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Table of Contents

Modulation of Apoptosis-Associated and DNA Repair Genes to Enhance Radiation Therapy

Introduction

Of the approximately 220,900 men in the United States diagnosed with carcinoma of the prostate (CaP) in 2003, approximately eight-five percent presented with presumed localized disease for which external beam radiation therapy (EBRT) was one of the few treatment options. While it is relatively effective, the failure rate still remains unacceptably high with a 5-year biochemical failure rate of 10-40%. The curative potential of radiation therapy still remains to be maximized.

Genes that may be critical in controlling radiosensitivity are several that function in the G2 cell cycle phase arrest checkpoint after cellular radiation damage, and DNA damage repair mechanisms. *p53* is a tumor suppressor gene that has a crucial role in the molecular pathway following genotoxic insult. It encodes a DNA-binding protein that transactivates genes that either influence survival through cell cycle arrest and DNA damage repair, or signal apoptosis in extensively damaged cells. Using a model system in which p53 function was conditionally restored to *p53*-null PC3 prostate cancer cells by stable transfection with a human CaP-derived temperature-sensitive *p53* (tsp53) mutant allele, we have shown that functional p53 significantly increased clonogenic survival ($p < 0.01$) following exposure to daily doses of 2 Gy IR, and contributed to a more sustained G2 arrest and increased G1 arrest in response to the multi-fraction regimen. These studies implicate the presence of wild-type (wt) *p53* with increased survival of CaP cells following fractionated exposure to radiation, suggesting that wt p53 in prostate tumor cells, found in approximately 65% of primary prostate cancers, may reduce the effectiveness of radiation therapy.

p53 regulated genes which contribute to survival after ionizing radiation are those that are active at the G2 phase checkpoint, such as *14-3-3σ* which is required to maintain G2 arrest, and the ribonucleotide reductase gene *p53R2* which ensures a nuclear supply of deoxyribonucleotides for DNA damage repair. Recent studies have revealed that the loss of *p53*-regulated *14-3-3σ* increases radiation sensitivity resulting from the inability to maintain G2 arrest, and that loss of *p53R2* increases sensitivity through decreased DNA repair ability, indicating potential roles in enhancing the radiation efficiency.

BCL2, an anti-apoptosis oncogene also plays an important role in radioresistance by suppression of apoptosis. Utilizing genetically engineered LNCaP prostate cancer sublines that either over-expressed Bcl2 (LNCaP/S22-d) or had down-regulated Bcl2 (LNCaP/AS17-f), we have shown that the radiation dose response curves (2 – 8 Gy) for the sublines differed significantly from the parental LNCaP (LNCaP/S22d: $p < 0.001$ and LNCaP/AS17-f: $p = 0.008$). The relative survival of the sublines revealed increased survival in the Bcl2 over-expressing cells, and decreased survival in the Bcl2 down-regulated cells. These data suggest a second potentially important therapeutic approach for enhancing radiosensitivity in prostate tumors via anti-sense oligonucleotide or other drug therapies that down-regulate Bcl2, which is abnormally overexpressed in approximately 25% of CaPs.

Thus, we hypothesized that down-regulation of wt *p53*, *p53R2*, and *BCL2* and induction of *14-3-3σ* in prostate tumors would increase the response to EBRT. To test this hypothesis, we are using *in vitro* and *in vivo* models and the gene silencing techniques, such as antisense and small interfering RNA. Further, we hypothesized that down-regulation of both *p53R2* and *BCL2* would have a more favorable response than down-regulation of *p53*, *BCL2*, or both.

Body

This initial annual report is focused on the first two tasks, which are the following:

Task 1. **To demonstrate that silencing of wild-type p53 sensitizes prostate tumor xenografts to external beam radiation (EBRT) (Months 1- 12)**

a. To develop and optimize the use of anti-*p53* RNA:DNA hybrid oligonucleotides (siHybrid) in vivo (Months 1-4)
b. To develop and optimize the use of 2 Gy radiation on growth of LNCaP tumor xenografts (Months 1-3)
c. To determine response to EBRT on xenografts treated with anti-*p53* siHybrid (Months 4-12)
d. Write the annual report (Month 11)

Task 2. **To demonstrate that silencing of BCL2 in prostate tumor xenografts will increase response to EBRT (Months 6- 18)**

a. To develop and optimize the use of antisense *BCL2* and anti-*BCL2* siHybrid *in vivo* (Months 6-14)
b. To determine response to EBRT on xenografts treated with anti-*BCL2* siHybrid (Months 12-24)

Task 1

a. To develop and optimize the use of anti-p53 RNA:DNA hybrid oligonucleotides (siHybrid) in vivo (Months 1-4)

To start, we needed to determine the optimal gene silencing method. In addition to traditional antisense with modified DNA oligonucleotides, an RNA-based method of gene silencing has been described known as RNA interference. This technology employs double-stranded small interfering RNA (siRNA) sequences of 21-23 nucleotides in length that have demonstrated highly effective gene silencing abilities. Our experiences with siRNA targeted to several genes of interest have resulted in rapid and potent gene silencing. Our colleagues at the Lawrence Livermore National Laboratory (LLNL) have developed second generation gene silencing methods using DNA-RNA hybrid inhibitors (siHybrids), which have increased potency and duration-of-effect. In this project, we proposed to test the siHybrid approach to target *p53*, *14-3-3σ, p53R2* and *BCL2* in human CaP cell lines, as well as in xenograft models. Unfortunately, the initial experiments targeting *p53* showed little efficacy of the siHybrids. Thus, our colleagues at LLNL are further investigating the specific target sequences within the *p53* gene to develop more effective siHybrids. While they are redesigning the *p53* siHybrids, we turned our attention to silencing the *BCL2* gene, which is the goal of Task 2a listed above.

Recently, using *BCL2* siRNA (Stealth: Invitrogen), we have successfully down-regulated the mRNA level of *BCL2* in the human prostate cancer cell lines LNCaP and CWR22R. Listed here are the sequences of the *BCL2* siRNA, which includes a proprietary modification of the RNA backbone, and the sequence of the control siRNA, which contains approximately the same GC content (45-55%) as the targeted *BCL2* siRNA (GC Control: 48%).

BCL2 siRNA: 5'-GGAGAUAGUGAUGAAGUACAUCCAU-3' and
5'-AUGGAUGUACUUCAUCACUAUCUCC-3'.

Shown in **Figures 1** and **2**, are the RT-PCR results from LNCaP and CWR22R, respectively. mRNA was extracted from cells on Day 2 (48hrs from transfection) using the trizol approach.

While the GC control-treated cells showed some reduction of *BCL2* mRNA, in the *BCL2* siRNA-transfected cells, levels of *BCL2* mRNA are nearly undetectable in both cell lines.

Analyses of the Bcl2 protein levels by Western blotting in the siRNA-treated cells, compared to untreated controls and the cells treated with the GC Control, has begun, with cells harvested for protein extracts and quantitation.

Since phenotypic effects on CWR22R cells were not observed at day three when cells were harvested for Western blotting, one plate of transfected cells was imaged on Day 5. On Day 2 transfected cells were similar in morphology to CWR22R cells before transfection (**Figure 3**). At Day 5 transfected cells appear flattened and elongated demonstrating a phenotypic effect. Levels of the Bcl2 protein, the cell cycling profile, and markers of apoptosis will next be examined in these treated cells at that timepoint.

b. To develop and optimize the use of 2 Gy radiation on growth of LNCaP tumor xenografts (Months 1-3)

In order to facilitate the *in vivo* study we needed to first test the number of 2Gy fractions delivered daily. Since the goal was to move forward with the *BCL2* studies, we chose the *BCL2* overexpressing, *p53*-null, human prostate tumor cells PC3 as the xenograft model. The goal of these studies was to reduce tumor size via radiation treatment to about 50% of untreated controls, while maintaining tumor viability. This would allow us to observe the effects of down-regulation *BCL2* in addition to radiation treatment. The cells were grown as xenografts in the BALB/c homozygous *nu/nu* mice. Once tumors were palpable (approximately 21 days), Group1 served as the control and received no radiation. Group 2 received one fraction of 2 Gy. Group 3 received a 2 Gy fraction of radiation on two consecutive days (2 X 2 Gy). Group 4 received three fractions of 2 Gy (3 X 2 Gy), and Group 5 received four fractions of 2 Gy (4 X 2 Gy). Shown in **Figure 4** are the tumor growth curves of PC3 xenografts untreated versus those treated with a single physiological 2 Gy dose of EBRT. This was determined to be the optimal dose that reduced tumor growth by approximately 50%.

Since the optimal dosage of EBRT has been determined and down-regulation of *BCL2* has been achieved, we have begun the efforts to study the effects of combining siRNA with radiation treatment.

c. To determine response to EBRT on xenografts treated with anti-p53 siHybrid (Months 4-12)

This task is awaiting the next design of the *p53* siHybrids. However, based on the success of the *BCL2* siRNA, we plan to begin experiments using the *p53* siRNA in the immediate future.

d. Write the annual report (Month 11)

This is the initial annual report.

Task 2

a. To develop and optimize the use of antisense BCL2 and anti-BCL2 siHybrid *in vivo* (Months 6-14)

The *in vitro* experiments were described above, and the *in vivo* studies are being initiated.

b. To determine response to EBRT on xenografts treated with anti-BCL2 siHybrid (Months 12-24)

Currently cells are being grown for the inoculation of the mice for the *in vivo* testing of the *BCL2* siRNA described above, alone and in combination with the 2Gy radiation in the *p53*-null PC3 xenograft model. Since we know that the mRNA is fully downregulated at Day 2, and that Bcl2 functions to prevent radiation-induced apoptosis approximately 24 hrs after EBRT, the tumors will be directly injected with the *BCL2* siRNA on Day 0, with 2 Gy of radiation delivered on Day 2, which will set the stage for the anticipated effects on Day 3. Tumor size will be monitored daily as in the xenograft radiation experiment above.

Finally, the other Tasks remain the same for this ongoing project and are the following:

Task 3. **To assess the down-regulation of p53R2 on p53-induced DNA repair in prostate tumor xenografts**

a. To develop and optimize the use of anti-p53R2 siHybrid in vivo (Months 16-20)
b. To determine response to EBRT on xenografts following treatment with anti-p53R2 siHybrid (Months 20-30)
c. Write the annual report (Month 23)

Task 4. **To determine response of prostate tumor xenografts to EBRT following treatment with combination anti-*BCL_2* and anti- *p53R2* RNA:DNA hybrid oligonucleotides (Months 28-35)**

a. Determine effectiveness of combination treatment with anti-p53R2 and anti-BCL2 prior to EBRT on xenografts (Months 28-32)
b. Compare results of combination anti-p53R2 and anti-BCL2 siHybrid against anti-p53 siHybrid alone (Months 30-35)

Task 5. **Final analyses, report writing, and manuscript preparation**

a. Final analyses of all data from the experiments comparing sensitivity of the approaches (Months 33-36)
b. Write the final report (Month 36)
c. Prepare manuscripts for publication (Months 33-36, and earlier if warranted)

Key Research Accomplishments

1. Establishment of gene silencing approach for *BCL2* in multiple human prostate cell lines *in vitro.*
2. Establishment of the optimal dose of a single 2 Gy dose of EBRT for assessing combination treatments of the PC3 xenografts.

Reportable Outcomes

None to date.

Conclusions

From these studies, we conclude that the *BCL2* siRNA is successfully hitting its target and down-regulating *BCL2* mRNA. Having established the optimal EBRT dose for the *in vivo* studies now allows the examination of the combinations of siRNA with radiation. Further, we

believe these studies have begun to lay the framework for enhancing radiation treatment for prostate cancer patients, which we anticipate will be tested in clinical trials.

Figure 1. RT-PCR results for LNCaP.

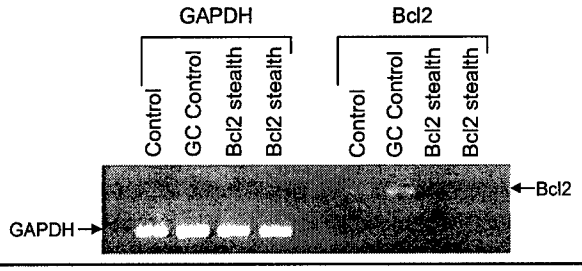

Figure 2. RT-PCR results for CWR22R.

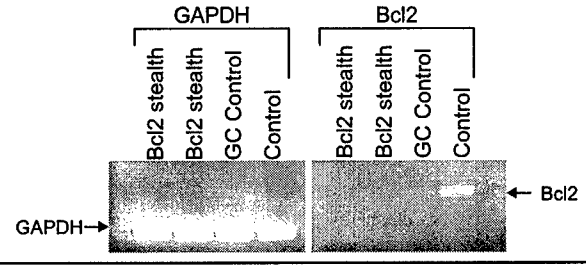

Figure 3. CWR22R following transfection with BCL2 siRNA.

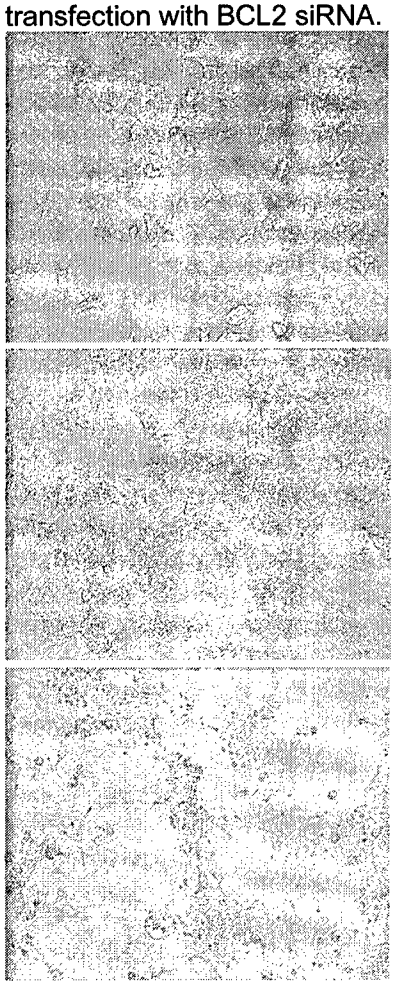

Figure 4. *in vivo* tumor growth following treatment with 2 Gy radiation in PC-3 xenograft model.