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# ADENOVIRUS-MEDIATED HERPES SIMPLEX VIRUS THYMIDINE KINASE GENE AND GANCICLOVIR THERAPY LEADS TO SYSTEMIC ACTIVITY AGAINST SPONTANEOUS AND INDUCED METASTASIS IN AN ORTHOTOPIC MOUSE MODEL OF PROSTATE CANCER

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**It is critical to develop new therapies, such as gene therapy, which can impact on both local and metastatic prostate cancer progression. We have developed an orthotopic mouse model of metastatic prostate cancer using a cell line (RM-1) derived from the mouse prostate reconstitution (MPR) model system. This mouse model closely simulates the anatomical and biological milieu of the prostate and allows for realistic testing of experimental gene therapy protocols. Adenovirus (ADV)-mediated transduction of the herpes simplex virus thymidine kinase (*HSV-tk*) gene in conjunction with ganciclovir (GCV) in this model led to significant suppression of growth and of spontaneous metastasis at 14 days post-tumor inoculation. Longer-term studies produced a significant survival advantage and a continued suppression of metastatic activity for treatment animals despite regrowth of the primary tumor. Challenge by injection of tumor cells into the tail vein following excision of treated and control s.c. primary tumors resulted in 40% reduction in lung colonization in the treatment group, indicating the possible production of systemic anti-metastatic activity following a single *in situ* treatment with ADV/*HSV-tk* + GCV in this model system.** ***Int. J. Cancer, 70:183–187, 1997.***
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Transduction of genes which activate prodrugs to produce cytotoxicity in infected cells is now considered a potential therapeutic strategy for cancer treatment. One specific cytotoxic gene therapy approach is viral transduction of the herpes simplex virus thymidine kinase (*HSV-tk*) gene followed by the systemic administration of ganciclovir (GCV) (Moolten, 1986). The *HSV-tk* gene product phosphorylates GCV, activating its potential to terminate DNA synthesis. This approach involves the ''bystander effect'', whereby the number of cells killed significantly exceeds the number of cells transduced with the foreign gene (Freeman *et al.,* 1993). Adenovirus (ADV)-mediated *HSV-tk* + GCV therapy has been used in various tumor model systems to achieve local control, variable prolongation of survival and, in some cases, significant cure rates (Bonnekoh *et al.,* 1995; Chen *et al.,* 1994, 1995; Eastham *et al.,* 1996; Elshami *et al.,* 1996; O'Malley, *et al.,* 1995; Perez-Cruet *et al.,* 1994). Previously, our group demonstrated that ADV/*HSV-tk* + GCV therapy in the mouse prostate cancer cell line RM-1 resulted in sensitivity to GCV both *in vitro* and *in vivo* using a s.c. tumor model (Eastham *et al.,* 1996). However, the effects of ADV/*HSV-tk* + GCV on metastatic activity were not addressed in these studies.

Orthotopic inoculation of tumor cells can result in potentiation of the malignant phenotype (local tumor growth, invasion and metastatic activity) when compared with the s.c. model (Fidler, 1990). Prostatic injection of the RM-1 cell line resulted in a metastatic model of mouse prostate cancer suitable to evaluate candidate gene therapy protocols with regard to metastatic progression. Using this model, we tested *in situ* injection of the primary prostatic tumor with ADV/*HSV-tk* followed by GCV therapy, with particular attention to the possible effects on metastatic activity. Our results indicate that this gene therapy protocol produces a significant reduction in spontaneous metastases and systemic activity against lung colonization following tail vein injection of tumor cells.

## MATERIAL AND METHODS

### *Mouse prostate cancer cell line*

The mouse prostate cancer cell line RM-1 was derived from a primary prostate tumor induced in the Zipras/*myc*-9-infected mouse prostate reconstitution (MPR) model system using C57BL/6 mice, as previously described (Thompson *et al.,* 1989; Baley *et al.,* 1995). Cells were grown in DMEM with 10% FBS, 10 mM HEPES, penicillin (100 U/ml) and streptomycin (100 mg/ml), passaged by trypsinization with 0.025% trypsin, and maintained with routine media changes. All chemicals for cell culture were obtained from GIBCO (Gaithersburg, MD).

### *Orthotopic tumor induction*

Following trypsinization, cells were counted and resuspended in Hank's buffered saline solution at designated concentrations for injection. For orthotopic tumor inoculation, syngeneic C57/BL6 mice were anesthetized with sodium pentobarbital. A low abdominal transverse incision was made and the dorso-lateral prostate exposed. Injection of 1,000 cells in 10 µl directly into the right or left lobe of the dorso-lateral prostate resulted in efficient and reproducible tumor formation and documented metastatic activity in pelvic, retroperitoneal and mesenteric lymph nodes by 14 days post-tumor inoculation (data not shown).

### *ADV vectors*

A replication-defective recombinant ADV carrying the *HSV-tk* gene under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter was prepared as previously described (Chen *et al.,* 1994). Likewise, a replication-defective recombinant ADV vector containing the bacterial β-galactosidase gene under the transcriptional control of the RSV-LTR promoter (ADV/*RSV-β-gal*) was prepared in a similar fashion as described previously (Stratford-Perricaudet *et al.,* 1992). Recombinant ADVs were isolated from a single plaque, expanded in the 293 cell line and purified by double cesium gradient ultracentrifugation (Graham and Pervec, 1991). Virus titer was determined by plaque assay in 293 cells and reported as plaque-forming units (PFU).

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In vivo *gene therapy*

Since tumors were metastatic by 2 weeks post-inoculation, we chose day 7 as a starting point for gene therapy studies. At this time point the primary cancers had essentially replaced the lobe of the prostate originally inoculated, averaging 10–15 $mm^3$, and there was no evidence of metastasis (data not shown). To facilitate direct injection of the ADVs, animals were anesthetized with sodium pentobarbital and the previous incision re-opened. The tumor was measured by a vernier caliper and directly injected with ADV. The incision was then closed with clips. Tumor volume prior to virus injection was calculated by the formula of a rotational ellipsoid: $m_1^2 \times m_2 \times 0.5236$, where $m_1$ represents the shorter axis and $m_2$ the longer axis (Janik *et al.,* 1975). Animals were divided into 2 groups to receive $5 \times 10^8$ PFU of either ADV/*RSV-tk* or ADV/*RSV-β-gal*. This dose of virus was based on dose-escalation studies presented earlier (Eastham *et al.,* 1996). Beginning the following day i.p. injections of GCV (10 mg/kg) or PBS were performed twice daily for 6 days. Animals euthanized on the 14th day post-tumor inoculation underwent a careful autopsy for gross metastasis. The primary tumor was removed and weighed. 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. All tissues were placed in formalin, paraffin-embedded, cut in 4–5 μm sections and stained with hematoxylin and eosin for histological examination.

Survival studies were set up utilizing the same 4 groups of animals in identical fashion. Study end-points were animal death or sacrifice when appearing in distress, as evidenced by lethargy, ruffled fur or weight loss. At autopsy, gross metastases were noted; the primary tumor was removed and weighed. The pelvic and retroperitoneal lymph nodes and lung samples were likewise processed for histological analysis regardless of gross appearance. An animal was scored as having metastasis if any lymph node and/or lung had microscopic evidence of metastasis. Conversely, an animal was scored as free of metastasis if there was no evidence of activity in any tissue examined microscopically.

*Tail vein inoculum challenge*

Tail vein inoculum challenges were performed to ascertain whether systemic anti-metastatic activity could be induced against a second tumor challenge following a single treatment with ADV/*RSV-tk* + GCV. Ideally, this experiment would be performed in the absence of the treated primary tumor. However, complete surgical removal of orthotopic tumors was not feasible due to the high risk of i.p. tumor spillage and the mortality encountered from the procedure. Therefore, these experiments were carried out in the s.c. model, as previously described (Eastham *et al.,* 1996). Briefly, tumors were established following s.c. injection of $4 \times 10^6$ RM-1 cells (day 0). ADV/*RSV-tk* vector was injected on day 2. Beginning the following day, either GCV or PBS was administered i.p. for 6 days. Under pentobarbital-induced anesthesia on day 9 post-tumor inoculation, s.c. tumors were surgically removed and 50,000 RM-1 cells were injected *via* the dorso-lateral tail vein. Animals were euthanized 2 weeks later, the lungs removed and fixed in Bouin's solution and individual visible lung metastases counted with the aid of a dissecting microscope.

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

## RESULTS

In vivo *gene therapy*

At 14 days post-tumor inoculation, the day following termination of GCV treatment, therapy with ADV/*RSV-tk* + GCV resulted in significant growth suppression by wet weight measurements ($p < 0.0001$, unpaired *t* test) (Fig. 1). As previously documented in the s.c. model (Eastham *et al.,* 1996), tumors treated with ADV/*RSV-tk* + GCV displayed large areas of necrosis with infiltration of leukocytes, though there were interspersed patches of viable cells. In contrast, control tumors were notable for a few scattered areas of necrosis lacking a strong, leukocytic infiltrate between large sheets of viable cells (data not shown). Furthermore, treatment with ADV/*HSV-tk* + GCV resulted in suppression of metastatic activity compared with controls. Overall, 71.4% (10/14) of the pooled controls had metastases compared with 12.5% (2/16) of the *HSV-tk* + GCV treatment group ($p = 0.0032$, Fisher's exact test) (Fig. 2). No macroscopic lung metastases were noted in any animal, though microscopic analysis of lung specimens showed tumor emboli in <5% of control animals screened. While no treatment animals had such tumor emboli, the incidence of lung metastasis was too low for realistic comparison.

Treatment with ADV/*RSV-tk* + GCV resulted in statistically significant prolongation of survival, with mean survival of 16.8 ± 0.15 days for controls and 18.9 ± 0.33 days for treated animals

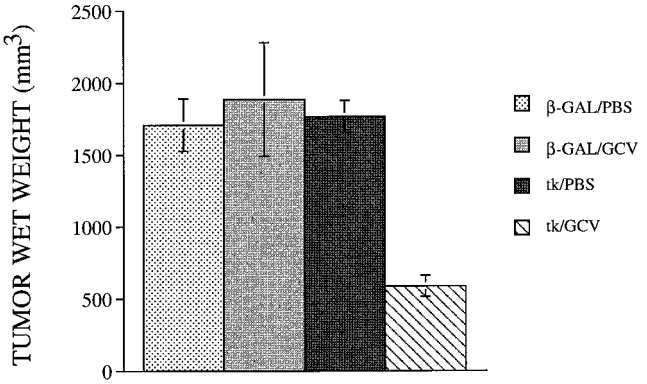


**FIGURE 1** – Primary tumor wet weight at 14 days. All animals appeared healthy at the time of death. Tumors treated with ADV/*RSV-tk* + GCV were significantly smaller than controls ($p < 0.0001$, *t* test) (*β-gal*/PBS: n = 6, *β-gal*/GCV: n = 4, *tk*/PBS: n = 4, *tk*/GCV: n = 16).

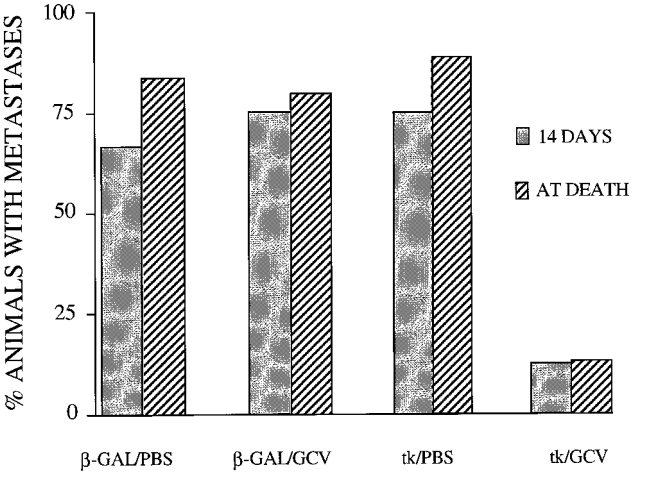


**FIGURE 2** – Percentage of animals with metastasis. At 14 days (solid bars), 71.4% of controls had metastases *vs.* 12.5% of treatment animals ($p = 0.0032$, Fisher's exact test), while at death (hatched bars) 81% of control animals had metastases *vs.* 13.7% of treatment animals ($p < 0.0001$, Fisher's exact test). All metastases documented were in pelvic, retroperitoneal and/or mesenteric lymph nodes. The presence or absence of metastasis was confirmed histologically.

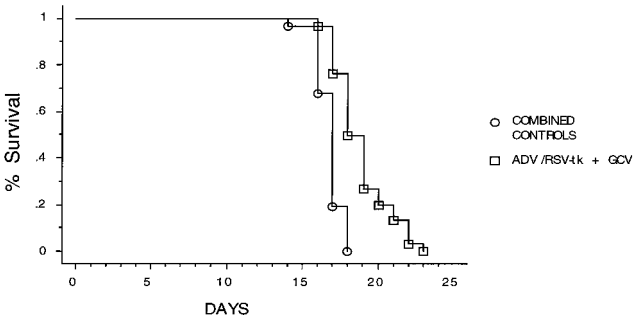


**FIGURE 3** – Kaplan-Meier survival curve in combined controls *vs.* ADV/*RSV-tk* + GCV-treated animals. Combined controls include animals treated with ADV/*RSV-tkβ-gal* + PBS (n = 13), ADV/*RSV-β-gal* + GCV (n = 10) and ADV/*RSV-tk* + PBS (n = 9). ADV/*RSV-tk* + GCV therapy led to significant prolongation of survival ($p < 0.0001$, Mantel-Cox rank test).

($p < 0.0001$, Mantel-Cox rank test) (Fig. 3). There was no difference in survival between the 3 control groups (data not shown). The Kaplan-Meier curve indicates a survival advantage for approximately 50% of the animals treated with ADV/*RSV-tk* + GCV. Examination of animals from the survival study revealed a continued suppression of metastasis: 81.2% (26/32) of pooled controls and 13.7% (4/29) of treatment animals had documented metastases ($p < 0.0001$, Fisher's exact test) (Fig. 2). In the control group, tumors with a wet weight above a threshold weight of 1,400 mg had an 82.5% (33/40) incidence of metastasis (Fig. 4). In contrast, only 13% (3/23) of the ADV/*RSV-tk* + GCV-treated animals with tumors greater than 1,400 mg had metastases, indicating that metastasis suppression continued despite primary tumor regrowth.

*Tail vein inoculum challenge*

Systemic challenge of RM-1 cells by tail vein injection following excision of the s.c. primary tumor revealed a significant difference in the number of lung metastases counted between animals treated with ADV/*RSV-tk* + GCV and ADV/*RSV-tk* + PBS: 63 ± 9.2 (range 26–109) *vs.* 100.5 ± 7.7 (range 71–117) ($p = 0.0098$, *t* test), (Fig. 5). On an individual basis, there was no correlation between response to therapy as judged by tumor wet weight at the time of removal prior to tail vein challenge and the number of lung metastases counted.

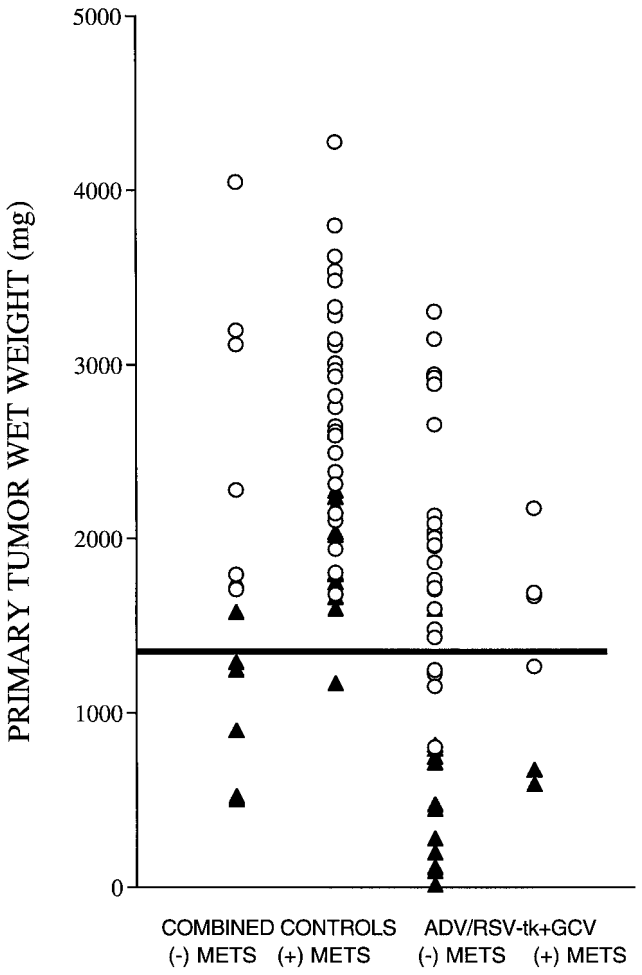


**FIGURE 4** – Scattergram of primary tumor wet weight *vs.* the presence of metastasis in combined controls *vs.* ADV/RSV-tk + GCV-treated animals. Line across graph indicates tumor wet weight (1,400 mg) above which 82.5% (33/40) of control animals had metastases. Treatment animals with tumors of this size had a 13% (3/23) incidence of metastasis (▲, animals killed at 14 days; ○, animals from the survival study). Each symbol represents one animal.

## DISCUSSION

Prostate cancer is now the most commonly diagnosed internal malignancy in men and will lead to an estimated 41,400 deaths in 1996 in the United States (Parker *et al.,* 1996). At the present time, potentially curative treatment options (radiation therapy and radical prostatectomy) for newly diagnosed patients are applicable only to those with localized disease. However, failure rates within 5 years, as manifested by a rising prostate-specific antigen (PSA) for patients undergoing radical surgery for presumed localized disease, range from 20% (Ohori *et al.,* 1995) to 57% (Zeitman *et al.,* 1994), indicating the presence of either local tumor recurrence or metastases or both. Neither of these conditions is amenable to cure by present therapeutic strategies. Therefore, there is a need to develop new therapies, such as gene therapy, for prostate cancer to more effectively control both local and distant disease. The direct introduction of therapeutic genes into prostatic lesions may offer new treatment options. We have previously reported that *HSV-tk* + GCV therapy produced extensive cytotoxicity and significantly enhanced survival using the RM-1 mouse prostate cancer cell in a non-metastatic s.c. model (Eastham *et al.,* 1996). Therefore, these studies did not include an analysis of possible anti-metastatic effects of this form of therapy.

To evaluate the possible anti-metastatic activity of ADV/*RSV-tk* + GCV therapy, we developed the orthotopic RM-1 model of metastatic prostate cancer using immunocompetent host animals. The RM-1 cell line was derived from an MPR-initiated, non-metastatic, primary prostate cancer and was demonstrated to be clonal with respect to the integrated recombinant retrovirus Zipras/*myc*-9, which initiated tumorigenesis (Baley *et al.,* 1995). Since the original primary tumor was not metastatic, it would appear that either the RM-1 clone had failed to manifest its metastatic potential *in vivo* or that *in vitro* culture selected for the metastatic phenotype. Orthotopic inoculation of the RM-1 cell line resulted in an aggressive model of prostate cancer, with distress or death of animals developing by 16–17 days post-tumor inoculation. In contrast to the s.c. model, orthotopic tumors resulted in documented metastatic activity in over 80% of animals by 16–17 days, with the highest activity in the pelvic and retroperitoneal lymph nodes and the lowest in the lung.

The use of ADV/*RSV-tk* + GCV in this aggressive orthotopic model resulted in significant local tumor growth suppression, with

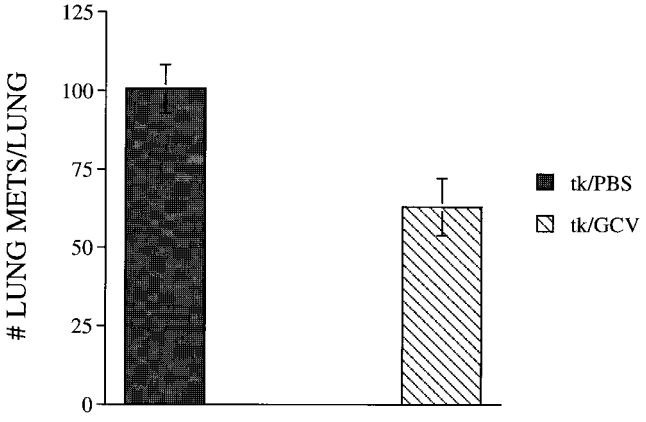


**FIGURE 5** – Average number of lung metastases following tail vein challenge. Subcutaneous tumors were generated and treated with ADV/*RSV-tk* + GCV (n = 10) or ADV/*RSV-tk* + PBS (n = 6). Tumors were excised following termination of GCV and challenged the same day with 50,000 RM-1 cells. Lung metastases were counted 14 days later ($p = 0.0098$, *t* test).

treatment tumors being 60% smaller than untreated controls at 2 weeks post-tumor inoculation. This growth suppression translated to a statistically significant prolongation of survival. However, these activities were not as impressive as those generated in the s.c. model of RM-1 tumors, which were approximately 80% smaller after a similar growth period following a single injection with the same dose of virus (Eastham *et al.,* 1996). A specific reason for the discrepancy between the s.c. and orthotopic models is unclear but may represent tissue-specific interactions, which can influence sensitivity to this form of therapy (Eastham *et al.,* 1996), and/or tissue-specific differences in immunity induction, which may be important in the efficacy of *HSV-tk* + GCV therapy (Cool *et al.,* 1996). It is notable that the prostate may be a relatively immunoprivileged site (Frost *et al.,* 1976; Gittes and McCullough, 1974; Vieweg *et al.,* 1994).

Our results demonstrate that a single *in situ* injection of ADV/*RSV-tk* + GCV into an established prostatic tumor can result in significant, though temporary, local growth suppression and lead to long-term suppression of spontaneous metastatic activity. This suppression occurs during the period of increasing metastatic activity in the control group (Fig. 2) and despite primary tumor regrowth above the threshold size for metastatic activity. To date, ADV-mediated HSV-tk pre-clinical studies have not addressed the effect of ADV/*RSV-tk* + GCV on spontaneous metastatic activity (Bonnekoh *et al.,* 1995; Chen *et al.,* 1994, 1995; Eastham *et al.,* 1996; Elshami *et al.,* 1996; O'Malley, *et al.,* 1995; Perez-Cruet *et al.,* 1994). The induction of activity against a challenge tumor following *HSV-tk* + GCV therapy utilizing other vector systems for delivery has been interpreted as evidence for induction of anti-metastatic activity (Barba *et al.,* 1994; Boviatsis *et al.,* 1994; Vile *et al.,* 1994). In the 9L rat brain model, animals cured of cerebral tumors are resistant to a second intracerebral tumor challenge (Barba *et al.,* 1994; Boviatsis *et al.,* 1994) or s.c. tumor challenge (Barba *et al.,* 1994). Treatment of s.c. melanomas which were subsequently removed also resulted in complete rejection or significantly delayed tumor development of a repeat s.c. challenge injection (Vile *et al.,* 1994). A direct effect of *HSV-tk* + GCV therapy on metastasis has been reported only following systemic delivery of retroviral HSV-tk to treat experimental metastases induced following tail vein injection of murine melanoma cells (Vile *et al.,* 1994).

In the RM-1 orthotopic model, the inhibition of spontaneous metastases by ADV/*RSV-tk* + GCV could result from the killing of pre-metastatic cells by the reduction, or possibly the elimination, of the pool of cells with metastatic potential. Alternatively, *HSV-tk* + GCV has been documented to directly result in the elimination of tumor vasculature (Ram *et al.,* 1994). Therefore, ADV/*RSV-tk* + GCV-mediated injury of angiogenic vessels, which has been implicated in the facilitation of metastasis in several cancers including prostate (Weidner *et al.,* 1993), could also lead to a disruption in the production of metastases. However, our data demonstrate that this suppressive activity continues despite tumor regrowth, suggesting that these mechanisms alone are unlikely to produce the longer-term anti-metastatic activity documented in this study and implicating the induction of systemic anti-metastatic activity.

To verify the presence of systemic anti-tumor activity, we chose to challenge treatment (ADV/*RSV-tk* + GCV) and control (ADV/*RSV-tk* + PBS) animals by tail vein injection with the parent RM-1 cell line to induce metastases following surgical removal of the s.c. primary tumor. The location of the primary tumor was changed due to the difficulty in safely excising an orthotopic primary tumor as we felt this experiment was more definitive if the challange was performed in the absence of the treated/control primary tumor. This experiment revealed a 40% reduction in lung colonization in animals whose primary tumor had been treated with ADV/*RSV-tk* + GCV compared with controls. Such significant systemic, anti-metastatic activity could result from the induction of anti-angiogenic substances such as angiostatin from the primary tumor (O'Reilly *et al.,* 1994) or possibly the induction of other tumor- and/or host-derived factors. Alternatively, and perhaps more likely, this treatment could induce immunological activity against tumor antigens.

The generation of immunological activity has been implicated in the efficacy of *HSV-tk* + GCV therapy in both primary and challenge tumors by several investigators (Barba *et al.,* 1994; Vile *et al.,* 1994). As documented in our study, treated tumors contain a strong infiltrate of lymphocytes (Barba *et al.,* 1994; Eastham *et al.,* 1996; Perez-Cruet *et al.,* 1994). In addition, *HSV-tk* + GCV can result in intra-tumoral production of cytokines such as IL-1α and IL-6 (Freeman *et al.,* 1995) and induce a CTL response against the parent cell line of a treated tumor (Chen *et al.,* 1995). The successful treatment of B16 lung metastases following systemic retroviral *HSV-tk* + GCV therapy cannot be duplicated when studies are performed in athymic nude mice (Vile *et al.,* 1994). Induction of an immune response, while failing to adequately control a large tumor, could suppress the development of spontaneous micrometastasis and underlie the anti-metastatic activity against a tail vein challenge as described in this study.

This study has demonstrated that a single *in situ* treatment with ADV/*RSV-tk* + GCV in an orthotopic mouse model of metastatic prostate cancer produces temporary control of local tumor growth yet can induce sustained anti-metastatic activity against both spontaneous metastases from the primary tumor and a challenge tail vein inoculum of parental tumor cells. Our data, therefore, indicate the presence of systemic anti-metastatic activity induced by ADV/*RSV-tk* + GCV therapy. In the clinical setting, ADV/*RSV-tk* + GCV may have a role as a neoadjuvant therapy to establish anti-metastatic activity prior to definitive local therapy. In addition, it is conceivable that this therapeutic approach would be useful in combating metastases in patients diagnosed with disseminated disease.

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## REFERENCES


BALEY, P.A., YOSHIDA, K., QIAN, W., SEHGAL, I. and THOMPSON, T.C., Progression to androgen insensitivity in a novel *in vitro* mouse model for prostate cancer. *J. Steroid Biochem. mol. Biol.,* **52,** 403–413 (1995).

BARBA, D., HARDIN, J., SADELAIN, M. and GAGE, F.H., Development of anti-tumor immunity following thymidine kinase-mediated killing of experimental brain tumors. *Proc. nat. Acad. Sci. (Wash.),* **91,** 4348–4352 (1994).

BONNEKOH, B., GREENHALGH, D.A., BUNDMAN, D.S., ECKHARDT, J.N., LONGLEY, M.A., CHEN, S.-H., WOO, S.L.C. and ROOP, D.R., Inhibition of melanoma growth by adenoviral-mediated HSV thymidine kinase gene transfer *in vivo. J. invest. Dermatol.,* **104,** 313–317 (1995).

BOVIATSIS, E.J., PARK, J.S., SENA-ESTEVES, M., KRAMM, C.M., CHASE, M., EFIRD, J.T., WEI, M.X., BREAKFIELD, X.O. and CHIOCCA, E.A., Long-term survival of rats harboring brain neoplasms treated with gamaclorir and a herpes simplex virus ?? that retains an intact thyridine kinase gene. *Cancer Res.,* **54,** 5745–5751 (1994).

CHEN, S.-H., CHEN, X.H.L., WANG, Y., KOSAI, K.-I., FINEGOLD, M.J., RICH, S.S. and WOO, S.L.C., Combination gene therapy for liver metastasis of colon carcinoma *in vivo. Proc. nat. Acad. Sci. (Wash.),* **92,** 2577–2581 (1995).

CHEN, S.-H., SHINE, H.D., GOODMAN, J.C., GROSSMAN, R.G. and WOO, S.L.C., Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo. Proc. nat. Acad. Sci. (Wash.),* **91,** 3054–3057 (1994).

COOL, V., PIROTTE, B., GERARD, C., DARGENT, J.-L., BAUDSON, N., LEVIVIER, M., GOLDMAN, S., HIDEBRAND, J., BROTCHI, J. and VELU, T., Curative potential of herpes simplex virus thymidine kinase gene transfer in rat with 9L gliosarcoma. *Hum. Gene Ther.,* **7,** 627–635 (1996).

EASTHAM, J.A., CHEN, S.-H., SEHGAL, I., YANG, G., TIMME, T.L., HALL, S.J., WOO, S.L.C. and THOMPSON, T.C., Prostate cancer gene therapy: herpes simplex virus thymidine kinase gene transduction followed by ganciclovir in mouse and human prostate cancer models. *Hum. Gene Ther.,* **7,** 515–523 (1996).

ELSHAMI, A.A., KUCHARCZUK, J.C., ZHANG, H.B., SMYTHE, W.R., HWANG, H.C., LITZKY, L.A., KAISER, L.R. and ALEBELDA, S.M., Treatment of pleural mesothelioma in an immunocompetent rat model utilizing adenoviral transfer of the herpes simplex virus thymidine kinase gene. *Hum. Gene Ther.,* **7,** 141–148 (1996).

FIDLER, I.J., Critical factors in the biology of human cancer metastasis. *Cancer Res.,* **50,** 6130–6138 (1990).

FREEMAN, S.M., ABBOUD, C.N., WHARTENBY, K.A., PACKMAN, C.H., KOEPLIN, D.S., MOOLTEN, F.L. and ABRAHAM, G.N., The ''bystander effect'': tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res.,* **53,** 5274–5283 (1993).

FREEMAN, S.M., RAMESH, R., SHASTRI, M., MUNISHI, A., JENSEN, A.K. and MARROGI, A.J., The role of cytokines in mediating the bystander effect using HSV-TK xenogeneic cells. *Cancer Lett.,* **92,** 167–174 (1995).

FROST, P., ROSE, N.R. and CHOE, B.-K., Immunology of prostate carcinoma—an overview. *Semin. Oncol.,* **3,** 107–113 (1976).

GITTES, R.F. and MCCULLOUGH, D.L., Occult carcinoma of the prostate: an oversight of immune surveillance—a working hypothesis. *J. Urol.,* **112,** 241–244 (1974).

GRAHAM, F. and PERVEC, L., Manipulation of adenoviral vectors. *In:* E.J. Murray (ed.) *Methods in molecular biology: gene transfer and expression protocols,* Humana Press, Clifton, NJ (1991).

JANIK, P., BRIAND, P. and HARTMANN, N.R., The effect of estrone-progesterone treatment on cell proliferation kinetics of hormone-dependent GR mouse mammary tumors. *Cancer Res.,* **35,** 3698–3704 (1975).

MOOLTEN, F.L., Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control study. *Cancer Res.,* **46,** 5276–5281 (1986).

OHORI, M., WHEELER, T.M., KATTAN, M.W., GOTO, Y. and SCARDINO, P.T., Prognostic significance of positive surgical margins in radical prostatectomy specimens. *J. Urol.,* **154,** 1818–1824 (1995).

O'MALLEY, B.W., JR., CHEN, S.-H., SCHWARTZ, M.R. and WOO, S.L.C., Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. *Cancer Res.,* **55,** 1080–1085 (1995).

O'REILLY, M.S., HOLMGREN, L., SHING, Y., CHEN, C., ROSENTHAL, R.A., MOSES, M., LANE, W.S., CAO, Y., SAGE, E.H. and FOLKMAN, J., Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell,* **79,** 315–328 (1994).

PARKER, S.L., TONG, T., BOLDEN, S. and Wingo, P.A., Cancer statistics, 1996. *CA Cancer J. Clin.,* **46,** 5–27 (1996).

PEREZ-CRUET, M.J., TRASK, T.W., CHEN, S.-H., GOODMAN, J.C., WOO, S.L.C., GROSSMAN, R.G. and SHINE, H.D., Adenovirus-mediated gene therapy of experimental gliomas. *J. Neurosci. Res.,* **39,** 506–511 (1994).

RAM, Z., WALBRIDGE, S., SHAWKER, T., CULVER, K.W., BAESE, R.M. and OLDFIELD, E.H., The effect of thymidine kinase transduction and ganciclovir therapy on tumor vasculature and growth of 9L gliomas in rats. *J. Neurosurg.,* **81,** 256–260 (1994).

STRATFORD-PERRICAUDET, L.D., MAKEH, I., PERRICAUDET, M. and BRIAND, P., Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. clin. Invest.,* **90,** 626–630 (1992).

THOMPSON, T.C., SOUTHGATE, J., KITCHENER, G. and LAND, H., Multi-stage carcinogenesis induced by *ras* and *myc* oncogenes in a reconstituted model. *Cell,* **56,** 917–930 (1989).

VIEWEG, J., ROSENTHAL, F.M., BANNERJI, R., HESTON, W.D.W., FAIR, W.R., GANSBACHER, B. and GILBOA, E., Immunotherapy of prostate cancer in the Dunning rat model: use of cytokine gene modified tumor vaccines. *Cancer Res.,* **54,** 1760–1765 (1994).

VILE, R.G., NELSON, J.A., CASTLEDEN, S., CHONG, H. and HART, I.R., Systemic gene therapy of murine melanoma using tissue specific expression of the *HSVtk* gene involves an immune component. *Cancer Res.,* **54,** 6228–6234 (1994).

WEIDNER, N., CARROLL, P.R., FLAX, J., BLUMENFIELD, W. and FOLKMAN, J., Tumor angiogenesis correlates with metastasis in invasive prostate cancer. *Amer. J. Pathol.,* **143,** 401–409 (1993).

ZEITMAN, A.L., EDELSTEIN, R.A., COEN, J.J., BABAYAN, R.K. and KRANE, R.J., Radical prostatectomy for adenocarcinoma of the prostate: the influence of preoperative and pathologic findings on biochemical disease-free outcome. *Urology,* **43,** 828–833 (1994).