Oncolytic Herpes Simplex Virus Vector G47Δ in Combination with Androgen Ablation for the Treatment of Human Prostate Adenocarcinoma

Hiroshi Fukuhara,[1,2] Robert L. Martuza,[1] Samuel D. Rabkin,[1] Yoshikazu Ito,[4] and Tomoki Todo[1,3]

Abstract

Purpose: The use of oncolytic herpes simplex virus type 1 is a promising stategy for cancer treatment. We constructed herpes simplex virus type 1 vector G47Δ by deleting the *α47* gene and the promoter region of *US11* from G207. We now report studies demonstrating the potential of G47Δ as a therapeutic modality for prostate cancer in combination with androgen ablation.

Experimental Design: The cytopathic activities of G47Δ at low multiplicities of infection was tested in human prostate cancer cell lines LNCaP, PC-3, and DU145 *in vitro*. Two androgen-dependent mouse s.c. tumor models, murine TRAMP and human HONDA, were used to investigate the *in vivo* efficacy of G47Δ in combination with androgen ablation.

Results: G47Δ at low multiplicities of infection showed more rapid tumor cell killing than G207 in LNCaP and DU145 *in vitro* and showed a 22-fold higher virus yield in a single-step growth experiment. *In vivo*, G47Δ treatment resulted in reduced tumor growth of established s.c. TRAMP and HONDA tumors and inhibited the growth of recurrent HONDA tumors that once regressed by androgen ablation therapy. In both TRAMP and HONDA tumor xenografts, the combination therapy of G47Δ with androgen ablation led to significantly enhanced inhibition of the tumor growth and prolonged survival.

Conclusions: These results suggest that oncolytic virus therapy with G47Δ can be usefully combined with androgen ablation therapy for the treatment of prostate cancer.

The use of oncolytic conditionally replicating herpes simplex virus type 1 (HSV-1) vectors is an attractive strategy for prostate cancer therapy (1). Oncolytic HSV-1 therapy uses the natural characteristics of the virus to kill host cells in the course of viral replication. Mutations in genes associated with virulence and/or viral DNA synthesis can limit virus replication only to transformed cells. G207, one of the first oncolytic HSV-1 vectors taken into clinical trials, was derived from HSV-1 strain F and has deletions in both copies of the *γ34.5* gene and a *lacZ* insertion inactivating the *ICP6* gene so as to permit replication within cancer cells that can complement these mutations but not in normal cells including neurons (2). Results from a phase I clinical trial of patients with recurrent malignant glioma showed radiological responses and indicated that intraneoplastic inoculation of G207 is safe at the highest dose tested [3×10^9 plaque forming units (pfu)] (3). The combination of an ability to kill cancer cells while sparing surrounding neural tissue is particularly appealing for prostate cancer therapy in which neural damage can be associated with incontinence and sexual dysfunction. G207 has been shown to be effective against human prostate cancer *in vitro* and *in vivo* following direct intraneoplastic inoculation as well as i.v. administration (4). In preclinical safety evaluation, G207 displayed no evidence of clinical disease, no shedding of infectious virus, and no spread of the virus into other organs when injected into the prostates of HSV-1–susceptible mice and nonhuman primates (5).

From G207, we constructed a multimutated, replication-competent HSV-1 vector, G47Δ, by creating a further deletion within the nonessential *α47* gene (6). This additional deletion has been shown to provide enhanced viral replication and to partially restore MHC class I expression in infected human cells. In this article, we show that (*a*) G47Δ shows enhanced antitumor activity in prostate cancer cells *in vitro* and *in vivo*; (*b*) combination therapy of G47Δ and androgen ablation has cooperative effects, resulting in greater inhibition of tumor growth than either therapy alone; and (*c*) G47Δ is also effective for those prostate cancers that once responded to androgen ablation but eventually became refractory and recurred.

Authors' Affiliations: [1]Molecular Neurosurgery Laboratory, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts; [2]Departments of Urology and [3]Neurosurgery, University of Tokyo, Tokyo, Japan; and [4]School of Health Sciences, Gunma University, Gunma, Japan

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Requests for reprints: Robert L. Martuza, Brain Tumor Research Center MGH-Simches Research Building, 185 Cambridge Street, CPZN-3800, Boston, MA 02114. Phone: 617-726-8583; Fax: 617-726-4814; E-mail: rmartuza@partners.org or toudou-nsu@umin.ac.jp.

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Materials and Methods

Cells. Human prostate cancer cell lines DU145, LNCaP, PC-3, mouse prostate cancer cell line TRAMP-C2 (7), and African green monkey kidney cell line Vero were obtained and maintained as described (4, 6, 8). The androgen-dependent human prostate cancer line HONDA was passaged in athymic mice as s.c. tumor fragments (9, 10).

In vitro *cytotoxicity studies.* *In vitro* cytotoxicity studies were done as described (6, 11). The number of surviving cells was counted daily with a Coulter Counter (Beckman Coulter, Fullerton, CA) and expressed as a percentage of mock-infected controls.

Single-step growth studies. Cells were seeded in six-well plates at 3×10^5 per well and infected with G47Δ, G207, or the laboratory strain HSV-1 (strain F) at two wells per virus at a multiplicity of infection (MOI) of 2. At 4, 8, 12, 22, or 30 hours after infection, the cells were scraped into the medium and lysed by three cycles of freeze and thawing. The progeny virus was titered on Vero cells by plaque assay as described (6).

Animal studies. Six-week-old male C57BL/6 mice and athymic mice (BALB/c *nu/nu*) were purchased from Harlan Laboratories (Indianapolis, IN) and the National Cancer Institute (Frederick, MD), respectively. Studies were done in mice 7 weeks of age or older. All animals were caged in groups of five or fewer. All animal procedures were approved by the Institutional Animal Care and Use Committee. Subcutaneous tumor therapy was done as described (6, 12).

Surgical castration or sham operation was done via transperineal approach in male C57BL/6 mice anesthetized with Nembutal as previously described (13). A 60-day-release flutamide (50 mg/kg/d; ref. 14) or placebo pellet (Innovative Research of America, Sarasota, FL) was implanted s.c. through a 1-cm incision on the flank into anesthetized male athymic mice.

Immunofluorescence microscopy. Five-micrometer formalin-fixed, paraffin-embedded section was applied for immunofluorescence. The sections were deparaffinized in xylene, rehydrated with serial ethanol, and rinsed in 10 mmol/L PBS. After incubation with normal rabbit serum for 30 minutes at room temperature, the sections were incubated with a rabbit polyclonal antibody against human prostate-specific antigen (ab946, 1:100 dilution; Abcam, Cambridge, United Kingdom) overnight at 4°C. The sections were then rinsed with PBS and labeled with a Cy3-labeled secondary antibody for 30 minutes at room temperature. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (10 μmol/L 4′,6-diamidino-2-phenylindole in 800 mmol/L trisodium citrate; Sigma, St. Louis, MO) for 30 minutes at room temperature.

Statistical analysis. All *in vitro* data and *in vivo* tumor volume data were evaluated by unpaired *t* test. Survival was described by Kaplan-Meier analysis and evaluated by Wilcoxon test.

Results

***Cytopathic effect of G47Δ* in vitro.** The cytolytic activity of G47Δ *in vitro* was compared with G207 in three human prostate cancer cell lines. G47Δ killed LNCaP cells more rapidly than G207 at low MOIs of 0.01 and 0.1 ($P < 0.05$ at MOI = 0.01 on days 1 to 3 and at MOI = 0.1 on days 1 and 2; Fig. 1A). DU145 cells were resistant to killing by both G207 and G47Δ at an MOI of 0.01 but were killed significantly more rapidly with G47Δ than G207 at an MOI of 0.1 ($P < 0.05$ on days 1-4). In PC-3 cells, both G207 and G47Δ showed >70% cell destruction within 4 days of infection with no significant difference.

Single-step growth studies. Deletion of the *α47* gene in G47Δ also places the late *US11* gene under control of the immediate-early *α47* promoter (6), which suppresses the reduced growth properties of *γ34.5*-deficient mutants (15). A single-step growth study was done to examine the *in vitro* replication capability of G47Δ in LNCaP cells in comparison with G207 and the parental laboratory strain HSV-1 (strain F). G47Δ showed better replication capability than G207 at all time points tested: The yield of progeny G47Δ was 22 times greater than G207 at 22 hours postinfection and 10 times greater at 30 hours (Fig. 1B). As expected, due to deletions that can be associated

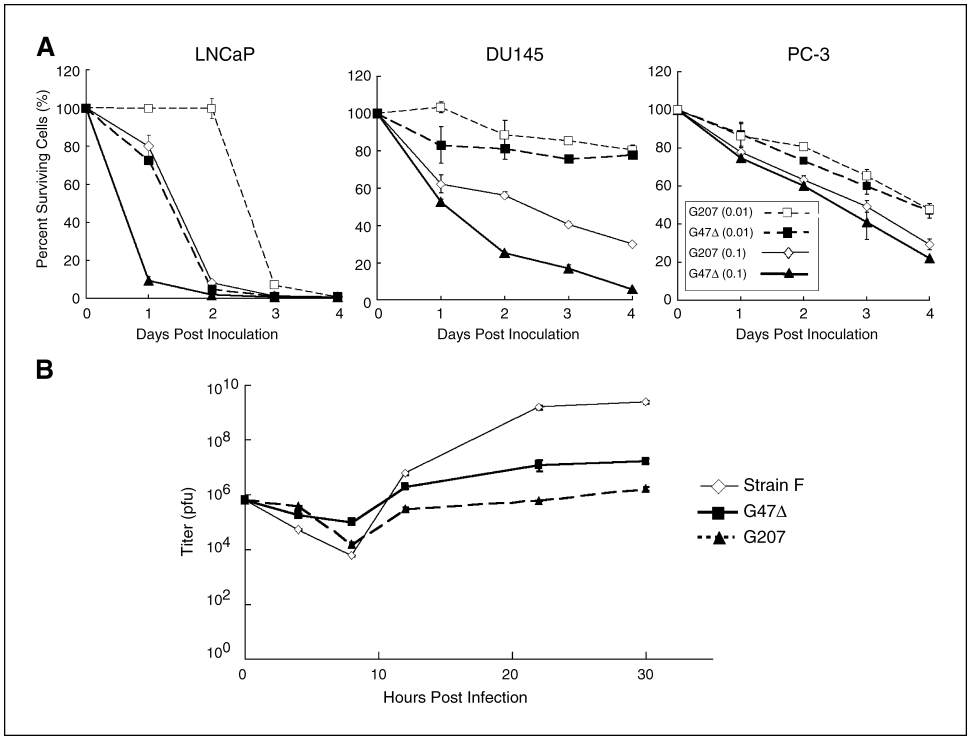

Fig. 1. *A,* cytopathic studies *in vitro*. G47Δ at low MOIs showed a more rapid tumor cell killing than G207 in LNCaP and DU145 cells. The number of surviving cells was counted daily and expressed as a percentage of mock-infected controls. Points, mean of triplicate wells; bars, SD. *B,* single-step growth studies. LNCaP cells were infected with the viruses at an MOI of 2 and the progeny virus was titered at the indicated time points. G47Δ (■) showed a 22-fold higher virus yield than G207 at 22 hours and a 10-fold higher yield at 30 hours (▲). ◇, Strain F. Points, mean of duplicate wells; bars, SD.

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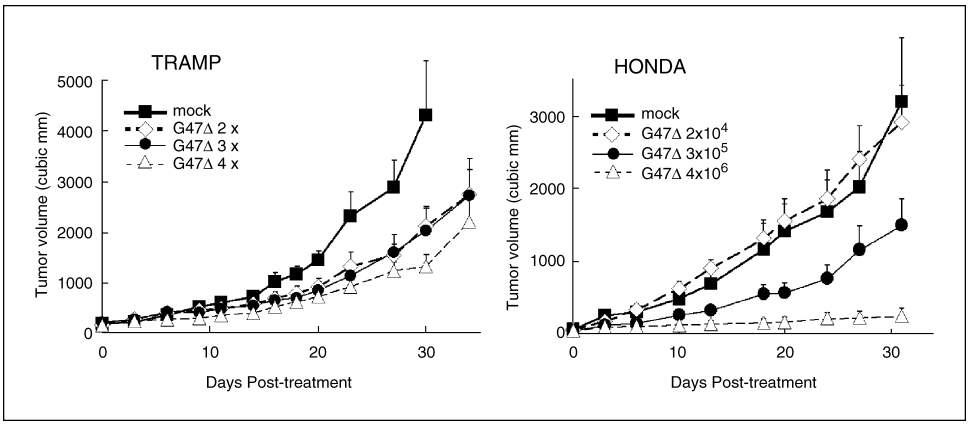

Fig. 2. Intraneoplastic injection of G47Δ *in vivo*. TRAMP-C2 mouse prostate cancer cells from culture (*left*) or HONDA human prostate cancer tumor fragments passaged *in vivo* (*right*) were implanted in male C57BL/6 or athymic mice, respectively. After allowing tumor growth, established tumors of 5 to 7 mm in diameter were inoculated with G47Δ. S.c. TRAMP-C2 tumors were treated by intraneoplastic inoculation of G47Δ (5×10^6 pfu) either twice (days 0 and 3), thrice (days 0, 3, and 6), or four times (days 0, 3, 6, and 9) or mock (PBS with 10% glycerol; days 0, 3, 6, and 9). S.c. HONDA tumors were treated intraneoplastically with G47Δ (2×10^4, 2×10^5, or 2×10^6 pfu) or mock on days 0 and 3. Bars, SE.

with attenuation, strain F replicated better than either mutated virus, with virus yields reaching ~100 times more than G47Δ (Fig. 1B).

Antitumor efficacy of G47Δ in vivo. Because of the increased growth and cytopathic activity of G47Δ versus G207 in the human prostate cancer cell culture studies, the efficacy of G47Δ was next tested in an immunocompetent mouse tumor model. TRAMP-C2 mouse prostate cancer cells (5×10^6) were injected s.c. into syngeneic C57BL/6 male mice. Mouse cells are more resistant to HSV-1 oncolysis than human cells; thus, several treatment paradigms involving multiple inoculations were tested. Seven days after TRAMP-C2 cell inoculation, animals bearing established s.c. tumors (6-7 mm in diameter) were inoculated intraneoplastically with G47Δ (5×10^6 pfu) either twice (days 0 and 3), thrice (days 0, 3, and 6), or four times (days 0, 3, 6, and 9). Animals inoculated four times with G47Δ showed the largest reduction in tumor growth compared with mock-treated control animals ($P < 0.05$ versus control on days 9-30; Fig. 2, *left*). In this model, we previously showed that three doses of G207 at 2×10^7 pfu were required to cause a significant tumor growth inhibition (8).

The efficacy of G47Δ was further tested in athymic mice bearing human prostate cancer xenografts. Tumor fragments of HONDA human prostate cancer were transplanted s.c. in male athymic mice. Seven days later, established s.c. tumors (5 mm in diameter) were inoculated intraneoplastically with mock or G47Δ at three different doses (2×10^4, 2×10^5, or 2×10^6 pfu) on days 0 and 3. Because of the higher susceptibility of human cells to HSV-1 oncolysis than murine cells, only two inoculations were done. Dose dependency was noted and, at the highest dose (2×10^6 pfu), G47Δ caused a significant reduction in tumor growth compared with mock ($P < 0.05$ versus control on days 3-31; Fig. 2, *right*). Two of six animals treated at 2×10^6 pfu showed a total regression of the tumor. In this model with the same treatment protocol, G207 was less efficacious than G47Δ and did not cause any cures at 2×10^6 pfu (Supplementary Fig. S1). These results show that the improved replication of G47Δ over G207 shown in the *in vitro* studies translates into improved antitumor efficacy *in vivo*.

Antitumor efficacy of G47Δ in recurrent prostate cancer-refractory to androgen ablation. Subcutaneous human prostate tumors (HONDA) transplanted into male athymic mice usually undergo a complete sustained remission after castration; however, a small proportion of such animals had a relapse of local tumor growth 7 to 10 weeks later. To mimic an often-encountered clinical setting where prostate cancers become refractory to androgen ablation therapy after a period of stable disease, those relapsed HONDA tumors were reimplanted s.c. into female athymic mice. These s.c. tumors grew despite the absence of androgen, and when they reached the size of 6 to 7 mm in diameter, they were treated with G47Δ by intraneoplastic inoculation (2×10^6 pfu, days 0 and 3). The G47Δ treatment successfully inhibited the growth of androgen-refractory tumors ($P < 0.05$ versus control on days 10-47; Fig. 3, *left*). The tumors that grew in female mice stained positively for prostate-specific antigen by immunohistochemistry, confirming that they were, in fact, prostate cancers (Fig. 3, *right*).

Concurrent use of G47Δ and androgen ablation. Whereas the above experiment explored the effects of G47Δ used sequentially in recurrences after androgen ablation, we also

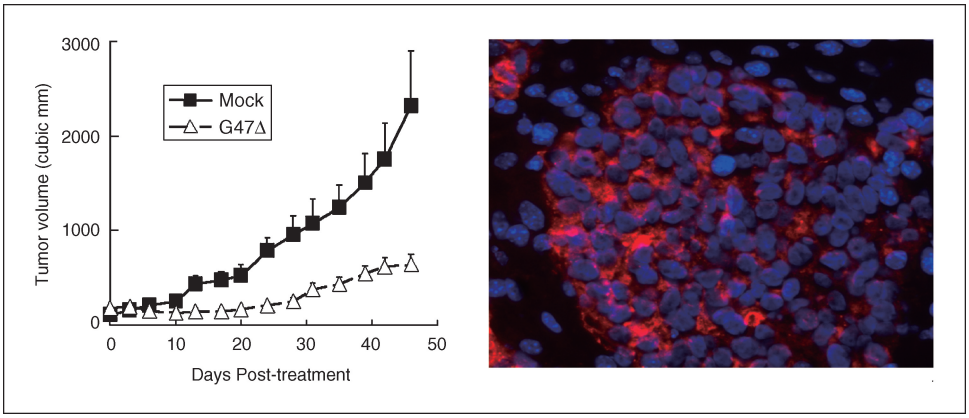

Fig. 3. Therapeutic effects of G47Δ in HONDA tumors that relapsed after androgen ablation therapy. Left, relapsed HONDA tumors were reimplanted in female athymic mice. After allowing tumor growth, established tumors of 5 to 7 mm in diameter were inoculated with G47Δ (2×10^6 pfu) or mock on days 0 and 3. Bars, SE. Right, prostate-specific antigen immunofluorescence of relapsed HONDA tumors after androgen ablation therapy. Tumor cells showed positive cytoplasmic immunostaining for prostate specific antigen (*red*). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*blue*). Original magnification, ×40.

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studied the *in vivo* efficacy of G47Δ used concurrently in combination with androgen ablation therapy for previously untreated tumors. It has been shown that the growth of s.c. TRAMP-C2 tumors in C57BL/6 mice is inhibited by castration but paradoxically accelerated by flutamide treatment (14). On the other hand, as noted above, the growth of s.c. HONDA tumors in athymic mice is strongly dependent on androgen such that castration often results in complete regression of tumors, but flutamide treatment only suppresses tumor growth, mimicking a partial effect as often seen in patients (16). Therefore, for combination therapy studies that might be analogous to clinical situations in humans, the murine TRAMP-C2 model was well suited for androgen ablation therapy by castration and the human HONDA model by flutamide treatment.

C57BL/6 male mice bearing established s.c. TRAMP-C2 tumors (5 mm in diameter) were treated with intraneoplastic G47Δ inoculations [5×10^6 pfu, days 0 and 3; intermediate growth inhibition from Fig. 2 (*left*)] in combination with surgical transperineal castration or sham operation (day 0). The combination therapy led to an enhanced inhibition of tumor growth, resulting in significantly smaller tumor volumes than castration ($P < 0.05$ on days 9-34) or G47Δ treatments alone ($P < 0.05$ on days 20-23 and 30; Fig. 4A, *left*).

Athymic male mice bearing established s.c. HONDA tumors (5 mm in diameter) were also treated with intraneoplastic G47Δ inoculations [2×10^5 pfu, days 0 and 3; intermediate growth inhibition from Fig. 2 (*right*)] in combination with 60-day-release flutamide (50 mg/kg/d) or placebo pellets (from day 0). The combination therapy again caused an enhanced inhibition of tumor growth, resulting in significantly smaller tumor volumes than flutamide alone ($P < 0.05$ on days 15-30) or G47Δ alone ($P < 0.05$ on days 23 and 30-34; Fig. 4A, *right*). At the G47Δ doses tested, Kaplan-Meier analyses showed that the combination therapy significantly prolonged survival of tumor-bearing animals in both models compared with monotherapies of androgen ablation and G47Δ treatment ($P < 0.05$; Fig. 4B). Similar results were obtained with G207 in combination with flutamide (Supplementary Fig. S2).

Discussion

Androgen ablation, either through surgical castration or administration of luteinizing hormone-releasing hormone analogs, forms the basis of the most common strategies for the management of locally advanced or metastatic prostate cancer (17). However, despite a favorable initial response in 70% to 80% of these patients, androgen ablation results in only 2% to 3% improvement of 5-year survival, and most patients eventually experience biochemical or clinical evidence of disease progression within a median time of 12 to 18 months (18). Second-line treatment options are limited, and most patients who present with metastatic disease die within 5 years of diagnosis (19). Previously, we reported that LNCaP tumors that recurred after radiation therapy remained sensitive to G207 therapy (4). Herein, we show that G47Δ treatment was efficacious for relapsed HONDA tumors that became refractory to androgen ablation. Thus, intraneoplastic administration of oncolytic HSV-1 vectors may be a useful second-line treatment modality for localized prostate cancer deposits that have progressed after either radiation therapy or androgen ablation.

Importantly, oncolytic virus therapy may also be considered in earlier-stage prostate cancer at the time that androgen ablation therapy is initiated. Our studies show that oncolytic HSV-1 therapy improves the outcome when combined with androgen ablation therapy. The combination of castration with G47Δ treatment resulted in tumor growth suppression that was significantly greater than either castration or G47Δ treatment alone in the mouse TRAMP-C2 s.c. model. Similarly, the combination treatment of flutamide with G47Δ resulted in significantly better tumor growth inhibition than either flutamide or G47Δ alone in the human HONDA s.c. model.

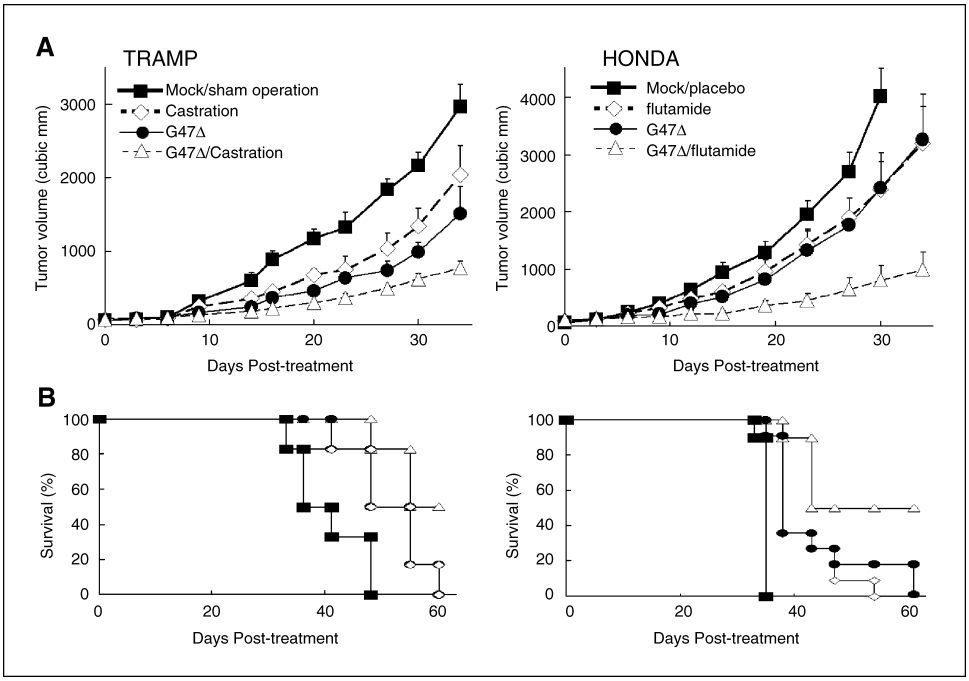

Fig. 4. *A,* therapeutic effects of G47Δ in combination with androgen ablation. Left, C57BL/6 male mice bearing established s.c. TRAMP-C2 tumors were treated with intraneoplastic G47Δ inoculations (5×10^6 pfu, days 0 and 3) in combination with surgical castration or sham operation (day 0). The combination treatment (△) was significantly more efficacious than castration alone (◇) or G47Δ alone (●), resulting in smaller tumor volumes ($P < 0.05$ for castration alone on days 9-34 and for G47Δ alone on days 20-23 and 30). Right, male athymic mice bearing established s.c. HONDA tumors were treated with intraneoplastic inoculations of 2×10^5 pfu of G47Δ on days 0 and 3 in combination with 60-day-long flutamide pellets implanted on day 0. The combination treatment (△) was significantly more efficacious than flutamide alone (◇) or G47Δ alone (●). $P < 0.05$ for G47Δ alone on days 15 to 30 and for flutamide alone on days 23 and 30 to 34. Bars, SE. *B,* Kaplan-Meier analyses of survival in experiments described above.

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Thus, both in mouse syngeneic and in human xenograft models of prostate cancer, the combination of androgen ablation with G47Δ results in superior tumor growth suppression and prolonged animal survival compared with either therapy alone. This enhanced inhibition of tumor growth was observed to be specific not only to G47Δ but also to G207.

Whereas our studies were done using intraneoplastic inoculation of viral vector and whereas such inoculations may benefit locally advanced disease, studies in other settings have shown that i.v. delivery of HSV-1 vectors is feasible and can cause regression of distant prostate cancer deposits (4). This would be a clinically relevant area for future investigation for treatment of widely metastatic prostate cancer with G47Δ in combination with androgen ablation. Whereas the syngeneic TRAMP-C2 model used in the study is relatively resistant to HSV-1 pathogenicity among murine models, the improved efficacy of G47Δ may reflect into a greater clinical effect in which human prostate cancer cells are more susceptible to HSV-1.

Recently, replication-competent adenovirus vectors have been tested for recurrent or high-risk prostate cancer in multiple clinical studies (20, 21). The results showed that intraprostatic delivery of oncolytic viruses was safe and could cause a reduction in the serum prostate-specific antigen levels, warranting a further development of this therapeutic approach.

In summary, the data presented suggest that oncolytic virus therapy using G47Δ in combination with androgen ablation is worthy of further exploration and development for the treatment of primary or recurrent prostate cancer.

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