.4 Storage

Prior to dilution, the vials of RPR/INGN 201 should be stored at $\leq -60^{\circ}\text{C}$ freezer. All study supplies must be kept in an appropriate locked room which can be accessed only by the investigator, or designated personnel.

3.5 Handling

After removing the RPR/INGN 201 vial from the freezer, it should be immediately placed on wet ice. Standard chemotherapy preparation precautions (gown, gloves, mask) and sterile technique should be followed while preparing the specific dose of RPR/INGN 201 required. Dose preparation should take place under a biosafety hood. A detailed handling procedure will be maintained in the Site Study File.

3.6 Dilution

The diluent used for dilution will be Dulbecco's phosphate-buffered saline. A dilution worksheet will be completed by the site staff for each dose prepared.

3.7 Stability

3.7.1 RPR/INGN 201 is stable at room temperature for up to 24 hours without loss of titer. It is recommended, however, that the vector is kept on wet ice before, during and after dilution and that administration of the vector should occur in a timely manner. RPR/INGN 201 may be allowed to reach room temperature just prior to administration.

3.7.2 The RPR/INGN 201 Dose Preparation Worksheet and Vector Administration case report form will document times of dose preparation and administration.

3.9 Drug Accountability

3.9.1 The study personnel responsible for study treatment dispensing are required to maintain adequate records of all study materials. These records include an acknowledgment stating that study materials have been received from Introgen, dispensing records, and shipping records for all unused materials returned to Introgen. Damaged and/or contaminated vials and vials that have been reconstituted but not administered, for any reason, must also be accounted for in the dispensing records. These steps will be coordinated through the study research nurse.

3.9.2 The study site personnel responsible for study treatment administration to the patient will record the date and the time the treatment is administered to the patient. It will be recorded if the treatment administration is interrupted or stopped.

4.0 PATIENT ELIGIBILITY

- 4.1 Prior external beam radiotherapy for clinical stage T1-T3 (Nx, M0) adenocarcinoma of the prostate.
- 4.2 Evidence of a rising PSA (3 consecutive rises on follow-up).
- 4.3 The PSA doubling time should be greater than 1 year.
- 4.4 A positive post-external beam radiotherapy prostate biopsy for adenocarcinoma.
- 4.5 No evidence of metastases by bone scan and CT-scan of the pelvis <1 month prior to enrollment.
- 4.6 No evidence of palpable extraprostatic extension at the time of enrollment.
- 4.7 PSA ≤ 10 ng/ml within 2 weeks of enrollment.
- 4.8 Transrectal prostate volume ≤ 65 cc, determined <1 month prior to enrollment.
- 4.9 Androgen ablation is permitted if it was for ≤ 6 mo and was stopped >1 year prior protocol enrollment. This includes natural hormones, such as PC-Spes. Patient agrees not to initiate hormone therapy within two years of completion of treatment, except under advisement of protocol physicians.
- 4.10 Zubrod Performance Status ≤ 2 .
- 4.11 No history of grade 3 radiation reaction to external beam radiotherapy
- 4.12 No history of HIV positivity or chronic hepatitis B or C infections.
- 4.13 No history of steroid medications of greater than two months duration. (Such patients are considered to have been treated with hormonal therapy).
- 4.14 Must be willing and able to avoid contact with severely immunodeficient persons.
- 4.15 Must be willing and able to practice effective barrier methods of contraception.

5.0 TREATMENT PLAN

- 5.1 Randomization
Ad5CMV-p53 plus I-125 seed implant versus I-125 implant alone
- 5.2 Stratification
 - 5.2.1 Androgen ablation in the past versus no androgen ablation
 - 5.2.2 Pre-implant PSA ≤ 2 ng/ml versus >2 ng/ml
- 5.3 Ad5CMV-p53 (RPR/INGN 201) Treatment
 - 5.3.1 The first RPR/INGN 201 treatment will be done immediately before the implant procedure, during the same anesthesia. There will be a total of 3 RPR/INGN 201 treatments: the second and third treatments will be spaced at approximately 2 week intervals, with the second between days 12-16 and the third between days 26-30 post-implant.
 - 5.3.2 RPR/INGN 201 will be administered via TRUS-guided percutaneous intraprostatic injections. Injections will be performed under local, general, or spinal anesthesia to minimize patient movement and discomfort. RPR/INGN 201 will be injected transperineally into the prostate under direct ultrasonographic visualization. RPR/INGN 201 will be administered in 4 – 6 divided injections in an attempt to inject the entire prostate gland with study vector. A total of 3×10^{12} viral particles will be injected in a volume of 3 mL. All interruptions during the vector administration procedure will be documented.
 - 5.3.3 Patients may be treated as outpatients. If the patient develops an upper respiratory

infection, the patient should wear a mask and avoid contact with immunosuppressed people and children under three years old until there is no clinical evidence of infection.

5.4 I-125 Seed Implant

5.4.1 Pre-planning: A transrectal ultrasound prostate volume study must be done prior to protocol enrollment to confirm that the prostate is ≤ 65 cc in size. Androgen ablation may not be used to shrink the prostate prior to seed implantation. After enrollment in the protocol the patient will undergo a transrectal ultrasound pre-planning study using a stepping unit designed to move the probe at 0.5-cm increments. The ultrasound measurements will be obtained from the base to the apex of the prostate with the patient in the lithotomy position and with the first horizontal row of the grid parallel to the posterior margin of the prostate. The sum of the measurements will correspond to the gross tumor volume (GTV) and will be equal to the clinical target volume (CTV). The PTV will include the prostate with a 2-5 mm margin. In addition to the prostate, the urethra will be delineated on the ultrasound images. There are two techniques that may be used for the implant. If pre-loaded needles are used, the ultrasound volume pre-plan should be obtained prior to day of the implant. Alternatively, the pre-planning study may be done in the operating room at the time of the implant if the Mick applicator is used. In the latter case, the nomogram devised by Stock and Stone (1998) will be used. The dosimetry will be based upon AAPM Report TG43 for I-125 seeds. The prescribed dose to the PTV is to be 110 Gy. Approximately 75% of the seeds will be placed peripherally and 25% centrally.

5.4.2 Implant procedure: The implants will be done using I-125 seeds, model 6711, with seed activity of 0.3 – 0.45 mCi. Prior to implantation a random selection of seeds shall be calibrated in the manner determined by the Department of Radiation Physics at M D Anderson Cancer Center.

The procedure will be performed as an outpatient procedure under either general or spinal anesthesia with the patient in lithotomy position. Transrectal ultrasound and fluoroscopy shall be available to verify needle and seed placement during and after the procedure. At the beginning of the procedure the patient will be treated with intravenous antibiotics and steroids (e.g., Gentamycin 80 - 120 mg and Decadron 10 mg), as determined by the attending physician.

Once positioned, the patient's perineum will be prepped and draped in a sterile fashion. Approximately 15cc of aerated KY Jelly or viscous lidocaine is injected into the urethra and held in place using a penile clamp. This allows for visualization of the urethra on ultrasound. Then the scrotum will be retracted anteriorly to move it out of the field and the perineum will be shaved and prepped. The stepping unit with the ultrasound probe and template will be attached to a stabilized platform. Then the ultrasound apparatus will be positioned so as to guide needles to the desired position. Verification of needle and seed placement will be accomplished using fluoroscopy and the ultrasound probe in longitudinal mode.

At the end of the procedure seed placement will be verified using ultrasound and fluoroscopy. Additional seeds may then be placed to adequately obtain the desired prescribed dose, at the discretion of the physician. For the purpose of this protocol seeds may be placed using either a Mick applicator or preloaded needles. During the procedure a record of seed placement and template position will be kept.

5.4.3 Post-implant procedure: Following the procedure cystoscopy may be performed as deemed necessary. Seeds located in the bladder and urethra will be removed. An in-dwelling Foley catheter will then be placed. Following recovery from anesthesia, the patient will undergo simulation obtaining orthogonal films, followed by CT scan of the pelvis on day 0 (within 24 hr of the implant). The patient will then be discharged from hospital with oral antibiotics (Ciprofloxacin 500mg q 12 hours for 10 days) and other medications as needed. A sheet of discharge instructions will be given to the patient at this time. The patient will be seen on post-procedure day one for post-procedure follow-up and evaluation. On post-procedure day one the Foley catheter will be removed and the patient will undergo a voiding trial. The patient will undergo an additional CT scan with retrograde urethrogram 3-5 weeks post-implant. This CT-scan will be done before the third Ad-p53 injection to avoid any transient effects of the Ad5CMV-p53 injection on prostate swelling.

5.4.4 Post-implant dosimetry: The effect of Ad5CMV-p53 injection on implant dosimetry is uncertain, but, is not likely to be a major problem. The 1-month CT-scan post-implant (before the 3rd Ad5CMV-p53 injection for the combined group) will be used to document the dosimetry of the implants and to compare the two groups.

The minimum target dose will be defined as the minimum dose at the periphery of the Evaluation Target Volume (ETV) which is the post-implant CT definition of the prostate as determined by CT obtained on or near post-implant day 30. The High Dose Volume will be defined as the volume enclosed by 200% of the prescribed dose. The Low Dose Volume will be defined as the volume encompassed by 100 Gy. The Maximum Urethral Dose will be defined as the maximum dose delivered to the prostatic urethra. The High Dose Urethral Dose Volume will be defined as that volume of the urethra receiving more than 200% of the prescribed dose. The following criteria will be used for evaluation:

Per Protocol: greater than or equal to 80% of the ETV receives at least 90% of the prescription dose.

Variation, Acceptable: greater than or equal to 50% of the ETV receives at least 90% of the prescription dose.

Deviation, Unacceptable: greater than or equal to 50% of the ETV receives less than 90% of the prescription dose.

6.0 PRETREATMENT AND TREATMENT EVALUATION

6.1 Informed consent obtained prior to any study-specific procedures.

- 6.2 Complete history and physical examination within 21 days of treatment.
 - 6.2.1 Medical history including diagnosis of primary disease and concurrent illnesses.
 - 6.2.2 Documentation of medications.
 - 6.2.3 Documentation of clinical signs and symptoms.
 - 6.2.4 Physical examination including height, weight, Zubrod performance status, vital signs (temperature/pulse/sitting blood pressure/respiration rate), digital rectal exam.
- 6.3 Post external beam radiotherapy prostate biopsy to confirm local tumor persistence after external beam radiotherapy.
- 6.4 Prostate tissue sample obtained and fixed in formalin for documentation of p53 mutation (not required for enrollment) and histology. Tissue sample may be from a previous biopsy.
- 6.5 Pretreatment blood tests ≤ 2 weeks prior to treatment.
 - 6.5.1 Complete blood count with differential and platelets, PT and PTT, ≤ 2 weeks, prior to treatment.
 - 6.5.2 Serum PSA.
 - 6.5.3 Calcium, sodium, potassium, chloride, total protein, albumin, creatinine, alkaline phosphatase, ASAT (SGOT), ALAT (SGPT), lactate dehydrogenase, urea, and total bilirubin.
 - 6.5.4 Hepatitis B & C Screen
- 6.6 Pretreatment urine tests/samples.
 - 6.6.1 Urinalysis including blood and protein.
 - 6.6.2 First morning urine samples (20 mL).
- 6.7 Pretreatment imaging
 - 6.7.1 Prostate transrectal ultrasound volume study within 3 months prior to treatment.
 - 6.7.2 Chest x-ray within 1 month prior to treatment.
 - 6.7.3 Bone scan ≤ 3 months prior to treatment.
 - 6.7.4 CT-scan pelvis ≤ 3 months prior to treatment.
- 6.8 EKG within 1 month prior to treatment.
- 6.9 Temperature log
 - 6.9.1 Study staff will review Patient Temperature Log instructions with patient and caregiver prior to preparation for procedure.
 - 6.9.2 Patient's oral temperature will be recorded one to two hours prior to vector administration and two hours (± 1 hour) after vector administration.
 - 6.9.3 Administration of pretreatment questionnaires including the UCLA Prostate Cancer Index (Litwin, et al 2000), FACT-P QOL, AUA Symptom Index and Late Effects Questionnaires.
- 6.10 Sextant transrectal ultrasound-guided prostate biopsies will be obtained the day after the implant and the first p53 injection. The tissue will be used to measure molecular response parameters.
- 6.11 A pelvic CT-scan will be performed within 24-hours after the implant to determine seed placement.
- 6.12 Patient monitoring for the first month after the implant & first p53 injection.
 - 6.12.1 Day 2 - 6: The patient or caregiver will record patient's oral temperature three times during waking hours: immediately upon rising, early afternoon (about 1 P.M.), and before retiring. Patient will be given a temperature log (Appendix G) on which to record his temperatures and medications for pain and fever.

- 6.12.2 Day 7 - 11: The patient or caregiver will record patient's oral temperature once per day during waking hours: immediately upon rising. Patient will record his temperature and medications for pain and fever as he did for Day 2 through Day 6.
- 6.12.3 Day 15 (± 1 day): prior to second vector administration
 - 6.12.3.1 Physical examination including weight, Zubrod performance status, vital signs (pulse/sitting blood pressure/respiration rate).
 - 6.12.3.2 Documentation of any changes in concomitant medications.
 - 6.12.3.3 Documentation of any adverse events.
 - 6.12.3.4 CBC with differential and platelet count. Serum PSA, calcium, sodium, potassium, chloride, total protein, albumin, creatinine, alkaline phosphatase, ASAT (SGOT), ALAT (SGPT), lactate dehydrogenase, urea, and total bilirubin.
 - 6.12.3.5 Urinalysis including blood and protein. First morning urine samples (30 mL) for CPE testing.
 - 6.12.3.6 Serum sample (10 mL) collected in one (1) serum separator tube (during Course 1 only).
 - 6.12.3.7 Study staff will review Patient Temperature Log instructions with patient and caregiver prior to preparation for procedure.
 - 6.12.3.8 Patient's oral temperature will be recorded one to two hours prior to vector administration and two hours (± 1 hour) after vector administration.
 - 6.12.3.9 Vector administration as per section 5.3
- 6.12.4 Day 16 through Day 20.
 - 6.12.4.1 The patient or caregiver will record patient's oral temperature three times during waking hours: immediately upon rising, early afternoon (about 1 P.M.), and before retiring. Patient will be given a temperature log (Appendix H) on which to record his temperatures and medications for pain and fever.
- 6.12.5 Day 21 through Day 25
 - 6.12.5.1 The patient or caregiver will record patient's oral temperature once per day during waking hours: immediately upon rising. Patient will record his temperature and medications for pain and fever as he did for Day 16 through Day 20.
- 6.12.6 Day 29 (± 1 day) prior to third vector administration
 - 6.12.6.1 Physical examination including weight, Zubrod performance status, vital signs (pulse/sitting blood pressure/respiration rate).
 - 6.12.6.2 Documentation of any changes in concomitant medications.
 - 6.12.6.3 Documentation of any adverse events.
 - 6.12.6.4 CBC with differential and platelet count. Serum PSA, calcium, sodium, potassium, chloride, total protein, albumin, creatinine, alkaline phosphatase, ASAT (SGOT), ALAT (SGPT), lactate dehydrogenase, urea, and total bilirubin.
 - 6.12.6.5 Urinalysis including blood and protein. First morning urine samples (30 mL) for CPE testing.
 - 6.12.6.6 Serum sample (10 mL) collected in one (1) serum separator tube (during Course 1 only).
 - 6.12.6.7 Study staff will review Patient Temperature Log instructions with patient and caregiver prior to preparation for procedure.

- 6.12.6.8 Patient's oral temperature will be recorded one to two hours prior to vector administration and two hours (\pm 1 hour) after vector administration.
- 6.12.6.9 Vector administration as per section 5.3
- 6.12.7 Day 30 through Day 34.
 - 6.12.7.1 The patient or caregiver will record patient's oral temperature three times during waking hours: immediately upon rising, early afternoon (about 1 P.M.), and before retiring. Patient will be given a temperature log (Appendix H) on which to record his temperatures and medications for pain and fever.
- 6.12.8 Day 35 through Day 39
 - 6.12.8.1 The patient or caregiver will record patient's oral temperature once per day during waking hours: immediately upon rising. Patient will record his temperature and medications for pain and fever as he did for Day 16 through Day 20.
- 6.13 Instruction in transmission-based precautions
 - 6.13.1 Patients will be instructed by the research nurse in good hygiene practices and transmission-based precautions.
 - 6.13.2 Instructions to avoid contact with immunodeficient individuals.
 - 6.13.3 Instructions to practice effective barrier methods of contraception.

7.0 POST-TREATMENT EVALUATION.

- 7.1 After treatment is completed (after last p53 injection), follow-up PSAs will be obtained at 2 weeks, 1 month, every 3 months for 2 years and every 6 months thereafter. History and physical examination will be done at 2 weeks, 1 month, 3 months, every 6 months for 2 years, and every 6 months thereafter. Rectal exam will not be done until 3 months after the last injection. Patients should be encouraged to mail copies of any PSA values from outside institutions to the research nurse.
- 7.2 The UCLA Prostate Cancer Index (Litwin, et al, 2000) and the FACT-P quality-of-life (FACT-P QOL: Esper et al, 1997) questionnaires Appendix B, will provide the determination of QOL from the patient's perspective. The AUA symptom index (Appendix B, Section 4) will also be obtained. The questionnaires will be administered prior to the implant, and at 1 month, 3 months, 6 months, 1 year and 2 years after completion of treatment. In addition, a more specific questionnaire designed to assess radiotherapy sexual, urinary, and bowel late side effects (Appendix B, Sections 2 & 3) will be administered. This questionnaire will be administered prior to the implant and 2 years after completion of therapy. The completed questionnaires should be mailed to Joy Phillips, RN, Department of Radiation Oncology (Box 97) within 7 days of completion.
- 7.3 An increasing palpable induration in the prostate should be considered suggestive of a local recurrence, and should be investigated by prostate biopsy.
- 7.4 If the PSA rises on three consecutive blood tests separated by 1.5-3 mo intervals or rises by ≥ 2 ng/ml on a single test (with confirmation by a repeat test), then bone scan, CT-pelvis, and prostate biopsy will be obtained. These tests will be done to document the site of first failure. If the tests are negative, it is recommended that the patient be observed for 1 year

and the tests repeated. Subsequent treatment is at the discretion of the Urologist and/or Radiation Oncologist caring for the patient.

7.5 Prostate biopsies in absence of a rising PSA: see section 8.3.1.

8.0 DATA COLLECTION.

8.1 All protocol patients must sign the consent and be enrolled with the Department of Radiation Oncology research division within 1 week of signing the consent

8.2 Toxicity: Treatment effects will be assessed at each follow-up visit. The acute toxicity GU and GI scales in Appendix D will be used to document the grade of reaction for the first 3 months after treatment. Six months following radiotherapy, the late toxicity GU and GI scales in Appendix E will be used. Any grade 3 or higher acute or chronic side effects must be reported to Alan Pollack, M.D., Ph.D., Department of Radiation Oncology (97) (see section 9.0).

8.3 Tumor Response: The main response parameter will be prostate biopsy at 1 and 2 years post-treatment. Biochemical response will be based on the nadir PSA level with a minimum follow-up of 2 years. Clinical response will also be documented.

8.3.1 In the absence of a rising PSA, the prostate will be biopsied at 1 and 2 years to determine if the tumor has been eradicated. The biopsies will be used to assess *pathologic response*, which is the main response endpoint. The biopsies will be classified as no tumor seen, atypical cells consistent but not diagnostic of carcinoma, adenocarcinoma with treatment effect, and adenocarcinoma without treatment effect; the first two are considered negative and the latter two positive based on prior data using a rising PSA as the main endpoint (Pollack et al, 2000).

8.3.2 *Clinical primary tumor response* will be measured by digital rectal exam and recorded in the following ways:

- (a) Estimate length (apex to base) and width of each nodule or tumor mass in centimeters and record in a diagram in the chart.
- (b) Qualitatively score the tumor volume relative to that on the prior examination as:
 - (i) Complete response: no palpable tumor.
 - (ii) Partial response: at least 50% decrease in the product of the length and width of the tumor mass (in case of more than one nodule, the sum of the products will be used).
 - (iii) Stable disease: changes too small to qualify for partial response or progression.
 - (iv) Progression: at least a 25% increase in the product of the length and width of tumor relative to the smallest volume recorded, or new extension of tumor beyond the capsule, or re-extension of tumor beyond the capsule after initial regression, or urinary obstructive symptoms with carcinoma found at TURP. In all cases of clinically suspected local failure, biopsy confirmation of carcinoma will be obtained.

We anticipate that nearly all patients will have a complete clinical response, i.e., disappearance of palpable prostate tumor, since this is typical with radiation treatment. Sometimes after seed implant there is residual firmness in the prostate and this will be documented.

- 8.3.3 Biochemical response will be determined by the nadir PSA level observed with a minimum follow-up of 2 years. In our experience, the most significant declines are seen within the first 2 years post-treatment, and subsequent drops are minimal. A nadir PSA of ≤ 0.5 ng/ml will be considered a favorable response and a PSA > 0.5 a negative response.

9.0 REPORTING OF ADVERSE OR UNKNOWN DRUG REACTIONS

Adverse or Unknown Reactions from RPR/INGN 201 and/or Radiotherapy (Appendix G). Any grade 3 reaction should be reported to the Principal Investigator (Dr. Alan Pollack) within 1 week of documentation. Any grade 4 – 5 or previously unknown reactions should be transmitted to the Principal Investigator within 24 hr by phone and to the Surveillance Committee in writing within 10 working days. A fatal event (grade 5 toxicity) that occurs within 30 days of completing treatment must likewise be reported to the Principal Investigator within 24 hr by phone and to the Surveillance Committee in writing within 10 working days.

10.0 STATISTICAL CONSIDERATIONS.

- 10.1 Response and Toxicity. The primary objective of this study is to assess efficacy in terms of pathologic response (negative 1 year prostate biopsy) and toxicity (\geq grade 3 late bladder or rectal morbidity) for Ad5CMV-p53 gene therapy plus radioactive I-125 seed implant versus seed implant alone in patients refractory to external beam radiotherapy. The purpose of the randomization is to provide unbiased estimators of the effects of adding gene therapy on response and toxicity as defined, as well as on all other variables of interest.

From historical data, the response rate for the seed implant regimen alone is about 30%. We need to demonstrate that the combined regimen (Ad5CMV-p53 gene therapy plus seed implant) is at least as efficacious as the seed implant regimen alone with a target increase in response rate to 50%. However, the study may be terminated early if the observed objective response is far less than the seed implant regimen alone or if excessive adverse events (more than 20%) are seen. Response will be determined by prostate biopsy at 1 year after the completion of therapy; a negative biopsy will evidence of response. An adverse event (toxicity) is defined as a Grade 3 or above late reaction.

The safety monitoring method of Bryant and Day (1995) will be used to incorporate both rates of clinical response and adverse events. Based on data from prior studies of seed implant alone, the response probability is about 30%. Therefore for the Ad5CMV-p53

gene therapy plus seed implant arm, an unacceptable response will have a probability of 0.30, an acceptable response probability of 0.50, an unacceptable toxicity probability of 0.3 and an acceptable toxicity probability of 0.1.

For the combined regimen arm, both response and toxicity rates will be assessed for the first 15 patients. If the number of responses at that time is fewer than 30% then an early termination of the trial is recommended due to inadequate response. If there are 30% or more responses and 30% or more toxicities then an early termination of the trial is recommended due to excessive toxicity. If the results at the time of the interim analysis are inconclusive, the trial will be extended to accrue an additional 22 patients for each arm.

From the total sample of 37 patients for each arm, if (a) there are 14 or fewer responses for the combined treatment arm, then the treatment is rejected due to inadequate response; (b) if there are 9 or more adverse events then the treatment is rejected due to excessive toxicities; (c) if there are more than 14 responses and fewer than 10 adverse events then the treatment is recommended for further consideration. With the above rules for the combined treatment arm, the overall type I errors are 2% (poor response and excessive toxicity), 10% (poor response and acceptable toxicity), 14% (good response and excessive toxicity), and a type II error will be 14% (Bryant and Day, 1995).

For estimation purposes, using a Bayesian argument, the proposed sample size is adequate to provide confidence intervals with at least 80% coverage probability. For example, consider the case of estimating the rate of grade >3 toxicity. Within each treatment arm, we may assume that the prior probability of an adverse event follows a Beta(0.2,0.2) distribution. If, for example, 7/37 of the patients respond, this will provide a posterior probability interval (0.10, 0.30) for toxicity with posterior coverage probability of 88.7%

- 10.2 We will correlate the main response variables (biopsy positivity, Psa nadir) with the biomarkers variables using standard univariate analysis and several multivariate data reduction techniques. Clustering methods, such as principal components analysis and hierarchical clustering, will be used to examine associations among biomarkers. After important prognostic variables associated with the dependent variable have been identified, all prognostic variables will be evaluated together via the ordinal logistic regression analysis to construct a quantitative model. Goodness-of-fit will be examined using deviance residuals and Hosmer-Lemeshow test. In parallel with the logistic regression analysis, we will also apply decision tree analysis (e.g., CART - Breiman et al 1984) and multivariate adaptive regression splines (MARS- Friedman and Roosen 1995) to construct nonparametric quantitative models. Both these latter methods are computer intensive and are especially useful for non-linear dependency for detecting outliers, and can handle missing values (for covariates). MARS in particular has more power and flexibility to model relationships that are nearly additive or involve interactions. The model can be represented in a form that separately identifies the additive contributions and those associated with the different multivariable interactions.

Biomarkers are often measured repeatedly over time. To model the change of biomarkers over time, we shall use either the generalized estimating equation approach by Liang and Zeger (1986) for both continuous and discrete data, or a generalized linear mixed model.

Data analyses will be performed in SPSS, SAS, SPlus, and CART.

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Appendix A

EXAMINATIONS, TESTS TO BE DONE, AND SCHEDULES:

<u>Tests</u>	<u>Prior to Implant</u>	<u>Prior to 2nd&3rd p53</u>	<u>F/U^a Visits</u>	<u>Biochem Failure</u>
History & Physical & concomittant meds	X	X	X	X
CBC, Diff, PLT'S	X	X		
PT/PTT	X	X		
SMA-12, Electrolytes, Mg	X	X		
Hepatitis B and C screen	X			
Urinalysis	X	X		
Morning urine sample	X	X		
Serum sample	X	X		
PSA	X	X	X	X
EKG	X			
Bone Scan	X			X
CT-Pelvis	X			X
Chest X-ray	X			X
Biopsy prostate ^b	X			X
QOL Questionnaires				
UCLA Prostate Cancer Index	X		X ^c	
FACT-P QOL	X		X ^c	
AUA Symptom Index	X		X ^c	
Late Effects Questionnaire	X		X ^d	

^a Follow-up at 2 weeks, 1 month, every 6 months for 2 years and every year thereafter.

^b Biopsy prostate for diagnosis before implant, at first sign of local failure or a rising PSA, and at 1 and 2 yr in the absence of failure.

^c Administer questionnaires at 1 month, 3 months, 6 months, 1 year and 2 years after completion of treatment .

^d Administer questionnaire at 2 years after completion of treatment.

13. Family communication about my illness is poor..... 0 1 2 3 4
14. I feel close to my partner (or the person who is my main support). 0 1 2 3 4
15. Have you been sexually active during the past year? No _____ Yes _____
 If yes: I am satisfied with my sex life. 0 1 2 3 4

16. Looking at the above 7 questions, how much would you say your SOCIAL/FAMILY WELL-BEING affects your quality of life?(circle one number)

0 1 2 3 4 5 6 7 8 9 10
 Not at all Very much so

RELATIONSHIP WITH DOCTOR

not at all a little bit some-what quite a lot very much

17. I have confidence in my doctor(s) 0 1 2 3 4
18. My doctor is available to answer my questions..... 0 1 2 3 4

19. Looking at the above 2 questions, how much would you say your RELATIONSHIP WITH THE DOCTOR affects your quality of life? (circle one number)

0 1 2 3 4 5 6 7 8 9 10
 Not at all Very much so

EMOTIONAL WELL BEING

not at all a little bit some-what quite a lot very much

20. I feel sad..... 0 1 2 3 4
21. I am proud of how I'm coping with my illness..... 0 1 2 3 4
22. I am losing hope in the fight against my illness..... 0 1 2 3 4
23. I feel nervous..... 0 1 2 3 4
24. I worry about dying..... 0 1 2 3 4
25. I worry that my condition will get worse..... 0 1 2 3 4

26. Looking at the above 6 questions, how much would you say your EMOTIONAL WELL-BEING affects your quality of life? (circle one number)

0 1 2 3 4 5 6 7 8 9 10
 Not at all Very much so

FUNCTIONAL WELL-BEING

not at all a little bit some-what quite a lot very much

27. I am able to work (include work at home)..... 0 1 2 3 4

28. My work (include work in home) is fulfilling..... 0 1 2 3 4

29. I am able to enjoy life..... 0 1 2 3 4

30. I have accepted my illness..... 0 1 2 3 4

31. I am sleeping well..... 0 1 2 3 4

32. I am enjoying the things I usually do for fun..... 0 1 2 3 4

33. I am content with the quality of my life right now..... 0 1 2 3 4

34. Looking at the above 7 questions, how much would you say your FUNCTIONAL WELL-BEING affects your quality of life? (circle one number)

0 1 2 3 4 5 6 7 8 9 10
 Not at all Very much so

ADDITIONAL CONCERNS

not at all a little some-bit quite a what very lot much

35. I am losing weight..... 0 1 2 3 4

36. I have a good appetite..... 0 1 2 3 4

37. I have aches and pains that bother me..... 0 1 2 3 4

38. I have certain areas of my body where I experience significant pain..... 0 1 2 3 4

39. My pain keeps me from doing things I want to do..... 0 1 2 3 4

Appendix B (Section 2 of 5)
Late Effects Questionnaire I (LEQI): Pre-Implant

Page 1 of 2

Medical Record # _____
(optional) Name _____

Bladder function

1. Since after radiotherapy, have you had a problem with dripping or leaking urine?
 yes no
a) Did you have any surgery to help stop dripping or leaking urine?
 yes no
b) Do you still have any problem at all with dripping or leaking urine?
 yes no
2. Dripping or leaking urine can happen at different times.
In the past month have you:
a) dripped or leaked urine when you coughed or sneezed?
 yes no
b) dripped or leaked urine when your bladder was full before you could get to the bathroom?
 yes no
3. If you drip or leak urine, about how much comes out?
 a few drops less than one tablespoon more than one tablespoon
4. If you drip or leak urine, how often does it occur in one day?
 less than once about once a day more than once a day
5. Some men wear pads, rubber pants, adult diapers, or a clamp to help with wetness.
Do you use anything like that now?
 yes no
6. a) Since radiotherapy, how many times do you get up to urinate at night?
 none/rarely once 2-3 times 4-5 times 6 or more
b) Compared to before radiotherapy, is this:
 the same more frequent less frequent
7. Since radiotherapy, have you noticed blood in your urine?
 never occasionally frequently
8. If you have noticed blood in the urine, have you had:
 tests to investigate the bleeding
 prescription medications to treat it (if so which _____)
 transfusions because of heavy bleeding (if so when _____)
 surgery or procedures because of bleeding (if so when _____)
9. Since radiotherapy, do you have burning on urination?
 never occasionally frequently
10. Since radiotherapy, is your urine stream:
 the same weaker stronger

Continued on next page.....

Bowel function

1. Since radiotherapy, have you noticed a change in your bowel function?
 no change mild change moderate change major change
2. a) How many bowel movements do you have a day?
 2-3 4-5 6 or more
- b) Compared to before radiotherapy, is this:
 the same more frequent less frequent
3. Are you concerned because your bowel movements are more urgent?
 yes no
4. Are you able to control your bowel movements without accidents?
 yes no
5. Do you take anti-diarrheal pills such as Lomotil or Immodium?
 never occasionally every week daily
6. Have you noticed any blood with bowel movements in the past 6 months?
 never once only occasionally at least once a week daily
7. If you have noticed blood with your bowel movements, have you had:
 tests to investigate the bleeding
 prescription medications to treat it (if so which _____)
 transfusions because of heavy bleeding (if so when _____)
 rectal surgery or procedures because of bleeding (if so when _____)

Sexual function

1. a) Before radiotherapy, could you have any full sexual erections when you were stimulated?
 yes no
- b) If no, could you have any partial erections?
 yes no
- c) How often were your erections firm enough to have intercourse?
 not at all a few times fairly often usually always
2. a) Since radiotherapy, have you had any full erections when you were stimulated?
 yes no
- b) If no, have you been able to have any partial erections?
 yes no
- c) How often were they firm enough to have intercourse?
 not at all a few times fairly often usually always
3. Since radiotherapy, have you tried treatments such as shots or penile injections, implant surgery, or vacuum suction devices to help your sexual function?
 yes no
4. a) Since radiotherapy, have you had a change in any chronic medications (for example: new blood pressure medication or higher dose)?
 yes no
- b) If yes, did this decrease your erections
 no decrease mild decrease moderate decrease major decrease

Appendix B (Section 3 of 5)
Late Effects Questionnaire II (LEQII): Post-Implant

Page 1 of 2

Medical Record # _____
(optional) Name _____

Bladder function

1. Over the last year, have you had a problem with dripping or leaking urine?
 yes no
a) Did you have any surgery to help stop dripping or leaking urine?
 yes no
b) Do you still have any problem at all with dripping or leaking urine?
 yes no
2. Dripping or leaking urine can happen at different times.
In the past month have you:
a) dripped or leaked urine when you coughed or sneezed?
 yes no
b) dripped or leaked urine when your bladder was full before you could get to the bathroom?
 yes no
3. If you drip or leak urine, about how much comes out?
 a few drops less than one tablespoon more than one tablespoon
4. If you drip or leak urine, how often does it occur in one day?
 less than once about once a day more than once a day
5. Some men wear pads, rubber pants, adult diapers, or a clamp to help with wetness.
Do you use anything like that now?
 yes no
6. a) How many times do you get up to urinate at night?
 none/rarely once 2-3 times 4-5 times 6 or more
b) Compared to one year ago, is this:
 the same more frequent less frequent
7. Have you noticed blood in your urine?
 never occasionally frequently
8. If you have noticed blood in the urine, have you had:
 tests to investigate the bleeding
 prescription medications to treat it (if so which _____)
 transfusions because of heavy bleeding (if so when _____)
 surgery or procedures because of bleeding (if so when _____)
9. Do you have burning on urination?
 never occasionally frequently
10. Compared to one year ago, is your urine stream:
 the same weaker stronger

Continued on next page.....

Bowel function

1. Have you noticed a change in your bowel function over the last year?
 no change mild change moderate change major change
2. a) How many bowel movements do you have a day?
 2-3 4-5 6 or more
- b) Compared to one year ago, is this:
 the same more frequent less frequent
3. Are you concerned because your bowel movements are more urgent?
 yes no
4. Are you able to control your bowel movements without accidents?
 yes no
5. Do you take anti-diarrheal pills such as Lomotil or Immodium?
 never occasionally every week daily
6. Have you noticed any blood with bowel movements in the past 6 months?
 never once only occasionally at least once a week daily
7. If you have noticed blood with your bowel movements, have you had:
 tests to investigate the bleeding
 prescription medications to treat it (if so which _____)
 transfusions because of heavy bleeding (if so when _____)
 rectal surgery or procedures because of bleeding (if so when _____)

Sexual function

1. a) Over the last year, have you had any full erections when you were stimulated?
 yes no
- b) If no, have you been able to have any partial erections?
 yes no
- c) How often were they firm enough to have intercourse?
 not at all a few times fairly often usually always
2. Over the last year, have you tried treatments such as shots or penile injections, implant surgery, or vacuum suction devices to help your sexual function?
 yes no
3. a) Over the last year, have you had a change in any chronic medications (for example: new blood pressure medication or higher dose)?
 yes no
- b) If yes, did this decrease your erections
 no decrease mild decrease moderate decrease major decrease

Appendix B (Section 4 of 5) AUA Symptom Index

Page 1 of 1

Medical Record # _____
(optional) Name _____

Over the last month, how many times, on average, do you urinate during the night?

0	1	2	3	4	5
None	Once	Twice	three times	four times	five or more

Over the past month, how often do you have the sensation of not completely emptying your bladder?

0	1	2	3	4	5
Never	less than $\frac{1}{4}$	less than $\frac{1}{2}$	$\frac{1}{2}$	more than $\frac{1}{2}$	always

Over the past month, how many times have you had to urinate less than two hours after you finished urinating?

0	1	2	3	4	5
Never	less than $\frac{1}{4}$	less than $\frac{1}{2}$	$\frac{1}{2}$	more than $\frac{1}{2}$	always

Over the past month, how often have you found that you stopped and started again several times when you urinated?

0	1	2	3	4	5
Never	less than $\frac{1}{4}$	less than $\frac{1}{2}$	$\frac{1}{2}$	more than $\frac{1}{2}$	always

Over the past month, how often have you found it difficult to postpone urination?

0	1	2	3	4	5
Never	less than $\frac{1}{4}$	less than $\frac{1}{2}$	$\frac{1}{2}$	more than $\frac{1}{2}$	always

Over the past month, how often have you had a weak urinary stream?

0	1	2	3	4	5
Never	less than $\frac{1}{4}$	less than $\frac{1}{2}$	$\frac{1}{2}$	more than $\frac{1}{2}$	always

Over the past month, how often have you had to push or strain to begin urination?

0	1	2	3	4	5
Never	less than $\frac{1}{4}$	less than $\frac{1}{2}$	$\frac{1}{2}$	more than $\frac{1}{2}$	always

Appendix B (Section 5 of 5)
 UCLA Prostate Cancer Index

Page 1 of 8 (UCLA)

1. The following questions are about activities you might do during a typical day. Does your health now limit you in these activities? If so, how much?

(Circle 1, 2, or 3 on each line)

	Yes, Limited <u>A Lot</u>	Yes, Limited <u>A Little</u>	No, Not Limited <u>At All</u>
a. Vigorous activities, such as running, lifting heavy heavy objects, participating in strenuous sports.	1	2	3
b. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf.	1	2	3
c. Lifting or carrying groceries.	1	2	3
d. Climbing several flights of stairs	1	2	3
e. Climbing one flight of stairs.	1	2	3
f. Bending, kneeling, or stooping	1	2	3
g. Walking more than a mile	1	2	3
h. Walking several blocks	1	2	3
i. Walking one block.	1	2	3
j. Bathing or dressing yourself	1	2	3

2. During the PAST 4 WEEKS, have you had any of the following problems with your work or other regular daily activities as a result of your PHYSICAL HEALTH?

(Please answer YES or NO for each question by circling 1 or 2 on each line.)

	<u>Yes</u>	<u>No</u>
a. Cut down the amount of time you spent on work or other activities.	1	2
b. Accomplished less than you would like.	1	2
c. Were limited in the kind of work or other activities	1	2
d. Had difficulty performing the work or other activities (for example, it took extra effort).	1	2

Appendix B (Section 5 of 5)

Page 2 of 8 (UCLA)

3. During the **PAST 4 WEEKS**, have you had any of the following problems with your work or other regular daily activities as a result of any **EMOTIONAL PROBLEMS**, such as feeling depressed or anxious?

(Please answer YES or NO for each question by circling 1 or 2 on each line)

	<u>Yes</u>	<u>No</u>
a. Cut down the amount of time you spent on work or other activities	1	2
b. Accomplished less than you would like	1	2
c. Didn't do work or other activities as carefully as usual	1	2

4. These questions are about how you feel and how things have been with you during the **PAST 4 WEEKS**. For each question, please give the one answer that comes closest to the way you have been feeling.
 How much of the time during the **past 4 weeks** . . .

(Circle one number on each line)

	<u>All of the Time</u>	<u>Most of the Time</u>	<u>A Good Bit of the Time</u>	<u>Some of the Time</u>	<u>A Little of the Time</u>	<u>None of the Time</u>
a. Did you feel full of pep?	1	2	3	4	5	6
b. Have you been a very nervous person?	1	2	3	4	5	6
c. Have you felt so down in the dumps that nothing could cheer you up?	1	2	3	4	5	6
d. Have you felt calm and peaceful?	1	2	3	4	5	6
e. Did you have a lot of energy?	1	2	3	4	5	6
f. Have you felt downhearted and blue?	1	2	3	4	5	6
g. Did you feel worn out?	1	2	3	4	5	6
h. Have you been a happy person?	1	2	3	4	5	6
i. Did you feel tired?	1	2	3	4	5	6

Appendix B (Section 5 of 5)

Page 3 of 8 (UCLA)

5. During the past 4 weeks, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?

All of the time 1
Most of the time. 2
Some of the time. . . 3 (Circle one number)
A little of the time. . 4
None of the time. . . 5

6. During the past 4 weeks, to what extent has your physical health or emotional problems interfered with your normal social activities with family, friends, neighbors, or groups?

Not at all. 1
Slightly. 2
Moderately. 3 (Circle one number)
Quite a bit 4
Extremely 5

7. How much bodily pain have you had during the past 4 weeks?

None. 1
Very mild 2
Mild. 3 (Circle one number)
Moderate. 4
Severe. 5
Very severe 6

8. During the past 4 weeks, how much did pain interfere with your normal work (including both work outside the home and housework)?

Not at all. 1
Slightly. 2
Moderately. 3 (Circle one number)
Quite a bit 4
Extremely 5

Appendix B (Section 5 of 5)

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9. Please choose the answer that best describes how true or false each of the following statements is for you.

(Circle one number on each line)

	Definitely <u>True</u>	Mostly <u>True</u>	Not <u>Sure</u>	Mostly <u>False</u>	Definitely <u>False</u>
a. I seem to get sick a little easier than other people.	1	2	3	4	5
b. I am as healthy as anyone I know	1	2	3	4	5
c. I expect my health to get worse. . .	1	2	3	4	5
d. My health is excellent	1	2	3	4	5

10. In general, would you say your health is:

- Excellent 1
- Very good 2
- Good. 3 (Circle one number)
- Fair. 4
- Poor. 5

11. Compared to one year ago, how would you rate your health in general now?

- Much better now than one year ago 1
- Somewhat better now than one year ago 2
- About the same. 3 (Circle one number)
- Somewhat worse now than one year ago. 4
- Much worse now than one year ago. 5

Appendix B. (Section 5 of 5)

URINARY FUNCTION

This section is about your urinary habits. Please consider **ONLY THE LAST 4 WEEKS.**

12. Over the past 4 weeks, how often have you leaked urine?

- Every day 1
- About once a week 2 (Circle one number)
- Less than once a week 3
- Not at all 4

13. Which of the following best describes your urinary control during the last 4 weeks?

- No control whatsoever 1
- Frequent dribbling 2 (Circle one number)
- Occasional dribbling 3
- Total control 4

14. How many pads or adult diapers per day did you usually use to control leakage during the last 4 weeks?

- 3 or more pads per day 1
- 1-2 pads per day 2 (Circle one number)
- No pads 3

15. How big a problem, if any, has each of the following been for you?
 (Circle one number on each line)

	<u>No</u> <u>Problem</u>	<u>Very Small</u> <u>Problem</u>	<u>Small</u> <u>Problem</u>	<u>Moderate</u> <u>Problem</u>	<u>Big</u> <u>Problem</u>
a. Dripping urine or wetting your pants	0	1	2	3	4
b. Urine leakage interfering with your sexual activity	0	1	2	3	4

16. Overall, how big a problem has your urinary function been for you during the last 4 weeks?

- No problem 1
- Very small problem 2
- Small problem 3 (Circle one number)
- Moderate problem 4
- Big problem 5

Appendix B (Section 5 of 5)

Page 6 of 8 (UCLA)

BOWEL HABITS

The next section is about your bowel habits and abdominal pain.
Please consider **ONLY THE LAST 4 WEEKS**.

17. How often have you had rectal urgency (felt like I had to pass stool, but did not) during the last 4 weeks?
- | | | |
|-----------------------------|---|---------------------|
| More than once a day | 1 | |
| About once a day | 2 | |
| More than once a week | 3 | (Circle one number) |
| About once a week | 4 | |
| Rarely or never | 5 | |
18. How often have you had stools (bowel movements) that were loose or liquid (no form, watery, mushy) during the last 4 weeks?
- | | | |
|---------------------------|---|---------------------|
| Never | 1 | |
| Rarely | 2 | |
| About half the time | 3 | (Circle one number) |
| Usually | 4 | |
| Always | 5 | |
19. How much distress have your bowel movements caused you during the last 4 weeks?
- | | | |
|-------------------------|---|---------------------|
| Severe distress | 1 | |
| Moderate distress | 2 | (Circle one number) |
| Little distress | 3 | |
| No distress | 4 | |
20. How often have you had crampy pain in your abdomen or pelvis during the last 4 weeks?
- | | | |
|-----------------------------|---|---------------------|
| Several times a day | 1 | |
| About once a day | 2 | |
| Several times a week | 3 | (Circle one number) |
| About once a week | 4 | |
| About once this month | 5 | |
| Rarely or never | 6 | |
21. Overall, how big a problem have your bowel habits been for you during the last 4 weeks?
- | | | |
|--------------------------|---|---------------------|
| Big problem | 1 | |
| Moderate problem | 2 | |
| Small problem | 3 | (Circle one number) |
| Very small problem | 4 | |
| No problem | 5 | |

Appendix B (Section 5 of 5)

SEXUAL FUNCTION

The next section is about your sexual function and sexual satisfaction. Many of the questions are very personal, but they will help us understand the important issues that you face every day. Remember, YOUR NAME DOES NOT APPEAR ANYWHERE ON THIS SURVEY. Please answer honestly about THE LAST 4 WEEKS ONLY.

22. How would you rate each of the following during the last 4 weeks?
 (Circle one number on each line)

		<u>Very Poor</u>	<u>Poor</u>	<u>Fair</u>	<u>Good</u>	<u>Very Good</u>
a.	Your level of sexual desire?	1	2	3	4	5
b.	Your ability to have an erection?	1	2	3	4	5
c.	Your ability to reach orgasm (climax)?	1	2	3	4	5

23. How would you describe the usual QUALITY of your erections?

- None at all 1
- Not firm enough for any sexual activity 2
- Firm enough for masturbation and foreplay only ... 3 (Circle one number)
- Firm enough for intercourse 4

24. How would you describe the FREQUENCY of your erections?

- I NEVER had an erection when I wanted one 1
- I had an erection LESS THAN HALF the time I wanted one .. 2
- I had an erection ABOUT HALF the time I wanted one 3 (Circle one number)
- I had an erection MORE THAN HALF the time I wanted one . 4
- I had an erection WHENEVER I wanted one 5

25. How often have you awakened in the morning or night with an erection?

- Never 1
- Seldom (less than 25% of the time). 2
- Not often (less than half the time) 3 (Circle one number)
- Often (more than half the time). 4
- Very often (more than 75% of the time). 5

Appendix B (Section 5 of 5)

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26. During the last 4 weeks did you have vaginal or anal intercourse?

- No 1
- Yes, Once 2 (Circle one number)
- Yes, More than Once 3

27. Overall, how would you rate your ability to function sexually during the last 4 weeks?

- Very poor 1
- Poor 2
- Fair 3 (Circle one number)
- Good 4
- Very good 5

28. Overall, how big a problem has your sexual function been for you during the last 4 weeks?

- No problem 1
- Very small problem 2
- Small problem 3 (Circle one number)
- Moderate problem 4
- Big problem 5

Appendix C
PERFORMANCE STATUS

Zubrod Scale*

- 0 Normal activity
- 1 Symptoms but nearly fully ambulatory
- 2 Some bed time but needs to be in bed less than 50% of normal daytime.
- 3 Needs to be in bed more than 50% of normal daytime.
- 4 Unable to get out of bed.

*Zubrod et al: J. Chronic Dis. 11:7, 1960.

Appendix D

Clinical Staging System (1992 AJCC)

T-Stage	Description
T1	Non-palpable tumor
T1a	Nonpalpable, 5% or less of TURP-resected tissue with cancer
T1b	Nonpalpable, more than 5% of TURP-resected tissue with cancer
T1c	Nonpalpable, needle-biopsy positive, no TURP
T2	Tumor palpably confined within the prostate
T2a	Palpable, size \leq 1/2 lobe
T2b	Palpable, size $>$ 1/2 lobe but \leq 1 lobe
T2c	Palpable, size $>$ 1 lobe
T3	Tumor palpably extends through the prostatic capsule
T3a	Palpable, unilateral capsule penetration
T3b	Palpable, bilateral capsule penetration
T3c	Palpable invading seminal vesicles
T4	Tumor is fixed or invades adjacent structures other than the seminal vesicles
T4a	Invasion of bladder neck, external sphincter, or rectum
T4b	Invasion of levator muscles and/or fixation to the pelvic wall

TURP: transurethral resection of prostate.

Appendix E

Acute Radiation Toxicity Grading

	Grade 1	Grade 2	Grade 3	Grade 4
Lower Gastro-intestinal	<p>Increased frequency or change in quality of bowel habits not needing medication. Rectal discomfort not requiring analgesics.</p>	<p>Diarrhea needing parasymphatholytic drugs (e.g. Lomotil). Mucous discharge infrequently requiring sanitary pads. Rectal pain needing analgesics or occasional narcotics. Mild rectal bleeding.</p>	<p>Diarrhea needing parenteral support. Severe mucous discharge requiring extended use of sanitary pads. Abdominal distention. Rectal pain requiring frequent narcotics. GI bleeding requiring one transfusion.</p>	<p>Acute or subacute obstruction. Fistula or perforation. GI bleeding requiring more than one transfusion. Abdominal pain or tenesmus requiring bowel diversion.</p>
Urinary	<p>Frequency or nocturia twice pretreatment habit. Dysuria not needing medication.</p>	<p>Frequency or nocturia less frequent than hourly. Dysuria, bladder spasm needing local anesthetic (e.g. Pyridium or occasional narcotics.). Infrequent gross hematuria. Temporary catheterization.</p>	<p>Frequency or nocturia hourly or more. Dysuria, pain or spasm needing frequent narcotics. Gross hematuria requiring one transfusion. Prolonged urinary obstruction due to prostate inflammation or clots requiring catheterization (including suprapubic).</p>	<p>Hematuria needing more than one transfusion. Hospitalization for sepsis due to obstruction and/or ulceration, or necrosis of the bladder.</p>

Appendix F

Delayed Radiation Toxicity Grading

	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Lower Gastro-intestinal	Excess bowel movements twice baseline. Slight rectal discharge or blood.	More than 2 antidiarrheals/week. Two or fewer coagulations for bleeding. Occasional steroids for ulceration. Occasional dilatation. Intermittent use of incontinence pads. Regular nonnarcotic or occasional narcotic for pain.	More than two antidiarrheals/day. At least one blood transfusion or more than two coagulations for bleeding. Steroids per enema. Hyperbaric oxygen for ulceration. Regular dilation. Persistent use of incontinence pads. Regular narcotic for pain.	Dysfunction requiring surgery. Perforation. Life threatening bleeding.	Fatal toxicity
Urinary	Nocturia twice baseline. Microscopic hematuria. Light mucosal atrophy and minor telanglectasia.	Moderate frequency. Nocturia more than twice baseline. Generalized telanglectasia. Intermittent macroscopic hematuria. Occasional blood transfusions. Two or fewer coagulations. Regular nonnarcotic or occasional narcotic for pain.	Severe frequency and dysuria. Nocturia more frequent than once every hour. Reduction in bladder capacity (150cc). Frequent hematuria. Frequent transfusions. More than one coagulation for hematuria. Regular narcotic for pain.	Severe hemorrhagic cystitis. Ulceration. Requirement for urinary diversion and/or cystectomy.	

Appendix G

Guidelines For Reporting Of Adverse Drug Reactions (ADRs) To The Surveillance Committee (IRB)

In general, ADRs are defined as:

- 1) PREVIOUSLY UNKNOWN TOXICITIES (not included in the list of known toxicities provided by the Division of Cancer Treatment (DCT);
- and 2) LIFE-THREATENING OR FATAL TOXICITIES (regardless of whether or not previously unknown).

The timely reporting of adverse drug reactions is required by the Food and Drug Administration (FDA). The reporting of adverse reactions is in addition to and does not supplant the reporting of toxicities as part of the report of the results of the clinical trial. The Surveillance Committee (IRB) must be notified of any significant life-threatening and/or serious adverse reactions or experiences regardless of cause on a timely basis and must be apprised of all adverse experiences by written report on a periodic and timely basis, at least annually.

1. Reporting ADRs occurring with Investigational Agents

Phase I Studies: Submit a written report within 10 working days to the Surveillance Committee

- Life-threatening events (Grade 4) which may be due to drug administration
- All fatal events (Grade 5) while on study (or within 30 days of treatment)
- First occurrence of any previously unknown clinical event (regardless of Grade)

2. Reporting ADRs Occurring with Commercial Drugs

Submit a written report to the Surveillance Committee within 10 working days.

Any increased incidence of a known ADR as reported in the package insert and/or the literature, and ADR which is both serious (life-threatening, fatal) and unexpected or any death on study if clearly related to commercial agent.

3. Devices in Clinical Research

Grade 4 and 5 toxicities

Submit a written report to the Surveillance Committee within 10 working days.

Note: Report event by telephone within 24 hours to study sponsor or FDA (if study is conducted under an institutional IND)

APPENDIX H Temperature Log

Page 1 of 4
 Protocol ID99-205

Patient Initials: _____
 Patient No.: _____

DATE OF TREATMENT _____ TREATMENT # 1 OF 3

(MM/DD/YY)

INSTRUCTIONS FOR PATIENT

TAKE YOUR TEMPERATURE AT THE FOLLOWING TIMES FOR THE FIRST FIVE DAYS AFTER TREATMENT (DAY 2 THROUGH DAY 6)

- . IMMEDIATELY WHEN YOU GET UP IN THE MORNING (FOR EXAMPLE : 7:00 AM)
- . EARLY AFTERNOON (FOR EXAMPLE : 1:00PM)
- . BEFORE YOU GO TO BED IN THE EVENING (FOR EXAMPLE: 9:00 PM)

THEN FOR AN ADDITIONAL FIVE DAYS (DAY 7 THROUGH DAY 11) TAKE YOUR TEMPERATURE IMMEDIATELY WHEN YOU GET UP IN THE MORNING (7AM)

RECORD THE DATE, THE ACTUAL TIME (INDICATING AM OR PM) YOU TOOK YOUR TEMPERATURE, AND THE TEMPERATURE IN THE BOXES BELOW

TEMPERATURE LOG

Post Treatment	Date of Collection (MM/DD/YY)	Time of Collection (Morning)	Temperature	Time of Collection (Afternoon)	Temperature	Time of Collection (Evening)	Temperature
Day 2	___/___/___		(F)		(F)		(F)
Day 3	___/___/___		(F)		(F)		(F)
Day 4	___/___/___		(F)		(F)		(F)
Day 5	___/___/___		(F)		(F)		(F)
Day 6	___/___/___		(F)		(F)		(F)
Day 7	___/___/___		(F)	NA	NA	NA	NA
Day 8	___/___/___		(F)	NA	NA	NA	NA
Day 9	___/___/___		(F)	NA	NA	NA	NA
Day 10	___/___/___		(F)	NA	NA	NA	NA
Day 11	___/___/___		(F)	NA	NA	NA	NA

APPENDIX H
 Temperature Log

Page 2 of 4
 Protocol ID99-205

Patient Initials: _____
 Patient No.: _____

DATE OF TREATMENT _____ TREATMENT # 2 OF 3

(MM/DD/YY)

INSTRUCTIONS FOR PATIENT

TAKE YOUR TEMPERATURE AT THE FOLLOWING TIMES FOR THE FIRST FIVE DAYS AFTER TREATMENT (DAY 16 THROUGH DAY 20)

- . IMMEDIATELY WHEN YOU GET UP IN THE MORNING (FOR EXAMPLE : 7:00 AM)
- . EARLY AFTERNOON (FOR EXAMPLE : 1:00PM)
- . BEFORE YOU GO TO BED IN THE EVENING (FOR EXAMPLE: 9:00 PM)

THEN FOR AN ADDITIONAL FIVE DAYS (DAY 21 THROUGH DAY 25) TAKE YOUR TEMPERATURE IMMEDIATELY WHEN YOU GET UP IN THE MORNING (7AM)

RECORD THE DATE, THE ACTUAL TIME (INDICATING AM OR PM) YOU TOOK YOUR TEMPERATURE, AND THE TEMPERATURE IN THE BOXES BELOW

TEMPERATURE LOG

Post Treatment	Date of Collection (MM/DD/YY)	Time of Collection (Morning)	Temperature	Time of Collection (Afternoon)	Temperature	Time of Collection (Evening)	Temperature
Day 16	___/___/___		(F)		(F)		(F)
Day 17	___/___/___		(F)		(F)		(F)
Day 18	___/___/___		(F)		(F)		(F)
Day 19	___/___/___		(F)		(F)		(F)
Day 20	___/___/___		(F)		(F)		(F)
Day 21	___/___/___		(F)	NA	NA	NA	NA
Day 22	___/___/___		(F)	NA	NA	NA	NA
Day 23	___/___/___		(F)	NA	NA	NA	NA
Day 24	___/___/___		(F)	NA	NA	NA	NA
Day 25	___/___/___		(F)	NA	NA	NA	NA

APPENDIX H Temperature Log

Page 3 of 4
 Protocol ID99-205

Patient Initials: _____
 Patient No.: _____

DATE OF TREATMENT

TREATMENT # 3 OF 3

(MM/DD/YY)

INSTRUCTIONS FOR PATIENT

TAKE YOUR TEMPERATURE AT THE FOLLOWING TIMES FOR THE FIRST FIVE DAYS AFTER TREATMENT (DAY 30 THROUGH DAY 34)

- . IMMEDIATELY WHEN YOU GET UP IN THE MORNING (FOR EXAMPLE : 7:00 AM)
- . EARLY AFTERNOON (FOR EXAMPLE : 1:00PM)
- . BEFORE YOU GO TO BED IN THE EVENING (FOR EXAMPLE: 9:00 PM)

THEN FOR AN ADDITIONAL FIVE DAYS (DAY 35 THROUGH DAY 39) TAKE YOUR TEMPERATURE IMMEDIATELY WHEN YOU GET UP IN THE MORNING (7AM)

RECORD THE DATE, THE ACTUAL TIME (INDICATING AM OR PM) YOU TOOK YOUR TEMPERATURE, AND THE TEMPERATURE IN THE BOXES BELOW

TEMPERATURE LOG

Post Treatment	Date of Collection (MM/DD/YY)	Time of Collection (Morning)	Temperature	Time of Collection (Afternoon)	Temperature	Time of Collection (Evening)	Temperature
Day 30	___/___/___		(F°)		(F°)		(F°)
Day 31	___/___/___		(F°)		(F°)		(F°)
Day 32	___/___/___		(F°)		(F°)		(F°)
Day 33	___/___/___		(F°)		(F°)		(F°)
Day 34	___/___/___		(F°)		(F°)		(F°)
Day 35	___/___/___		(F°)	NA	NA	NA	NA
Day 36	___/___/___		(F°)	NA	NA	NA	NA
Day 37	___/___/___		(F°)	NA	NA	NA	NA
Day 38	___/___/___		(F°)	NA	NA	NA	NA
Day 39	___/___/___		(F°)	NA	NA	NA	NA

II

**Phase II Prostate
 Initial Evaluation Form**

PLACE LABEL HERE

Revision

Institution

Institution No.

Patient's Name

Patient's I.D. No.

Instructions: Submit this form at time of patient's entry on study. Use -1 for unknown or not applicable unless otherwise specified in the codetable.

DE

1 _____ Patient's DOB

2 GENERAL HEALTH HISTORY

A ZUBROD PERFORMANCE STATUS AT ENTRY TO STUDY (See appendix II in protocol)

B HAS PATIENT HAD ANOTHER MALIGNANCY (other than study site)
 1 No
 2 Yes (complete all items in Q2B)
 9 Unknown

SITE

----- DATE ERADICATED (mm/yyyy) If not NED from second malignancy, leave date blank.

C INTERCURRENT DISEASE
 1 No, skip to 3
 2 Yes, complete all
 9 Unknown

CARDIOVASCULAR

DIABETES

HYPERTENSION

OTHER, Specify _____

3 PRIOR TREATMENT FOR CANCER (prior to entry on study)

1 No
 2 Yes (complete section)
 9 Unknown

PRIOR SURGERY FOR PROSTATE CANCER (excluding diagnostic biopsy and lymph node sampling)

----- DATE OF SURGERY
 mm/dd/yyyy

SPECIFY TYPE OF SURGERY

PRIOR RADIOTHERAPY

----- DATE COMPLETED
 mm/dd/yyyy

DETAILS

PRIOR HORMONES greater than 2 months prior to study treatment

1 yes
 2 no

HAS PATIENT STARTED HORMONES FOR PROSTATE CANCER?

1 No
 2 Yes

A: DOSE TO DATE

B: START DATE

ZOLADEX -----

LUPRON -----

FLUTAMIDE -----

CASODEX -----

HISTOPATHOLOGIC INFORMATION

A _____ DATE MALIGNANCY CONFIRMED
 mm/dd/yyyy

B METHOD OF CONFIRMATION

1 Needle biopsy
 2 T.U.R.
 3 Prostatectomy
 4 Other, specify _____
 9 Unknown

C HISTOLOGIC TYPE

1 Adenocarcinoma
 2 Other, specify _____
 9 Unknown

D PRIMARY GLEASON PATTERN OF MALIGNANCY (Gleason classification 1-5, 9=unknown)

E SECONDARY GLEASON PATTERN OF MALIGNANCY (Gleason classification 1-5, 9=unknown)

F COMBINED GLEASON SCORE (Unknown=99)

I1

REVISION

PLACE LABEL HERE

D WAS LYMPH NODE SAMPLING DONE
 1 No (Complete ^F)
 2 Yes (Complete ^E)
 9 Unknown

Institution Institution No.
 Patient's Name Patient's I.D. No.

E METHOD OF LYMPH NODE SAMPLING
 0 Not applicable
 1 Laparotomy
 2 Laparoscopy
 3 Percutaneous biopsy
 4 Other, specify _____

History of HIV positivity
 1 No
 2 Yes

____/____/____ DATE PELVIC NODE SAMPLING
 mm/dd/yyyy

History of chronic Hepatitis B or C infections
 1 No
 2 Yes

F WAS PELVIC CT OR MRI DONE
 1 No
 2 Yes

History of grade 3 radiation reaction to external beam radiotherapy
 1 No
 2 Yes

LABORATORY VALUES AT TIME OF ENTRY (See protocol for requirements)

G 1 Not done
 2 Normal (specify value)
 3 Abnormal, elevated (specify value and lab range*)
 4 Abnormal, < normal (specify value and lab range*)
 9 Unknown

Transrectal prostate volume ≤ 65cc
 1 No
 2 Yes

PROSTATIC SPECIFIC ANTIGEN (PSA) (See protocol for requirements)

TIME POINTS
 1 Pre-hormones
 2 Study entry
 3 Initial diagnosis

DATE mm/dd/yyyy	PSA VALUE	TIME POINTS

DISEASE EVALUATION

A PALPABLE TUMOR
 1 No
 2 Yes, one lobe
 3 Yes, both lobes
 4 Yes, NOS
 9 Unknown

TUMOR BEYOND CAPSULE AS DETERMINED BY CLINICAL EXAMINATION
 1 No
 2 Yes
 9 Unknown

Appendix I (Section 1 of 8)

I1

REVISION

PLACE LABEL HERE

10 TNM (See protocol)

A

CODED CLINICAL T CLASSIFICATION

- 0 T0
- 1 T1, NOS
- 2 T1a
- 3 T1b
- 4 T1c
- 5 T2, NOS
- 6 T2a
- 7 T2b
- 8 T2c
- 10 T3, NOS
- 11 T3a
- 12 T3b
- 13 T3c
- 14 T4, NOS
- 15 T4a
- 16 T4b
- 9 TX

B

RADIOGRAPHIC N CLASSIFICATION

C

PATHOLOGIC N CLASSIFICATION

- 0 N0
- 1 N1
- 2 N2
- 3 N3
- 9 NX

D

METASTASES

- 0 M0
- 1 M1
- 2 M1a
- 3 M1b
- 4 M1c
- 9 MX

E

WAS BONE SCAN DONE

- 1 No
- 2 Yes

*COMMENTS _____

*Signature _____

Date form completed _____

Institution

Institution No.

Patient's Name

Patient's I.D. No.

INTROGEN THERAPEUTICS, INC.

Patient Initials: _____

Protocol No.: ID99-205

Patient No.: _____

DEMOGRAPHICS/DISEASE HISTORY
 SCREEN/BASELINE

Date of Enrollment: ___/___/___
 MM DD YY

DEMOGRAPHIC DATA

Birthdate	Ethnic Origin
___/___/___ (MM/DD/YY)	<input type="checkbox"/> White <input type="checkbox"/> Hispanic <input type="checkbox"/> Black <input type="checkbox"/> Other (specify): _____ <input type="checkbox"/> Asian

DIAGNOSIS OF PRIMARY CANCER

Date of Initial Diagnosis	Clinical Stage of Disease	Staging at Initial Diagnosis
___/___/___ (MM/DD/YY)	<input type="checkbox"/> Stage T1c or T2a (Gleason 8-10) <input type="checkbox"/> T2b-T2c (Gleason ≥ 7, PSA>10) <input type="checkbox"/> T3	T ___ N ___ M ___

CURRENT DIAGNOSIS (if different than primary)

Same as primary

Date	Clinical Stage of Disease	Current Stage
___/___/___ (MM/DD/YY)	<input type="checkbox"/> Stage T1c or T2a (Gleason 8-10) <input type="checkbox"/> T2b-T2c (Gleason 7, PSA>10) <input type="checkbox"/> T3	T ___ N ___ M ___

PRIOR UROLOGICAL TREATMENT

Record all prior treatments for urological problems

Check if None

Date of Treatment (MM/DD/YY)	Treatment
1 ___/___/___	
2 ___/___/___	
3 ___/___/___	
4 ___/___/___	
5 ___/___/___	

Appendix I (Section 3 of 8)

INTROGEN THERAPEUTICS, INC.

Patient Initials:

Protocol No.: ID99-205

Patient No.: _____

MEDICAL HISTORY
 SCREEN/BASELINE

Date of Exam: ___/___/___
 MM DD YY

Does the patient have a history of any of the following? Check one box for each condition. If checked Yes, specify in space provided.

Body Region/System	Yes	No	Un- known	If checked Yes, provide specifics of disease and inclusive dates
1 Alcoholism				
2 Allergies				
3 Bronchopulmonary disease				
4 Cardiovascular disease				
5 Dermatological disease				
6 Diabetes				
7 Drug abuse				
8 HEENT disease				
9 Endocrine disease (other than diabetes)				
10 Gastrointestinal disease				
11 Hematological disease				
12 Hepatobiliary disease				
13 Immunological disease				
14 Musculoskeletal disease				
15 Neoplastic disease				
16 Neurological disease				
17 Psychiatric disease				
18 Urinogenital disease				
19 Other (specify)				

Patient Initials:

Protocol No.: ID99-205

Patient No.: _____

VITAL SIGNS/PHYSICAL EXAM
 SCREEN/BASELINE

Date of Exam: ___/___/___
 MM DD YY

VITAL SIGNS

Temperature (F°)	Heart Rate (beats/min)	Blood Pressure (mm/Hg)	Respiration Rate (breaths/min)	Perf. Status (Zubrod)	Weight (kg)	Height (cm)

PHYSICAL EXAM

Check one box on each line. If abnormal, specify

Body Region/System	Normal	Abnormal	Not Done	If Abnormal, Specify
1 Mental Status	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
2 Neurological	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
3 Skin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
4 HEENT	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
5 Chest & Back	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
6 Lungs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
7 Cardiovascular	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
8 Abdomen	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
9 Genitourinary	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
10 Musculoskeletal	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
11 Lymph Nodes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
12 Other (specify): _____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	

Appendix I (Section 5 of 8)

INTROGEN THERAPEUTICS, INC.

Patient Initials:

Protocol No.: ID99-205

Patient No.: _____

PROSTATE ASSESSMENT
 SCREEN/BASELINE

Date of Exam: / /
 MM DD YY

DIGITAL RECTAL EXAMINATION

Check if ND

Consistency of Prostate	Tumor Measurement (cm)	Location of Tumor (check all that apply)	Seminal Vesicle Involvement		Extracapsular Extension
			Right	Left	
<input type="checkbox"/> Hard <input type="checkbox"/> Soft	 <hr/> <input type="checkbox"/> Not Palpable	<input type="checkbox"/> Rt Apex <input type="checkbox"/> Lt Apex <input type="checkbox"/> Rt Lateral <input type="checkbox"/> Lt Lateral <input type="checkbox"/> Rt Base <input type="checkbox"/> Lt Base <input type="checkbox"/> Not palpable <input type="checkbox"/> Other (specify below)	<input type="checkbox"/> Palpable <input type="checkbox"/> Not Palpable <input type="checkbox"/> Unknown	<input type="checkbox"/> Palpable <input type="checkbox"/> Not Palpable <input type="checkbox"/> Unknown	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> NA

Appendix I (Section 6 of 8)
 INTROGEN THERAPEUTICS, INC.

Patient Initials:

Protocol No.: ID99-205

Patient No.: _____

PROSTATE ASSESSMENT
 SCREEN/BASELINE

Date of Exam: / /
 MM DD YY

TRUS EXAMINATION

Check if ND

Location of Tumor (Check all that apply)	Tumor Measurement (cm x cm x cm)	Seminal Vesicle Involvement	
		Right	Left
1. Rt Apex <input type="checkbox"/>	_____ x _____ x _____ <input type="checkbox"/> ND	<input type="checkbox"/> Yes	<input type="checkbox"/> Yes
2. Rt Mid-zone <input type="checkbox"/>		<input type="checkbox"/> No	<input type="checkbox"/> No
3. Rt Base <input type="checkbox"/>		<input type="checkbox"/> Unknown	<input type="checkbox"/> Unknown
4. Lt Apex <input type="checkbox"/>			
5. Lt Mid-zone <input type="checkbox"/>			
6. Lt Base <input type="checkbox"/>			
7. Other (specify below) <input type="checkbox"/> _____			

Extracapsulary	Tumor Response
<input type="checkbox"/> Yes	<input type="checkbox"/> Baseline
<input type="checkbox"/> No	<input type="checkbox"/> Complete Response
<input type="checkbox"/> NA	<input type="checkbox"/> Partial Response
	<input type="checkbox"/> Stable Disease
	<input type="checkbox"/> Progression
	<input type="checkbox"/> Not Evaluable

INTROGEN THERAPEUTICS, INC.

Patient Initials:

Protocol No.: ID99-205

Patient No.: _____

SCREENING DIAGNOSTICS

Date (MM/DD/YY)	Procedure	Result	Clinically Significant Abnormalities
____/____/____	<input type="checkbox"/> Chest X-Ray	<input type="checkbox"/> Normal <input type="checkbox"/> Abnormal, not clinically significant <input type="checkbox"/> Abnormal, clinically significant (if checked, describe in next column) <input type="checkbox"/> ND	_____ _____ _____ _____ _____
____/____/____	<input type="checkbox"/> Bone Scan	<input type="checkbox"/> Normal <input type="checkbox"/> Abnormal, not clinically significant <input type="checkbox"/> Abnormal, clinically significant (if checked, describe in next column) <input type="checkbox"/> ND	_____ _____ _____ _____ _____
____/____/____	<input type="checkbox"/> EKG	<input type="checkbox"/> Normal <input type="checkbox"/> Abnormal, not clinically significant <input type="checkbox"/> Abnormal, clinically significant (if checked, describe in next column) <input type="checkbox"/> ND	_____ _____ _____ _____ _____
____/____/____	<input type="checkbox"/> CT Pelvis	<input type="checkbox"/> Normal <input type="checkbox"/> Abnormal, not clinically significant <input type="checkbox"/> Abnormal, clinically significant (if checked, describe in next column) <input type="checkbox"/> ND	_____ _____ _____ _____ _____

INTROGEN THERAPEUTICS, INC.

Patient Initials:

Protocol No.: ID99-205

Patient No.: _____

VITAL SIGNS/PHYSICAL EXAM
 TREATMENT # _____ OF 3

Date of Exam: ___/___/___
 MM DD YY

VITAL SIGNS

Temperature (°F)	Heart Rate (beats/min)	Blood Pressure (mm/Hg)	Respiration Rate (breaths/min)	Perf. Status (Zubrod)	Weight (kg)	Height (cm)

PHYSICAL EXAM

Check one box on each line. If abnormal, specify

Body Region/System	Normal	Abnormal	Not Done	If Abnormal, Specify
1 Mental Status	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
2 Neurological	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
3 Skin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
4 HEENT	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
5 Chest & Back	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
6 Lungs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
7 Cardiovascular	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
8 Abdomen	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
9 Genitourinary	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
10 Musculoskeletal	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
11 Lymph Nodes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
12 Other (specify): _____	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	

10/24/97

Signature: _____

10/24/97

INTROGEN THERAPEUTICS, INC.

Page 2 of 2

Patient Initials:

Protocol No.: ID99-205

Patient No.: _____

CONCOMITANT MEDICATIONS

List all over-the-counter and prescription medications taken 14 days prior to study treatment, during study treatment, and 40 days after the last dose. None

Drug Name (Generic or Brand Name)	Dose	Route	Indication	Start Date (DD/MM/YY)	Stop Date ² (DD/MM/YY)
3				/ /	/ / <input type="checkbox"/> Continuing
14				/ /	/ / <input type="checkbox"/> Continuing
15				/ /	/ / <input type="checkbox"/> Continuing
16				/ /	/ / <input type="checkbox"/> Continuing
17				/ /	/ / <input type="checkbox"/> Continuing
18				/ /	/ / <input type="checkbox"/> Continuing
9				/ /	/ / <input type="checkbox"/> Continuing
0				/ /	/ / <input type="checkbox"/> Continuing
1				/ /	/ / <input type="checkbox"/> Continuing
2				/ /	/ / <input type="checkbox"/> Continuing
3				/ /	/ / <input type="checkbox"/> Continuing
4				/ /	/ / <input type="checkbox"/> Continuing

Specify routes as: IV = Intravenous
 TOP = Topical

PO = By Mouth
 IP = Intraperitoneal

SC = Subcutaneous
 INH = Inhalation

IM = Intramuscular

Appendix J(Section 1 of 2)

INTROGEN THERAPEUTICS,INC.

Patient Initials: _____

Patient No.: _____

Protocol ID99-205: Ad5-p53 plus I-125 seed implant vs I-125 seed implant alone

Treatment Date: _____ Anesthesia: _____ Pt. Weight : _____

Ad5-p53 Injection # _____ of (3) 1) Local _____ Zubrod: _____

Ad5-p53 dose/vol. _____ 2) General _____

Start Time (24hr clock) : _____ 3) Spinal _____

Stop Time (24hr clock) : _____

Total volume administered : _____ Vital Signs: Temp. PR RR BP Time
(sitting)

Number of punctures : _____ 2 hrs. before vector adm: = _____

Number of Injections : _____ 2hrs. after vector adm: = _____

Location of Puncture Site	Puncture Site:	Tissue Obtained
1. Right Apex	Yes ___ No ___	Yes ___ No ___
2. Right Mid-Zone	Yes ___ No ___	Yes ___ No ___
3. Right Base	Yes ___ No ___	Yes ___ No ___
4. Left Apex	Yes ___ No ___	Yes ___ No ___
5. Left Mid- Zone	Yes ___ No ___	Yes ___ No ___
6. Left Base	Yes ___ No ___	Yes ___ No ___
7. Other	Yes ___ No ___	Yes ___ No ___

COMMENTS: If treatment was interrupted, indicate reason in comments field

Signature: _____ Date: _____

Appendix J (Section 2 of 2)

Protocol ID99-205: Ad5-p53 plus I-125 seed implant vs I-125 seed implant alone

Study Medication Administration

Patient Initials _____ MDACC # _____ Date _____

Dose # _____ of (3) Lot # _____ Part# _____

Was Ad5-p53 administered? Yes _____ No _____

If No was checked indicate reason in the comments field.

Dose / Volume administered : _____

Total Volume administered : _____

Number of punctures: _____

Number of injections : _____

Start time of procedure (24 hr clock) _____

Stop time of procedure (24 hr clock) _____

Was procedure completed? Yes ___ No ___

COMMENTS: _____

Patient Initials:

Protocol No.: INT-

Patient No.: _____

VITAL SIGNS/PHYSICAL EXAM
 FOLLOW UP FORM

Date of Exam: ___/___/___
 MM DD

VITAL SIGNS

Temperature (°F)	Heart Rate (beats/min)	Blood Pressure (mm/Hg)	Respiration Rate (breaths/min)	Perf. Status (Zubrod)	Weight (kg)	Height (cm)

PHYSICAL EXAM

PSA _____ DATE _____

Check one box on each line. If abnormal, specify

Body Region/System	Normal	Abnormal	Not Done	If Abnormal, Specify
1 Mental Status	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
2 Neurological	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
3 Skin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
4 HEENT	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
5 Chest & Back	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
6 Lungs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
7 Cardiovascular	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
8 Abdomen	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
9 Genitourinary	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
10 Musculoskeletal	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
11 Lymph Nodes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	
12 Other (specify):	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	

Signature: _____



DEPARTMENT OF THE ARMY

US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

13 Feb 02

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statements for Accession Document Numbers listed at enclosure be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Request for Change in Distribution Statements

Accession Document Numbers

ADB261483

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