

BIOLOGY CONTRIBUTION

EFFICACY AND TOXICITY OF REPLICATION-COMPETENT ADENOVIRUS-MEDIATED DOUBLE SUICIDE GENE THERAPY IN COMBINATION WITH RADIATION THERAPY IN AN ORTHOTOPIC MOUSE PROSTATE CANCER MODEL

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Purpose: The purpose of this study was to evaluate the efficacy and toxicity of replication-competent adenovirus-mediated double suicide gene therapy in an adjuvant setting with external beam radiation therapy (EBRT) in an experimental prostate cancer model in preparation for a Phase I clinical study in humans.

Methods: For efficacy studies, i.m. DU145 and intraprostatic LNCaP C4-2 tumors were established in immune-deficient mice. Tumors were injected with the lytic, replication-competent Ad5-CD/TKrep adenovirus containing a cytosine deaminase (CD)/herpes simplex virus thymidine kinase (HSV-1 TK) fusion gene. Two days later, mice were administered 1 week of 5-fluorocytosine + ganciclovir (GCV) prodrug therapy and fractionated doses of EBRT (trimodal therapy). Tumor control rate of trimodal therapy was compared to that of EBRT alone. For toxicology studies, immune-competent male mice received a single intraprostatic injection (10^{10} vp) of the replication-competent Ad5-CD/TKrep adenovirus. Two days later, mice were administered 4 weeks of 5-fluorocytosine + GCV prodrug therapy and 56 Gy EBRT to the pelvic region. The toxicity of trimodal therapy was assessed by histopathologic analysis of major organs and clinical chemistries.

Results: In both the i.m. DU145 and intraprostatic LNCaP C4-2 tumor models, trimodal therapy significantly improved primary tumor control beyond that of EBRT alone. In the DU145 model, trimodal therapy resulted in a tumor growth delay (70 days) that was more than twice that (32 days) of EBRT alone. Whereas EBRT failed to eradicate DU145 tumors, trimodal therapy resulted in 25% tumor cure. In the LNCaP C4-2 tumor model, EBRT slowed the growth of intraprostatic tumors, but resulted in no tumor cures, and 57% of the mice developed retroperitoneal lymph node metastases at 3 months. By contrast, trimodal therapy resulted in 44% tumor cure and reduced significantly the percentage (13%) of lymph node metastases relative to EBRT alone. Overall, trimodal therapy was associated with little toxicity. A comparison of the major histopathologic findings among the treatment groups indicated that most of the locoregional (prostate, seminal vesicles, urinary bladder) pathology was attributable to the combined effects of the Ad5-CD/TKrep vector and EBRT and that the prodrugs contributed little to this effect. Importantly, trimodal therapy did not exacerbate inflammation of the rectum and intestines beyond that of EBRT alone.

Conclusion: Together, the results support the thesis that replication-competent adenovirus-mediated double suicide gene therapy may be a safe and effective adjuvant to EBRT and provide a sound scientific rationale for human trials. © 2002 Elsevier Science Inc.

Prostate cancer, Gene therapy, Adenovirus, Radiation therapy.

INTRODUCTION

Prostate cancer is the most common cancer in men in the United States. Although conventional therapies (surgery and radiation therapy) produce a high rate of cure for patients with early-stage disease, a significant fraction of

these cancers recur, and each therapy results in significant morbidity (1). The prognosis for androgen-independent prostate cancer is much worse, because there is no effective treatment, and a vast majority of these patients eventually succumb to the disease. There is a real need to develop new therapies that would reduce the morbidity associated with

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conventional therapies, decrease the incidence of local tumor recurrence, and improve the outlook for recurrent and androgen-independent cancer.

Although prolonged survival is common, disease control after external beam radiation therapy (EBRT) is only modest for locally advanced but nonmetastatic cancers (Stage T2–4), as documented by clinical examination, prostate biopsy, and serum prostate specific antigen (PSA) (2–5). For example, using rigorous PSA criteria, 40% of patients with Stage T3–4 tumors are disease free at 10 years (5). Clinical local failure, which significantly underestimates tumor local control, occurs in about 30%–50% of patients at 5 years and in up to 75% at 10 years (5). It has been modeled that more than one half of distant recurrences originate in association with a local recurrence (6), making local control an important end point. Given the prevalence of prostate cancer, the clinical benefits of improving the efficacy of EBRT are substantial in decreasing local recurrences, possibly decreasing distant recurrence, increasing survival, and providing a more effective and less morbid alternative to radical surgery.

Although tumor control in locally advanced prostate cancer is directly related to radiation dose (7), even a considerable dose of 70 Gy or higher seems to be inadequate to eradicate tumor clonogens in patients with bulky disease. Increasing the prescription dose beyond 70 Gy, however, is associated with an increased likelihood of long-term complications (8). Grade ≥ 2 rectal complications (bleeding and/or mucous discharge that requires medical treatment) in the range of 30% has been reported for central prostate doses of >74 Gy using three-dimensional conformal therapy. Therefore, increasing tumor cell killing by biochemical means is necessary to enhance whatever technical benefits can be achieved. Furthermore, biologic enhancement may be many times more effective than the gain that can be achieved by increasing the radiation dose.

With these goals in mind, we have been exploring the possibility of using gene therapy as a means to improve the effectiveness of EBRT. We have developed a novel, trimodal approach involving oncolytic viral, double suicide gene and EBRT (9, 10). Our approach uses a modified, replication-competent adenovirus (Ad5-CD/TKrep) to deliver a pair of therapeutic suicide genes to tumors. Our preclinical studies have demonstrated that the Ad5-CD/TKrep virus itself generates a potent antitumor effect by replicating in and preferentially destroying cancer cells. The therapeutic effect of the Ad5-CD/TKrep virus can be significantly enhanced by invoking two suicide gene systems (CD/5-FC [5-fluorocytosine] and HSV-1 TK/GCV [ganciclovir]), which render malignant cells sensitive to specific pharmacologic agents and sensitizes them to radiation (11–18).

The safety and efficacy of Arms 1 (Ad5-CD/TKrep viral therapy) and 2 (double suicide gene therapy) of our trimodal approach were recently evaluated in a Phase I trial (BB-IND 8436) of local recurrence of prostate cancer after definitive radiation therapy (19). This trial has been completed with excellent results. As a prerequisite for follow-up studies in

which Ad5-CD/TKrep viral and double suicide gene therapies will be used in an adjuvant setting with EBRT in men with locally advanced prostate cancer, we examined the efficacy and toxicity of our trimodal approach in two experimental prostate cancer models in the mouse. The results support the notion that replication-competent adenovirus-mediated double suicide gene therapy may be a safe and effective adjuvant to EBRT in the clinic.

METHODS AND MATERIALS

Adenoviruses and cell lines

The replication-competent Ad5-CD/TKrep (9, 10) and replication-defective Ad5-FGFR adenoviruses (9, 20) have been described previously. Viruses were titered on HEK 293 cells using standard procedures. DU145 cells were obtained from the American Type Culture Collection and grown in Dulbecco's Modified Essential Medium (DMEM) with 10% fetal bovine serum (FBS). LNCaP C4-2 cells were obtained from Dr. Leland Chung and were grown in DMEM with 10% FBS.

In vitro cytopathic effect assays

Viral cytopathic effect assays were performed as described previously (9). Briefly, cells (2×10^4 /well, 24-well plate) were either mock infected or infected with increasing amounts of Ad5-CD/TKrep virus in 0.2–0.4 mL DMEM containing 2% FBS. After 1 h, the virus was removed, and cells were maintained in growth medium. Duplicate plates were fixed and stained with crystal violet 5 and 9 days postinfection. For prodrug sensitivity assays, cells (1×10^6 , 60-mm dish) were either mock infected or infected with Ad5-FGFR adenovirus at an moi (multiplicity of infection) of 10. After 1 h, the cells were detached by trypsinization and immediately replated in triplicate at low density (1×10^4 and 1×10^3 cells/60-mm dish) in growth medium. Cells were maintained in growth medium containing varying concentrations of 5-FC or GCV until staining. The media was changed every 2 days. Colonies were stained with crystal violet and counted 10 days after replating.

In vivo efficacy studies

All animal studies were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital. Female athymic (nu/nu) CD-1 mice (Charles River Laboratories) 5 to 6 weeks old (20–22 g) were used for i.m. DU145 tumor studies. DU145 tumors were established by inoculating 2×10^6 cells prepared in 0.9% NaCl and 15% Matrigel in the right gastrocnemius muscle (i.m.). Upon reaching approximately 120 mm³, tumors were injected with 5×10^9 vp (10^8 pfu) Ad5-CD/TKrep or phosphate-buffered saline (PBS) (50 μ L) for 5 consecutive days (Days 0–4). Viral injections were distributed evenly throughout the tumor. Tumor dimensions were measured every 2–3 days. Volumes of i.m. leg tumors were determined using the following formula (21): $d'^3 - (0.6)^2 d' = \text{volume (cm}^3\text{)}$, where d' is the average diameter of the tumored leg (cm),

and the product $(0.6)^{2d}$ is the correction factor for normal leg volume. Mice were killed humanely when the tumor volume exceeded the predetermined end point of $5 \times (600 \text{ mm}^3)$ the initial tumor volume.

Male SCID mice (20–22 g) 5–6 weeks old were used for the intraprostatic LNCaP C4-2 tumor studies. Sterile surgical procedures were used throughout. Mice were anesthetized with Nembutal (60 mg/kg), and an incision was made in the lower abdomen. The seminal vesicles and urinary bladder were retracted gently, exposing the dorsolateral prostate. LNCaP C4-2 cells (1×10^6 , 15% Matrigel, 10 μL) were injected into the right dorsolateral lobe of the prostate with the aid of a dissecting microscope. The incision was closed with surgical clamps. Blood (100 μL) was obtained weekly by supraorbital bleeding to monitor serum PSA levels. Serum PSA was measured using the Tandem PSA kit (Hybritech). When PSA levels reached 10 ng/mL, mice were injected with adenovirus or saline. Mice were anesthetized with Nembutal, and the previous incision was reopened. Ad5-CD/TKrep virus (10^9 vp, 2×10^7 pfu; 10 μL) was injected into the intraprostatic tumor with the aid of a dissecting microscope (Day 0). The incision was closed with surgical clamps. Blood was obtained weekly thereafter for measurement of serum PSA levels. When PSA levels reached ≥ 1000 ng/mL ($\sim 2 \text{ cm}^3$ tumor), mice were killed humanely, and a necropsy was performed. The entire abdominal cavity was inspected carefully for the presence of metastases. The intraprostatic LNCaP C4-2 tumor and retroperitoneal lymph nodes were removed and weighed. Retroperitoneal lymph nodes were dissected and examined carefully under a dissecting microscope for metastases.

Administration of prodrugs

At the conclusion of the viral inoculations, mice in the prodrug treatment groups received daily i.p. injections of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day), as indicated in the figures.

External beam radiation therapy

Intramuscular, rather than s.c., DU145 tumors were used to minimize stretching of the skin, which can lead to unacceptable ulceration after irradiation. Radiation was delivered to the tumored leg 2 h after prodrugs were given for that day. Mice were anesthetized with Nembutal (60 mg/kg), and a single dose of ^{60}Co γ -radiation (8 Gy) was delivered to the tumor-bearing leg on Day 7, as described previously (10). In the intraprostatic LNCaP C4-2 tumor model, mice were anesthetized with Nembutal and placed on their stomachs on a Styrofoam platform that conformed to their body. Mice were administered 3.5 Gy of ^{137}Cs γ -irradiation to their left (1.75 Gy) and right (1.75 Gy) pelvic region on Days 4 and 7 (7 Gy total). A lead collimator was used to deliver a 1-cm beam, protecting most of the body. Rotating mice 180° halfway through the exposure minimized the effect of beam attenuation on absorbed dose. To determine the radiation absorbed dose to the pelvic region, thermoluminescent dosimeters (TLDs) were placed

in dead mice, and the absorbed dose was measured as a function of distance along the central axis of the collimator and across the collimator width along the central plane of mice. TLD cubes (LiF TLD-100, $1 \times 1 \times 1 \text{ mm}^3$, [Bicron, Solon, OH]) were placed end to end within a low-density polyethylene tube, which was placed within dead mice and irradiated. A Harshaw Model 2000D Automated TLD Reader was used to read the TLDs after irradiation. Each TLD was followed through the annealing cycle, radiation, and reading to account for individual sensitivities. Within the target volume (pelvic region), radiation-absorbed dose was measured to be within 5% of the prescribed dose.

Toxicology study

Ninety male C57BL/6 mice (20–22 g) were injected intraprostatically with either saline (Groups 1, 2, and 7) or Ad5-CD/TKrep virus (10^{10} vp in 10 μL , Groups 3–6), as described above. Mice that were scheduled to receive prodrugs (Groups 5–7) were given daily injections of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) i.p. for 4 weeks (Days 3–30). Other groups received saline in place of prodrugs. Mice that were scheduled to receive radiation (Groups 2, 4, and 6) received 4 Gy of ^{137}Cs γ -irradiation to their left (2 Gy) and right (2 Gy) pelvic region twice per week for 7 weeks for a total dose of 56 Gy. Mice were examined for a number of toxicologic parameters, such as body weight taken before injection and at each necropsy time point. General observations were noted daily, and gross observations were made at time of necropsy. On Days 4, 31, and 53, either a partial or full set of tissues was taken for histopathologic observations. Clinical chemistries were also examined to determine blood cell levels and liver-specific enzymes at Days 31 and 53. The study (HFHS-002) was conducted at the Henry Ford Health Systems. Clinical pathology was performed by Laboratory Corporation of America (Burlington, NC). Histopathology was performed by Experimental Pathology Laboratories Inc. (Herndon, VA). Statistical analyses were conducted by the Department of Biostatistics and Epidemiology at the Henry Ford Health Systems. Good laboratory practices were used throughout.

RESULTS

Sensitivity of human prostatic adenocarcinoma cells to replication-competent Ad5-CD/TKrep adenovirus and suicide gene systems in vitro

The sensitivity of human DU145 and LNCaP C4-2 prostatic adenocarcinoma cells to the cytopathic effects of the Ad5-CD/TKrep virus were examined *in vitro* at varying moi. Both cell lines were efficiently killed by the Ad5-CD/TKrep virus with LNCaP C4-2 cells being approximately 10-fold more sensitive than DU145 cells (Fig. 1). The greater sensitivity of LNCaP C4-2 cells was in part because of greater infection efficiency *in vitro* (not shown).

To assess the sensitivity of these prostatic cell lines to the cytotoxic effects of the CD/5-FC and HSV-1 TK/GCV suicide gene systems in the absence of the Ad5-CD/TKrep

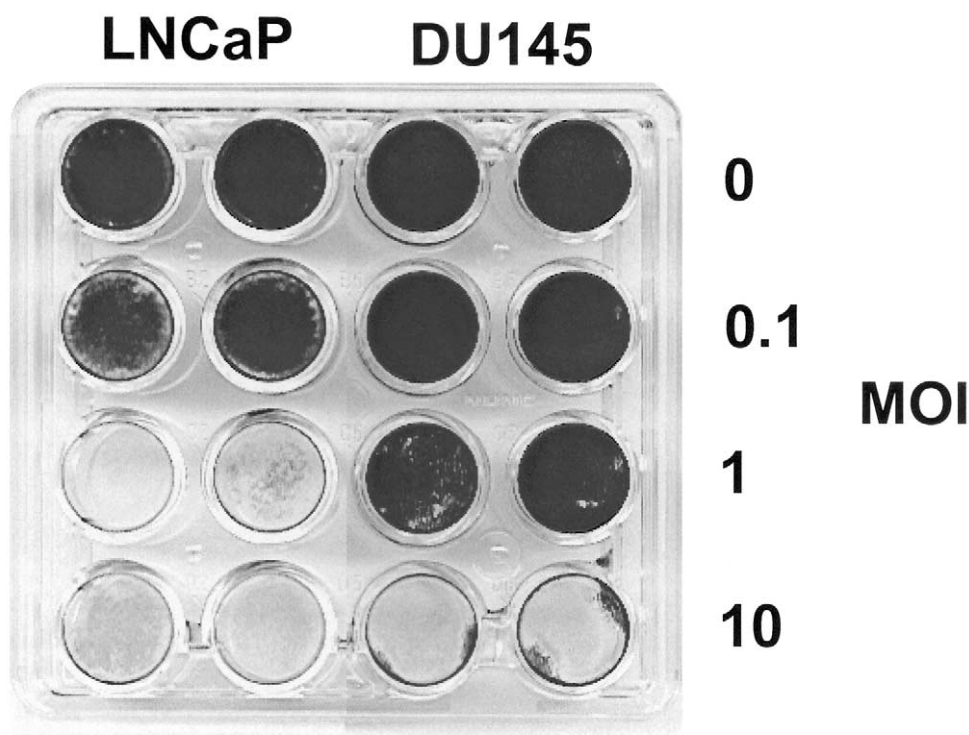


Fig. 1. Cytopathic effect of Ad5-CD/TKrep virus toward human prostate adenocarcinoma cells. DU145 and LNCaP C4-2 cells (2×10^4 /well, 24-well plate) were either mock infected or infected with increasing amounts of Ad5-CD/TKrep virus as indicated. Cells were fixed and stained with crystal violet 5 days postinfection. For each cell line, duplicate wells are shown. The assay was repeated over a dozen times with similar results (MOI, multiplicity of infection).

viral cytopathic effect, cells were infected with a replication-defective version of Ad5-CD/TKrep, Ad5-FGNR (20), and exposed to varying concentrations of 5-FC and GCV for 24 h. Under the condition used (moi = 100), approximately

50% and 90% of DU145 and LNCaP C4-2 cells were infected, respectively (not shown). Both cell lines displayed a concentration-dependent sensitivity to the suicide gene systems (Fig. 2). LNCaP C4-2 cells were much more sen-

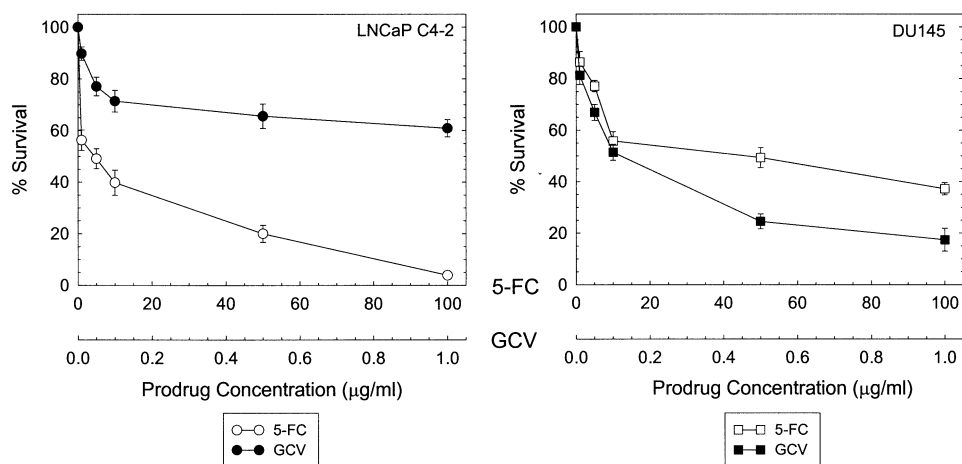


Fig. 2. Sensitivity of DU145 and LNCaP C4-3 cells to CD/5-FC and HSV-1 TK/GCV suicide gene systems. Cells (1×10^6 , 60-mm dish) were either mock infected or infected with the replication-defective Ad5-FGNR adenovirus (9, 20) at an moi of 10. After 1 h, cells were detached by trypsinization and immediately replated in triplicate at low density (1×10^4 and 1×10^3 cells/60-mm dish) in growth medium without, or with, varying concentrations of 5-FC or GCV. Colonies were stained with crystal violet and counted 10 days later. The data points represent the mean \pm standard deviation. With the low moi used, there was <10% cytotoxicity due to the viral vector itself. The studies were repeated three times with similar results.

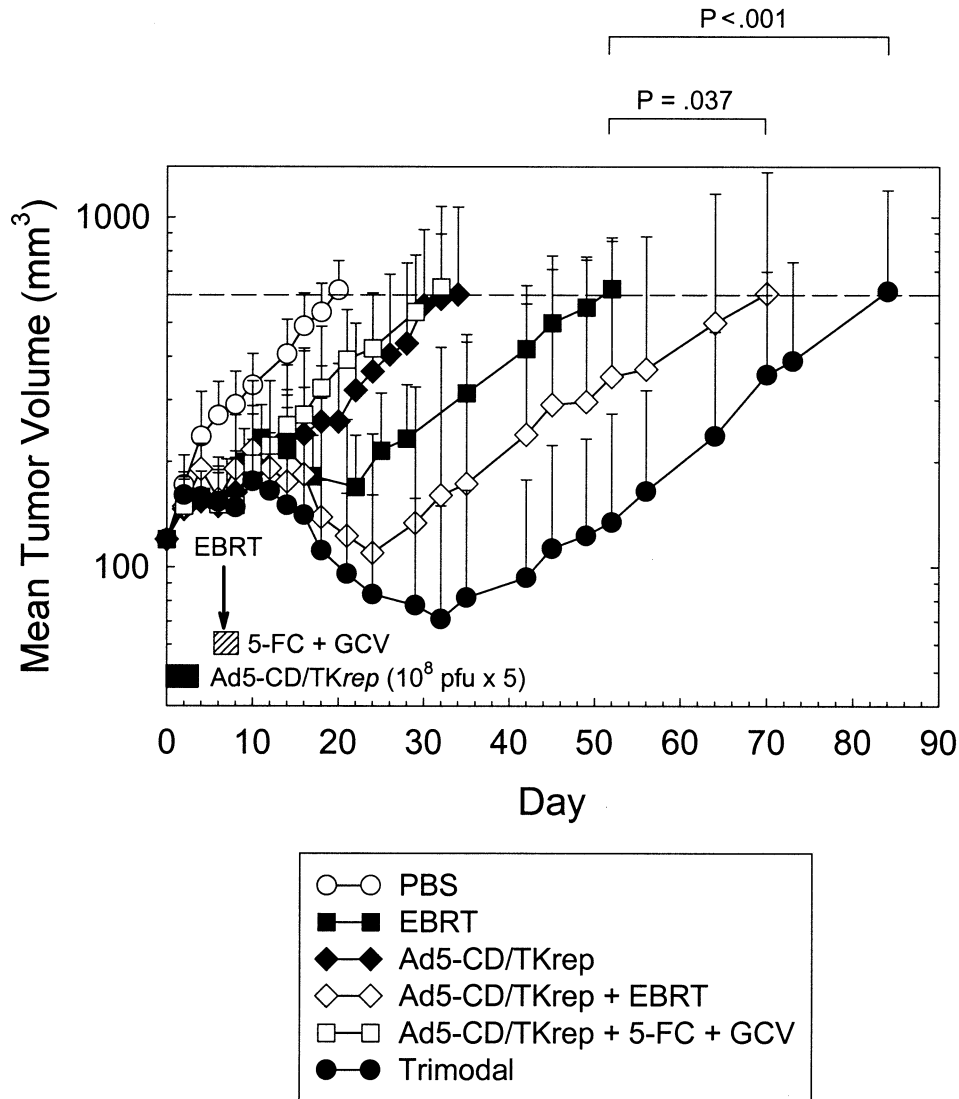


Fig. 3. Response of i.m. DU145 tumors to trimodal therapy. Intramuscular DU145 tumors (120 mm^3) were injected with saline or $5 \times 10^9 \text{ vp}$ (10^8 pfu) Ad5-CD/TKrep ($50 \mu\text{L}$) for 5 consecutive days (Days 0–4, solid black bar). Beginning on Day 6, mice in prodrug-treated groups received 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) prodrug therapy for 4 days (Days 6–9, hatched bar). Mice in radiation-treated groups received a single dose of 8 Gy to their tumored leg on Day 7. The data points represent the mean \pm standard deviation. The dashed line represents the predetermined end point of five times (600 mm^3) the initial tumor volume. The studies were repeated twice with similar results.

sitive to the CD/5-FC than the HSV-1 TK/GCV enzyme/prodrug system, which may be the result of a better bystander effect of the CD/5-FC system with these cells.

In vivo efficacy of Ad5-CD/TKrep-mediated double suicide gene therapy in combination with EBRT

The ability of Ad5-CD/TKrep viral therapy either alone or in combination with double suicide gene therapy to enhance the effectiveness of EBRT was evaluated in two prostate tumor models: i.m. DU145 and intraprostatic LN-CaP C4-2. In the DU145 model, Ad5-CD/TKrep viral therapy alone slowed tumor growth significantly ($p = 0.048$, analysis of variance [ANOVA]) relative to controls, resulting in a tumor growth delay of 14 days (Fig. 3, Table 1). Despite the sensitivity of DU145 cells to both suicide gene

systems *in vitro* (Fig. 2), the addition of double prodrug therapy (Ad5-CD/TKrep + 5-FC + GCV) did not improve tumor control beyond that of the Ad5-CD/TKrep virus alone *in vivo*. Combining Ad5-CD/TKrep viral therapy with

Table 1. Results with intramuscular DU145 tumor model

Group	Tumor growth delay (days)	% cure
PBS	NA	0 (0/10)
EBRT	32	0 (0/8)
Ad5-CD/TKrep	14	0 (0/10)
Ad5-CD/TKrep + EBRT	50	0 (0/8)
Ad5-CD/TKrep + 5-FC + GCV	12	0 (0/8)
Trimodal therapy	70	25 (2/8)

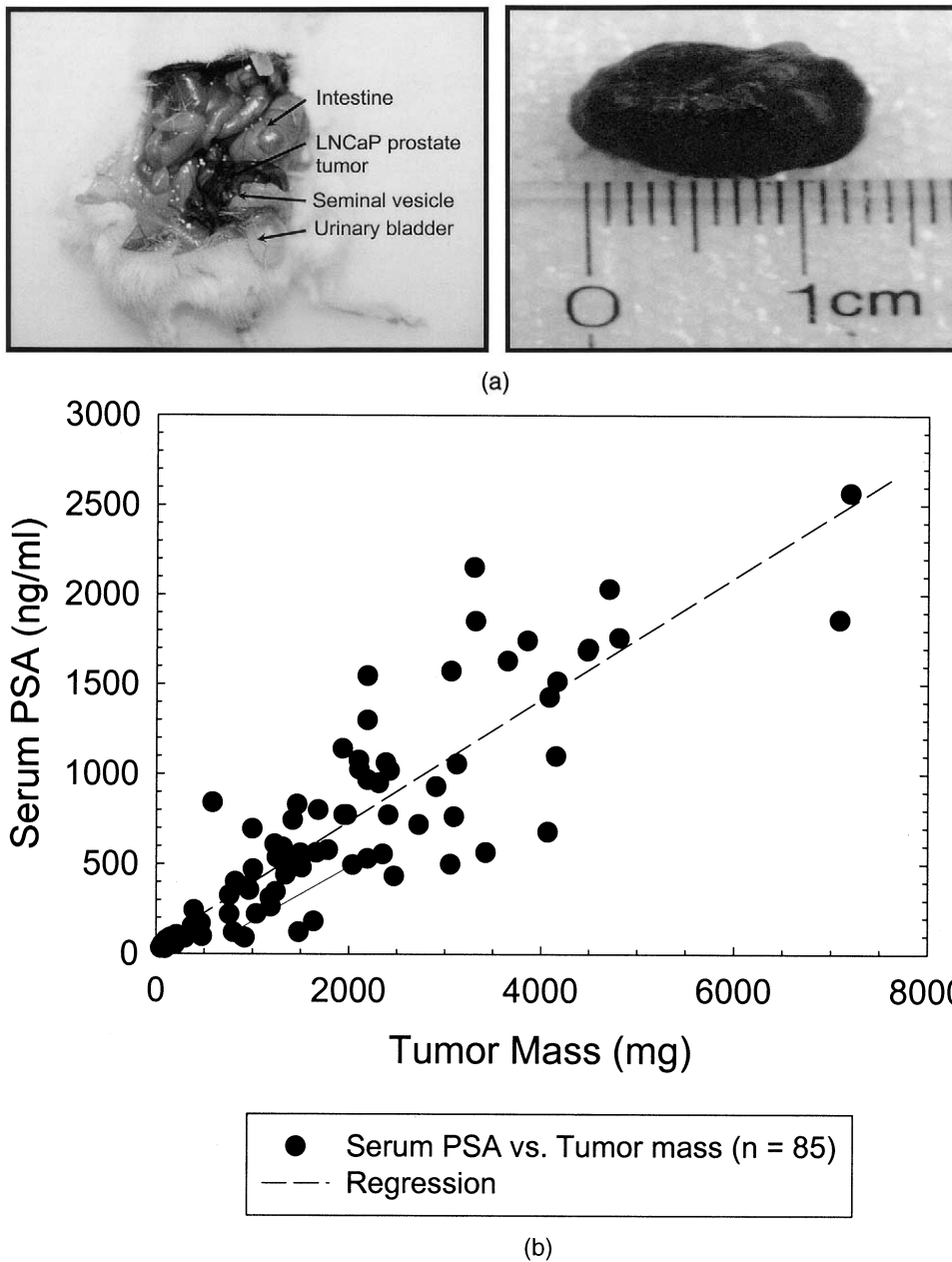
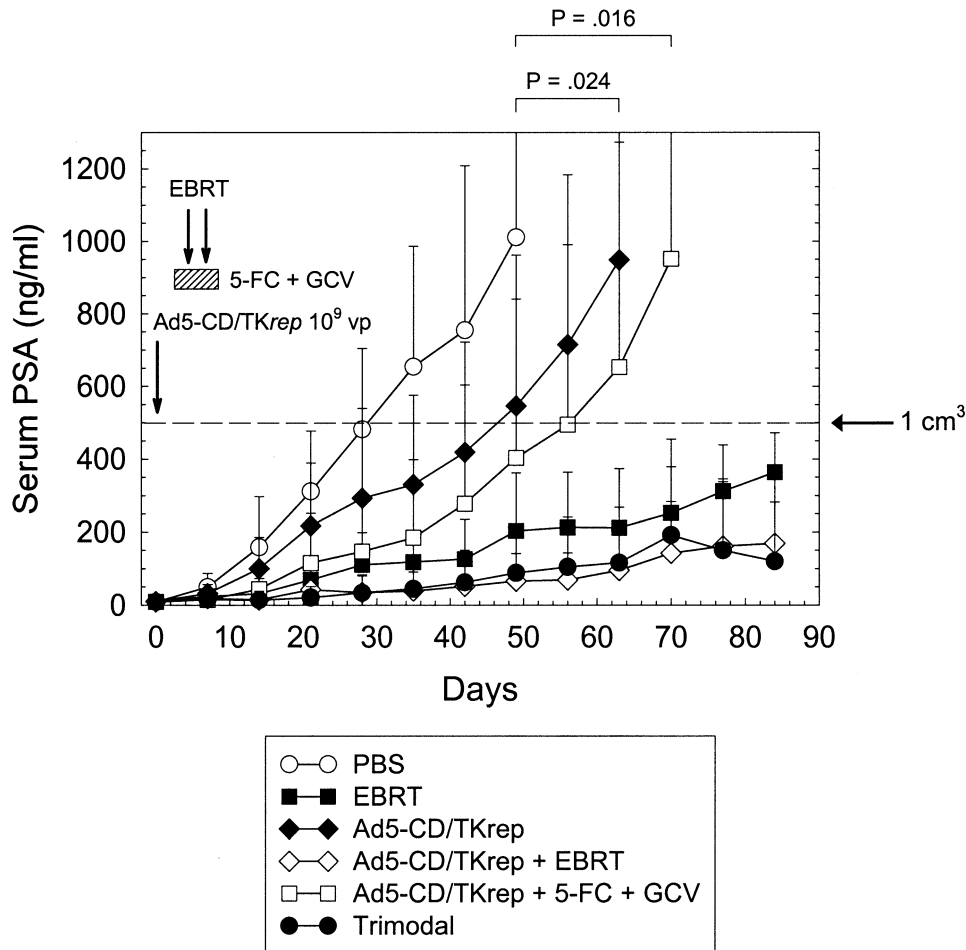


Fig. 4. (a) Picture of intraprostatic LNCaP C4-2 tumor before and after resection. (b) Serum PSA levels correlate well with tumor mass in the intraprostatic LNCaP C4-2 tumor model. Intraprostatic tumors were removed at various times after inoculation, and tumor mass and serum PSA were measured. The correlation coefficient is 0.87. (c) Response of intraprostatic LNCaP C4-2 tumors to trimodal therapy. When serum PSA levels reached approximately 10 ng/mL (Day 0), mice received a single intratumoral injection of saline or Ad5-CD/TK rep virus (10^9 vp, 2×10^7 pfu). Two days later, mice in prodrug-treated groups received 5-FU (500 mg/kg/day) and GCV (30 mg/kg/day) for 7 days (Days 3–9, hatched bar). Mice in radiation-treated groups received 3.5 Gy to their pelvic region on Days 4 and 7 (7 Gy total). The data points represent the mean \pm standard deviation. The dashed line represents the predetermined end point of 500 ng/mL PSA (1 cm^3 tumor). The studies were repeated twice with similar results.

EBRT (Ad5-CD/TK rep + EBRT) improved tumor control significantly relative to EBRT alone ($p = 0.037$, ANOVA), although neither treatment resulted in any cures (Fig. 3, Table 1). Interestingly, despite the fact that double suicide gene therapy did not enhance the antitumor effect of the Ad5-CD/TK rep virus in the absence of EBRT, it did improve tumor control beyond that of the Ad5-CD/TK rep virus when used in an adjuvant setting with EBRT (trimodal

therapy vs. Ad5-CD/TK rep + EBRT, $p = 0.022$; trimodal therapy vs. EBRT, $p < 0.001$, ANOVA). Moreover, whereas no mono or dual therapies resulted in any tumor cure, trimodal therapy resulted in 25% tumor cure.

To more closely mimic the clinical situation, the ability of Ad5-CD/TK rep viral and double suicide gene therapies to enhance the efficacy of EBRT was examined in the intraprostatic LNCaP C4-2 tumor model (22). Human LNCaP



(c)

Figure 4. (Cont'd)

C4-2 tumors grow well in the prostate of immune-deficient male mice, reaching 1 cm³ in approximately 6 weeks (Fig. 4a). LNCaP C4-2 tumors produce PSA, and serum PSA levels correlate well ($r = 0.87$) with primary tumor mass in this experimental model (Fig. 4b). Thus, as in the clinic, tumor response can be evaluated by following serum PSA levels. Moreover, because intraprostatic LNCaP C4-2 tumors metastasize to retroperitoneal lymph nodes (with latent period of 2 to 3 months), the effect of the various treatments on metastatic tumor control can also be evaluated.

Mice were administered a single intraprostatic injection of the Ad5-CD/TKrep virus (10⁹ vp) when serum PSA levels averaged 10 ng/mL. Higher viral doses could not be administered intraprostatically to immune-deficient mice, because some of the injected virus leaks into the abdominal cavity, resulting in infection of the liver serosa. This leads to severe hepatotoxicity and death in mice receiving 5-FC and GCV prodrug therapy (dose causing 50% lethality [LD₅₀] ~ 10¹⁰ vp). Ad5-CD/TKrep viral therapy alone and the combination of Ad5-CD/TKrep viral + 5-FC + GCV prodrug therapies resulted in significant tumor growth delays of 18 and 28 days and

8% and 14% tumor cure, respectively (Fig. 4c, Table 2). Although EBRT was effective at slowing the growth of intraprostatic LNCaP C4-2 tumors, it resulted in no tumor cure, and 57% of mice developed retroperitoneal lymph node metastases by 3 months (Fig. 4c, Table 2). The efficacy of EBRT was enhanced significantly by the addition of Ad5-CD/TKrep viral therapy (Ad5-CD/TKrep + EBRT) and the Ad5-CD/TKrep viral and double suicide gene therapy combination (trimodal therapy). At Week 12, the mean PSA level of the Ad5-CD/TKrep + EBRT and trimodal therapy group were both significantly ($p < 0.05$, ANOVA) lower than that of the EBRT group. Importantly, whereas EBRT alone resulted in no tumor cure, Ad5-CD/TKrep + EBRT and trimodal therapy resulted in 29% ($p = 0.048$, Fisher's exact test) and 44% ($p = 0.007$, Fisher's exact test) tumor cure, respectively. Although the addition of double prodrug therapy (i.e., trimodal therapy) did not improve primary tumor control beyond that of the Ad5-CD/TKrep + EBRT combination in this model, it did result in the greatest tumor cure and reduced significantly the frequency of lymph node metastases when used in an adjuvant setting with EBRT (trimodal therapy vs. EBRT, $p = 0.019$, Fisher's exact test).

Table 2. Results with intraprostatic LNCaP C4-2 tumor model

Group	Tumor growth delay (days)	Serum PSA @ Week 12	% tumor cure*	% RLN mets @ necropsy†
PBS	NA	>>1000	0 (0/14)	7 (1/14)
EBRT	ND	367 ± 118	0 (0/14)	57 (8/14)
Ad5-CD/TK rep	18	>1000	8 (1/13)	8 (1/13)
Ad5-CD/TK rep + EBRT	ND	123 ± 88	29 (5/17)	35 (6/17)
Ad5-CD/TK rep + 5-FC + GCV	28	>1000	14 (2/14)	36 (5/14)
Trimodal therapy	ND	103 ± 69	44 (7/16)	13 (2/16)

Abbreviations: NA = not applicable; ND = cannot be determined from the data because the group mean never reached the predetermined end point of 500 ng/mL (1 cm³ tumor) within the 90-day time frame of the study.

*Tumor cure is defined as having a serum PSA of <1 ng/mL at Week 12.

†The percentage of animals with retroperitoneal lymph node (RLN) metastases in the PBS and Ad5-CD/TK rep -treated groups is low, because these animals had to be killed, because their tumor burden before metastases had time to develop (latent period of 2 to 3 months).

Toxicity associated with trimodal therapy

The effectiveness of all cancer therapies must be weighed carefully against their risks (i.e., toxicities). To assess the toxicity of trimodal therapy in a preclinical model, immune-competent C57BL/6 male mice were administered a single intraprostatic injection of the Ad5-CD/TK rep virus (10¹⁰ vp) followed by 4 weeks of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) prodrug therapy and 56 Gy of EBRT (14 × 4 Gy) to the pelvic region (Fig. 5). Every possible mono and dual therapy combination was also evaluated for comparison. On a weight basis, the viral dose level (5 × 10¹¹ vp/kg) is 40 times the highest dose level (10¹² vp) used in a recent Phase I study (BB-IND 8436) in which the toxicity of Ad5-CD/TK rep viral and double suicide gene therapies was

evaluated in men with local recurrence of prostate cancer (19), and it is 40 times that to be used in the follow-up trial with EBRT (BB-IND 9852). The prodrug dose levels are three times, and the duration four times, those administered in this Phase I study. The radiation regimen (14 × 4 Gy, 2 doses per week for 7 weeks) was designed to produce the same biologic effect as standard EBRT for localized prostate cancer (35 × 2 Gy, 5 doses per week for 7 weeks) (23). Daily radiation doses cannot be administered to mice, because they eventually succumb to repeated anesthesia.

The mice were killed on Day 4 (early time point), Day 31 (end of prodrug therapy course), and Day 53 (1 week after last dose of EBRT), and a histopathologic evaluation of the major organs (prostate, seminal vesicles, urinary bladder, testes,

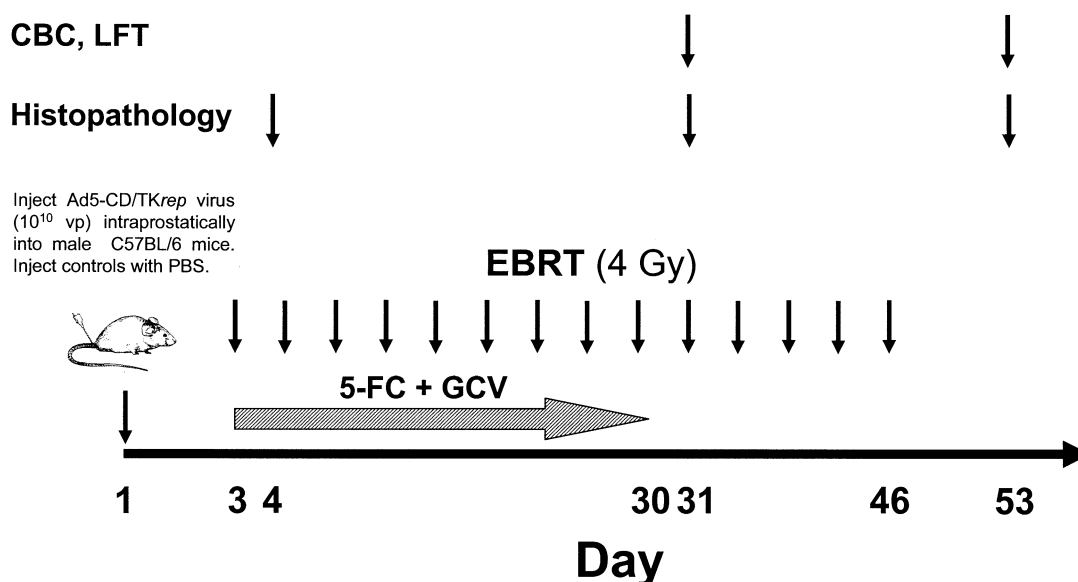


Fig. 5. Design of trimodal toxicology study. Male C57BL/6 mice were injected intraprostatically with either saline or Ad5-CD/TK rep virus (10¹⁰ vp) on Day 1. Mice that were scheduled to receive prodrugs were given daily injections of 5-FC (500 mg/kg/day) and GCV (30 mg/kg/day) for 4 weeks (Days 3–30). Mice that were scheduled to receive radiation received 4 Gy of γ -irradiation to their pelvic areas twice per week for 7 weeks for a total dose of 56 Gy. Mice were examined for a number of toxicologic parameters, including body weight taken before injection and at each necropsy time point. General observations were noted daily, and gross observations were made at time of necropsy. At Days 4, 31, and 53, either a partial or full set of tissues was taken for histopathologic observations. Clinical chemistries were examined to determine blood cell levels and liver-specific enzymes at Days 31 and 53.

Table 3. Major histopathologic findings on Day 53

Group	Treatment	Death	Major pathologic findings*	Score
1	Vehicle (PBS)	0	No abnormalities	0
2	EBRT	0	PR - minimal chronic inflammation (1/6)	0.17
			SV - minimal chronic inflammation (1/6)	0.17
			UB - no abnormalities (0/6)	0
			SI - no abnormalities (0/6)	0
			CO - no abnormalities (0/6)	0
			LV - no abnormalities (0/6)	0
			TS - severe degeneration (6/6)	5.0
3	Ad5-CD/TKrep	0	PR - no abnormalities (0/6)	0
			SV - no abnormalities (0/6)	0
			UB - no abnormalities (0/6)	0
			SI - no abnormalities (0/6)	0
			CO - no abnormalities (0/6)	0
			LV - minimal chronic inflammation of serosa (1/6)	0.17
			TS - minimal/mild degeneration (3/6)	0.67
4	Ad5-CD/TKrep + EBRT	0	PR - minimal/mild chronic inflammation (6/6)	1.3
			SV - minimal chronic inflammation of serosa (5/6)	0.83
			UB - minimal chronic inflammation of serosa (4/6)	0.67
			SI - no abnormalities (0/6)	0
			CO - no abnormalities (0/6)	0
			LV - no abnormalities (0/6)	0
			TS - severe degeneration (6/6)	5.0
5	Ad5-CD/TKrep + 5-FC + GCV	0	PR - minimal chronic inflammation (1/6)	0.17
			SV - no abnormalities (0/6)	0
			UB - no abnormalities (0/6)	0
			SI - no abnormalities (0/6)	0
			CO - no abnormalities (0.6)	0
			LV - minimal chronic inflammation of serosa (6/6)	1.0
			TS - severe degeneration (5/6)	4.2
6	Trimodal therapy	0	PR - minimal/mild chronic inflammation (5/6)	1.4
			SV - minimal/mild chronic inflammation of serosa (5/6)	1.2
			UB - minimal chronic inflammation of serosa (2/6)	0.4
			SI - no abnormalities (0/6)	0
			CO - no abnormalities (0/6)	0
			LV - minimal/mild inflammation of serosa (5/6)	1.6
			TS - severe degeneration (5/6)	5.0
7	5-FC + GCV	0	TS - severe degeneration (6/6)	5.0

Abbreviations: PR = prostate; SV = seminal vesicles; UB = urinary bladder; SI = small intestine; CO = colon/rectum; LV = liver; TS = testes.

*Numbers in parentheses indicate the number of animals showing some pathology/total number of animals in that group. Histopathology was scored on a 1 to 5 scale; 1 = minimal; 2 = light/mild; 3 = moderate; 4 = moderately severe; 5 = high/severe. Average histopathologic scores are in column 5.

small and large intestines, including rectum, and kidney and liver) was performed (Fig. 5). Complete blood counts and liver function tests were performed at the Day 31 and 53 time points.

The major histopathologic findings at Day 53 are summarized in Table 3. The most notable change was the presence of minimal to mild chronic inflammation in groups that received the Ad5-CD/TKrep vector. The inflammation was often present along the serosal membranes of several abdominal organs (seminal vesicle, urinary bladder, liver) or within the organ parenchyma, as was the case with the prostate gland (Fig. 6). Chronic inflammation was noted at the Day 4, 31, and 53 time points; however, less inflammation was present at Days 31 and 53 than at Day 4. Chronic

inflammation was characterized by the presence of mononuclear cells and very subtle fibrosis. Occasionally, polymorphonuclear inflammatory cells indicative of an ongoing active response were present. The pattern of inflammation was considered to be a low-grade, and somewhat regional, form of peritonitis. In groups that received the vector and prodrugs (Groups 5 and 6), inflammation of the liver serosa was associated with gross observations of lobe deformities (rounded and occasionally fused). These lesions were observed on Days 31 and 53 only. Importantly, the liver parenchyma in all groups appeared normal.

Small and large intestine, including rectum (Fig. 6), appeared normal in all groups at all time points. Severe testicular degeneration and atrophy were observed at the Day 31 and 53

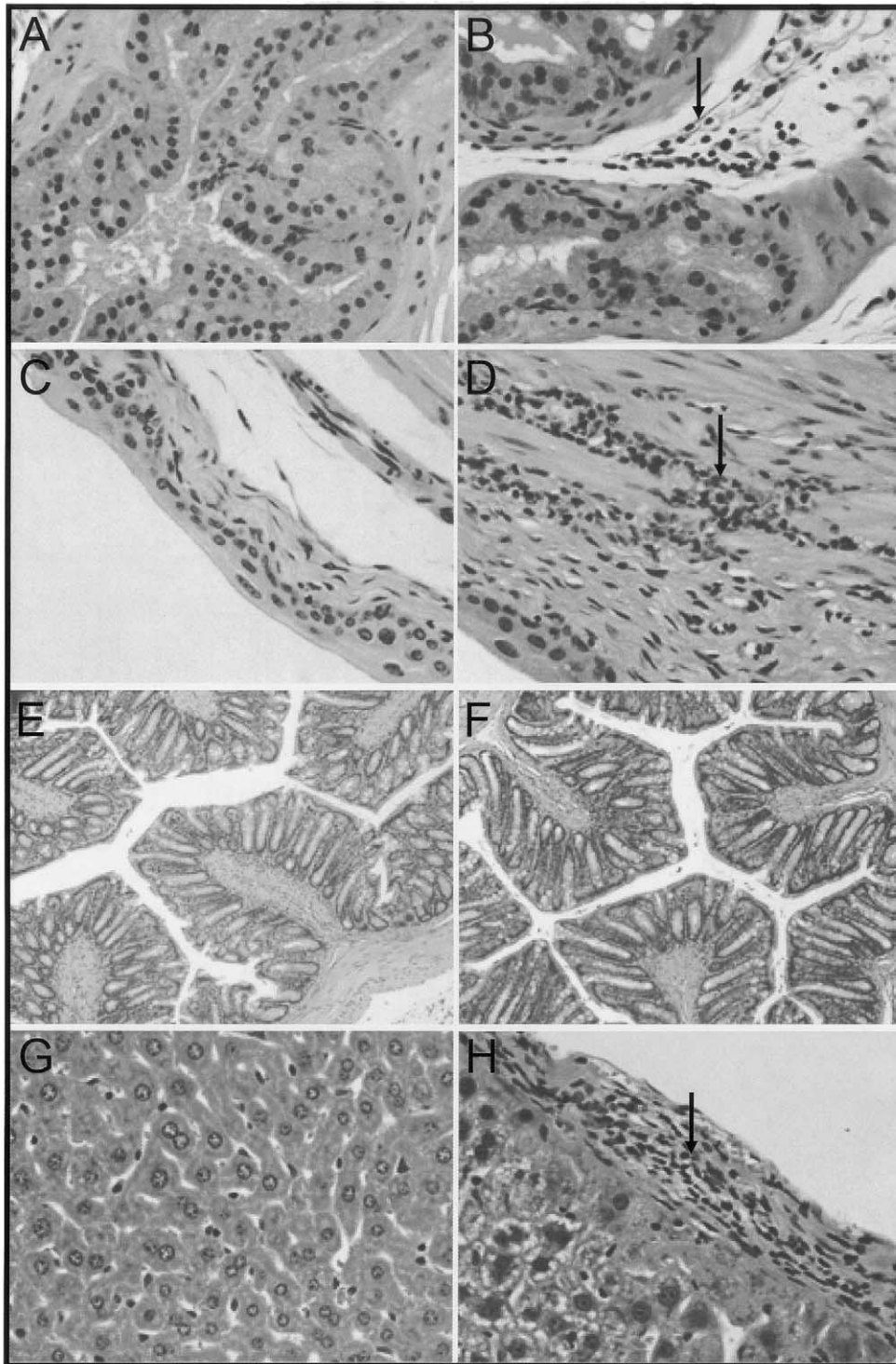


Fig. 6. Comparison of histopathology of major organs in trimodal therapy vs. control group on Day 53. Control group: A, C, E, G; trimodal therapy group: B, D, F, H. All tissue sections shown were stained with hematoxylin and eosin and photographed at 125 \times , except for rectum, which was photographed at 25 \times . Prostate (A, B), urinary bladder (C, D), rectum (E, F), liver (G, H). Areas of inflammation are indicated by the arrows.

time points in all groups that received prodrugs and/or EBRT (Groups 2, 4, 5, 6, and 7). This effect of the 5-FC and GCV prodrugs was observed previously and is unrelated to administration of the adenoviral vector (24).

A comparison of the histopathologic findings among the

treatment groups indicated that most of the locoregional (prostate, seminal vesicles, urinary bladder) inflammation was attributable to the combined effects of the Ad5-CD/TKrep vector and EBRT and that the prodrugs contributed little to this effect (Table 3, compare Group 6 to Group 4).

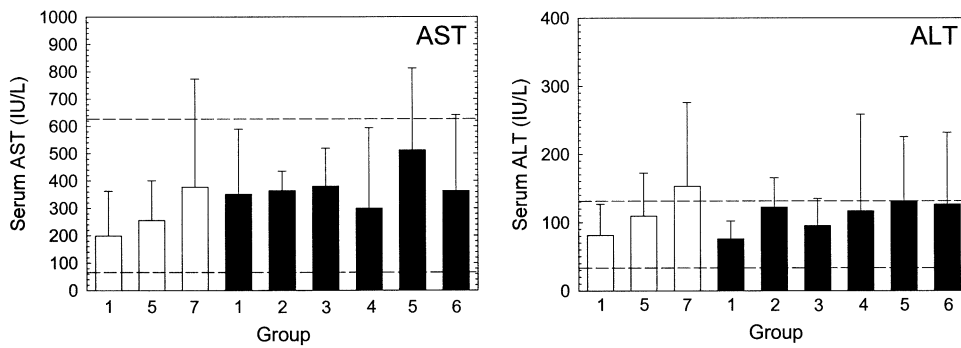


Fig. 7. Comparison of liver transaminase levels on Day 31 (open bars) and Day 53 (solid bars). Bars represent the mean \pm standard deviation. The dashed lines represent the normal range for adult male C57BL/6 mice.

Indeed, the combined effects of the Ad5-CD/TK rep vector and EBRT may have been synergistic (Compare Group 4 to Groups 2 and 3). Double prodrug therapy did seem to exacerbate inflammation of the liver serosa (Table 3, compare Group 6 to Group 4 and Group 5 to Group 3).

There were no significant differences in liver function tests (AST, ALT, bilirubin, albumin, alkaline phosphatase) among the treatment groups at the Day 31 and 53 time points (Fig. 7). At the Day 53 time point, there was a statistically significant decrease in red blood cell counts in the trimodal therapy group (Group 6, $6.36 \pm 0.36 \times 10^6/\text{mm}^3$) vs. the control group (Group 1, $8.35 \pm 0.14 \times 10^6/\text{mm}^3$) ($p = 0.032$, ANOVA). There was also a statistically significant increase in eosinophils in the EBRT group (Group 2, $0.049 \pm 0.032 \times 10^3/\text{mm}^3$) vs. the control group (Group 1, $0.004 \pm 0.01 \times 10^3/\text{mm}^3$) ($p = 0.042$, ANOVA). No other significant differences were noted in the clinical chemistries.

DISCUSSION

Using two experimental prostate cancer models, we demonstrate here that Ad5-CD/TK rep viral and double suicide gene therapies are effective adjuvant therapies to EBRT. In both the i.m. DU145 and intraprostatic LNCaP C4-2 tumor models, the addition of Ad5-CD/TK rep viral therapy significantly improved primary tumor control relative to EBRT alone. A further improvement in primary (DU145) and metastatic (LNCaP C4-2) tumor control was achieved with the addition of double prodrug therapy (trimodal therapy). The results raise the possibility that such novel therapies may be effective adjuvant therapies to EBRT in the clinic (9, 10, 25, 26).

In the absence of EBRT, the addition of double prodrug therapy did not improve tumor control beyond that of Ad5-CD/TK rep viral therapy in either model. These results cannot be explained by a lack of sensitivity to the CD/5-FC and HSV-1 TK/GCV suicide gene systems, because both DU145 and LNCaP C4-2 cells proved to be sensitive to these systems *in vitro*. Moreover, we have observed that DU145 tumors are sensitive to double suicide gene therapy *in vivo* after infection with a replication-defective version of Ad5-CD/TK rep , Ad5-FGNR (20), resulting in a tumor growth delay similar to that of

Ad5-CD/TK rep viral therapy alone (unpublished results). More likely explanations are (1) the suicide gene systems may be inhibiting Ad5-CD/TK rep viral replication *in vivo*, which has been demonstrated previously *in vitro* (9, 10), or (2) in these particular tumor models, the Ad5-CD/TK rep virus is more efficient at destroying tumor cells *in vivo* than are the CD/5-FC and HSV-1 TK/GCV suicide gene systems, and therefore the combined effects of these modalities are not much different than the virus itself. *In vitro* cytopathic effect assays have demonstrated that DU145 and LNCaP C4-2 cells are more sensitive to the replication-competent Ad5-CD/TK rep virus than eight other (A549, C33A, Hep3B, HT-29, SK-OV-3, PC-3, U251, U343) human tumor cell lines tested (not shown). Moreover, in contrast to what we have observed with many other human tumor cell lines (9, 10), the addition of the 5-FC and/or GCV prodrugs does not increase the extent of DU145 and LNCaP cell kill *in vitro* beyond that of the Ad5-CD/TK rep virus itself (not shown). Whether the marked sensitivity of DU145 and LNCaP C4-2 prostatic cells to replication-competent adenoviruses accurately reflects the general sensitivity of human prostate tumors *in vivo* is not known. It is worth noting, however, that human PC-3 prostate adenocarcinoma cells are relatively insensitive to the Ad5-CD/TK rep virus, and the effects of the suicide gene systems can be demonstrated with these cells (9). More importantly, we demonstrated previously that implementation of the CD/5-FC and HSV-1 TK/GCV suicide gene systems can improve primary tumor control beyond that of Ad5-CD/TK rep viral therapy in other tumor models (e.g., C33A) where the virus is less destructive (10). Thus, in the absence of EBRT, use of the CD/5-FC and HSV-1 TK/GCV suicide gene systems may be beneficial only in situations where the targeted tumor exhibits moderate to low sensitivity to replication-competent adenoviruses, or when viral replication and cytopathicity is limited by environmental factors (e.g., immune system, surrounding fibrotic tissue, etc.).

By contrast, the beneficial effects of the CD/5-FC and HSV-1 TK/GCV systems when used in an adjuvant setting with EBRT are well documented (9–20, this report). In the DU145 model, trimodal therapy resulted in a significantly better tumor growth delay and tumor cure than EBRT alone and the Ad5-CD/TK rep + EBRT combination. Although tri-

modal therapy did not improve primary tumor control beyond that of the Ad5-CD/TK rep + EBRT combination in the intraprostatic LNCaP C4-2 model, it did result in better metastatic tumor control and proved to be far superior to EBRT alone. Together, the results suggest that the concentration of toxic metabolites required for radiosensitization may be lower than that required for chemosensitization *in vivo*. Indeed, we demonstrated previously that double suicide gene therapy can result in marked radiosensitization *in vivo*, even with a prodrug therapy regimen that does result in chemosensitization (10). Similar observations have been made with other radiosensitizers, such as gemcitabine (27). Thus, when implemented in an adjuvant setting with EBRT, CD/5-FC and HSV-1 TK/GCV suicide gene systems may be beneficial, even in situations where their chemotherapeutic effects are not evident.

For any cancer therapy to have value in the clinic, it must exhibit greater destructiveness toward malignant than normal tissue (i.e., therapeutic index). Trimodal therapy was associated with no premature deaths and little overall toxicity. As expected, mice that received trimodal therapy exhibited minimal to mild inflammation of the prostate, most of which seemed to be the result of the combined effects of the viral vector and EBRT. Because human replication-competent adenoviruses replicate less efficiently in mouse than in human prostate cells (24), it is possible that the preclinical model used here underestimates the pathology that would occur in humans. It is important to keep in mind, however, that most of the pathologies associated with adenoviral infection seem to be attributable to viral gene expression and the resulting host immune response and are unrelated to virus-induced cytotoxicity (28, 29; see "Discussion" in Ref. 24).

Trimodal therapy was also associated with minimal to mild inflammation of the serosa linings of the seminal vesicles, urinary bladder, and liver. We believe this event is an artifact of the preclinical model used and will not be observed in humans. The mouse dorsolateral prostate is very small (about 0.1% of human prostate gland), and much of the injected vector leaks into the abdominal cavity, resulting in a regional form of peritonitis. Except for the prostate, the parenchyma of all major abdominal organs appeared normal. In a recently completed Phase I study that evaluated the toxicity of Ad5-CD/TK rep viral and double suicide gene therapies in men with local recurrence of prostate cancer, the fact that no peritonitis was observed supports this thesis (19). Only 4 of 16 (25%) patients exhibited a transient elevation in SGOT (Grade 1), occurring on Day 3 and resolving by Day 5, raising the possibility that some of the injected vector may have disseminated to the liver. Ad5-CD/TK rep viral DNA was detected in the blood of all patients as far out as Day 76 (19). Although our preclinical data indicate that double suicide gene therapy does not contribute significantly to locoregional inflammation (whereas EBRT did), it did exacerbate inflammation of the liver serosa. Although hepatotoxicity is a major concern of all gene therapy trials, our recently completed Phase I study (BB-IND 8436) demonstrated that intraprostatic injection of the Ad5-CD/TK rep virus (up to 10^{12} vp, 1.25×10^{10} vp/kg),

followed by 7 days of 5-FC and GCV prodrug therapy, results in little or no hepatotoxicity in humans (19). Because the liver is well outside of the prescribed radiation field, there is little reason to believe that the addition of EBRT (i.e., trimodal therapy) will result in greater hepatotoxicity. Thus, we expect that trimodal therapy will be associated with little hepatotoxicity in humans.

It is interesting to compare the pathologic findings among the various treatment groups. When used independently, EBRT and the Ad5-CD/TK rep vector resulted in little (prostate) or no (seminal vesicles, urinary bladder, small/large intestines) chronic inflammation on Day 53. However, when these two modalities were combined, there was a noticeable increase in locoregional inflammation (although still minimal/mild) that did not seem to be significantly different from that of trimodal therapy. These results seem to suggest that most of the locoregional inflammation observed with trimodal therapy is attributable to the combined effects of the Ad5-CD/TK rep viral vector and EBRT and that the suicide gene systems contributed little to this effect. Although the cellular and humoral immune responses to adenoviral infection have been well studied (30, 31), little is known about the effect that EBRT might have on the immune response to gene therapy vectors (and vice versa). Both adenoviral infection and EBRT induce the local production of several cytokines (e.g., IL-1 α , IL-1 β , IL-6, IFN γ and TNF α), some of which potentiate the cellular immune response, as well as the toxic effects of radiation (32–34). Moreover, repeated radiation doses can lead to sustained cytokine gene expression (35). Because locoregional, and not systemic, toxicity is likely to be dose limiting with our trimodal therapy approach, further investigation of the immune response to adenoviral vectors and EBRT and of how these factors interact and contribute to locoregional toxicity may be warranted.

The dose-limiting toxicities of EBRT in the treatment of prostate cancer are small bowel/rectal damage (bleeding); bladder complications, including urinary frequency, dysuria, hematuria, infections, and incontinence; and impotence. Of these, rectal damage is the most serious. In the preclinical study described here, the urinary bladder, small bowel, and rectum were all within the radiation field. Despite this, the small intestine and rectum in all groups, including trimodal therapy group, appeared normal. Relative to EBRT, trimodal therapy was associated with increased inflammation of the urinary bladder serosa. However, this inflammation was minimal and, as discussed above, is likely the result of leakage of vector into the abdominal cavity. Despite these encouraging preclinical results, we acknowledge that Ad5-CD/TK rep viral and double suicide gene therapies may exacerbate locoregional toxicity of EBRT in humans; we plan to monitor these side effects closely in our next clinical trial (BB-IND 9852). It is our hope that Ad5-CD/TK rep viral and double suicide gene therapies will be demonstrated to be a safe and effective adjuvant therapy to EBRT in the clinic and may provide another therapeutic option for the treatment of human cancer.

REFERENCES

1. Oesterling J, Fuks Z, Lee C, Scher H. In: DeVita VT, Hellman S, Rosenberg SA, editors. *Cancer: Principles and practice of oncology*. Philadelphia: JB Lippincott; 1997. p. 1322–1386.
2. Stephenson RA, Smart CR, Mineau GP, James BC, Janerich DT, Dibble RL. The fall of incidence of prostate cancer. *Cancer* 1995;77:1342–1348.
3. Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics, 1997. *A Cancer Journal for Clinicians* 1997;47:5–27.
4. Coleman CN, Beard C, Gelman R, Kantoff P. Rate of relapse following treatment for localized prostate cancer: A critical analysis of retrospective reports. *Int J Radiat Oncol Biol Phys* 1993;28:303–313.
5. Zagars G, Pollack A, von Eschenbach A. Prognostic factors for clinically localized prostate carcinoma: Analysis of 938 patients irradiated in the PSA era. *Cancer* 1997;79:1370–1380.
6. Fuks Z, Leibel SA, Wallner KE, *et al.* The effect of local control on metastatic dissemination in carcinoma of the prostate: Long-term results in patients treated with I-125 implantation. *Int J Radiat Oncol Biol Phys* 1991;21:537–542.
7. Hanks G, Martz K, Diamond J. The effect of dose on local control of prostate cancer. *Int J Radiat Oncol Biol Phys* 1998;15:1299–1305.
8. Smit W, Helle P, Van Putten W, *et al.* Late radiation damage in prostate cancer patients treated by high dose external radiotherapy in relation to rectal dose. *Int J Radiat Oncol Biol Phys* 1990;18:23–29.
9. Freytag SO, Rogulski KR, Paielli DL, Gilbert JD, Kim JH. A novel three-pronged approach to selectively kill cancer cells: Concomitant viral, double suicide gene, and radiotherapy. *Hum Gene Ther* 1998;9:1323–1333.
10. Rogulski KR, Wing M, Paielli DL, Gilbert JD, Kim JH, Freytag SO. Double suicide gene therapy augments the therapeutic efficacy of an oncolytic adenovirus through enhanced cytotoxicity and radiosensitization. *Hum Gene Ther* 2000;11:67–76.
11. Kim JH, Kim SH, Brown SL, Freytag SO. Selective enhancement by an antiviral agent of the radiation-induced cell killing of human glioma cells transduced with HSV-tk gene. *Cancer Res* 1994;54:6053–6056.
12. Kim JH, Kim SH, Kolozsvary A, Brown SL, Kim OB, Freytag SO. Selective enhancement of radiation response of herpes simplex virus thymidine kinase transduced 9L gliosarcoma cells *in vitro* and *in vivo* by antiviral agents. *Int J Radiat Oncol Biol Phys* 1995;33:861–868.
13. Khil M, Kim JH, Mullen CA, Kim SH, Freytag SO. Radiosensitization by 5-fluorocytosine of human colorectal carcinoma cells in culture transduced with cytosine deaminase gene. *Clin Cancer Res* 1995;2:53–57.
14. Rogulski KR, Kim JH, Kim SH, Freytag SO. Glioma cells transduced with an *E. coli* CD/HSV-1 TK fusion gene exhibit enhanced metabolic suicide and radiosensitivity. *Hum Gene Ther* 1997;8:73–85.
15. Kim SH, Kim JH, Kolozsvary A, Brown SL, Freytag SO. Preferential radiosensitization of 9L glioma cells transduced with HSV-TK gene by acyclovir. *J Neurooncol* 1997;33:189–194.
16. Rogulski KR, Zhang K, Kolozsvary A, Kim JH, Freytag SO. Pronounced anti-tumor effects and tumor radiosensitization of double suicide gene therapy. *Clin Cancer Res* 1997;3:2081–2088.
17. Gable M, Kim JH, Kolozsvary A, Khil M, Freytag SO. Selective *in vivo* radiosensitization by 5-fluorocytosine of human colorectal carcinoma cells transduced with the *E. Coli* cytosine deaminase (CD) gene. *Int J Radiat Oncol Biol Phys* 1997;41:883–887.
18. Kim JH, Kolozsvary A, Rogulski KR, Khil M, Freytag SO. Double suicide fusion gene: A selective radiosensitization of 9L glioma in rat brain. *Cancer J Scient Am* 1998;4:364–369.
19. Freytag SO, Khil M, Stricker H, *et al.* 2002. Phase I study of replication-competent adenovirus-mediated double suicide gene therapy for the treatment of locally recurrent prostate cancer. *Cancer Res* 2002;62:4968–4976.
20. Xie Y, Gilbert JD, Kim JH, Freytag SO. Efficacy of adenovirus-mediated CD/5-FC and HSV-1 TK/GCV suicide gene therapies concomitant with p53 gene therapy. *Clin Cancer Res* 1999;5:4224–4232.
21. Alfieri A, Hahn E. An *in situ* method for estimating cell survival in a solid tumor. *Cancer Res* 1978;38:3006–3011.
22. Sato N, Gleave M, Bruchovsky N, Rennie P, Beraldi E, Sullivan L. A metastatic and androgen-sensitive human prostate cancer model using intraprostatic inoculation of LNCaP cells in SCID mice. *Cancer Res* 1997;57:1584–1589.
23. Ellis F. Dose, time and fractionation: A clinical hypothesis. *Clin Radiol* 1969;20:1–7.
24. Paielli DL, Wing M, Rogulski KR, *et al.* Evaluation of the biodistribution, toxicity, and potential of germ line transmission of a replication-competent human adenovirus following intraprostatic administration in the mouse. *Molecular Ther* 2000;1:263–274.
25. Rogulski KR, Freytag SO, Zhang K, *et al.* Anti-tumor activity of ONYX-015 is influenced by p53 status and is augmented by radiotherapy. *Cancer Res* 2000;60:1193–1196.
26. Chen Y, DeWeese T, Dilley J, *et al.* CV706, a prostate cancer-specific adenovirus variant, in combination with radiotherapy produces synergistic antitumor efficacy without increasing toxicity. *Cancer Res* 2001;61:5453–5460.
27. Shewach DS, Hahn TM, Chang E, Hertel LW, Lawrence TS. Metabolism of 2',2'-difluoro-2'-deoxycytidine and radiation sensitization of human colon carcinoma cells. *Cancer Res* 1994;54:3218–3223.
28. Ginsberg H, Moldawer L, Sehgal P, *et al.* A mouse model for investigating the molecular pathogenesis of adenovirus pneumonia. *Proc Natl Acad Sci U S A* 1991;88:1651–1655.
29. Prince G, Potter D, Jenson A, Horswood R, Chanock R, Ginsberg H. Pathogenesis of adenovirus type 5 pneumonia in Cotton rats (*Sigmodon hispidus*). *J Virol* 1993;67:101–111.
30. Yang Y, Hildegund E, Wilson J. MHC class I-restricted cytotoxic T lymphocytes to viral antigens destroy hepatocytes in mice infected with E1-deleted recombinant adenoviruses. *Immunity* 1994;1:433–442.
31. Yang Y, Xiang Z, Ertl H, Wilson J. Upregulation of class I histocompatibility complex antigens by interferon γ is necessary for T-cell-mediated elimination of recombinant adenoviruses-infected hepatocytes *in vivo*. *Proc Natl Acad Sci U S A* 1995;92:7257–7261.
32. Hallahan D, Spriggs D, Beckett M, Kufe D, Weichselbaum R. Increased tumor necrosis factor alpha mRNA after cellular exposure to ionizing radiation. *Proc Natl Acad Sci U S A* 1989;86:10104–10107.
33. Chiang C, McBride W. Radiation enhances tumor necrosis factor alpha production by murine brain cells. *Brain Res* 1991;566:265–269.
34. Syljuasen R, Belldegrun A, Tsao C, Withers H, McBride W. Sensitization of renal carcinoma to radiation using alpha interferon (IFNA) gene transfection. *Radiat Res* 1997;148:443–448.
35. Hong J, Chiang C, Tsao C, Lin P, McBride W, Wu C. Rapid induction of cytokine gene expression in the lung after single and fractionated doses of radiation. *Int J Radiat Biol* 1999;75:1421–1427.