Prostate Cancer Gene Therapy: Comparison of Adenovirus-mediated Expression of Interleukin 12 with Interleukin 12 plus B7-1 for in Situ Gene Therapy and Gene-modified, Cell-based Vaccines


ABSTRACT

We have documented previously that adenovirus-mediated interleukin 12 (IL-12) gene therapy is effective for orthotopic tumor control and suppression of pre-established metastases in a preclinical prostate cancer model (Y. Nasu et al., Gene Ther., 6: 338–349, 1999). In this report, we directly compare the effectiveness of an adenovirus that expresses both IL-12 and the costimulatory molecule B7-1 (AdmIL-12/B7) with one that expresses IL-12 alone (AdmIL-12) using the poorly immunogenic RM-9 orthotopic murine model of prostate cancer. We document AdmIL-12/B7-mediated secretion of IL-12 and increased surface expression of B7-1 in infected RM-9 tumor cells. A significant reduction in orthotopic tumor size and increased survival was demonstrated in mice treated with a single orthotopic injection of AdmIL-12/B7 compared with AdmIL-12 or controls. Six of 19 animals treated with AdmIL-12/B7 survived long term with apparent eradication of the primary tumor in contrast to one of 38 animals in the AdmIL-12-treated group. Orthotopic treatment of tumors with both vectors led to an infiltration of both CD4+ and CD8+ immunoreactive cells, with AdmIL-12/B7 treatment having a more prolonged infiltration of CD8+ cells. AdmIL-12/B7 was also more effective than AdmIL-12 or controls at suppression of pre-established metastases. We further developed a vaccine model based on s.c. injection of infected, irradiated RM-9 cells and found that both AdmIL-12 and AdmIL-12/B7 are effective at suppressing the development and growth of challenge orthotopic tumors using this protocol.

INTRODUCTION

Despite breakthroughs in the early detection of prostate cancer using PSA and improvements in radical surgery technique, prostate cancer continues to be a significant cause of morbidity and mortality in the Western world. Although radical retropubic prostatectomy has demonstrated a 76% progression-free probability at 10 years in patients with clinically organ-confined disease, patients with advanced pathological staging after surgery have demonstrated only a 48% progression-free survival at 5 years (1, 2). In addition, the two currently available, potentially curative treatment modalities for localized prostate cancer are not without significant morbidity (3). There are no efficacious treatments for patients who present with locally advanced or metastatic diseases or in cases of recurrent prostate cancer after definitive treatment. Hormonal therapy offers palliation, but most patients who live long enough will develop hormone-resistant disease and succumb to their cancer.

To develop new therapeutic approaches for prostate cancer, we have conducted extensive preclinical studies of various adenovirus-mediated gene therapy protocols. We have evaluated “suicide gene therapy” extensively in preclinical studies using adenovirus-mediated expression of the HSV-tk gene, followed by the systemic administration of the prodrug GCV studies (4–8). In our orthotopic models of prostate cancer, tumors are established by intraprostatic injection of well-characterized prostate cancer cell lines. Approximately 1 week later, the tumor is injected directly with the therapeutic adenoviral vector, and the animals are followed for 2–3 weeks. HSV-tk + GCV gene therapy led to marked growth inhibition of the local prostate cancer with suppression of spontaneous and induced metastases using two-cell mouse prostate cancer cell lines, RM-1 and RM-2 (5, 6, 8). In these studies, antimitastatic activity consistent with the induction of an antitumor immune response was also associated with HSV-tk + GCV therapy. These preclinical studies led to the first clinical trial of in situ gene therapy for prostate cancer, which was shown to be safe and suggested that in situ
gene therapy with HSV-tk + GCV could suppress serum PSA levels in men who have local recurrence of prostate cancer after radiotherapy (9). We have subsequently accrued additional patients and documented alterations of the PSA doubling times and the presence of activated CD8 immunocyte populations in the peripheral blood of patients who received gene therapy.

Human (10) and mouse (11, 12) prostate cancer cells are generally considered to be poorly immunogenic despite the presence of antigens that may be tumor specific (13). This lack of immune stimulation may be overcome by enhancing the presentation of the tumor antigens to T cells and by delivering immunostimulatory cytokines. Modification of the immune response against cancer using specific cytokines may prove effective against cancers such as prostate cancer. IL-12, a heterodimeric cytokine, was found to be elevated after HSV-tk + GCV therapy in melanoma models (14) and has potent antitumor properties in several preclinical models of cancer (15–17). However, administration of cytokines systemically to treat cancers has resulted in significant toxicity in both preclinical studies and early human trials. Using gene therapy to deliver immunomodulatory genes directly into tumors is one technique to limit the toxicity associated with systemic administration of cytokines. We reported the efficacy of a single injection of an adenovirus expressing IL-12 to treat a poorly immunogenic orthotopic murine model of prostate cancer. Treatment of the primary tumor resulted in a reduction of primary tumor weight by 58%, a significant survival advantage, and a reduction in the number of pre-established lung metastases with minimal toxicity (11). The mechanism of efficacy in our system appeared to involve NK-mediated cytolysis, increased activation of nitric oxide synthase in macrophages and, potentially, T-cell activities.

Antigen presentation requires binding of processed peptides complexed with MHC molecules to the T-cell receptor. Professional antigen-presenting cells express costimulatory molecules such as B7-1 (CD80) that interacts with its specific receptor, CD28, expressed on T cells to generate long-lasting antitumor responses (18). B7-1 is poorly expressed on most tumor cells; thus, genetically engineered tumor cells that express B7-1 have been studied as whole-cell vaccines in several preclinical models (19, 20). In addition to its costimulatory role in activation of T cells, B7-1 has also been shown to enhance nonspecific NK-mediated cytolysis of a poorly immunogenic murine lung carcinoma (21).

In this study, we compared the efficacy of adenovirus-mediated expression of IL-12 and B7-1 (AdmIL-12/B7) to an adenovirus expressing IL-12 alone (AdmIL-12) and a control adenovirus (Ad/CMV/βgal) in the treatment of a poorly immunogenic prostate cancer cell line, RM-9. We document IL-12 secretion and enhanced surface expression of B7-1 after in vitro infection with AdmIL-12/B7. In our orthotopic model, we show a dose-dependent reduction of tumor weight that is significantly better than AdmIL-12 alone. This vector also offered a significant survival advantage that involved long-term survivors/apparent cures and reduced the number of pre-established lung metastases. We also developed a model for cell-based vaccine therapy with irradiated RM-9 cells infected with either AdmIL-12 or AdmIL-12/B7 and demonstrated therapeutic activity of both vectors using this protocol.

MATERIALS AND METHODS

Adenoviral Vectors. The adenoviral vectors were kindly provided by Dr. Frank Graham of McMaster University (Ontario, Canada). The construction of the AdmIL-12 and AdmIL-12/B7 has been described previously (22, 23). Briefly, the AdmIL-12 vector has the expression cassette for the p35 subunit of murine IL-12 is inserted in the E1 region and the p40 subunit cassette in the E3 region. Expression of each IL-12 cDNA is driven by the human CMV immediate-early gene promoter and terminated by the polyadenylation signal of SV 40. In the AdmIL-12/B7 vector, the IL-12 subunit cDNAs p40 and p35 are both located in the E1 region separated by the encephalomyocarditis virus internal ribosome entry site and are expressed from a polycistronic message. The B7-1 cDNA is inserted in E3. Both the IL-12 and B7-1 genes are under the control of the MCMV IE promoter and terminated by the polyadenylation signal of SV40. The recombinant adenoviruses were isolated from a single plaque, expanded in a 293 cell line, and purified by double cesium gradient ultracentrifugation. Virus titers were determined by plaque assay in 293 cells and reported as PFUs. As a control virus vector, Ad/CMV/βgal was prepared as described previously (11).

Characterization of in Vitro Infected Cells. Adenoviral infection of the mouse prostate cell line, RM-9, with AdmIL-12 and AdmIL-12/B7 was tested in vitro to evaluate for direct cytotoxicity, IL-12 secretion, and expression of cell surface markers. The RM-9 prostate cancer cell line was derived from the mouse prostate reconstruction model system using C57BL/6 mice as described previously (24, 25). Triplicate aliquots of 10,000 RM-9 cells were plated on 24-well plates in DMEM with 10% fetal bovine serum, 10 mM HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin and allowed to adhere for 24 h. The medium was then aspirated, and the adherent cells were washed gently with PBS. The cells were then infected with AdmIL-12, AdmIL-12/B7, or Ad/CMV/βgal at a MOI of 100 using serum-free media (DMEM, 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1% BSA). The medium was gently aspirated after 2 h and replaced with 1 ml of serum containing DMEM as described above. Cell counts were measured in triplicate 24, 48, 72, and 96 h after transduction to evaluate for direct cytotoxicity of the vectors on the RM-9 cell line. Medium was collected from each of the wells at 24, 48, 72, and 96 h and assayed for murine IL-12 by ELISA (BioSource International, Camarillo, CA). Cell suspensions of 5 × 10^5 infected and parental RM-9 prostate cancer cells were incubated for 30 min on ice with biotin-conjugated antimume MHC class I, II, B7-1, and B7-2 antibodies (PharMingen, San Diego, CA) and then with fluorescein-conjugated avidin (Pierce Corp., Rockford, IL). The cells were washed, and 10,000 cells were analyzed on EPICS XL-MCL (Coulter Electronics, Westbrook, Connecticut, USA) using the EPICS software (Coulter Electronics, Westbrook, Connecticut, USA).
ME) for expression of MHC class I, II, B7-1, and B7-2 antigen by mean fluorescence intensity as described previously (11).

Orthotopic Tumor Induction. After trypsinization, RM-9 cells were counted and resuspended in HBSS. For orthotopic tumor inoculation, syngeneic C57/B6 mice were anesthetized with sodium pentobarbital. A low transverse abdominal incision was made, and the dorsolateral prostate was exposed. Injection of 5000 cells in 10 μl directly into the right lobe of the dorsolateral prostate resulted in efficient and reproducible orthotopic tumor formation. The tumors were allowed to grow for 7–8 days prior to treatment. At this time point, most mice had tumors ranging between 5 and 15 mm³, as measured with calipers.

In Vivo IL-12/B7-1 Gene Therapy. Previously, Nasu et al. (11) had determined the optimal therapeutic dose of AdmIL-12 to be 1 × 10⁸ using this orthotopic model of prostate cancer. To determine an optimal therapeutic viral dose of AdmIL-12/B7, escalating viral doses from 2.5 × 10⁸ to 3 × 10⁹ PFUs of AdmIL-12/B7 were directly injected into established prostate tumors 7–8 days after tumor cell inoculation. Ad/CMV/βgal, and PBS were used as controls. A 27-gauge needle was placed through an intracapsular, transprostatic tract prior to tumor penetration to minimize leakage of virus at the time of needle withdrawal. The needle point was then moved around within the tumor during injection to maximize the area exposed to the vectors. A volume of <25 μl was used for all virus injections.

Animals were euthanized on the 14th day after virus injection or at seven different time points in a kinetic analysis (1, 3, 5, 7, 10, and 14 days after viral injection). For survival analysis studies, animals were evaluated at death, or they were euthanized when appearing in distress, as evidenced by lethargy, ruffled fur, or weight loss. All animals underwent a careful necropsy for gross metastasis. The primary tumor and spleen were removed and weighed. Blood was collected, allowed to clot, and serum frozen at −80°C for analysis of IL-12 as described above. The spleen was placed in sterile medium until the splenocytes could be harvested to perform flow cytometry. NK cell activity assays. In addition, the pelvic and retroperitoneal lymph nodes and samples of lung were excised, regardless of gross appearance, and along with a portion of the primary tumor processed for histological analysis. Samples of tissue were placed in formalin, paraffin embedded, cut into 4–5-μm sections, and stained with H&E for histological examination or immersed in Tissue-Tec OCT medium and quick frozen in liquid N₂.

All mice were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care, and all animal studies were conducted in accordance with the principles and procedures outlined in the NIH’s Guide for the Care and Use of Laboratory Animals.

Pre-established Metastases and Treatment. In our model of preexisting lung metastases, mice receive a tail vein injection of RM-9 cells (100,000 cells in 100 μl of HBSS) to induce lung metastases at the same time as orthotopic tumor inoculation with 5000 cells. Six days after inoculation, animals were randomized to receive a single injection of either AdmIL-12, AdmIL-12/B7, PBS, or a control vector, Ad/CMV/βgal, directly into the orthotopic prostate tumor. In previous studies, the presence of lung metastases has been confirmed at the time of virus injection (11). Animals with preexisting metastases were sacrificed on the eighth day after virus injection, at which time the lungs were weighed and lung metastases were counted with the aid of a dissecting microscope by two blinded observers.

Vaccination Protocol. Vaccination consisted of three s.c. injections at weekly intervals of one million irradiated RM-9 cells either uninfected or infected with adenovirus. Control vaccination consisted of injection of HBSS. The RM-9 cells were infected in vitro at an MOI of 100 1 day prior to irradiation. The next day cells were trypsinized, centrifuged, and resuspended in HBSS at 1 × 10⁶ cells/ml and then irradiated with 300 Gy with a gamma cell irradiator. The cells were then centrifuged and resuspended in HBSS at a concentration of 1 × 10⁶ cells/100 μl and injected s.c. in the right upper abdominal quadrant. This dose of irradiation was determined to prevent further cell growth either in vitro or in vivo but did not affect the ability of the adenovirus-infected, irradiated cells to continue to express IL-12 in vitro (not shown). Three weeks after the last vaccination, the mice were challenged with orthotopic injection of 5000 RM-9 cells as described above for orthotopic tumor induction. The mice were sacrificed 22 days later and evaluated for prostate tumor formation, and the wet weights of tumors were recorded.

Quantitative Immunohistochemistry. Frozen tissues were sectioned with a cryostat, and sections reacted with CD4, CD8, and F4/80 antibodies and visualized as described previously (11). Computer-assisted image analysis was used to determine the number of immunopositive cells/mm² (11).

In Vitro Cytolytic Assays. NK cell lytic activity was measured in the splenocytes harvested from the different treatment groups. NK activity was determined at several time points after treatment with 5³Cr-labeled YAC cells as the target cells. A standard assay was used as described previously (11). Various effector (splenocytes):target cell (YAC) ratios were used. The YAC cell line was obtained from American Type Culture Collection.

Statistical Analysis. ANOVA was used to compare tumour weights. Survival analysis was with the log-rank test (Mantel-Cox test). All statistical analyses were performed with Statview 4.02 (Abacus Concepts, Berkeley, CA).

RESULTS

Growth and IL-12 Production after in Vitro Transduction. RM-9 cells were infected with Ad/CMV/βgal, AdmIL-12, or AdmIL-12/B7-1 at a MOI of 100 to determine whether there was direct cytotoxicity from infection and to measure IL-12 production. There was no significant difference in the growth of any of the cells lines compared with noninfected RM-9 cells (Fig. 1a). In vitro IL-12 production was higher in the AdmIL-12/B7-infected cells compared with AdmIL-12-infected cells (at 24, 48, and 72 h after infection) and controls (Fig. 1b), which is consistent with previous reports in a breast cancer cell line (23).

Flow Cytometry. We have documented previously that RM-9 cells have poor surface expression of MHC class I (11), demonstrating that it is a poorly immunogenic prostate cancer
IL-12/B7 Gene Therapy for Prostate Cancer

408

15% of mice treated with the 3 dose demonstrated the greatest efficacy. However, approximately 10

-10 8 . At the time of treatment, tumors were consistently between 5

the tumors were excised and weighed. The 3
tumor was performed at various doses between 2.5

cells into the right dorsal lateral lobe of the prostate. Seven days

prostate cancer. Tumors were initiated by injecting 5000 RM-9

toxicity ratio of AdmIL-12/B7 in treating orthotopic models of

A dose escala-

cell line similar to human prostate cancer (10) and other similarly
derived mouse prostate cancer cell lines (12). In Table 1,

we show that RM-9 cells have low expression of B7-1 and B7-2. However, infection with AdmIL-12/B7 led to an up-regulation
of B7-1 surface expression by 3.1 times compared with parental
RM-9 cells (mean fluorescence increased from 2.9 to 9.0; Fig.
2). AdmIL-12/B7 infection did not affect MHC I, MHC II, or
B7-2 surface expression. Infection of cells with AdmIL-12 alone
did not alter surface expression of MHC I, II, B7-1, or B7-2.

In Situ Treatment of Orthotopic Tumors with AdmIL-

B7 Efficacy Compared with AdmIL-12. A dose escalation
study was performed to determine the maximal efficacy:
toxicity ratio of AdmIL-12/B7 in treating orthotopic models of
prostate cancer. Tumors were initiated by injecting 5000 RM-9
cells into the right dorsal lateral lobe of the prostate. Seven days
later, a single injection of the AdmIL-12/B7 virus directly into the
tumor was performed at various doses between 2.5 × 10 7 and 3 × 10 8 . At the time of treatment, tumors were consistently between 5

and 15 mm 3 . Two weeks after treatment, mice were sacrificed, and
the tumors were excised and weighed. The 3 × 10 8 PFU dose demonstrated the greatest efficacy. However, approximately 10–
15% of mice treated with the 3 × 10 8 PFU demonstrated signs of
toxicity including decreased activity, ruffled fur, and lethargy. In

addition, they also were found to have increased amounts of ascites
at sacrifice and larger spleen weights. This is similar to the results
reported previously for AdmIL-12 at high doses (11). The 2.5 × 10 7 , 5 × 10 7 , and 1 × 10 8 doses of AdmIL-12/B7 demonstrated
efficacy without signs of toxicity. The 2.5 × 10 7 PFU dose was
significantly less efficacious than the 1 × 10 8 PFU dose (P =
0.04). The 5 × 10 7 and 1 × 10 8 PFU doses demonstrated similar
efficacies. Both doses produced a significant reduction in tumor
size compared with AdmIL-12 at the optimal therapeutic dose (P <
0.0001; Fig. 3).

In Situ AdmIL-12/B7 Prolongs Survival. A single in situ injection of 1 × 10 8 PFU AdmIL-12/B7 into tumors 7 days
after initiation resulted in a significant survival advantage com-
pared with controls (Ad/CMV/βgal; 40 days versus 28 days;
P = 0.0093). There was no significant survival advantage of
AdmIL-12/B7 (1 × 10 8 PFU) over AdmIL-12 (40 versus 37
days; P = 0.3876). However, the 5 × 10 7 PFU dose of AdmIL-
12/B7 demonstrated a significant survival advantage compared
with AdmIL-12 (48 days versus 37 days; P = 0.0019; Fig. 4). More than 20% of the animals survived over an extended time
period. All animals had measurable tumors at the time of ad-
enviral vector injection. Six of 19 animals were examined at
day 50 for the presence of tumor, and there was no evidence of
malignancy within the prostate gland, suggesting that these
animals had been cured of their disease. In contrast, only 1 of 38
animals injected with AdmIL-12 demonstrated long-term sur-
vival without evidence of disease.

Suppression of Pre-established Lung Metastases. AdmIL-12 and AdmIL-12/B7 (at 5 × 10 7 and 1 × 10 8 PFU)
demonstrated a significant reduction in pre-established lung
metastases compared with Ad/CMV/βgal; 40 days
(P < 0.0001; Fig. 5). Both doses of AdmIL-12/B7 (5 × 10 7 and 1 × 10 8 PFU)
demonstrated a trend toward enhancement of ant metastatic
effect compared with AdmIL-12, but this did not reach statisti-
cal significance (P = 0.18 and P = 0.52).

Kinetic Analysis of Treatment. Orthotopic RM-9 tu-
mors were injected with 1 × 10 8 PFUs of AdmIL12 or Ad/
CMV/βgal or 5 × 10 8 PFUs of AdmIL-12/B7, and tumor
development was closely monitored in animals by sequentially
sacrificing a limited number of mice (n = 3/time point). In Fig.
6A, we show that IL-12/B7 suppressed tumor growth compared
with control or AdmIL-12-injected tumors. The spleen from
each animal was weighed at the time of sacrifice, and as seen in
Fig. 6B, the AdmIL-12/B7-treated animals had the largest in-
ncrease in spleen weight, with a maximum increase somewhat
delayed compared with the AdmIL-12-treated animals. Consistent
with our previous study (11), serum IL-12 levels peaked 1
day after adenoviral vector injection with either AdmIL-12 or
AdmIL-12/B7 (Fig. 6C). With equivalent vector doses (1 × 10 8 ),
a somewhat larger but more variable peak serum level of
IL-12 was obtained with the AdmIL-12 vector. The serum levels
at subsequent time points were similar for the two vectors at this
dose. The optimal therapeutic dose of AdmIL-12/B7 (5 × 10 8 )
yielded lower serum IL-12 levels.

To probe the mechanism of action of the two vectors, we
analyzed specific tumor-infiltrating immunocytes using quanti-
tative immunohistochemistry (Fig. 7). There was a 2–3-fold
increase in the number of CD4-reactive T cells on day 7 after
either vector (Fig. 7A). The number was slightly larger in the
AdmIL-12 group compared with the AdmIL-12/B7 group. An increase in CD8-reactive T cells was even more pronounced at day 7 after both vectors (Fig. 7B). The AdmIL-12/B7 induced a somewhat greater increase in CD8+ cell numbers at day 7, and this increase was sustained through day 14 in contrast to the AdmIL-12 group in which there was a substantial decrease in CD8 counts by day 14. The levels of cells reactive with the macrophage-selective antibody (F4/80) were elevated for both treatment groups on day 2 relative to the control Ad/CMV/bgal (on day 14) and to the control levels observed previously at the time of injection (11). The number of F4/80-positive cells in AdmIL-12-treated tumors initially decreased more rapidly than the AdmIL-12/B7 group after the day-2 peak (Fig. 7C).

NK Activity. Splenocytes harvested from mice with orthotopic tumors injected with AdmIL-12 or AdmIL-12/B7 (5 × 10⁷ PFU) demonstrated increased levels of lytic activity against YAC cells on day 2 relative to the control Ad/CMV/bgal (on day 14) and to the control levels observed previously at the time of injection (11). The number of F4/80-positive cells in AdmIL-12-treated tumors initially decreased more rapidly than the AdmIL-12/B7 group after the day-2 peak (Fig. 7C).

Cell-based Vaccination. Three weekly injections of irradiated, AdmIL-12/B7-infected RM-9 cells protected 33% (3 of 9) of the mice vaccinated from an orthotopic challenge with live RM-9 cells 3 weeks later (Fig. 9A). Vaccination with AdmIL-12-infected RM-9 cells protected 20% (8 of 10) of the mice. No mice vaccinated with HBSS alone or irradiated, uninfected cells were tumor free. The weight of the orthotopic tumors in the animals with tumors also suggested that vaccination with adenovirus-infected cells generated a systemic immune response that suppressed the growth of the orthotopic challenge tumors (Fig. 9B). There was no significant difference in tumor weight between the two vaccine

<table>
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<th>Cells/Vector</th>
<th>Rat IgG % Pos</th>
<th>MHC I % Pos</th>
<th>MFI</th>
<th>MHC II % Pos</th>
<th>B7-1 % Pos</th>
<th>MFI</th>
<th>B7-2 % Pos</th>
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<td>9.7</td>
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<tr>
<td>RM-9/AdmIL-12/B7</td>
<td>1.1</td>
<td>1.6</td>
<td>34</td>
<td>4</td>
<td>8.1</td>
<td>2.4</td>
<td>74.3</td>
<td>9</td>
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Fig. 2  Flow cytometric analysis for expression of surface B7-1 (CD80) antigen in RM-9 cells (left tracing) and RM-9 cells infected with AdmIL-12/B7 (right tracing).

Fig. 3 Orthotopic tumor weight 14 days after vector injection (21 days after orthotopic injection of 5000 RM-9 cells). AdmIL-12/B7 vector injection reduced the tumor size most effectively. See text for statistical details. Bars, SD.

Fig. 4 Survival of animals with orthotopic tumors. Kaplan-Meier survival plot for animals that received injections of each vector. Six animals injected with AdmIL-12/B7 (5 × 10⁷ PFU) were alive and healthy on day 50. Close examination did not reveal any prostatic tumor remaining.
treatments, but both were significantly smaller than controls ($P < 0.02$).

DISCUSSION

We have reported previously that adenovirus-mediated IL-12 gene therapy was effective in a preclinical model for prostate cancer (11). We determined that in situ treatment of orthotopic RM-9 tumors with $1 \times 10^8$ PFU of AdmIL-12 was optimal for reducing tumor size and generating a local immune response that involved NK cells, macrophages, and T cells. In addition, a systemic response provided protection from experimental metastases from tail vein inoculation of tumor cells in a pre-established metastasis protocol. In this report, we have extended these studies by comparing the in situ AdmIL-12 protocol with a similar protocol that involved an adenovirus that expresses both subunits of IL-12 together with the costimulatory molecule B7-1. We also compared the efficacy of AdmIL-12 versus AdmIL-12/B7 in a cell-based vaccine protocol using infected, irradiated RM-9 cells. We demonstrate that orthotopic delivery of AdmIL-12/B7 may have a higher potency than AdmIL-12 because lower titers are required to achieve suppression of orthotopic tumor weight (Fig. 3) or pre-established lung metastases (Fig. 5). Although in vitro production of IL-12 appeared to be increased in the AdmIL-12/B7 vector (Fig. 1B), this did not translate into increased serum levels in vivo after orthotopic injection into RM-9 tumors (Fig. 6C). Therefore, the therapeutic benefits of AdmIL-12/B7 are not likely to be explained as simply increased production of IL-12. A possible mechanism of increased potency was revealed by the sustained intratumoral CD8 response after AdmIL-12/B7 injection (Fig. 7B). Although a lower titer of AdmIL-12/B7 may achieve a similar therapeutic response as AdmIL-12, careful monitoring of the safety of this vector is warranted because high doses tended to produce increased spleen weight compared with AdmIL-12 (Fig. 6B) at a lower dose ($5 \times 10^7$ PFU orthotopically).

Although there are numerous studies that have used IL-12 and/or B7-1 under various conditions, there are very few that have directly compared IL-12 and IL-12 + B7.1 using adenoviral vector-mediated in situ transduction of these therapeutic, immunostimulatory genes. One study to which our current study can be compared is that in which AdmIL-12 and AdmIL-12/B7...
were compared directly in an orthotopic mouse model of breast cancer. In this study, AdmIL-12/B7 was significantly more effective in delaying orthotopic tumor growth and inducing partial or complete regression. Furthermore, AdmIL-12/B7 in situ gene therapy also protected against a second inoculation of tumor cells when rechallenged 2–3 months later in those animals that had experienced complete primary tumor regression (23). However, in these studies, the breast cancer cell line was derived from transgenic mice in which breast cancer was induced by the potent immunogenic polyoma middle T antigen (23). In our studies, we used RM-9 metastatic mouse prostate cancer cells that have been shown previously to be of low immunogenicity (11) and therefore similar in that regard to human prostate cancer (10).

An additional study that compared IL-12 with IL-12 and B7 using a stable transfection protocol in the CMT93 mouse colorectal tumor was also able to demonstrate that the combination of IL-12 + B7 induced significantly higher levels of systemic protection against inoculation with live CMT93 cells relative to IL-12 or B7 alone (26). However, in this study, the CMT93 alone cells alone were also able to induce systemic antitumor immunity, and thus, the pronounced superior effect of IL-12 + B7 compared with IL-12 alone may also be related to intrinsic immunogenicity of these cells. In a liver tumor cell line that expressed low MHC class I and no MHC class II, ex vivo retroviral transduction of IL-12 + B7 was more effective than either IL-12 or B7 alone, but treating established s.c. tumors with retrovirus producing lines did not reveal a difference between IL-12 and IL-12 + B7 (27). Depletion studies suggested that CD4+ T cells appeared to be the major contributor to the antitumor effect of B7 and both CD4+ and CD8+ T cells were implicated in the effect of IL-12 alone. However, the combination of IL-12 and B7 depended mainly on CD4+ T cells with non-T cells also contributing. The increased efficacy and biological activities associated with IL-12 + B7 compared with IL-12 alone documented in this study of prostate cancer could

Fig. 7 Kinetic analysis of immune effector cells in frozen sections of tumor tissues. Frozen sections were reacted with anti-CD4 (A), anti-CD8 (B), or macrophage-selective F4/80 antibody (C). Each section was then analyzed by computer-assisted image analysis, and the number of immunopositive cells was expressed per mm2 of tumor tissue.

Fig. 8 Lysis of YAC cells by splenocytes from animals with orthotopic RM-9 tumors injected with the indicated virus. Animals were sacrificed 1 day (■) or 3 days (■) after virus injection.

Fig. 9 Vaccination with three weekly injections of irradiated, infected cells s.c. induces protection from tumor challenge with orthotopic RM-9 cells 3 weeks later. A, the percentage of animals without tumors 21 days after injection of 5000 RM-9 cells orthotopically. B, weight of orthotopic tumors at the time of sacrifice in those animals with tumors. Bars, SD.
reflect a difference in the mechanisms of antitumor effects resulting from the tumor type or possibly the tumor site, i.e., s.c. versus prostate. Although the results of these earlier studies are highly informative, in studies of prostate cancer it is important to compare the results of IL-12 with IL-12 + B7 in situ gene therapy in a model of documented low immunogenicity as we have done. Indeed, this is the first study to make these comparisons in such a prostate cancer model system.

The AdmIL-12/B7 vector may also be useful in cell-based vaccine approaches for prostate cancer. Although our study used only one vaccination schedule, we were able to induce an immune response that led to rejection of an orthotopic tumor in one-third of the animals that received the IL-12/B7 vaccine. The IL-12 vaccine was also effective with 20% of the animals protected. These studies are encouraging and suggest that both viruses may prove useful in developing further optimized cell-based vaccines for prostate cancer. Both vectors are capable of inducing NK cells after orthotopic injection into tumors (Fig. 8). Additional studies will be required to determine whether specific levels of NK or CTLs are induced by the cell-based vaccination protocols.

In prostate cancer, a clinical trial with a vaccine derived from autologous tumor cells infected with a retrovirus expressing granulocyte/macrophage-colony stimulating factor has demonstrated that this approach is safe and capable of eliciting systemic immune responses as detected by development of a delayed-type hypersensitivity response (28). Three of eight patients also generated antibodies that recognized specific proteins that were present in their prostate cancer as well as in prostate cancer cell lines and normal prostate epithelium. A limitation of this strategy is the inability to grow sufficient numbers of prostate tumor cells in vitro to provide the cancer cells for genetic modification via a retroviral vector and use as a vaccine. Other vaccine type approaches for prostate cancer have relied on delivery of autologous dendritic cells pulsed with prostate membrane-specific antigen (29). Reductions in serum PSA have been reported in ~30% of the treated patients, even those with hormone-refractory metastatic disease (29, 30). The vaccine approach that we describe here uses recombinant adenoviral vectors to transduce the cell-based vaccines. For prostate cancer, this approach is novel and might have unique value clinically for prostate cancer cell-based vaccines that have proven exceedingly difficult because of the difficulty in culturing prostate cancer cells in vitro (28).

Overall, AdmIL-12/B7 is the most effective single-vector combination in situ gene therapy strategy that we have tested thus far in our preclinical model systems. When used as an in situ approach, this therapy was more effective in comparison to IL-12 alone in suppressing localized tumor growth, as well as in models of pre-established lung metastases. It is remarkable that 6 of 19 animals treated with AdmIL12/B7 survived long term with apparent eradication of their disease in contrast to 1 of 38 animals treated with AdmIL12. In additional protocols, we also demonstrated that AdmIL-12/B7 (as well as AdmIL-12) was effective in generating systemic antitumor immunity using a cell-based vaccine strategy. On the basis of these studies, IL-12 + B7 gene therapy approaches should now be considered in both in situ and vaccine strategies as a single-vector gene therapy approach or potentially in combination with cytotoxic therapies that could potentially contribute to an immune response (8). Further development of these approaches hopefully will lead to more rational and effective therapies for this devastating disease.

REFERENCES


Prostate Cancer Gene Therapy: Comparison of Adenovirus-mediated Expression of Interleukin 12 with Interleukin 12 plus B7-1 for in Situ Gene Therapy and Gene-modified, Cell-based Vaccines

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