Triple Combination of Oncolytic Herpes Simplex Virus-1 Vectors Armed with Interleukin-12, Interleukin-18, or Soluble B7-1 Results in Enhanced Antitumor Efficacy

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Abstract

Conditionally replicating herpes simplex virus-1 (HSV-1) vectors are promising therapeutic agents for cancer. Insertion of therapeutic transgenes into the viral genome should confer desired anticancer functions in addition to oncolytic activities. Herein, using bacterial artificial chromosome and two recombinase-mediated recombinations, we simultaneously created four “armed” oncolytic HSV-1, designated vHsv-B7.1-1g, vHsv-interleukin (IL)-12, vHsv-IL-18, and vHsv-null, which express murine soluble B7-1 (B7.1-1g), murine IL-12, murine IL-18, and no transgene, respectively. These vHsv vectors possess deletions in the γ34.5 genes and contain the green fluorescent protein gene as a histochemical marker and the immunostimulatory transgene inserted in the deleted ICP6 locus. The vHsv showed similar replicative capabilities in vitro. The in vivo efficacy was tested in A/J mice harboring s.c. tumors of syngeneic and poorly immunogenic Neuro2a neuroblastoma. The triple combination of vHsv-B7.1-1g, vHsv-IL-12, and vHsv-IL-18 exhibited the highest efficacy among all single vHsv or combinations of two viruses. Combining 1 × 10^5 plaque-forming units each of the three armed viruses showed stronger antitumor activities than any single armed virus at 3 × 10^5 plaque-forming units in inoculated tumors as well as in noninoculated remote tumors. Studies using athymic mice indicated that this enhancement of antitumor efficacy was likely mediated by T-cell immune responses. The combined use of multiple oncolytic HSV-1 armed with different immunostimulatory genes may be a useful strategy for cancer therapy.

Replication-competent viral vectors are useful tools for the treatment of malignant tumors, because they can serve as oncolytic bioreagents as well as vectors that provide amplified gene delivery within the tumor. Herpes simplex virus-1 (HSV-1) is suited for clinical application, because it infects a wide variety of cell types, it exhibits strong cytotoxicity, circulating antibodies do not affect the cell-to-cell spread of the virus, and antiviral drugs are available (1). Oncolytic HSV-1 vectors have one or more genetic mutations in the viral genome, which restricts the viral replication to tumor cells, and therefore kill the host tumor cells without harming the normal tissue (2).

One of the advantages of HSV-1 vectors is the capacity to incorporate large and/or multiple transgenes within the viral genome (3). Aside from the extent of replication capability within the tumor, the efficacy of an oncolytic HSV-1 can be augmented by inducing antitumor immunity (4). Therefore, the genes of immunomodulatory molecules are potential candidates for “arming” oncolytic HSV-1 vectors. In situ expression of interleukin (IL)-12 or soluble B7-1 (B7.1-1g) when combined with G207, a double-mutated oncolytic HSV-1 currently used in clinical trials, significantly enhanced antitumor efficacy (5, 6). Replication-competent HSV-1 vectors expressing IL-12 or granulocyte macrophage colony-stimulating factor showed better efficacy than unarmed control vectors in various experimental tumor models (7–11).

Engineering new recombinant HSV-1 vectors using conventional homologous recombination techniques had been a laborious task that required time-consuming processes of selection and structure confirmation. Bacterial artificial chromosome (BAC) is a single-copy plasmid that can stably retain a large size (~300 kb) DNA as an insert (12). BAC plasmids have been used to propagate the entire HSV-1 genome in Escherichia coli, allowing an easy genetic manipulation (13, 14). In this article, we use a new BAC-using method for generating “armed” oncolytic HSV-1 vectors with the backbone of MGH-1, an oncolytic HSV-1 vector with the genome structure identical to G207 (i.e., deletions in both copies of the γ34.5 gene and a lacZ insertion inactivating the ICP6 gene; ref. 2). The method also uses two recombinase systems (FLP/FRT and Cre/loxP) to allow

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precise insertion of a desired transgene into the ICP6 locus of MGH-1 and an excision of the BAC sequences from the final structure. The method enabled us to create four different oncolytic HSV-1 vectors unarmed or armed with soluble B7-1 (B7-1-Ig), IL-12, or IL-18 simultaneously within a considerably short time, which further enabled us to compare the effect of the different transgenes expressed in the same oncolytic HSV-1 backbone. In a poorly immunogenic Neuro2a s.c. tumor model, combined intraneoplastic administration of the three armed oncolytic HSV-1 vectors (1/3 dose each) resulted in the highest in vivo efficacy compared with any other combination of armed or unarmed vectors with the same total dose and led to eradication of inoculated tumors as well as remote noninoculated tumors.

### Materials and Methods

#### Cells and viruses

Vero (African green monkey kidney) and Neuro2a (murine neuroblastoma) cell lines were purchased and maintained as described previously (6). MGH-1 has the structure identical to G207 (2) and was constructed from strain F as described (15). Virus titers were described previously (6). MGH-1 has the structure identical to G207 (2).

#### Vector construction

The vector construction method using HsvQuik system is described (17). Briefly, the pTIE4/5 shuttle plasmid has a HSV immediate-early promoter IE4/5, a multiple cloning site, a bovine growth hormone gene polyadenylate sequence, the ampicillin resistance gene (Amp), a loxP site, a B6Kk ori sequence, and a FRT site (Supplementary Fig. S1). The HsvQuik1 is a BAC plasmid and was created by a homologous recombination replacing the lacZ gene and adjacent 764-bp sequence within the ICP6 gene of MGH-1 with a 9.4-kb sequence consisting of BAC sequences {F ori and the chloramphenicol resistance gene (CmR), a loxP site, a FRT site, a red fluorescent protein cDNA, and an enhanced green fluorescent protein (EGFP) cDNA.

The 2.4-kb EcoRV/NotI fragment from pB7.1g (6) was inserted into the EcoRV/NotI site within the multiple cloning site of pTIE4/5 shuttle plasmid. The 2.3-kb Spel-AflII fragment from pTI-12 p40-ires-p35 (5) was inserted into the NheI/AflII site within the multiple cloning site of pTIE4/5 shuttle plasmid. The 0.55-kb EcoRI fragment from pCEV3-FN-β-IL-18 (ref. 16; gift from Dr. Isao Hara, Department of Urology, Kobe University, Kobe, Japan) was inserted into the EcoRI site within the multiple cloning site of pTIE4/5 shuttle plasmid. The entire sequence of each shuttle plasmid with the transgene (B7-1-Ig, IL-12, or IL-18) or the control shuttle plasmid without a transgene was inserted into the FRT site of HsvQuik1 by coelectroporation with pFTP-T, a plasmid that expresses tetracycline (Tc)-inducible FLP and a tetracycline selection marker. Ampicillin and chloramphenicol double-resistant colonies were selected and the genomic structures of the resultant recombinant plasmids that express tetracycline (Tc)-inducible FLP and a tetracycline selection marker were confirmed by restriction enzyme digestion.

Each HsvQuik1 transgene DNA was purified from E. coli and cotransfected with the pc-nCre plasmid that expresses Cre recombinase into Vero cells using LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. A transient expression of Cre recombinase results in excision of the sequence between the two loxP sites (containing F ori, γ ori, Amp gene, Cmr gene, and red fluorescent protein). Five days later, the viruses were harvested and passaged on Vero cells, and virus plaques negative for red fluorescence were further selected and purified through three-time limiting dilutions on Vero cells. The structures of the recombinant viruses were confirmed by Southern blot analyses. Virus DNA was digested with HindIII, transferred to a nylon membrane, and probed with cDNA for murine IL-12, murine IL-18, or murine B7-1-Ig from the shuttle plasmids. The hybridized DNA bands were visualized using AlkPhos Direct Labeling and Detection System with CDP-Star (Amersham, Piscataway, NJ). The resultant recombinant virus contains deletions in both copies of the γ34.5 gene, a deletion within the ICP6 gene, the ICP6-GFP fusion driven by the endogenous ICP6 promoter, and a therapeutic transgene driven by the IE4/5 promoter. The vectors expressing soluble murine B7-1-Ig, murine IL-18, or murine IL-12, or no transgene were designated as vHsv-B7.1-Ig, vHsv-IL-12, vHsv-IL-18, and vHsv-null, respectively (Fig. 1). Virus stocks were purified and concentrated as described (19).

#### Virus yield studies

Vero or Neuro2a cells (4 × 10^5) were seeded on six-well plates. After a 24-hour incubation at 37°C, cells were infected with vHsv-B7-1-Ig, vHsv-IL-12, vHsv-IL-18, vHsv-null, or MGH-1 at a multiplicity of infection (MOI) of 0.01 (for Vero) or 0.1 (for Neuro2a) and further incubated at 37°C for 48 hours (20). Progeny viruses were titered on Vero cells.

#### In vitro cytotoxicity studies

In vitro cytotoxicity studies were done as described (4). Neuro2a cells (2 × 10^5) were seeded on six-well plates and incubated at 37°C overnight. Virus or mock was inoculated onto cells in a volume of 0.7 mL for 1 hour. The inoculum was then removed and cells were incubated at 34.5°C in DMEM supplemented with 1% heat-inactivated FCS. The number of surviving cells was counted daily with a Coulter counter (Beckman Coulter, Miami, FL) and expressed as a percentage of mock-infected controls. Our basic studies indicated that the viable cell count obtained by the above method is consistent with that determined by trypan blue exclusion.

#### In vitro detection of transgene expression

Vero cells (1 × 10^5) were seeded onto 24-well plates and incubated at 37°C overnight. The pTIE4/5 shuttle plasmids (pTIE4/5 B7-1-Ig, pTIE4/5 IL-12, and pTIE4/5 IL-18; 0.5 μg each) were transfected to cells using LipofectAMINE Plus reagent according to the manufacturer’s instruction. The cells were further incubated at 37°C in 0.6 mL DMEM supplemented with 1% heat-inactivated FCS for 48 hours. For the recombinant viruses, Vero cells were inoculated with the virus (MOI = 1) or mock extract and incubated at a nonpermissive temperature of 39.5°C in 0.6 mL DMEM supplemented with 1% heat-inactivated FCS. We observed that the cytopathic activities of the viruses were remarkably attenuated at 39.5°C. The supernatant was collected daily and cytokine concentrations were determined by standard methodology (16).
measured (for IL-12 and IL-18) or cells were fixed with 4% paraformaldehyde in PBS (for B7.1-Ig). IL-12 concentration was measured by mouse IL-12 p70 Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN), and IL-18 concentration was measured by mouse IL-18 ELISA kit (MBL, Nagoya, Japan). B7.1-Ig expression was determined by immunohistochemical staining using a biotin-conjugated antihuman IgG. Fc antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:50 dilution) as described (6).

In vitro IFN-γ stimulation studies. Spleen cells were harvested from a naïve 6-week-old female A/J mouse. In 96-well plates, RBC-depleted spleen cell suspension (1.5 × 10^5 cells per well) were cultured with conditioned medium of Vero cells transfected with a shuttle plasmid (pT IE4/5 IL-12 or pT IE4/5 IL-18) or mock in 200 μL complete medium (RPMI 1640 supplemented with 10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5 μg/mL fungizone, 2 mM L-glutamine, 1 mM L-sodium pyruvate, 0.1 mM L-nonessential amino acids, 50 μM L-2-mercaptoethanol) for 24 hours at 37°C. Recombinant mouse IL-16 (MBL, Nagoya, Japan) was used as positive control. After centrifugation at 2,000 rpm for 10 minutes, the IFN-γ concentration in the medium was measured by mouse IFN-γ ELISA kit (Endogen, Inc., Woburn, MA).

Animal studies. Six-week-old female A/J mice or athymic mice (BALB/c nu/nu) were purchased from the National Cancer Institute (Frederick, MD). All animal procedures were approved by the Institutional Committee on Research Animal Care.

Subcutaneous tumor therapy. Subcutaneous tumors were generated by injecting 5 × 10^6 Neuro2a cells s.c. into the bilateral or left flank(s) of 6-week-old female A/J mice or athymic mice (BALB/c nu/nu). When s.c. tumors reached ~5 mm in diameter 5 to 6 days after implantation, a standard size used in our previous studies using this model (6, 20), animals were randomized, and mock or virus [8 × 10^2 to 10^6 plaque-forming units (pfu)] in 20 μL PBS containing 10% glycerol was inoculated into left tumors. Viral administration was repeated 3 days later. Mock-infected extract (mock) was prepared from virus buffer–infected cells using the same procedures as those used for virus inoculum. Tumor growth was determined by measuring the tumor volume (length × width × height) thrice weekly (4). Animals were sacrificed when the maximum diameter of the tumor on either side reached 20 mm.

In vivo measurement of IL-12 and IL-18. S.c. Neuro2a tumors were generated in the left flank of female A/J mice. When tumors reached ~5 mm in diameter, vHSV-IL-12, vHSV-IL-18 (2 × 10^6 pfu), or mock virus extract in 20 μL PBS containing 10% glycerol was inoculated into the tumor. On days 1, 4, and 7, blood was collected by retro-orbital bleeding from two mice per group, after which the mice were euthanized and tumors were harvested. Tumors were homogenized in ice-cold PBS, sonicated, and centrifuged, and the cytokine concentration in the supernatant was measured by ELISA. Samples from each mouse were measured in triplicates. The detection limits for IL-12 were 12.5 pg/mL and 0.5 pg/mg for serum and tumor homogenates, respectively, and those for IL-18 were 125 pg/mL and 5 pg/mg, respectively.

Rechallenge studies. Mice whose established s.c. Neuro2a tumors regressed after vHSV treatment as well as age-matched naïve female A/J mice were used. Neuro2a cells (5 × 10^5) were injected s.c. into the right flank, and tumor growth was observed as described above. The animals were followed for 60 days.

Statistical analysis. All in vitro data and in vivo tumor volume data were evaluated by unpaired t-test.

Results

Generation and in vitro characterization of vHSV vectors. Using the HsvQuik system, we generated four oncolytic HSV-1 vectors unarmed or armed with soluble murine B7-1, murine IL-12, or murine IL-18, termed vHSV-null, vHSV-B7.1-Ig, vHSV-IL-12, and vHSV-IL-18, respectively. These vHSV vectors had a 1-kb deletion in both copies of the g34.5 gene and a 764-bp deletion within the ICP6 gene (Fig. 1). The transgene driven by the I4E/5 promoter and the GFP gene as a marker were inserted in the deleted ICP6 locus. The four vectors were constructed in parallel, and the final products were obtained within 6 months, after which the structures were confirmed by Southern blot analyses (Supplementary Fig. S2).

The virus yield obtained 48 hours after infection of Vero cells at a MOI of 0.01 for vHSV-null, vHSV-B7.1-Ig, vHSV-IL-12, and vHSV-IL-18 were 5.6 × 10^5, 4.2 × 10^5, 6.4 × 10^5, and 2.8 × 10^5 pfu, respectively. The results were considered to reflect both replicative and infective capabilities, and there was no significant difference among the vHSV vectors. The virus yield of the parental MGH-1 in Vero cells was 2.9 × 10^6 pfu, 5- to 10-fold greater than vHSV vectors. In Neuro2a murine neuroblastoma cells, the ratio of progeny virus recovery (recovery/input) 48 hours after infection was 0.10 for vHSV-null and 0.021 for MGH-1, both considerably lower than the yields obtained in Vero cells. We further tested the in vitro cytopathic activity of vHSV vectors in Neuro2a cells. At a MOI of 0.1, all vHSV vectors showed comparable cytopathic activities, killing ~70% to 80% of the cells by day 4 (Fig. 2A). In contrast to the results from virus yield studies, vHSV-null (MOI = 0.1) killed Neuro2a cells more rapidly than MGH-1 (P < 0.001, days 1-4; Fig. 2B).

Fig. 2. Cytopathic effect of vHSV vectors and MGH-1 on Neuro2a murine neuroblastoma cells in vitro. Neuro2a cells were plated into six-well plates at 2 × 10^5 per well. After a 24-hour incubation, cells were infected with virus at MOI = 0.1 or virus buffer (mock). The number of surviving cells was counted daily and expressed as a percentage of mock-infected control. A, vHSV-null and three armed vHSV vectors showed comparable cytopathic effects in vitro. B, MGH-1 killed Neuro2a cells less rapidly than vHSV-null (P < 0.001, days 1-4). Points, mean of triplicates; bars, SD.
The in vitro expression of the transgenes arming the vHsv vectors was checked in Vero cells. After infection with vHsv-IL-12 at a MOI of 1, the IL-12 concentration of the conditioned medium at 24, 48, and 72 hours was 7.84, 21.2, and 23.7 ng/mL, respectively. Similarly with vHsv-IL-18, the IL-18 concentration at 24, 48, and 72 hours was 0.649, 1.32, and 1.45 ng/mL, respectively. The IL-12 or IL-18 expressed by the transgenes was confirmed to have an intact bioactivity by the ability of conditioned medium of Vero cells transfected with pT IE4/5 IL-12 or pT IE4/5 IL-18 to stimulate IFN-γ secretion from mouse spleen cells (data not shown). The expression of soluble murine B7-1 by vHsv-B7.1-Ig was

![Graphs](image-url)
confirmed by immunostaining for human IgG Fc in Vero cells 48 hours after infection (data not shown).

**In vivo efficacy of vHsv vectors.** To characterize the in vivo performance of vHsv vectors, we first tested the efficacy of vHsv-null in comparison with the parental MGH-1. As an animal tumor model, we used A/J mice and syngeneic Neuro2a neuroblastoma cells that form s.c. tumors with ~100% efficiency, grow rapidly, and rarely ulcerate. A/J is one of the most susceptible inbred mouse strain to HSV-1 infection (21), and Neuro2a cells are poorly immunogenic and moderately susceptible to oncolytic HSV-1 infection and replication (6, 9, 20). Subcutaneous Neuro2a tumors were established in both flanks of A/J mice and the left-sided tumors were treated with intraneoplastic inoculations of mock, MGH-1, or vHsv-null (2 × 10⁶ pfu) on days 0 and 3 (n = 9 per group). Whereas both MGH-1 and vHsv-null caused growth inhibition of the inoculated tumors compared with mock, vHsv-null exhibited significantly greater efficacy than MGH-1 (P < 0.05, days 6-13). The vHsv-null treatment also caused growth inhibition of the contralateral noninoculated tumors (P < 0.05, days 10-13; Fig. 3A), whereas the MGH-1 treatment showed no effect (P = 0.15, day 13). We showed previously that, in immunocompetent mice, oncolytic HSV-1 inoculated into s.c. tumors was not detected in remote noninoculated tumors (4). The vHsv-null and MGH-1 have similar basic structures, but a major difference is that GFP is used as a histochemical marker in vHsv-null versus lacZ in MGH-1. Because GFP is known for its immunogenicity (22, 23), we investigated whether the increased antitumor efficacy of vHsv-null over MGH-1 involves T-cell responses. Bilateral s.c. Neuro2a tumors were generated in athymic mice, and the left tumors alone were treated with mock, MGH-1, or vHsv-null in a similar manner as in A/J mice. In athymic mice, there was no difference in efficacy between MGH-1 and vHsv-null in the inoculated side, and none of the treatments affected the growth of contralateral tumors (data not shown). The result suggested that GFP expressed in vHsv vectors played a role in increasing the antitumor efficacy potentially via T-cell-mediated immune responses.

To determine the effective dose range for vHsv vectors, A/J mice with unilateral s.c. Neuro2a tumors were treated with intraneoplastic inoculations of vHsv-null at three different doses (2 × 10⁵, 4 × 10⁵, and 8 × 10⁵ pfu) on days 0 and 3 (n = 9 per group). Inoculations with vHsv-null at 4 × 10⁵ pfu or higher significantly inhibited the growth of Neuro2a tumors in a dose-dependent manner (Fig. 3B). Therefore, to investigate the effect of transgenes expressed by the vHsv vectors, we initially selected 1 × 10⁵ pfu, a dose at which vHsv-null would exhibit a minimal effect. A/J mice with unilateral s.c. Neuro2a tumors were treated with intraneoplastic inoculations of mock, vHsv-null, vHsv-B7.1-Ig, vHsv-IL-12, or vHsv-IL-18 on days 0 and 3 (n = 9 per group). The treatment with vHsv-IL-12 resulted in a significantly greater tumor growth inhibition than other treatments (P < 0.05 versus each group, days 5-14; Fig. 3C). Both vHsv-B7.1-Ig and vHsv-IL-18 showed a marginal but significant tumor growth inhibition compared with mock (P < 0.05, days 9-14). Next, we chose a higher dose (2 × 10⁶ pfu) and did a study using A/J mice with bilateral s.c. Neuro2a tumors, mainly focusing on the antitumor effect in the contralateral noninoculated tumors. All vHsv vectors, inoculated intraneoplastically on days 0 and 3, significantly and equally inhibited the growth of inoculated tumors (n = 6 per group). Also in the contralateral side, all vHsv vectors significantly suppressed the tumor growth compared with mock (P < 0.01, days 10-15 for vHsv-IL-12 and vHsv-IL-18 and days 13-15 for vHsv-B7.1-Ig; P < 0.05, days 13-15 for vHsv-null), and there was no significant difference among the four vHsv vectors (Fig. 3D).

To investigate the kinetics of in vivo transgene expression, serum and intratumoral concentrations of IL-12 and IL-18 were determined 1, 4, and 7 days after a single intraneoplastic inoculation of vHsv-IL-12 and vHsv-IL-18 (2 × 10⁶ pfu), respectively, in A/J mice bearing s.c. Neuro2a tumors (n = 2 per group). The intratumoral IL-12 gradually decreased but was detectable on all days tested through day 7; however, serum IL-12 was detectable only on day 1 (Fig. 4). IL-18 was detectable in both serum and tumor on all days tested, although gradually decreased by day 7. There was no difference in size between vHsv-IL-12-inoculated and vHsv-IL-18-inoculated tumors. Neither IL-12 nor IL-18 was detected in serum and tumor in mock-treated or vHsv-null-treated mice.

**Enhancement of in vivo efficacy by combinations of vHsv vectors.** Certain combinations of immunostimulatory molecules, such as IL-12/B7-1 and IL-12/IL-18, have been reported to act synergistically (24–26); therefore, we further investigated whether combining vHsv vectors could enhance the antitumor efficacy. First, each armed vHsv vector (1 × 10⁵ pfu) was administered alone or in combination with others, and the total administered virus amount was adjusted to 3 × 10⁵ pfu (except mock) by adding necessary amounts of vHsv-null. The left tumors of A/J mice bearing bilateral s.c. Neuro2a tumors were treated with intraneoplastic inoculations on days 0 and 3 (n = 8 per group; Fig. 5A). In the inoculated side, all armed vHsv vectors, alone or combined, showed significant tumor growth inhibition (P < 0.05, days 6-13). The combination of vHsv-IL-12 and vHsv-IL-18 showed a significantly greater antitumor efficacy than other combinations of two armed vectors (P < 0.05, days 6-13). In the contralateral noninoculated tumors, an enhancement of antitumor efficacy was observed when the left tumors were
treated with vHsv-IL-12 plus vHsv-IL-18 or all three vectors combined, the latter treatment being more efficacious than the former ($P < 0.05$, days 8-13).

We further investigated, using the same animal model and treatment protocol, whether the combination of the three armed vHsv vectors ($1 \times 10^5$ pfu each) is more efficacious than a single vector given at $3 \times 10^5$ pfu. The combination treatment exhibited a significantly greater antitumor efficacy than any single vector treatment on both inoculated and noninoculated sides ($P < 0.05$ versus any group for both sides, days 9, 11, and 15; Fig. 5B), showing that, for the enhancement of antitumor efficacy, it is the combination that is important rather than the total amount of armed oncolytic HSV-1 vectors.

To investigate whether the enhancement of antitumor efficacy by the combination therapy requires T cells, s.c. Neuro2a tumors were generated in the left flanks of athymic mice and inoculated with $3 \times 10^5$ pfu of each vHsv vector or the combination of all three armed vHsv vectors ($1 \times 10^5$ pfu of each) on days 0 and 3 ($n = 6$ per group). Both the vHsv-IL-12 group and the combination group showed a significantly greater antitumor effect compared with other treatment groups ($P < 0.05$, days 7-11 for both vHsv-IL-12 and combination groups). In contrast to the results using A/J mice, vHsv-null, vHsv-B7.1-Ig, and vHsv-IL-18 showed no significant effect, suggesting that T-cell-mediated responses are largely responsible for the antitumor effect exhibited by these vectors at this dose. The superior antitumor efficacy of vHsv-IL-12 to other vectors in athymic mice is compatible with the known function of IL-12 that involves activation of natural killer cells (27). The combination therapy, however, did not increase the antitumor efficacy compared with the vHsv-IL-12 alone treatment, indicating that the enhancement effect by combining with vHsv-IL-18 and vHsv-B7.1-Ig may involve T-cell-mediated immune responses (Fig. 6A).

Furthermore, we tested whether the strong antitumor efficacy by the combination therapy observed in the contralateral noninoculated tumors in A/J mice involves T-cell immune responses. Bilateral s.c. Neuro2a tumors were generated in
athymic mice, and the left tumors alone were treated with intraneoplastic inoculations of mock, vHsv-null (3 × 10^5 pfu), or the three armed vHsv vectors combined (1 × 10^5 pfu each) on days 0 and 3 (n = 8 per group). In the inoculated side, reproducing the result from the previous experiment, vHsv-null showed no effect at this dose, whereas the combination resulted in a significant growth inhibition (P < 0.05 versus mock and vHsv-null, days 6-10; Fig. 6B). In the contralateral noninoculated tumors, however, the combination therapy caused no significant effect on tumor growth, indicating that the enhancement of the antitumor efficacy on remote tumors requires T cells.

To see whether arming of oncolytic HSV-1 vectors with immunostimulatory genes could provide stronger protective immunity, we pooled the A/J mice whose s.c. Neuro2a tumors were cured by treatment with vHsv vectors and rechallenged them with a s.c. injection of 5 × 10^6 Neuro2a cells. All of 10 naive 3-month-old A/J mice, used as control, showed rapid growth of s.c. tumors. Two of 5 vHsv-null-treated mice showed tumor growth, whereas only 1 of 15 armed vHsv vector-treated mice did (P = 0.1, Fisher’s test), suggesting that the in situ expression of immunostimulatory genes may provide enhanced antitumor protective immunity.

**Discussion**

The present system (termed HsvQuik) using BAC and the two recombinases (FLP and Cre) has multiple advantages over conventional homologous recombination techniques for the construction of armed oncolytic HSV-1 vectors, such as an insertion of desired transgenes into the intended locus with high probabilities and precision and selection of recombinant BAC plasmid clones in E. coli. The HsvQuik system allowed us to create four different oncolytic HSV-1 vectors (three armed and one unarmed control) simultaneously in a relatively short period. The transgene expression mediated by oncolytic HSV-1 vectors is transient in a host cell due to eventual cell destruction, yet the expression level within the tumor may be high due to an amplified gene delivery (28). Moreover, the destruction of tumor cells during viral oncolysis elicits specific antitumor and antiviral immune responses. Therefore, unlike replication-defective viral and nonviral vectors, it is difficult to predict the effect of the transgene expression in oncolytic HSV-1 vectors, especially if it might affect host immune responses, viral replication, and/or viral spread. The HsvQuik system allowed screening and simultaneous comparison of the in vivo effects of armed vectors.
effects of various transgenes expressed in the same oncolytic HSV-1 backbone.

Antitumor immune responses play important roles in the in vivo antitumor activities of oncolytic HSV-1 vectors, which supports the usage of immunostimulatory genes for arming the HSV-1 vectors (4, 6, 29, 30). We compared the efficacy of oncolytic HSV-1 vectors armed with murine soluble B7-1, murine IL-12, or murine IL-18 using HSV-1-sensitive A/J mice and syngeneic, poorly immunogenic Neuro2a tumors and showed that vHsv-IL-12 was the most efficacious at a low dose of 1 × 10^4 pfu. The superior efficacy was unlikely due to higher expression levels of IL-12 in vivo, because the serum and intratumoral concentration levels of IL-12 exceeded those of IL-12. It has been reported that NV1042, an oncolytic HSV-1 vector expressing murine IL-12, was more efficacious than a murine granulocyte macrophage colony-stimulating factor–expressing oncolytic HSV-1 vector with the same backbone (NV1034) in certain animal tumor models (10). In athymic mice, only vHsv-IL-12 (3 × 10^5 pfu) but not other vHsv vectors showed a significant growth suppression of the inoculated tumors, indicating that the augmented efficacy by IL-12 expression may be mediated, at least in part, via non-T-cell mechanisms, including an activation of natural killer cells (27), which may be a functional advantage over soluble B7-1 and IL-18 at low dose levels. In addition, it has been suggested that antitumor actions of NV1042 were partly exhibited via antiangiogenic effects of IL-12 (31). In A/J mice at a high dose of 2 × 10^5 pfu, the efficacy of a single armed vHsv vector was not significantly different from that of vHsv-null in both inoculated and noninoculated tumors regardless of the transgene, indicating that an addition of a single immunostimulatory gene expression may not significantly augment the efficacy of oncolytic HSV-1 vectors when a sufficient amount is administered. HSV-1 vectors have inherent ability to down-regulate MHC class I expression of infected host cells, which may undermine the immunostimulatory potentials of oncolytic HSV-1 vectors armed with cytokines. We have shown previously that tumor cells infected with an s47-deficient HSV-1 vector had enhanced MHC class I expression and showed increased stimulation of immune cells (20).

One of the most important advantages of using the HsvQuik system was that it allowed us to study the effect of combining the multiple armed oncolytic vectors created simultaneously. We showed that the triple combination of vHsv-B7.1-lg, vHsv-IL-12, and vHsv-IL-18 exhibited the highest efficacy among all single vHsv vectors or combinations of two vectors against the poorly immunogenic tumors. Combining 1 × 10^5 pfu each of the three armed vectors showed stronger antitumor activities than any single armed vector at 3 × 10^5 pfu in both inoculated and noninoculated sides, leading to “cures” in two of eight animals. In athymic mice, the enhancement of antitumor efficacy caused by the combination was reduced in inoculated tumors compared with A/J mice and abolished in remote noninoculated tumors. These data implicated that the enhanced efficacy resulted from the interactions between the three different immunostimulatory molecules expressed within the tumor that may have worked favorably to augment T-cell-mediated antitumor immune responses. In theory, coinfection of tumor cells with multiple oncolytic HSV-1 vectors can lead to generation of novel recombinants in vivo, which needs investigating if such therapeutic strategy were to be applied clinically.

A simultaneous usage of three oncolytic vectors armed with different immunostimulatory genes has never been reported, but certain combinations of two immunostimulatory molecules have been known to act synergistically in experimental cancer immunotherapy. The synergistic effects of IL-12 and IL-18 have been well documented (32). Although the combination of IL-12 and IL-18 caused severe toxicity when administered systemically with excessive increase in serum IFN-γ (33–37), a local expression of one combined with systemic administration of the other could enhance the antitumor efficacy with less toxicity (26, 38, 39). We observed that the combination of vHsv-IL12 and vHsv-IL18 exhibited a stronger antitumor effect on remote noninoculated tumors than any other combinations of two vectors. A combination of tumor cells expressing B7-1, a membrane-bound, potent costimulatory factor, and a local or systemic administration of IL-12 has been shown to cause enhanced stimulation of CTLs and a long-term protective immunity (40–42). We have shown previously that in situ expression of the soluble type of B7-1 in the context of G207 caused a strong augmentation of T-cell-mediated antitumor immune responses that occurred in the brain as well as in the periphery (6). We observed, however, that a combination with vHsv-B7.1-lg did not significantly enhance the efficacy of vHsv-IL-12 at the doses tested.

The HsvQuik system has been proven to be a powerful tool for screening the effect of transgenes inserted into oncolytic HSV-1 vectors. Transgene candidates are not limited to foreign therapeutic genes but include a HSV-1 gene, such as γ34.5, driven by a tumor-specific promoter (43). Some features of HsvQuik may benefit further improvement: One such feature is the use of GFP as a histochemical marker in the final product, because the effect of GFP expression may not be small enough to ignore when evaluating the effect of immunostimulatory gene expressions. GFP has been reported to show substantial cytotoxicity as well as immunogenicity (22, 23, 44). The in vitro cytopathic effect of vHsv-null in Neuro2a cells was greater than expected from virus yield study results compared with MGH-1, a major difference between vHsv-null being that it has lacZ instead of GFP. The vHsv-null showed a significantly greater efficacy than MGH-1 in both treated and remote tumors in A/J mice, but no difference in efficacy was observed in athymic mice. The antitumor efficacy of armed vHsv vectors in A/J mice was not significantly different from that of vHsv-null at higher doses. These unexpected results may be accounted for, at least in part, by the direct cytotoxic effect and/or immunogenicity of GFP. Alternatively, GFP expression may facilitate tumor cell killing that leads to release of tumor antigens, which can result in cross-priming of T cells to recognize and kill Neuro2a cells via systemic immunity. On the other hand, GFP has been used in other oncolytic HSV-1 vectors, and its expression may be beneficial for cancer therapy, if in fact it adds to antitumor activities without compromising safety (45, 46).

The replicative capabilities of vHsv vectors were remarkably lower than MGH-1 in Vero cells for reasons yet to be elucidated. Although the structures of vHsv vectors were confirmed by Southern blot analyses, a small possibility remains as to an MGH-1, which may be a functional advantage over soluble B7-1 and γ34.5, driven by a tumor-specific promoter (43). Some features of HsvQuik may benefit further improvement: One such feature is the use of GFP as a histochemical marker in the final product, because the effect of GFP expression may not be small enough to ignore when evaluating the effect of immunostimulatory gene expressions. GFP has been reported to show substantial cytotoxicity as well as immunogenicity (22, 23, 44). The in vitro cytopathic effect of vHsv-null in Neuro2a cells was greater than expected from virus yield study results compared with MGH-1, a major difference between vHsv-null being that it has lacZ instead of GFP. The vHsv-null showed a significantly greater efficacy than MGH-1 in both treated and remote tumors in A/J mice, but no difference in efficacy was observed in athymic mice. The antitumor efficacy of armed vHsv vectors in A/J mice was not significantly different from that of vHsv-null at higher doses. These unexpected results may be accounted for, at least in part, by the direct cytotoxic effect and/or immunogenicity of GFP. Alternatively, GFP expression may facilitate tumor cell killing that leads to release of tumor antigens, which can result in cross-priming of T cells to recognize and kill Neuro2a cells via systemic immunity. On the other hand, GFP has been used in other oncolytic HSV-1 vectors, and its expression may be beneficial for cancer therapy, if in fact it adds to antitumor activities without compromising safety (45, 46).

The replicative capabilities of vHsv vectors were remarkably lower than MGH-1 in Vero cells for reasons yet to be elucidated. Although the structures of vHsv vectors were confirmed by Southern blot analyses, a small possibility remains as to an unintended mutation being introduced during the BAC plasmid construction. The parental MGH-1 is considerably attenuated in situ, which may be a functional advantage over soluble B7-1 and γ34.5, driven by a tumor-specific promoter (43). Some features of HsvQuik may benefit further improvement: One such feature is the use of GFP as a histochemical marker in the final product, because the effect of GFP expression may not be small enough to ignore when evaluating the effect of immunostimulatory gene expressions. GFP has been reported to show substantial cytotoxicity as well as immunogenicity (22, 23, 44).
the γ34.5 and ICP6 genes. The low rate of replication may be beneficial for evaluating the effect of transgene expressions in experimental animals. In clinical practice, however, to obtain an efficient therapeutically effective from a small portion of vector-infected tumor cells, it may be preferable to retain good replicative capability, providing a large amplification of gene delivery as well as effective oncolytic activities. Furthermore, it is important for the backbone structure of armed oncolytic HSV-1 vectors to have a large therapeutic window by keeping the viral replication highly selective to tumor cells, because the transgene expression might increase the toxicity. A triple-mutated oncolytic HSV-1 vector, G47Δ, constructed from G207 by creating a further deletion within the z47 gene and the overlapping US11 promoter, was shown to have enhanced viral replication in tumor cells while preserving the safety features (20). A BAC-mediated oncolytic armed HSV-1 vector construction system with G47Δ as the backbone has been developed (47).

Published studies have shown that suppression of innate immunity can enhance the oncolytic virus replication and antitumor efficacy (48, 49). Whether the expression of the immunostimulatory transgenes significantly affected the in vivo replication of vHSV vectors is yet to be determined. A possibility remains that immune stimulation is beneficial when the oncolytic virus replicates relatively poorly. We observed that systemic administration of immunostimulatory cytokines did not alter the intratumoral replication of G47Δ. The results suggest that immune stimulation would be beneficial for oncolytic virus therapy if an effective T-cell-mediated antitumor immunity can be obtained.

We show that a combined use of multiple oncolytic HSV-1 vectors armed with different immunostimulatory genes can be a useful strategy for cancer therapy. We also show that the time-consuming processes of creating recombinant HSV-1 vectors can be overcome by using BAC and recombinase-mediated recombination. We believe that the development of armed oncolytic HSV-1 vectors is important not only to improve the efficacy but also to cope with a wide variation of cancer types, progression stages, or routes of administration.

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References


# Clinical Cancer Research

## Triple Combination of Oncolytic Herpes Simplex Virus-1 Vectors Armed with Interleukin-12, Interleukin-18, or Soluble B7-1 Results in Enhanced Antitumor Efficacy

Yasushi Ino, Yoshinaga Saeki, Hiroshi Fukuhara, et al.


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