Chapter 15

IMMUNO-GENE THERAPY FOR METASTATIC PROSTATE CANCER

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Abstract: It has long been appreciated that a significant number of prostate cancers will not progress to clinical significance, yet some prostate cancers disseminate rapidly leading to lethal metastatic disease. Therefore, a reasonable goal of prostate cancer research is to develop novel alternative interventions and adjuvant therapeutic approaches that would suppress local tumor growth and/or impact pre-existing metastatic disease. This chapter will describe the potential for gene therapy to achieve this goal. We will review both pre-clinical and clinical studies using *in situ* gene therapy to generate a systemic immune response. These therapeutic strategies include a cytotoxic approach using the HSV-*tk* gene combined with the prodrug ganciclovir, the immune modulator gene for interleukin-12, and a novel gene that is both cytotoxic and immunomodulatory, Related to Testes-specific, Vespid and Pathogenesis proteins-1 (RTVP-1).

Key words: gene therapy, prostate cancer, immune therapy, metastasis

1. INTRODUCTION

Although the frequency of detection and treatment of prostate cancer has increased dramatically in recent years, this malignancy remains a serious threat to the lives of US men. During the last decade, a sharp increase was detected in the age-adjusted rate of mortality for prostate cancer, but fortunately this increase has begun to decline (1). However, mortality from prostate cancer remains at an unacceptably high rate. Currently available therapies for prostate cancer are limited, i.e., potentially curative localized therapy (radical prostatectomy or irradiation) or palliative androgen ablation therapy for advanced disease. The ability of radical prostatectomy or irradiation therapy to significantly reduce prostate cancer mortality must await the results of ongoing

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clinical trials, yet on the basis of current rates of biochemical, i.e., serum prostate-specific antigen (PSA), failure it appears that they will not be sufficient as single modalities. In patients treated with radical prostatectomy, increased serum PSA levels indicative of local tumor recurrence and/or metastases occurs within five years in 20% (2) to 57 % (3) of the men. The apparent inadequacy of the treatment options for presumed localized disease appears to be related, in part, to the presence of occult micrometastases at the time of diagnosis and treatment. In general there is a relationship of tumor volume with metastatic progression, however, relatively small tumors that are confined to the prostate may also seed metastases (4, 5). These clinical observations have been supported by the results of *in vivo* experiments which indicate that metastases do not necessarily originate from the most abundant clone of malignant cells at the primary site (6). An additional confounding problem with prostate cancer is that the prevalence of histologic cancers with low malignant potential is high, about 40 % in men over 50 (7, 8), suggesting that many of the cancers detected and treated may in fact be "clinically unimportant" (7). Indeed, data from some studies have shown that 10 %-26 % of nonpalpable cancers detected by PSA screening are "clinically unimportant" based on pathologic criteria, e.g., less than 0.5 cc, Gleason sum $\leq$6, and disease confined to the prostate (9–12). The detection of "clinically unimportant" cancers and their treatment with potentially harmful therapy, such as radical prostatectomy and irradiation therapy, may not be a reasonable therapeutic decision in many cases. It would be useful to have safe treatments that can supplement the current treatments for localized prostate cancer. In addition and perhaps more importantly, a treatment that provides effective anti-metastatic activity will be required to substantially reduce the mortality from this disease. Novel approaches, such as gene/immunotherapy, may provide this anti-metastatic activity. In this chapter we will review the preclinical studies which provide the rationale for anti-metastatic prostate cancer gene therapy and describe the ongoing clinical trials with these objectives in mind.

2. STRATEGIES FOR METASTATIC PROSTATE CANCER THERAPY

2.1 Conceptual Framework

Metastatic prostate cancer typically presents as disseminated multifocal disease. It is therefore often assumed that to achieve effective anti-metastatic activity against such disseminated lesions will require a systemic delivery approach. Systemic delivery of small molecules or antibodies has progressed

rapidly for several cancers and has great potential as an anti-metastatic therapy. Systemic gene therapy must attain specificity for metastatic cells and several viral and non-viral strategies have been proposed to accomplish this. Various systemic immunotherapy approaches have been developed and tested in clinical trials with limited success for metastatic prostate cancer. As an alternative to systemic delivery we are of the opinion that immunomodulatory gene therapy delivered to localized prostate cancer lesions may achieve systemic antitumor effects that impact on disseminated disease. This *in situ* immunomodulatory gene therapy approach has been termed an "active vaccine."

2.2 Systemic Gene Therapy

Several groups have constructed adenoviral vectors for gene therapy that are prostate cancer selective (13–18) with the goal of using them systemically to transduce a cytotoxic gene or selectively replicate and lyse prostate cancer cells. Although this approach is attractive and very promising, the currently available vectors for gene therapy may not have the capacity to achieve complete infection of disseminated metastatic cell deposits within an acceptable toxicity profile. Indeed, it has been suggested that the systemic delivery of currently available adenoviral vectors may be inappropriate (19). Several investigators have used liposomes for systemic gene delivery in animal models (reviewed in 20, 21); however, it is unclear whether liposomes can successfully target genes to all sites where metastases develop.

2.3 Immunomodulation by Cytokine Gene Delivery

Enhancement of antigen presentation by stimulation of antigen presenting cells is exemplified by the use of granulocyte macrophage-colony stimulating factor (GM-CSF) gene modified cancer cell vaccination. A promising preclinical study with an anti-tumor vaccine comprised of irradiated autologous GM-CSF secreting-Dunning rat prostate carcinoma cells (22) led to a clinical trial. Eight of eleven patients with prostate cancer were treated with autologous GM-CSF-secreting, irradiated tumor cell vaccines prepared by *ex vivo* retroviral transduction of GM-CSF into surgically harvested cells (23). Insufficient cells were obtained from the other three patients. The treatment resulted in dendritic cell and macrophage infiltration at the injection site and activation of T-cells and B-cells against prostate cancer antigens, representing both Th1 and Th2 T cell response. No systemic side effects were reported. The major limitation of immunotherapy

for prostate cancer using similar approaches is poor recovery and growth of cancer cells from clinical specimens.

Many immunomodulatory gene therapy approaches target the enhancement of the cellular response through the delivery of specific cytokine genes. In our opinion the *in situ* delivery of cytokine genes has several potential advantages and relatively few disadvantages. The presence of viable tumor cells at the site of treatment allows for viable tumor cells and peptides derived from these cells to act as the vaccine, thus this approach has been termed "*in situ* adenoviral vector-mediated gene-modified active vaccination." Below we discuss specific cytokines that are promising candidates for this therapeutic strategy.

2.4 Prostate Cancer as a Model System for Gene Therapy

As summarized at a recent conference on gene therapy and immunology, the choice of vector for a gene therapy trial should also take into account the delivery route, transcriptional regulation, target cell and clinical status of the patient in order to minimize toxicity and maximize efficacy (24). The selection of an appropriate gene delivery system is critical for treatment success. In theory the ideal gene delivery system for metastatic prostate cancer would transfer a therapeutic gene to all cancer cells while causing no adverse events and sparing healthy tissues. In practice, however, it is highly unlikely that such an ideal scenario can be achieved using currently available technologies. Fortunately some alternative approaches do not rely on achieving this level of initial targeting. Localized, *in situ*, prostate cancer gene therapy can have distant effects mediated by factors such a bystander effect or induction of host immunity. *In situ* gene therapy involving the direct injection of a viral vector into the tumor is well suited for prostate cancer for a number of reasons. Prostate cancer is highly accessible to gene transfer relative to many other tumor sites. Transrectal ultrasound guidance is a routinely used imaging modality that has proved exceedingly useful not only for biopsies but also for other clinical applications. Gene therapy can be administered through ultrasound guided needle injections directly into hypoechoic sites of presumed tumor foci and the spread of vector during injection monitored to a certain extent. The expendability of the prostate in older men and the relatively slow growth of local prostate tumors make the combined use of *in situ* gene therapy with neoadjuvant/adjuvant approaches such as surgery or irradiation therapy an attractive therapeutic option. *In situ* gene therapy for prostate cancer may result in anti-metastatic benefits since the generation of cytotoxic activities against localized prostate cancer

may generate immunological activities affecting not only the primary tumor but also metastatic disease. We have pioneered a gene therapy approach using adenoviral vector delivery *in situ* for prostate cancer and will review the preclinical and clinical progress we have made with so-called "suicide gene therapy" using delivery of the Herpes Simplex Virus (HSV) thymidine kinase (*tk*) gene followed by a course of the pro-drug ganciclovir (GCV) or valacyclovir and our preclinical studies using immunomodulatory gene therapy.

3. *IN SITU* HSV-TK+GCV GENE THERAPY

3.1 Preclinical Evaluation

We developed and used preclinical mouse models of metastatic prostate cancer to demonstrate that extensive cytotoxic activity could be generated *in vivo* using adenoviral vector-mediated HSV-*tk* gene transfer followed by a course of systemic GCV. This gene therapy protocol established that a single vector injection not only suppressed the growth of local orthotopic mouse prostate cancer through necrotic and apoptotic activities, but also significantly reduced the size and number of pre-established metastatic lung foci (25–27) and reviewed in (28). Further preclinical studies demonstrated a satisfactory toxicological profile for this gene therapy protocol (29). We have recently developed additional vectors and compared the use of strong non-specific promoters such as the Rous Sarcoma Virus (RSV) or cytomegalovirus (CMV) promoters with one derived from the caveolin-1 (cav-1) gene that confers a unique specificity for prostate cancer and tumor associated endothelial cells (30). We have also expanded our toxicological evaluations to address the potential mechanisms of hepatoxicity of specific adenoviral vectors (31).

HSV-*tk* + GCV has also found to be effective when combined with other forms of therapy. In an androgen responsive prostate cancer model, castration therapy enhanced the effectiveness of HSV-*tk*+GCV gene therapy (27). *In vitro* and *in vivo* studies designed to evaluate the combination of gene therapy with radiation therapy demonstrated additive, if not synergistic, effects in cancer cell killing and in animal survival for the combined therapy when compared to either treatment alone (32, 33). Although the specific mechanism of action of systemic HSV-*tk*-stimulated anti-metastatic activity in prostate cancer models is still under investigation, it is believed that this involves a non-specific immune cell response of natural killer mainly (NK) cells (34, 35). Systemic depletion of NK cells in mice with orthotopic

prostate tumors before and during the course of HSV-*tk*+GCV gene therapy confirmed the importance of this cell type in both local tumor control and control of pre-established lung metastases (35). In other models with retrovirus transduced cancer cells, systemic T cell responses have also been implicated (36–38).

3.2 Clinical Evaluation

Baylor College of Medicine conducted the first *in situ* gene therapy Phase I clinical trial for human prostate cancer and demonstrated safety of *in situ* HSV-*tk* gene therapy. In this clinical trial men with biochemical recurrence of localized prostate cancer following radiation therapy received a single injection of the adenoviral vector. Some toxicity was observed at the highest dose (1×10^{11} IU) and there were indications of efficacy as serum PSA levels in 3 of 18 patients were suppressed by 50 % or more (39). This clinical trial was extended to an additional 18 patients with most receiving a dose of 1×10^{10} IU. Additional safety studies confirmed that this dose was safe even when administered at multiple sites or when repeated for up to three times (40). Further analysis of the patients in this gene therapy protocol indicated that this experimental treatment led to an increased PSA doubling time, a significantly increased mean percentage PSA reduction, and a significantly increased mean time to return to initial PSA following initial or repeat vector injections. An immune component in the response to this gene therapy protocol was evidenced by increased levels of activated (HLA DR+) CD8+ T cells in the peripheral blood following treatment, and interestingly, an increase in the density of CD8+ T cells in post-treatment biopsies. This latter observation was correlated with an increased number of apoptotic cells (41).

Having demonstrated the safety and potential efficacy of HSV-*tk* + GCV gene therapy in men with a biochemical recurrence of their prostate cancer after radiation therapy we expanded the clinical trial to a group of men with newly diagnosed prostate cancer with clinical markers that suggested high grade disease and who elected to undergo a radical prostatectomy. In this neo-adjuvant trial, the *in situ* gene therapy was delivered four to six weeks before surgery. The availability of the radical prostatectomy specimen allowed a clear demonstration that *in situ* gene therapy induced local inflammation within prostate cancer foci with an increased infiltration of CD4 and CD8 T cells (42). Remarkably, this form of therapy induced necrosis within prostate cancer lesions in preference to adjacent normal prostatic tissues (42). Further studies confirmed that HSV-*tk*+GCV gene

therapy treatment led to increased numbers of HLA DR+ $CD8^+$ T cells in the peripheral blood of these men.

An additional Phase I-II trial in progress combines two to three doses of HSV-*tk* with standard of care radiotherapy and replaces intravenous GCV with the oral bioequivalent drug valacyclovir. Men in this trial were stratified to three groups, low stage disease (PSA <10 ng/ml, biopsy Gleason score <7 and clinical stage T1-T2a), high stage disease (PSA >10 ng/ml, biopsy Gleason score >7 and clinical stage T2b-T3), or stage D1 (regional lymph node metastases). The latter two groups also received concurrent hormonal therapy. Mild hematologic and hepatic abnormalities could be attributable to the gene therapy while genitourinary and gastrointestinal side effects were typical radiation-related side effects. There was no added toxicity attributable to the combination therapeutic approach (43).

4. *IN SITU* INTERLEUKIN-12 GENE THERAPY

4.1 Preclinical Evaluation

Based on our preliminary data from adenoviral vector-mediated HSV-*tk* + GCV clinical trials suggesting a role for Interleukin-12 (IL-12) in the immune response (unpublished data) and other promising results indicating the potential for IL-12 mediated cancer gene therapy we considered IL-12 as the next step in the development of gene/immunotherapy for prostate cancer. We have conducted preclinical studies using adenoviral vectors that overexpress IL-12 in a highly aggressive mouse model of prostate cancer. Orthotopic tumors were established by injecting only 5000 cells of the mouse prostate cancer cell line RM-9 directly into the prostate. One week later when the tumor has achieved a size of around 10 to 20 mm^3 it is injected with the adenoviral vector and the animals observed for the next two weeks. At the end of this period they are sacrificed and the treatment effects evaluated. Initial studies used doses ranging from $1 \times 10^7$to 3×10^8 PFU per tumor. Systemic toxicity was not observed at doses of 1×10^8 or lower. However, in the 3×10^8 group some mice developed increased amounts of ascites but no lethality was observed. A dose-related splenomegaly was seen at the time of sacrifice in all treated animals. To further evaluate potential toxicity we performed a kinetic analysis with measurements of spleen weight and serum concentration of IL-12 during a period of ten days after vector injection. Serum IL-12 was maximal one day after vector injection and enlargement of the spleen was seen after a lag time of several days, with the maximum spleen size observed on day 7. No serum IL-12 or splenomegaly

was seen in animals injected with a control vector (Adv/CMV/βgal) or PBS. The AdIL-12 induced splenomegaly was reversible, as a gradual decrease of the serum IL-12 strongly correlated with a return to normal spleen size (44).

Compared to controls AdIL-12 significantly suppressed localized tumor growth as well as spontaneous and experimental metastatic activities (35, 44, 45). Metastatic cells were detected in the pelvic lymph nodes in 50 % of the animals with orthotopic tumors treated with AdIL12 compared to 83 % of control animals (44). In the pre-established lung metastasis model 100,000 RM-9 mouse prostate cancer cells are injected into the tail vein at the same time that an orthotopic tumor is established. The orthotopic tumor is injected with adenoviral vector with either IL-12 or βgal six days later and the number of lung metastases evaluated eight days after that. Animals in the AdIL-12 treatment group had 16 ± 1 lung metastases compared to 62 ± 3 in the Adv/CMV/βgal treatment group (44). We also evaluated the ability of adenoviral vector-mediated IL-12 treatment of orthotopic prostate tumors to prolong survival. Mean survival for the control group injected with Adv/CMV/βgal (n=36) was 23.4 ± 0.8 days, while in the AdIL-12 treatment group (n=38) the mean survival was 28.9 ± 1.2 days. This survival advantage for the AdmIL-12 group was significant ($p < 0.0001$) and one animal that appeared to be a long term survivor was sacrificed on day 50 and found to have only microscopic tumor.

Several techniques were used to determine the immune effector cells that contributed to the antitumor and/or antimetastatic activity. We observed enhanced NK cell cytolytic activity during the first 7 days following AdIL-12 injection. Immunohistochemical analysis of tumor specimens revealed that antitumoral activities likely resulted to a large extent from 1) enhanced NK lytic activity during the shortly after virus injection, 2) enhanced macrophage activities such as NOS activation and 3) supportive cytokine production and possible cytolytic activities of $CD4^+$ and/or $CD8^+$ T cells. Multiple immune activities, that potentially could develop into a systemic anti-tumor immune response, involving the generation of memory T cells were evident, and the results of analysis of distant antimetastatic activity in response to local injection of AdmIL-12 further supported this notion.

4.2 Clinical Studies

These encouraging preclinical data led us to construct a replication-defective adenoviral vector containing the human IL-12 genes and evaluate it for clinical trials. The mRNA's for both IL-12 subunits (p35 and p40) were induced in the human lymphoblast cell line NC-37 (ATCC CCL 214) with

phorbol 12,13-dibutyrate. The p35 and p40 cDNAs were used to construct an adenoviral vector with the encephalomyocarditis virus IRES. Single plaques were purified and used to generate an original virus preparation termed AdhIL-12. This virus was then used to manufacture a clinical grade version under GMP conditions by the Baylor Center for Cell and Gene Therapy Vector Core Facility. The seed vector and production lot to be used in the clinical trials was then extensively tested for quality control. Biological activity of the vector was confirmed by infection of cells in culture and evaluating the media after 48 hours for the presence of IL-12 by enzyme linked immunoassay.

Our IRB and FDA approved Phase I study involves direct intratumoral injection of a replication deficient adenoviral vector mediating expression of the human IL-12 gene in patients with prostate cancer who can be either hormone naïve or hormone refractory. Men who have a biopsy-proven recurrence of prostate cancer in association with a rising serum PSA level following radiotherapy will be eligible for enrollment. Men with a recurrence of their prostate cancer following radical prostatectomy will be eligible if they have metastatic disease either with the a soft tissue component suitable for vector injection. Patients with metastatic prostate cancer who are hormone refractory do not have any standard treatment available that has proven to be highly efficacious in eradicating the tumor with a reasonable degree of safety. For patients who are hormone naïve, hormonal ablation is only a palliative measure and hormone resistance develops over time in most patients. Therefore, the potential risks of the protocol seem justifiable and reasonable. The objective of the study is to assess the safety of direct intraprostatic injection of an adenoviral vector mediating expression of the IL-12 gene. Acute and long-term toxicity resulting from this treatment will be evaluated. The initial dose will be 1×10^{10} viral particles injected directly into the prostate under ultrasound guidance or into soft tissue metastases. This dose is essentially equivalent to the effective dose used in animal studies and below the maximal tolerated dose for mice. Since the human prostate weighs 20 to 50 g, whereas the mouse prostate is only 15 to 30 mg, we do not anticipate any toxic effects at this low dose. Doses will be increased by 1/2 log increments to a maximal dose of 5×10^{12} viral particles or until unacceptable toxicity is observed. Additional laboratory analyses such as detection of specific immune cells in the peripheral blood and evaluation of tissue samples for apoptosis, angiogenesis, and immune cell infiltrates will be performed for research purposes to evaluate potential mechanisms of action.

5. ADDITIONAL *IN SITU* IMMUNOMODULATORY GENE THERAPY APPROACHES

5.1 IL-12 + Co-stimulatory Molecules

Another immunomodulatory approach is to deliver genes encoding co-stimulatory molecules capable of promoting activation of T cells to a cytotoxic state (45). The enhancement of T-cell response using this approach is exemplified by experiments involving B7-1 gene therapy. B7-1 is poorly expressed on most tumor cells surface (46). The B7-1 gene combined with the IL-12 gene on an adenoviral vector (AdmIL-12/B7) was compared with IL-12 gene therapy alone in the RM-9 orthotopic murine prostate cancer model (45). Animals treated with AdmIL-12/B7 lived slightly longer 40 days versus 37 days with AdIL-12 alone at a dose of 1×10^8 PFU and a lower dose of AdmIL-12/B7 was even more effective with a mean survival time of 48 days with 20 % having no evidence of tumor on day 50. Interestingly, the therapeutic benefits of the lower dose of AdmIL-12/B7 also appeared to extend to anti-metastatic activities. As depicted in Figure 1 both IL-12 and IL-12/B7 gene therapy led to suppression of pre-established metastases. The number of lung metastases in the animals treated with AdmIL-12/B7 at 5×10^7 was reduced to only 7.8 ± 3.7 whereas the higher dose (1×10^8) yielded slightly more metastases with 12.9 ± 2 and AdmIL-12 was the least effective with 17.2 ± 3.8 lung metastases. This suppression of lung metastases was significant in all treated mice compared to controls ($P \leq 0.001$)

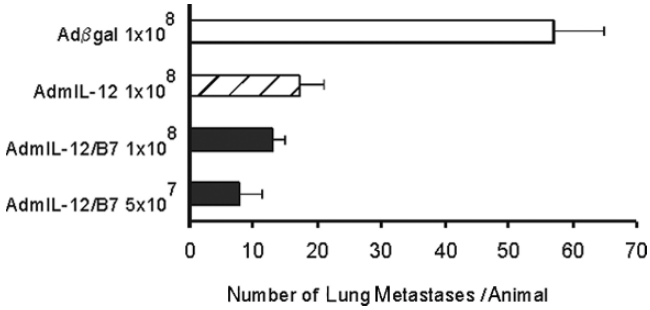

Figure 1. Anti-metastatic activity of *in situ* delivery of adenoviral vectors to orthotopic tumors. Lung metastases were established be tail vein injection at the time of orthotopic tumor establishment. Six days later the orthotopic tumors were transduced with the indicated adenoviral vector. Adapted from (69) .

but the differences between each treatment did not achieve significance (45). As with AdmIL-12 studies (Section 4.1), there was an increase in the number of immune cells in the tumor tissues with intratumoral CD4+ and CD8+ T lymphocyte infiltration similar seven days after injection but AdmIL-12/B7 treated tumors sustained the level of CD8+ cells longer after injection (45). Although a lower titer of AdmIL-12/B7 achieved a similar therapeutic response as AdmIL-12, a careful toxicological study of this vector is warranted; since higher doses tended to generate increased spleen weights compared to AdmIL-12.

The AdmIL-12/B7 vector may also be useful in cell-based vaccine approaches for prostate cancer. We tested a vaccination schedule with subcutaneous injections of transduced, irradiated cells every week for three weeks and were able to induce an immune response that led to rejection of an orthotopic tumor in one third of the mice which received the IL-12/B7 vaccine The IL-12 vaccine was also effective with one fifth of the mice protected from subsequent challenge with an orthotopic tumor (45). These studies are encouraging and suggest that both viruses may prove useful developing further optimized cell-based vaccines for prostate cancer.

5.2 IL-12 Combined with HSV-*tk*+GCV

AdIL-12 has also been directly compared with AdHSV-*tk*+GCV in the RM-9 mouse prostate cancer model (35). AdIL-12 alone had somewhat superior activity with in regard to local cytotoxicity, overall prolongation of survival time and antimetastatic effects. Combining both therapies at an optimal dose for each resulted in significantly increased suppression of orthotopic tumor growth but did not have any greater antimetastatic effects or improve survival time compared to AdIL-12 alone. HSV-*tk*+GCV therapy induced higher levels of necrosis in the orthotopic tumors compared to AdmIL-12 or combination therapy. Single and combination therapies all produced similar increases in apoptotic index in the local tumor. Quantitative immunohistochemical analysis of tumor-infiltrating immune cells indicated that HSV-*tk*+GCV therapy alone led to detectable increases in iNOS-positive cells, CD4+ and CD8+ T-cells and moderately increased numbers of F4/80 (macrophage selective)-positive cells. In contrast, AdmIL-12 elicited a highly robust pattern of tumor infiltration for all four immune cell markers. Combination therapy yielded results similar to AdIL-12 alone. Interestingly, local injection with AdHSV-*tk*+GCV induced splenocyte-derived NK cell cytolytic activities with a maximal response 6 days following treatment, whereas AdmIL-12 injection produced significantly higher NK activity with a maximal response 2 days following injection.

The combined treatment produced a higher systemic NK response over the entire 14-day treatment period. Depletion of NK cells *in vivo* demonstrated that this immune cell subpopulation was responsible for early locally cytotoxic activities induced by AdHSV-*tk*+GCV but not AdmIL-12 and that NK activities were largely responsible for activities against pre-established metastases demonstrated by both gene therapy protocols.

6. FUTURE DIRECTIONS - RTVP-1 GENE THERAPY

The overiding goal of our gene therapy program is to generate an "active vaccine" *in situ* through the cytotoxic effects acting either directly or indirectly. As discussed above there is preclinical and clinical evidence for the direct cytotoxic effects of HSV-*tk*+GCV gene therapy and the preclinical studies suggest that IL-12 gene therapy induces cytotoxicity indirectly through NK cell and T cell activities. It will likely be necessary to develop a greater understanding of the molecular basis of prostate cancer metastasis to design highly effective anti-metastatic therapies. The molecular changes that underlie stepwise development of malignancy from normal cells have been studied extensively during the last two decades (reviewed in 47). However, only recently have investigations been focused on genes that are specifically involved in regulation of the metastatic phenotype. Interestingly, numerous studies support a metastasis suppressor role for p53 in prostate cancer (reviewed in 48). Therefore p53-regulated genes or downstream targets of p53 may provide novel agents for future clinical trials.

To identify prostate cancer-related genes under the transcriptional regulation of p53, we used infected a p53 deficient mouse prostate cancer cell line (6) with an adenoviral vector that expresses wild-type p53 (49) used differential display-PCR to compare RNA expression with a control vector expressing β-galactosidase. Using this approach, we isolated numerous sequences that were known to be under p53 control including cyclin G, epoxide hydrolase, and MDM2. In addition, we isolated genes that had not been previously associated with p53 regulation. One of the sequences we identified encoded the mouse homologue for RTVP-1 (Related to Testes-specific, Vespid and Pathogenesis proteins) (50). In our efforts to understand the regulation of the mRTVP-1 gene by p53, we isolated genomic mRTVP-1 and sequenced a significant portion of this gene (~11 kb), including 3.5 kb of the promoter region, exon 1, intron 1, exon 2 and intron 2. Sequence analysis revealed four potential p53 binding sites that were located in

exon 1 and intron 1. Co-transfection studies and electrophoretic mobility shift analyses led to the detection of site in intron 1 as the sole p53-specific binding site. Our results conclusively determined that mRTVP-1 is a direct p53 target gene.

We also evaluated expression of RTVP-1 in mouse and human cell lines. Northern blotting analysis of non-transformed and transformed mouse and human cell lines expressed relatively low levels of RTVP-1 mRNA. Three cell lines including two non-transformed cell lines (MEF p53 +/+ and HUVEC) and a transformed human cell line (CCD-11) that contains wild type p53 expressed significant levels of RTVP-1 mRNA. However, in general, there was no evidence for linkage between the expression of wild-type p53 and RTVP-1. Western blotting analyses revealed somewhat higher levels of RTVP-1 protein than expected from the northern blots and RTVP-1 protein levels were not related to p53 or p21 protein levels. These blotting studies and induction studies with γ-irradiation or doxorubicin indicate p53 independent regulation of RTVP-1.

Overexpression of mRTVP-1 resulted in apoptosis in multiple cancer cell lines, suggesting that mRTVP may play a role in inhibition of malignant growth and progression through its pro-apoptotic activities. In our initial report we overexpressed RTVP-1 by either transfection of a plasmid or by transduction with an adenoviral vector and induced apoptosis in various cancer cell lines *in vitro*. We further showed that the signal peptide was important for RTVP-1-mediated apoptosis suggesting that the secreted form of RTVP-1, in part, mediated these activities. Overall, our results indicated that RTVP-1 is a uniquely acting p53 effector gene with therapeutic potential. Although the human RTVP-1 cDNA was originally cloned from human glioma tissue (51, 52) and was subsequently reported to be expressed in differentiated macrophages (53) the functional significance of RTVP-1 expression and the potential for gene therapy applications of RTVP-1 have not been reported. Preliminary studies in an orthotopic model suggest that adenoviral vector mediated RTVP-1 transduction leads to suppression of tumor growth and lung metastasis and a prolongation of survival (54). These therapeutic responses may be associated with RTVP-1 mediated activities other than apoptosis as increased numbers of specific tumor-infiltrating immune cells were also observed in these studies (54). Thus, RTVP-1 may be considered as a potential therapeutic gene for prostate cancer and potentially other malignancies based on its pro-apoptotic and immunostimulatory properties.

7. CONCLUSIONS

An overiding concept/goal of our gene therapy program is to generate an "active vaccine" *in situ* through direct (e.g., HSV-*tk*+GCV) or indirect (e.g., NK activity via IL-12 stimulation) tumor cell cytotoxicity. Cytotoxicity combined with the capacity to induce the activation of a Th1 response (e.g., IL-12) may lead to a systemic antitumor response that impacts on metastatic disease. A novel gene we have identified, RTVP-1, may be an optimal therapeutic gene for use in "active vaccine" protocols based on its potential for direct (apoptosis induction) and indirect (immune cell recruitment and activation) activities. Because of the general difficulty of generating and maintaining specific antitumor immunity, we anticipate that combination gene(s) and vaccine(s) protocols will ultimately be required to generate therapeutically significant results. Therefore, the identification of novel tumor antigens that could be useful as vaccines alone or in combination with immunostimulatory gene therapy protocols should be actively pursued.

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