Jpn. J. Cancer Res. **91**, 1339–1344, December 2000

Oncolytic Viral Therapy for Human Prostate Cancer by Conditionally Replicating Herpes Simplex Virus 1 Vector G207

Masafumi Oyama,[1, 2] Takashi Ohigashi,[2] Michio Hoshi,[1, 3] Masaru Murai,[2] Keiichi Uyemura[1] and Takahito Yazaki[1, 3, 4]

[1]Department of Physiology, [2]Department of Urology and [3]Department of Neurosurgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582

Over the last few years, a conditionally replicating herpes simplex virus 1 (HSV-1) vector, G207 has been used for the treatment of several malignant tumors. In this article we evaluate the antitumoral effect of G207 against prostate cancer *in vitro* and *in vivo*. The susceptibility of the human prostate cancer cell lines, DU145 and PC3 to G207 at a multiplicity of infection (MOI) of 0.1 was examined. In addition, the growth characteristics of G207 were assessed. Athymic mice with s.c. tumors were inoculated *in vivo* intraneoplastically with 1×10^7 plaque-forming units (PFU) of G207. For the pathological analyses, s.c. tumors were stained with X-gal. DU145 and PC3 were efficiently destroyed by G207 within 7 days. The viral yields of G207 increased time-dependently. *In vivo*, the intraneoplastic inoculation of G207 induced a significant inhibition of the tumor growth. The mean tumor growth ratio was significantly inhibited in the G207-treated tumors (DU145, $P<0.0001$; PC3, $P<0.001$ versus controls). In a pathological study, many *lacZ*-positive cells were diffusely present in the G207-treated tumors. G207 showed a significant antitumoral effect against human prostate cancer cell lines, and thus may be considered a useful agent for the treatment of prostate cancer.

Key words: Prostate neoplasms — Herpes simplex virus — Oncolytic virus — Replication-competent virus — Gene therapy

A previous study has proven the efficacy of G207 for the treatment of prostate cancer,[1)] even though G207 had originally been developed for the treatment of primary malignant brain tumors.[2)] It is important to demonstrate that decreased growth of tumor cells after injection of G207 is definitely due to viral replication and an oncolytic effect in order to show the efficient spread of viral therapy through its target. However, no studies have ever reported the *in vitro* replicatability of G207 in prostate cancer cells. In addition, no histochemical analysis has ever performed to visualize the distribution of G207 in prostate cancer cells.

Prostate cancer is the most common neoplasm in males and the second most common cause of cancer death in the United States.[3)] The current therapy for patients with localized disease (stage T1 and T2) is either a radical prostatectomy or radiation therapy. The 5- and 10-year disease-free survival of patients following a prostatectomy or radiation is 84% and 72%,[4)] or 87% and 77%,[5)] respectively. The survival for patients with localized prostate cancer therefore is not poor. However, prostatectomy and/or radiation may cause a number of complications, such as erectile dysfunction and/or incontinence.[6)] In addition, surgery may be invasive in elderly cases, patients with a poor cardiac function, or those with severe renal dysfunction, and so forth. Androgen withdrawal therapy is another treatment option. Although endocrine therapy is initially effective in more than 70% of patients, prostate cancer tends to begin to proliferate again in most cases within several years. Once the cancer becomes resistant to the endocrine therapy, there is no further reliable therapy. Therefore, the development of alternative less invasive approaches is important for the treatment of prostate cancer.

Corrective, immunomodulating cytoreductive, and suicide gene therapy approaches have to date been studied by many investigators,[7)] and various viral vectors have been used to deliver foreign genes into the target cells. However, most viral vectors are replication-incompetent and have major limitations regarding their spread to all cancer cells. Furthermore, a bystander effect cannot be expected in such patients because only small numbers of cancer cells are infected. On the other hand, the *E1B* gene-attenuated adenovirus, which can replicate in *p53*-mutated tumor cells, has been considered as a novel gene therapy strategy over the last few years.[8, 9)] However, *p53* mutations appear to be low-frequency events in primary prostate cancer.[10)] These limitations suggest that prostate cancer is a worthwhile target for cytolytic viral therapy.

The use of conditionally replicating HSV-1 vector G207 may be a particularly promising treatment modality. For possible clinical trials with replication-competent viruses, it is essential that adequate safeguards are employed. G207 has several important safety advantages as follows:

[4] To whom correspondence should be addressed.
E-mail: yazakit@med.keio.ac.jp

(1) one kilobase deletions of both *γ34.5* loci, which is the major viral determinant for neurovirulence[11] and also blocks the host response to infection.[12] (2) *Escherichia coli lacZ* gene insertion, which inactivates the *ICP6* gene. The *ICP6* gene encodes the large subunit of HSV ribonucleotide reductase, which is a key enzyme for viral DNA synthesis and is required for efficient viral replication in nondividing cells, but not in most dividing cells.[2, 13] These multiple mutations attenuate neurovirulence and thus make a reversion to the wild-type unlikely. As a result of its characteristic of replication only in dividing cells, the cytopathic effects of G207 are limited to neoplastic cells, and accordingly G207 for prostate cancer therapy has the ability to amplify itself and spread throughout tumor cells, thereby resulting in their destruction. Therefore, G207 seems to be an attractive vector with potential value as a less-invasive treatment for prostate cancer. The purpose of this paper is to explore the replicatability of G207 in prostate cancer cells *in vitro* and the spread rate of G207 in prostate cancer cells *in vivo*.

MATERIALS AND METHODS

Cell culture and virus Human-derived prostate cancer cell lines, DU145 and PC3, were utilized in this study. They were purchased from the American Type Culture Collection (ATCC). These cell lines were cultured in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (IFBS). All cell lines were maintained at 37°C in humidified 5% CO_2 with penicillin and streptomycin (Sigma Chemical Co., St. Louis, MO) added to the medium.

G207 was constructed as described previously[2]; both copies of the *γ34.5* gene were deleted and then a *lacZ* gene was inserted into the *ICP6* gene. G207 was propagated in African green monkey kidney (Vero) cells, which were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% IFBS. Vero cells were infected with G207 at a multiplicity of infection (MOI) of 0.02 at 37°C. The infected cells were harvested 2 days later, after the total cytopathic effect (CPE) occurred. The viral pellet was resuspended in virus buffer (150 m*M* NaCl-20 m*M* Tris, pH 7.5) after low-speed centrifugation (1500 rpm for 10 min at 4°C). G207 was subjected to freeze-thaw lysis and sonication three times to release the virus from the cell fraction and then was purified by high-speed centrifugation (3000 rpm for 10 min at 4°C), and stored at −80°C. Mock extracts were identically prepared, except for the fact that the virus buffer was used in place of the virus at the infection step. The viral titers were determined by a standard plaque assay on Vero cells as described previously.[2, 14]

Cytotoxicity in cell culture To evaluate the *in vitro* susceptibility to G207, each cell line was plated onto six-well dishes at 1×10^5 cells per well. Twenty-four hours later, each cell was infected with G207 at an MOI of 0.1, and maintained in RPMI 1640 with 5% IFBS at 37°C. The controls consisted of mock-infected extract. On days 1, 2, 4, 6, and 7 postinfection, the numbers of viable cells were counted by a hemocytometer, using the trypan blue exclusion method.

Viral growth curves To determine the potential of G207 to replicate in each cell line, we used a single-step growth analysis. Subconfluent monolayers of DU145 and PC3 were plated in six-well dishes, infected with G207 at a MOI of 0.1 in 0.7 ml of phosphate-buffered saline (PBS) containing 1% IFBS for 1 h at 37°C, while rocking the dishes for the first 10 min. The virus inoculum was removed after 60 min. The infected cells were then maintained in RPMI 1640 with 5% IFBS at 37°C in a 5% CO_2 humidified atmosphere. The infected cells were collected with a cell scraper at 3, 6, 12, 24, and 48 h postinfection, and then subjected to three cycles of freeze-thaw/sonication. After centrifugation, the supernatants were plated on confluent Vero cells. The virus yield at each time point was determined as described above. Vero cells were fixed and stained with methanol (Nacalai Tesque, Inc., Kyoto) and Giemsa stain (Merck, Tokyo). The plaques were counted by light microscopy and expressed as the number of plaque-forming units (PFU)/ml.

Animal studies Six-week-old male athymic BALB/c nude mice (*nu*/*nu*), purchased from Japan SLC Inc., Shizuoka, were kept in groups of five, housed in sterile cages, and all mice had free access to autoclaved food and water. All animal procedures were approved by the Laboratory Animal Center, School of Medicine, Keio University. Regarding the surgical procedures, each mouse was anesthetized with an i.p. injection of a 0.25–0.30 ml solution consisting of 84% bacteriostatic saline, 10% sodium pentobarbital (50 mg/ml; Abbott Laboratories, Chicago, IL), and 6% ethyl alcohol.

S.c. tumor therapy and X-gal staining The tumor cells (1×10^6) were s.c. implanted in the flanks of the mice. When the tumors reached a maximum diameter of approximately 5 mm, the mice were randomly divided into two groups (n=5/group) and then inoculated intraneoplastically with either 1×10^7 PFU of G207 in 30 μl of virus buffer or with 30 μl of mock-infected extract. The tumor size was calculated by external caliper measurements every other day. The condition of all animals was checked twice daily. The tumor volumes (V) were calculated using the following formula: $V=0.5(l\times w^2)$, where l is the long axis, and w is the short axis. The tumor growth ratio was determined as $0.5(l\times w^2)$day $n/0.5(l\times w^2)$day 0. The mean tumor volume was analyzed using the unpaired t test.

For pathological studies, tumor-bearing mice (tumors >10 mm in diameter) were treated with a single injection

of 1×10^7 PFU of G207 and then sacrificed on day 4 postinjection. When the s.c. tumors were excised, the tumor specimens were fixed in 0.5% formaldehyde (Nacalai Tesque, Inc.) and 0.5% glutaraldehyde (Nacalai Tesque, Inc.) in PBS for 24 h at 4°C. The tumors were then placed in a substrate solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; TaKaRa, Tokyo), 5 m*M* potassium ferricyanide, 5 m*M* potassium ferrocyanide, and 2 m*M* magnesium chloride in PBS for 3 to 6 h at 37°C, washed with PBS, and then soaked in 30% sucrose for 24 to 48 h. After having been frozen in OCT Compound (Miles, Elkhart, IN) in liquid nitrogen, the tumors were sectioned (8 μm) on a cryostat. The sections were mounted onto gelatin-coated glass slides. The slides were washed with PBS, stained with X-gal again overnight, and then counterstained with hematoxylin and eosin solution.

RESULTS

***In vitro* cytopathic effect of human prostate cancer cells** To evaluate the susceptibility of the human prostate cancer cell lines to G207, DU145 and PC3 were infected with G207 at an MOI of 0.1. These cells were efficiently destroyed by G207 within 1 week. The cytopathic effect appeared from day 1 postinfection, and 99% or greater cytotoxicity was demonstrable at day 7 postinfection (Fig. 1).

***In vitro* growth characteristics of G207 in human prostate cancer cells** To assess the replicative ability of G207 in the above susceptible cells, the viral single-step growth curves were analyzed. The cells were infected at an MOI of 0.1 and then harvested at 3, 6, 12, 24, and 48 h postinfection. The progeny virus yields were determined by titration on Vero cells. Infection into either DU145 or PC3 resulted in a similar viral yield (Fig. 2). The viral yields of G207 increased exponentially, depending on the length of time postinfection.

***In vivo* treatment of s.c. tumor-bearing athymic mice** We next studied whether or not the direct injection of G207 significantly suppressed the xenograft tumor growth in prostate cancer cell lines. BALB/c mice were injected s.c. with 1×10^6 DU145 or PC3 cells and then were inoculated intraneoplastically with 1×10^7 PFU of G207 or mock extract when the tumors had reached a size of approximately 5 mm in diameter. Growing tumors (>5 mm in diameter) were evident within 2 weeks. For the DU145 tumors, the tumor growth ratio of G207-treated tumors was significantly inhibited at all time points, when compared to that of the controls ($P<0.0001$; unpaired t test; Fig. 3A). No significant difference was observed in the diameter of the PC3 tumors between the G207-treated tumors and the controls for the first 2 days after the injection of G207. From day 4, a significant suppression of tumor growth in G207-treated tumors was evident at all time points, when compared to that of control tumors treated with mock extract ($P<0.001$; unpaired t test; Fig. 3B).

We next directly assessed the G207 infection in DU145 and PC3 tumors by a histologic study. The tumor-bearing mice, treated with either G207 or mock extract, were sacrificed on day 4 postinoculation. The tumors were stained with X-gal to determine the extent of β-galactosidase

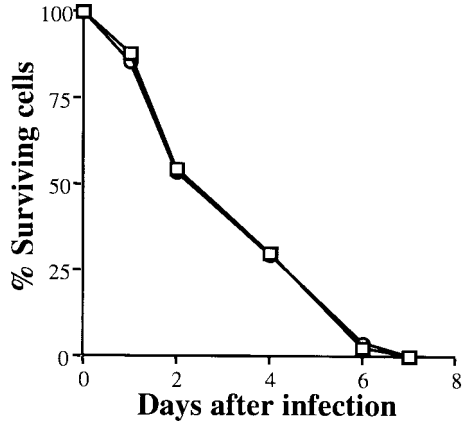

Fig. 1. The susceptibility of human-derived prostate cancer cell lines to G207 *in vitro*. Two human prostate cancer cell lines (DU145 and PC3) were infected with G207 at an MOI of 0.1. Each data point (mean of triplicate wells) indicates the percentage of surviving cells compared with the number of cells in the control (mock-infected) on days 1–7 postinfection. Symbols: ○, DU145; □, PC3.

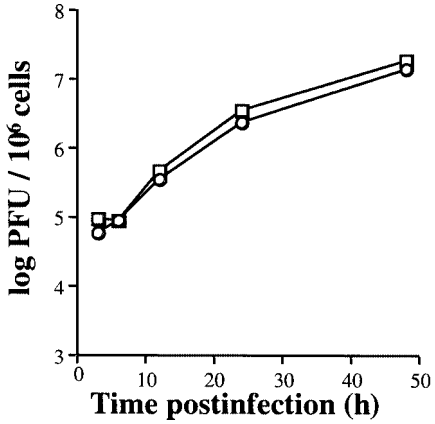

Fig. 2. The single-step growth curve of G207 on DU145 and PC3 cells. Monolayers of DU145 or PC3 cells, in six-well dishes, were infected at an MOI of 0.1 with serum-starved Vero cells. At each time point after infection, virus progeny was harvested and titrated on Vero cells. Symbols: G207 yields in DU145 (○), or PC3 (□).

expression. The intense blue color in cells following X-gal staining revealed the location of G207 infection. For both the DU145 and PC3 cells, the G207-treated tumors showed a large number of lacZ-positive cells that extended diffusely from the injection sites toward the periphery of the tumor (Fig. 4); however, the mock-treated tumors showed no β-galactosidase expression (data not shown).

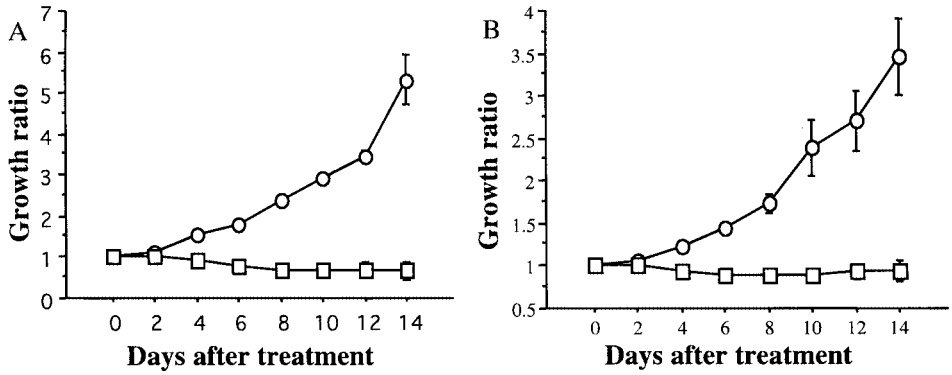

Fig. 3. The effect of the intraneoplastic injection of G207 on s.c. tumors, (A) DU145 and (B) PC3. The mean tumor growth rate of both DU145 and PC3 was significantly inhibited in the G207-treated tumors compared to the control tumors treated with mock extract. Symbols: □, G207; ○, mock.

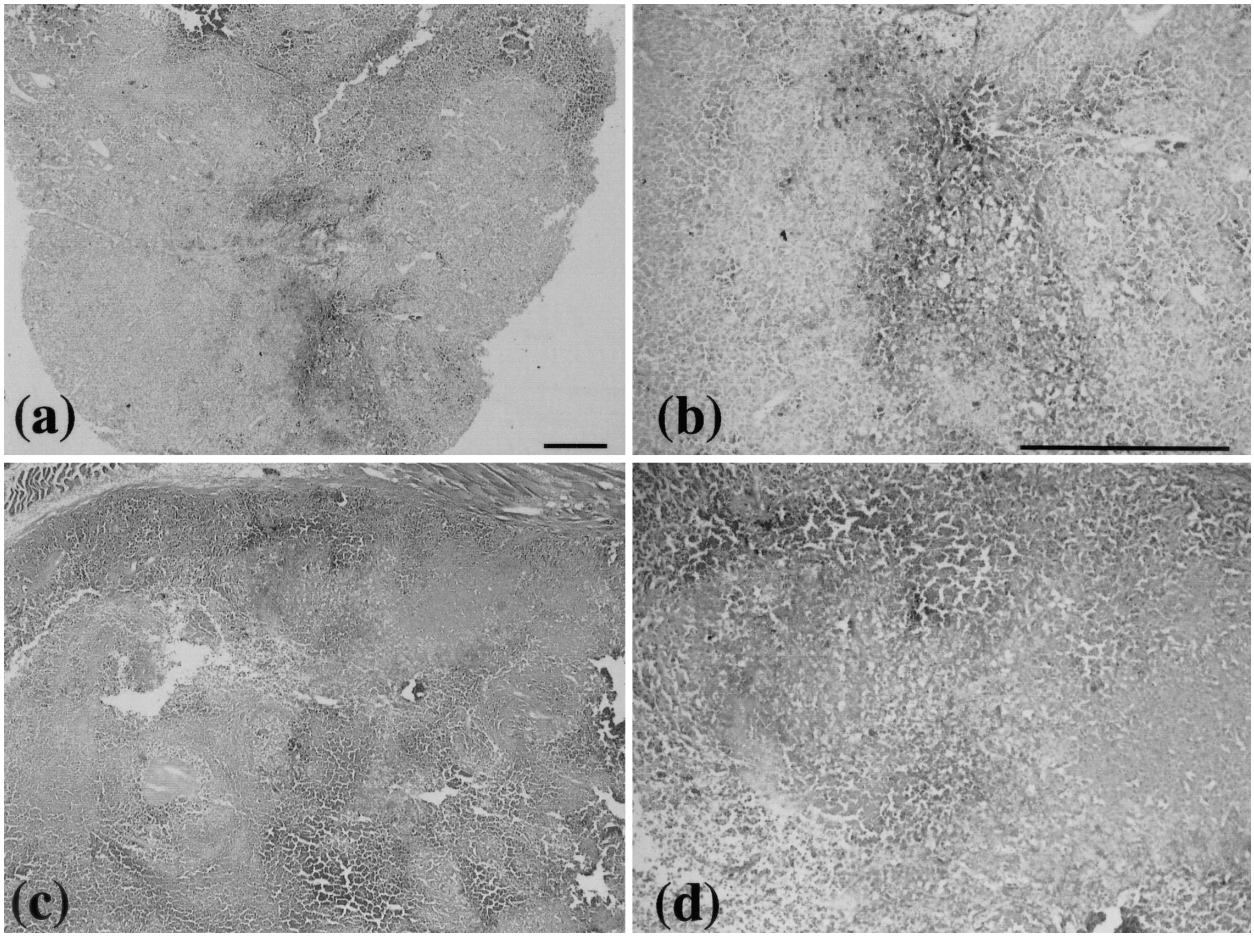

Fig. 4. Pathological examinations of G207-treated s.c. prostate cancer cells (DU145, a, b; PC3, c, d). (Original magnifications: ×40, a, c; ×100, b, d). The rate of *lacZ* expression was determined by X-gal staining. Scale bars=1 mm.

DISCUSSION

Over the past few years a considerable number of studies on the conditionally replicating HSV-1 vector, G207, have been reported, especially for brain tumors, and now phase II clinical trials for recurrent malignant gliomas are in progress in the USA. G207 has the following characteristics: (1) hypersensitivity to anti-herpetic drugs, which would allow prevention of any unexpected viral spread by the use of acyclovir/ganciclovir during the treatment, (2) temperature sensitivity, which would inactivate the viral replication in the case of fever and HSV encephalitis, (3) selectivity for rapidly dividing cells, thereby targeting viral spread to cancer cells, (4) markedly attenuated neuropathogenicity, (5) a marker gene (*lacZ*), which can track the viral replication in the tumor and (6) little likelihood of reversion to the wild type.[2)] These advantages are especially noteworthy for human trials. Our results and previous studies,[1, 15–17)] therefore, suggest that G207 should be considered for clinical trials in the treatment of several malignant tumors.

We demonstrated that G207 effectively destroyed prostate cancer cells both *in vitro* and *in vivo*. The effective replication of G207 in prostate cancer cells is verified by the single-step growth analysis and histochemical studies. In addition, the intraneoplastic injection of G207 induced a significant tumor growth inhibition. These results show that the antitumoral effect of G207 in fighting prostate cancer is similar to that in malignant brain tumors.[2, 17)]

G207 has already been shown to be safe. The i.v. administration of G207 (10^7 PFU) was shown to cause no toxicity in athymic mice.[18)] The intracerebral inoculation of up to 10^9 PFU of G207 was also found to be safe in the owl monkey.[19)] Furthermore, a recent report showed that G207 induces an antitumoral effect even in immunocompetent mice and, as a result, the herpes simplex virus (HSV) immune status is not considered to affect G207 therapy.[20)] The findings of these reports underpin the argument for human trials of G207 for prostate cancer.

The majority of patients with hormone-dependent prostate cancer, even at an advanced stage, might be controllable by endocrine therapy. However, a number of hormone-dependent cancers become hormone-independent. At the present time, hormone-refractory prostate cancer is incurable. Our experimental data therefore appear to justify further investigation of oncolytic viral therapy against hormone refractory prostate cancer. This modality using the oncolytic virus may prove to be an effective treatment for prostate cancer in the not too distant future.

ACKNOWLEDGMENTS

We thank Drs. Robert L. Martuza, Samuel D. Rabkin, and William D. Hunter for their valuable scientific assistance. We would also like to express our appreciation to Asako Harada, Hiroshi Endo, Kumiko Nakamura, and the entire staff of the Departments of Physiology and Urology, School of Medicine, Keio University for their technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan to TY and MO, grants from the Ministry of Health and Welfare, Japan to TY, a Grant-in-Aid from the Tokyo Biochemical Research Foundation to TY, and research grants for lifesciences and medicine from the Keio University Medical Science Fund to TY.

(Received May 15, 2000/Revised August 16, 2000/Accepted September 19, 2000)

REFERENCES

1) Walker, J. R., McGeagh, K. G., Sundaresan, P., Jorgensen, T. J., Rabkin, S. D. and Martuza, R. L. Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector G207. *Hum. Gene Ther.*, **10**, 2237–2243 (1999).
2) Mineta, T., Rabkin, S. D., Yazaki, T., Hunter, W. D. and Martuza, R. L. Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas. *Nat. Med.*, **1**, 938–943 (1995).
3) Landis, S. H., Murray, T., Bolden, S. and Wingo, P. A. Cancer statistics, 1999. *CA Cancer J. Clin.*, **49**, 8–31, 31 (1999).
4) Paulson, D. F., Moul, J. W. and Walther, P. J. Radical prostatectomy for clinical stage T1-2N0M0 prostatic adenocarcinoma: long-term results. *J. Urol.*, **144**, 1180–1184 (1990).
5) Bagshaw, M. A., Cox, R. S. and Ramback, J. E. Radiation therapy for localized prostate cancer. Justification by long-term follow-up. *Urol. Clin. North Am.*, **17**, 787–802 (1990).
6) Eastham, J. A. and Scardino, P. T. Radical prostatectomy. *In* "Campbell's Urology," ed. P. C. Walsh, A. B. Retik, E. D. Vaughan and A. J. Wein, pp. 2547–2564 (1998). W. B. Saunders, Philadelphia, PA.
7) Herman, J. R. and Lerner, S. P. Current status of gene therapy for prostate and bladder cancer. *Int. J. Urol.*, **4**, 435–440 (1997).
8) Bischoff, J. R., Kirn, D. H., Williams, A., Heise, C., Horn, S., Muna, M., Ng, L., Nye, J. A., Sampson-Johannes, A., Fattaey, A. and McCormick, F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science*, **274**, 373–376 (1996).
9) Heise, C., Sampson-Johannes, A., Williams, A., McCormick, F., Von Hoff, D. D. and Kirn, D. H. ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented

by standard chemotherapeutic agents. *Nat. Med.*, **3**, 639–645 (1997).
10) Bookstein, R., MacGrogan, D., Hilsenbeck, S. G., Sharkey, F. and Allred, D. C. p53 is mutated in a subset of advanced-stage prostate cancers. *Cancer Res.*, **53**, 3369–3373 (1993).
11) Chou, J., Kern, E. R., Whitley, R. J. and Roizman, B. Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture. *Science*, **250**, 1262–1266 (1990).
12) Chou, J. and Roizman, B. Herpes simplex virus 1 gamma(1)34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. *Proc. Natl. Acad. Sci. USA*, **91**, 5247–5251 (1994).
13) Goldstein, D. J. and Weller, S. K. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant. *J. Virol.*, **62**, 196–205 (1988).
14) Martuza, R. L., Malick, A., Markert, J. M., Ruffner, K. L. and Coen, D. M. Experimental therapy of human glioma by means of a genetically engineered virus mutant. *Science*, **252**, 854–856 (1991).
15) Toda, M., Rabkin, S. D. and Martuza, R. L. Treatment of human breast cancer in a brain metastatic model by G207, a replication-competent multimutated herpes simplex virus 1. *Hum. Gene Ther.*, **9**, 2177–2185 (1998).
16) Carew, J. F., Kooby, D. A., Halterman, M. W., Federoff, H. J. and Fong, Y. Selective infection and cytolysis of human head and neck squamous cell carcinoma with sparing of normal mucosa by a cytotoxic herpes simplex virus type 1 (G207). *Hum. Gene Ther.*, **10**, 1599–1606 (1999).
17) Yazaki, T., Manz, H. J., Rabkin, S. D. and Martuza, R. L. Treatment of human malignant meningiomas by G207, a replication-competent multimutated herpes simplex virus 1. *Cancer Res.*, **55**, 4752–4756 (1995).
18) Sundaresan, P., Hunter, W. D., Martuza, R. L. and Rabkin, S. D. Attenuated, replication-competent herpes simplex virus type 1 mutant G207: safety evaluation in mice. *J. Virol.*, **74**, 3832–3841 (2000).
19) Hunter, W. D., Martuza, R. L., Feigenbaum, F., Todo, T., Mineta, T., Yazaki, T., Toda, M., Newsome, J. T., Platenberg, R. C., Manz, H. J. and Rabkin, S. D. Attenuated, replication-competent herpes simplex virus type 1 mutant G207: safety evaluation of intracerebral injection in nonhuman primates. *J. Virol.*, **73**, 6319–6326 (1999).
20) Chahlavi, A., Rabkin, S., Todo, T., Sundaresan, P. and Martuza, R. Effect of prior exposure to herpes simplex virus 1 on viral vector-mediated tumor therapy in immunocompetent mice. *Gene Ther.*, **6**, 1751–1758 (1999).