Triple Gene-Deleted Oncolytic Herpes Simplex Virus Vector Double-Armed with Interleukin 18 and Soluble B7-1 Constructed by Bacterial Artificial Chromosome–Mediated System

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Abstract

Conditionally replicating herpes simplex virus type 1 (HSV-1) vectors are promising therapeutic agents for cancer. Certain antitumor functions may be added to oncolytic activities of recombinant HSV-1 vectors by inserting transgenes into the viral genome. Because conventional homologous recombination techniques had required time-consuming processes to create “armed” oncolytic HSV-1 vectors, we established an innovative construction system using bacterial artificial chromosome and two recombinase systems (Cre/loxP and FLPe/FRT). Using G47Δ, a safe and efficacious oncolytic HSV-1 with triple gene mutations, as the backbone, this system allowed a rapid generation of multiple vectors with desired transgenes inserted in the deleted ICP6 locus. Four oncolyic HSV-1 vectors, expressing murine interleukin 18 (mIL-18), soluble murine B7-1 [B7-1-immunoglobulin (B7-1-Ig)], both, or none, were created simultaneously within 3 months. In vitro, all newly created recombinant vectors exhibited virus yields and cytopathic effects similar to the parental G47Δ. In two immunocompetent mouse tumor models, TRAMP-C2 prostate cancer and Neuro2a neuroblastoma, the vector expressing both mIL-18 and B7-1-Ig showed a significant enhancement of antitumor efficacy via T-cell–mediated immune responses. The results show that “arming” with multiple transgenes can improve the efficacy of oncolytic HSV-1 vectors. The use of our system may facilitate the development and testing of various armed oncolytic HSV-1 vectors.

Discussion

The key to developing useful oncolytic herpes simplex virus type 1 (HSV-1) vectors is to acquire high antitumor efficacy without compromising safety, obtaining as wide therapeutic window as possible. G207 is one of the first oncolytic HSV-1 vectors used in clinical trials (1) and has deletions in both copies of the γ34.5 gene and a lacZ insertion inactivating the ICP6 gene (2). The double mutations permit viral replication within cancer cells that can complement these mutations but not in normal cells. G207, however, may be considerably attenuated not only for the pathogenicity but also for the tumor cell killing capability compared with wild-type HSV-1. G47Δ was constructed by creating a further deletion within the zγ7 gene and the overlapping US11 promoter of the G207 genome (3). This additional deletion conferred enhanced viral replication in tumor cells and partial restoration of MHC class I expression in infected human cells, resulting in drastic improvement of antitumor efficacy while preserving the safety features.

One of the advantages of HSV-1 vectors is the capacity to incorporate large and/or multiple transgenes within the viral genome. Aside from the extent of replication capability within the tumor, the efficacy of an oncolytic HSV-1 depends on the extent of antitumor immunity induction (4, 5). Therefore, the genes of immunomodulatory molecules would be reasonable candidates for “arming” oncolytic HSV-1 vectors. Conventionally, recombinant HSV-1 vectors have been constructed using homologous recombination techniques, which required time-consuming processes of selection and structure confirmation. Bacterial artificial chromosome (BAC) enables manipulation of large eukaryotic sequences such as oversized HSV-1 ampiclons and HSV-1 genomes in plasmids (6–11). In this article, we used BAC and two recombinase systems (Cre/loxP and FLPe/FRT) to develop a method that allowed a rapid, reliable, and simultaneous construction of multiple “armed” oncolytic HSV-1 vectors using G47Δ as the backbone.

Materials and Methods

Cells and viruses. Vero, Neuro2a, Prt14-2, and TRAMP-C2 cells were cultured as described (3). G47Δ was grown in Vero cells and virus titers were determined as described (2, 3).

Generation of BAC-G47Δ plasmid. BAC-G47Δ was created by a homologous recombination of G47Δ DNA and pBAC-ICP6EF, a plasmid that contains the insertion sequences of the ICP6 coding region. Transfections were done on Vero cells by using 0.9 μg of DNA composed of a 1:1 mixture of G47Δ DNA purified by Na/I method, pBAC-ICP6EF (undigested), and pBAC-ICP6EF linearized with AscI digestion, with Lipofectamine (Invitrogen, Carlsbad, CA). At a 30% to 50% cytopathic effect, recombinant viruses forming green fluorescent protein (GFP)–positive plaques were selected and further passaged in Vero cells (Supplementary Fig. S1). After three rounds of GFP-positive and lacZ-negative selection, circular virus DNA from infected Vero cells was isolated (Supplementary Fig. S2).

Construction of shuttle vectors. The shuttle vector pVec9 was constructed to contain a 45 bp FRT adaptor (5′-GATCCGAAGTTCTCTATA-CTTCTAGAATAGGAACCTGCGG-3′), a 50 bp loxP adaptor (5′-AGCT- TATAAATGTGTTATGATCTATACGAAGTTATCCATGGCTGCA-3′), and a 45 bp FRT adaptor (5′-GATCCGAAGTTCTCTATA-CTTCTAGAATAGGAACCTGCGG-3′).
the lacZ gene from pcDNA6/Eun-lacZ (Clontech, Mountain View, CA), an expression cassette with the cytomegalovirus (CMV) promoter and bovine growth hormone poly(A) from pVPZ2/myc-His2 (Invitrogen), a 3.989-bp fragment of the λ HindIII DNA, and a multiple cloning site sequence of AvrII, Stul, and NolI (GATCCCTTCGATGTCGCTAC/AAGCGGGCGCTCCGGC).

A 2.4-kb HindIII-NolI fragment containing the extracellular domain of B7-1 and the Fe portion of human immunoglobulin G (IgG) gene from B7.1-plg (5) was inserted into the AvrII site of pVec9 to generate murine B7-1-Ig (mB7-1-Ig)/Vec9. A 3.82-bp EcoRI fragment containing a mouse IFN-β signal sequence and a mature interleukin 18 (IL-18) sequence from pCEXV3/hybrid IL-18 (18) was provided by Dr. Isao Hara, Kobe University, Kobe, Japan; ref. 14) was inserted into the Stul site of pVec9 to generate mL18/Vec9. A 3.3-kb fragment containing the hybrid IL-18 gene inserted into the polylinker region of pRES (Clontech) and the B7-1-Ig gene was inserted into the AvrII site of pVec9 to generate IL18-B7/Vec9.

Reconstitution of BAC-G47Δ virus. Mutagenesis of the BAC-G47Δ plasmid was done by a two-step replacement procedure. Mixtures of BAC-G47Δ plasmid (1.5 µg) and mB7-1-Ig/Vec9, mL18/Vec9, IL18-B7/Vec9, or empty Vec9 (150 ng each) was incubated with Cre recombinase (NEB, Ipswich, MA) at 37°C for 30 minutes in 10 µL of solution and was electroporated into E.coli DH10B. To select those that contained the mutant BAC plasmid, the bacteria were streaked onto LB plates containing Cm (15 µg/mL) and Kan (10 µg/mL) and incubated at 37°C overnight. DNA structures of the recombinant BAC-G47Δ/Vec9 plasmids were confirmed by gel analyses following endonuclease digestions (Supplementary Fig. S3).

Transfections were done on Vero cells by using 2 µg of BAC-G47Δ/Vec9 DNA and 0.5 µg of pCAGGSFlpeIRES with 15 µL of Lipofectamine. Transfected cells were incubated in DMEM/10% FCS at 37°C overnight, then medium was replaced with DMEM/1% heat-inactivated FCS the next day, and incubation was continued for several days until plaques appeared. The progeny viruses were selected for GFP negativity by an inverted fluorescence microscope and for lacZ positivity by X-gal staining. Three rounds of limiting dilution were done to pick out a single clone. Recombinant vectors were harvested and the structure of the viral DNA was confirmed by endonuclease digestions and Southern blot analyses.

In vitro cytotoxicity studies and virus yield studies. In vitro cytotoxicity studies were done as described (2, 3). 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. For viral yield studies, Vero cells were seeded on six-well plates at 3 × 10⁶ per well. Wells were infected with four clones each of G47Δ-empty, G47Δ-mIL18, G47Δ-mB7-1-Ig, and G47Δ-IL18/B7 in duplicate wells at a multiplicity of infection (MOI) of 0.01 for 48 hours. G47Δ was used as a control. After 48 hours of infection, the cells were scraped and lysed by three cycles of freezing and thawing. The progeny virus was titrated on Vero cells by a plaque assay as described (2, 3). Results represent the average of duplicates.

Immunocytochemistry and ELISA. Cells were plated in 24-well plates and incubated at 37°C for 24 hours. Cells in duplicate wells were

**Figure 1.** A, a schema describing the system for constructing armed oncolytic HSV-1 vectors with the G47Δ backbone. The desired transgene for arming is inserted into the multiple cloning site of the shuttle vector (pVec9). The first step is to insert the entire sequence of the shuttle vector into theloxP site of BAC-G47Δ by a Cre-mediated recombination, followed by an electroporation into E.coli DH10B. The second step is to cotransfect the cotinogene with a plasmid expressing FLPRe onto Vero cells to excise the BAC sequence flanked by the FRT sites. The objective armed oncolytic HSV-1 vectors appear as GFP-negative and lacZ-positive.

BAC-G47ΔVec9

**Figure 2.** A schematic diagram describing the system for constructing armed oncolytic HSV-1 vectors with the G47Δ backbone. The desired transgene for arming is inserted into the multiple cloning site of the shuttle vector (pVec9). The first step is to insert the entire sequence of the shuttle vector into theloxP site of BAC-G47Δ by a Cre-mediated recombination, followed by an electroporation into E.coli DH10B. The second step is to cotransfect the cotinogene with a plasmid expressing FLPRe onto Vero cells to excise the BAC sequence flanked by the FRT sites. The objective armed oncolytic HSV-1 vectors appear as GFP-negative and lacZ-positive.

**Figure 3.** A schematic diagram describing the system for constructing armed oncolytic HSV-1 vectors with the G47Δ backbone. The desired transgene for arming is inserted into the multiple cloning site of the shuttle vector (pVec9). The first step is to insert the entire sequence of the shuttle vector into theloxP site of BAC-G47Δ by a Cre-mediated recombination, followed by an electroporation into E.coli DH10B. The second step is to cotransfect the cotinogene with a plasmid expressing FLPRe onto Vero cells to excise the BAC sequence flanked by the FRT sites. The objective armed oncolytic HSV-1 vectors appear as GFP-negative and lacZ-positive.

**Figure 4.** A schematic diagram describing the system for constructing armed oncolytic HSV-1 vectors with the G47Δ backbone. The desired transgene for arming is inserted into the multiple cloning site of the shuttle vector (pVec9). The first step is to insert the entire sequence of the shuttle vector into theloxP site of BAC-G47Δ by a Cre-mediated recombination, followed by an electroporation into E.coli DH10B. The second step is to cotransfect the cotinogene with a plasmid expressing FLPRe onto Vero cells to excise the BAC sequence flanked by the FRT sites. The objective armed oncolytic HSV-1 vectors appear as GFP-negative and lacZ-positive.

**Figure 5.** A schematic diagram describing the system for constructing armed oncolytic HSV-1 vectors with the G47Δ backbone. The desired transgene for arming is inserted into the multiple cloning site of the shuttle vector (pVec9). The first step is to insert the entire sequence of the shuttle vector into theloxP site of BAC-G47Δ by a Cre-mediated recombination, followed by an electroporation into E.coli DH10B. The second step is to cotransfect the cotinogene with a plasmid expressing FLPRe onto Vero cells to excise the BAC sequence flanked by the FRT sites. The objective armed oncolytic HSV-1 vectors appear as GFP-negative and lacZ-positive.
infected with each virus and further incubated at 39.5°C for 18 hours. Cells were fixed and immunostained for B7-1-Ig as described (5). The IL-18 concentration of the media was measured using a mouse IL-18 ELISA kit (MBL, Nagoya, Japan). Results represent the average of duplicates.

Animal studies. Six-week-old male C57BL/6 mice (Harlan Laboratories, Indianapolis, IN), female A/J mice (National Cancer Institute [NCI], Frederick, MD), and male/female athymic (BALB/c nu/nu) mice (NCI) were used. All animal procedures were approved by the Institutional Committee on Research Animal Care. S.c. tumor therapy was done as described (4). Statistical analysis was done by unpaired t test.

Results and Discussion

The established system involves two steps (Fig. 1A). The first step requires BAC-G47Δ, a plasmid of the G47Δ genome with the BAC-containing sequence inserted into the deleted ICP6 locus flanked by loxP and FRT sites. Also required is the shuttle vector (pVec9), a replication-conditional plasmid that contains the lacZ gene (without a promoter), loxP and FRT sites, a CMV promoter, and a multiple cloning site where the desired transgene is cloned (pVec9). The first step of this system is to insert the entire sequence of the shuttle vector into the loxP site of BAC-G47Δ by a Cre-mediated recombination (15). It is designed so that, after the recombination, lacZ is placed under the ICP6 promoter of G47Δ and expressed. The transgene cassette is placed in the downstream of lacZ, driven in the opposite direction (Fig. 1B). The second step is to cotransfect the cointegrate with a plasmid expressing FLPe onto Vero cells to excise the BAC sequence flanked by the FRT sites (16, 17). The lambda stuffer sequence is included in the shuttle vector so that, without a successful excision of the BAC sequence, there is no virus formation due to an oversized genome (16, 17). The objective recombinant HSV-1 vector is obtained by harvesting G47Δ empty plaques and isolated by limiting dilution. The entire procedure is typically done within 3 months.

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To test and use the system, we used the murine IL-18 (mIL-18) gene, the B7-1-Ig gene, both genes connected by the equine CMV internal ribosomal entry site (IRES) sequence, or no transgene to simultaneously create four different HSV-1 vectors, G47Δ-mIL-18, G47Δ-B7-1-Ig, G47Δ-IIL18/B7, and G47Δ-empty, respectively. The resultant vectors should have triple gene deletions in the ε34.5, ICP6, and ε47 genes, and the transgene driven by the CMV promoter and the lacZ gene driven by the ICP6 promoter both inserted in the deleted ICP6 locus. More than 99% of virus plaques formed after the FLPe recombination were both GFP negative and lacZ-positive plaques and isolated by limiting dilution. The entire procedure is typically done within 3 months.

Figure 2. Southern blotting analyses confirming the structures of the BAC-G47Δ plasmid, the BAC-G47Δ/pVec9-empty plasmid, and the recombinant G47Δ-empty virus. After HindIII, XhoI, or KpnI digestion, DNA fragments were separated by electrophoresis on 0.6% agarose gels in 1× Tris-borate-EDTA buffer for 14 to 18 hours at 2.5 V/cm. One of DNA fragments of EcoRI-digested pcDNA6E/Uni-lacZ corresponding to the lacZ sequence was used as the hybridization probe.

Because no significant difference was observed among clones, the first clone from each HSV-1 vector was used for further analyses. The in vitro cytopathic activities of the four oncolytic HSV-1 vectors were evaluated in mouse cell lines TRAMP-C2 (prostate cancer) and Neuro2a (neuroblastoma). Whereas mouse cells are generally less susceptible to HSV-1 infection than Vero cells, all four vectors killed tumor cells as rapidly as the parental G47Δ in both cell lines when infected at an MOI of 0.1 (Fig. 3A). The transgene expression of G47Δ-IIL18/B7 and G47Δ-mIL-18 was detected in all mouse cell lines tested (Table 1B).

Figure 1. (A) The first step of this system is to insert the entire sequence of the shuttle vector into the loxP site of BAC-G47Δ by a Cre-mediated recombination (15). The objective recombinant HSV-1 vector is obtained by harvesting G47Δ empty plaques and isolated by limiting dilution. The entire procedure is typically done within 3 months.

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The in vivo efficacy of the armed oncolytic HSV-1 vectors was screened in two immunocompetent mouse tumor models, TRAMP-C2 tumors in syngeneic C57BL/6 mice and Neuro2a tumors in syngeneic A/J mice (Fig. 3B and C). When established s.c. tumors reached ~6 mm in diameter, mock, G47Δ, G47Δ-empty, G47Δ-mIL-18, G47Δ-B7-1-Ig, or G47Δ-IIL18/B7 [5 × 10⁵ plaque-forming units (pfu) for TRAMP-C2 and 5 × 10⁶ pfu for Neuro2a] was inoculated into the tumor on days 0 and 3. In the TRAMP-C2 model, whereas all HSV-1 vectors caused a significant inhibition of tumor growth compared with mock, the G47Δ-IIL18/B7 treatment showed the greatest efficacy, resulting in a significantly smaller
tumor size than the treatment with G47Δ-mIL-18 or G47Δ-B7-1-Ig (P < 0.05 versus G47Δ-mIL-18 on days 19 and 23 and versus G47Δ-B7-1-Ig on days 23 and 26; Fig. 3B). Also in the Neuro2a model, in which tumors grow more aggressively than TRAMP-C2, all HSV-1 vectors caused a significant inhibition of tumor growth compared with mock. Only the G47Δ-II.18/B7 treatment was significantly more efficacious than G47Δ and G47Δ-empty (P < 0.05 on day 17; Fig. 3C). When athymic mice harboring s.c. Neuro2a tumors were treated in the same manner, there was no difference in efficacy between armed oncolytic vectors and unarmed control vectors, indicating that the enhancement of antitumor efficacy by arming with the IL-18 and/or B7-1-Ig gene(s) requires T cells (data not shown).

Our system has several important advantages over previously reported methods that use BAC to manipulate HSV-1 genomes (9, 11). The most time-consuming process for generating recombinant HSV-1 has been the selection of a correctly structured clone among, literally, millions of candidates after homologous recombination. We drastically improved the probability of a precise recombination occurrence by using recombinase systems. In fact, 14 of 16 of BAC-G47Δ/shuttle vector clones after the first step (Cre recombination) possessed the expected insert and over 99% of the clones after the second step (FLPe recombination) had the expected phenotype (Supplementary Fig. S1). We also used multiple devices for easy selection of correct recombinants. In addition to

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of cancer types, progression stages, or routes of administration. We believe our system facilitates the progress of such cancer therapeutics development.

Acknowledgments

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References


Figure 3. The efficacy of armed oncolytic HSV-1 vectors (G47Δ-transgene). A, cytopathic effect of the recombinant oncolytic HSV-1 vectors in vitro. TRAMP-C2 or Neuro2a cells were plated into six-well plates at 2 × 105 per well. After a 24-hour incubation, cells were infected with G47Δ, G47Δ-empty, G47Δ-mIL-18, G47Δ-B7-1-Ig, or G47Δ-IL18/B7 at an MOI of 0.1 or without virus (Control). The number of surviving cells was counted daily and expressed as a percentage of mock-infected controls. Points, mean of triplicates; bars, SD. B, in vivo efficacy of armed oncolytic HSV-1 vectors in male C57BL/6 mice harboring TRAMP-C2 mouse prostate cancer (n = 6 per group). When established s.c. tumors in the left flank reached ~ 6 mm in diameter, mock, G47Δ, G47Δ-empty, G47Δ-mIL-18, G47Δ-B7-1-Ig, or G47Δ-IL18/B7 (5 × 105 pfu) was inoculated into the tumor on days 0 and 3. The G47Δ-IL18/B7 treatment showed the greatest efficacy, resulting in a significantly smaller tumor size than the treatment with G47Δ-mIL-18 or G47Δ-B7-1-Ig (P < 0.05 versus G47Δ-mIL-18 on days 19 and 23 and versus G47Δ-B7-1-Ig on days 23 and 26). C, in vivo efficacy of armed oncolytic HSV-1 vectors in female A/J mice harboring s.c. Neuro2a mouse neuroblastoma (n = 6 per group). Animals were treated in the same manner as above (5 × 105 pfu). Only the G47Δ-IL18/B7 treatment was significantly more efficacious than G47Δ and G47Δ-empty (P < 0.05 on day 17). Tumor volume = length × width × height (mm). Bars, SE.


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