Prostate Cancer Radiosensitization in Vivo with Adenovirus-mediated p53 Gene Therapy

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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 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 on day 8. 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 mm^3 were calculated as 10.7 ± 0.7 (± 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 enhancement 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 PSA 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 untested. 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...
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℃ 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 x 105) 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 33% every 2 days.

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 enhancement 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 μl 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 p53null. 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 (Fig. 1), as compared with Ad5-Luc control vector. The data indicate that Ad5-p53 treatment resulted in functional p53 expression in vivo.
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 $\gamma$-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 mm$^3$, 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
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 over 56 ng/ml. The more meaningful end points of the 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.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Absolute delay (n)</th>
<th>Delay from saline control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>10.7 ± 0.7 (4)</td>
<td></td>
</tr>
<tr>
<td>Ad5-pA</td>
<td>9.8 ± 2.1 (3)</td>
<td>0</td>
</tr>
<tr>
<td>Ad5-p53</td>
<td>15.6 ± 1.6 (5)</td>
<td>4.9</td>
</tr>
<tr>
<td>PBS + 5 Gy</td>
<td>15.4 ± 2.2 (5)</td>
<td>4.7</td>
</tr>
<tr>
<td>Ad5-pA + 5 Gy</td>
<td>14.6 ± 1.5 (5)</td>
<td>3.9</td>
</tr>
<tr>
<td>Ad5-p53 + 5 Gy</td>
<td>31.4 ± 5.3 (5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different (P < 0.05) from other groups by one-way ANOVA (Scheffe test).

<sup>b</sup> Enhancement factor, 3.4.

**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<sup>wildtype</sup> tumors. For p53<sup>wildtype</sup> 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<sup>wildtype</sup> expression has been variable; some reports have described radiosensitization of p53<sup>wildtype</sup> 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<sup>wildtype</sup> LNCaP line.
rising PSA profile is the earliest and most sensitive end point in serum PSA as a measure of failure to control tumor growth. The efficacy of intraprostatic 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 PBS alone, 5 Gy group was significantly different (P < 0.05) from the PBS alone group by one-way ANOVA (Scheffe test). If the PSA rate of failure was significantly lower in the Ad5-p53 plus 5 Gy group, as compared with each of the other groups (P0.001). Treatment failure was defined as two consecutive rises in PSA or a single rise of >1.5 ng/ml at 6 weeks after treatment. The failure rates were significantly different overall by trended χ2 (P ≤ 0.001).

The rising PSA method 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 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.

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

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

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.

to about the same degree as for the p53null 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 p53null and p53wildtype 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 radiotherapy, was calculated to be >3-fold, relative to the controls. A similar effect was observed for p53wildtype 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

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Failure free</th>
<th>Failure free</th>
<th>Failure free</th>
<th>Failure free</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0% (0)</td>
<td>100% (5)</td>
<td>0% (0)</td>
<td>100% (5)</td>
</tr>
<tr>
<td>PBS + 5 Gy</td>
<td>20% (2)</td>
<td>80% (8)</td>
<td>30% (3)</td>
<td>70% (7)</td>
</tr>
<tr>
<td>Ad5-pA alone</td>
<td>0% (0)</td>
<td>100% (10)</td>
<td>10% (1)</td>
<td>90% (9)</td>
</tr>
<tr>
<td>Ad5-pA + 5 Gy</td>
<td>20% (1)</td>
<td>80% (4)</td>
<td>20% (1)</td>
<td>80% (4)</td>
</tr>
<tr>
<td>Ad5-p53</td>
<td>37% (3)</td>
<td>73% (8)</td>
<td>36% (4)</td>
<td>64% (7)</td>
</tr>
<tr>
<td>Ad5-p53 + 5 Gy</td>
<td>71% (10)</td>
<td>29% (4)</td>
<td>79% (11)</td>
<td>21% (3)</td>
</tr>
</tbody>
</table>

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 χ2 (P ≤ 0.001).

c The rate of failure was significantly lower in the Ad5-p53 plus 5 Gy group, as compared with each of the other groups (χ2; P < 0.05).
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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REFERENCES


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