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Review Article

New generation, conditionally replicating herpes simplex virus G47Δ as a potential backbone vector for expressing foreign proteins

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Abstract

The use of replication-competent viruses that can selectively replicate, spread *in situ* and lyse malignant cells is an attractive strategy for treating cancer. G207 is a multimutated, conditionally replicating HSV-1 vector, and recent clinical trials in recurrent malignant glioma patients showed its safety when inoculated into human brain tumors. Whereas G207 has been shown to be effective in various types of cancer in animal models, its replication capability in tumor cells is considerably attenuated compared with wild-type HSV-1. In order to improve the antitumor efficacy, we have recently constructed a new generation vector, G47Δ, by creating an additional deletion in the *α47* gene of G207. G47Δ showed enhanced tumor cell killing and increased major histocompatibility complex class I expression in infected human cells. This paper reviews the circumstances that led to the development of G47Δ and the features of the vector that may be suitable as a backbone for expressing foreign molecules.

Introduction

The use of replication-competent viruses that can selectively replicate, spread *in situ*, and lyse malignant cells is an attractive strategy for treating cancer (1). It has been more than a decade, since genetically engineered herpes simplex virus type 1 (HSV-1) has been introduced as a suitable means for oncolytic virus therapy (2). However, development of replication-competent HSV-1 vectors adequate for clinical application has not been an easy task. The pathogenicity of the vector needs to be attenuated enough not to cause any damage to normal human tissue, while retaining its replication capability in tumor cells. G207 is a multimutated, conditionally-replicating HSV-1 vector created with an emphasis on safety (3), and recent clinical trials in recurrent malignant glioma patients showed that G207 can in fact be safely inoculated into human brains (4). Whereas G207 has shown efficacy in various types of cancer in animal models (5), its replication capability in tumor cells is also considerably attenuated compared with wild-type HSV-1. In order to improve the antitumor efficacy, we have recently constructed a new generation vector, G47Δ, by creating an additional deletion in the *α47* gene of G207 (6). G47Δ showed enhanced tumor cell killing and increased major histocompatibility complex (MHC) class I expression in infected human cells. In this paper, we review the circumstances that led to the development of G47Δ and the features of the vector that may be suitable as a backbone for expressing foreign molecules.

HSV-1 as a therapeutic bioreagent

Conditionally-replicating HSV-1 is an effective means for treating cancer (1). Oncolytic HSV-1 therapy uses the natural characteristics of the virus to kill host cells in the course of viral replication. Mutations by genetic engineering in the genes associated with virulence and/or viral

DNA synthesis can limit the virus to replicate only in transformed cells. Recent studies have demonstrated that oncolytic activities by HSV-1 vectors can also induce antitumor immune responses (7-9). HSV-1 has many advantages as an oncolytic vector (1,10): (i) It infects a variety of tumor cell types. (ii) Total cell killing can be achieved by a relatively low multiplicity of infection (MOI). (iii) Circulating anti-HSV-1 antibody does not affect the cell-to-cell spread of the virus, therefore a vector can be administered repeatedly in immunocompetent hosts. (iv) The large genome (~152 kb) is well characterized and contains many nonessential genes that can be mutated or replaced with large-sized transgenes (11), which is in contrast with adenovirus whose genome size (35 kb) limits the size of transgenes. (v) Antiherpes drugs, such as aciclovir and ganciclovir, are available and can be used for optional termination of the therapy (12, 13). (vi) Unlike retrovirus, HSV-1 DNA remains episomal and is not integrated into the cellular genome. (vii) There are mice and nonhuman primates that are susceptible to HSV-1 and can be used for preclinical evaluation for safety and efficacy.

Since genetically engineered HSV-1 was first described in 1991 as a tool for treating malignant glioma (2), several replication-competent HSV-1 vectors have now been used in patients. Besides G207, a *γ34.5*-deleted HSV-1, strain 1716, has been tested for safety in patients with malignant glioma (4, 14) and also in melanoma patients (15). A phase I trial is under way with an attenuated HSV-1, NV1020, originally designed for HSV-1 vaccination, for patients with liver metastasis of colon cancer in which the virus is administered into the hepatic artery (16). So far, all these trials have demonstrated the feasibility of using replication-competent HSV-1 vectors for treating cancer patients.

G207: a multimutated, replication-competent HSV-1 vector

G207 was constructed from recombinant HSV-1 (R3616) that originates in HSV-1 laboratory strain F and has deletions in both copies of the *γ34.5* gene, the major determinant of HSV-1 neurovirulence (3). G207 also has an insertion of the *E. coli LacZ* gene in the infected-cell protein 6 (ICP6) coding region (UL39), inactivating ribonucleotide reductase, a key enzyme for viral DNA synthesis in nondividing cells but not in dividing cells. The double mutation confers favorable properties on G207 for cancer therapy: (i) It replicates selectively in cancer cells and causes no damage to normal tissues. (ii) The chance of reverting to wild-type is minimal. (iii) The reporter gene *LacZ* allows easy histochemical detection of the replicating virus. (iv) G207 is hypersensitive to ganciclovir/aciclovir.

G207 was first shown to inhibit the tumor growth and/or prolong the survival in athymic mice harboring malignant glioma in the brain or under the skin when inoculated intraneoplastically (3). Since then, G207 has shown efficacy in a variety of immunoincompetent and -competent animal tumor models (5). Those tumors include malignant meningioma (17), breast cancer (18), colorectal cancer (19), prostate adenocarcinoma (20), head and neck cancer (21), bladder cancer (22), gastric cancer (23), ovarian cancer (24), neuroblastoma (25), hepatic cancer (26), gallbladder cancer (26) and malignant melanoma (6).

G207 is significantly less toxic than wild-type HSV when injected into the brain and prostate of mice and nonhuman primates (3, 27-29). G207 was the first replication-competent HSV-1 vector used in the US in humans and was tested for safety in patients with recurrent malignant glioma. The dose-escalation study starting at 1×10^6 plaque forming units (pfu) demonstrated that intracerebral inoculation of G207 was safe at doses up to 3×10^9 pfu (4). The results warrant further clinical trials for brain tumors as well as other types of cancer. Phase Ib/II studies for malignant glioma are under way.

Antitumor immunity induction by G207

The tumor cell killing effect of G207 depends on the extent that tumor cells can support G207 replication. Thus there is a wide range of variation among different cell lines (5). The antitumor effect also depends on the extent of antitumor immunity induction in the course of G207 oncolytic activity (7-9). One way to enhance the antitumor efficacy of G207 while retaining the safety features is to harness this action of inducing antitumor immune responses. A potential contradicting consequence is that enhancing immune responses could inhibit viral replication within the tumor, leading to a reduced cytopathic effect. With adenovirus vectors, it has been shown that antiviral immune responses act adversely to the therapeutic efficacy (30, 31). Innate immunity may also limit the therapeutic efficacy of HSV-1 vectors (32).

In several immunocompetent animal tumor models, G207 has been shown to induce specific and systemic antitumor immunity (7, 9, 26). Intraneoplastic inoculation of G207 into a s.c. N18 neuroblastoma in syngeneic A/J mice inhibited the growth of remote tumors in the brain as well as in the periphery via systemic antitumor immune responses (7). The therapy also conferred tumor-specific protective immunity with long-term memory, which was demonstrated by cured animals protected from a s.c. rechallenge with N18 cells but not Sal/N cells (7). Antitumor immunity was associated with an elevation of specific cytolytic T lymphocyte (CTL) activity against tumor cells (7). A similar antitumor effect of G207 on remote tumors via systemic immune responses was observed in other bilateral s.c. tumor models such as CT26 colorectal carcinoma in BALB/c mice (9), M3 melanoma in DBA/2 mice (9) and KIGB-5 gallbladder carcinoma in Syrian hamsters (26). In the CT26 model, intraneoplastic G207 inoculation induced $CD8^+$ T cells that recognized a dominant tumor antigen in an MHC class I-restricted fashion (9).

Defective HSV-1 vectors expressing immunostimulatory molecules

Defective HSV-1 vectors have been used to introduce immunostimulatory genes into tumor cells both *ex vivo* and *in situ* (33). A replication-incompetent HSV-1 vector expressing interleukin (IL)-2 was effective in inducing antitumor immune responses against head and neck metastases of renal carcinoma in animal models (34). An HSV-1 amplicon vector expressing murine granulocyte-macrophage colony stimulating factor (GM-CSF) or IL-2 was used in combination with i.p. injections of interferon-γ (IFN-γ) in an animal hepatoma model. The combination therapy was more effective than any treatment alone. Complete elimination of the tumor was observed in 4 of 12 animals receiving GM-CSF/IFN-γ and 8 of 11 animals given IL-2/IFN-γ (35). An HSV-1 amplicon vector expressing IL-2 also showed a significant suppression of the growth of lung squamous cell carcinoma implanted s.c. in animals (36, 37). Treatment with the IL-2 vector caused a retardation of the growth of tumors remote from vector inoculation sites and led to a significant improvement in animal survival. In a bilateral s.c. tumor model of murine melanoma, intraneoplastic inoculation of a defective HSV-1 vector encoding murine GM-CSF significantly inhibited the growth of both the inoculated and noninoculated contralateral tumors (38).

An HSV-1 amplicon can be combined with oncolytic HSV-1 vectors by using an oncolytic HSV-1 vector as a helper virus when generating the defective HSV-1 vector. Several amplicon vectors expressing immunostimulatory molecules have been used in combination with G207 (39-41). When a mixture of G207 and a defective vector expressing IL-12, for example, is inoculated into the tumor, tumor cells infected with G207 allow the virus to replicate and are ultimately destroyed, further spreading progeny G207 to surrounding tumor cells. On the other hand, tumor cells infected with the defective vector produce IL-12 and recruit immune cells, which augment the antitumor immune response elicited by the oncolytic activity of G207. Intraneoplastic inoculation of the IL-12 defective vector with G207 significantly augmented the antitumor immune response compared with G207 alone in a s.c. CT26 tumor model (40). Increases in tumor-specific CTL activity and production of IFN-β by splenocytes were observed (40). A defective HSV vector (dvB7Ig) expressing a soluble form of B7-1, one of the most potent costimulatory molecules (42), was also used in combination with G207 (41). Soluble B7-1 was designed as a fusion protein of the extracellular domain of B7-1 and the Fc portion of IgG, so that it is secreted by tumor cells rather than expressed on the cell surface. The *in vivo* efficacy was tested in the poorly-immunogenic murine neuroblastoma, Neuro2a, in A/J mice. Intraneoplastic inoculation of dvB7Ig/G207 at a low titer successfully inhibited the growth of established s.c. tumors despite the fact that the expression of B7-1-Ig was detected in only 1% or less of tumor cells at the inoculation site; treatment also prolonged the survival of mice bearing intracerebral tumors. Inoculation of dvB7Ig/G207 induced a significant influx of $CD4^+$ and $CD8^+$ T cells into the tumor. *In vivo* depletion of immune cell subsets further revealed that the antitumor effect required $CD8^+$ T cells but not $CD4^+$ T cells. Treatment with dvB7Ig/G207 conferred tumor-specific protective immunity in cured animals.

Replication-competent HSV-1 vectors for amplified gene delivery

The use of replication-competent vectors for transgene expression has multiple attractive advantages over defective vectors: i) continuous generation of a high-titer, homogenous vector stock is possible which allows manufacturing of a large amount with a better quality control; ii) amplified gene delivery can be obtained *in vivo;* and iii) transgene expression may lower administering doses required therefore decreasing toxicity. On the other hand, potential demerits of using replication-competent vectors for expressing foreign proteins are that the transgene expression may: i) be shorter in duration than defective vectors due to destruction of the host cell by viral replication; ii) increase the toxicity of the vector; and iii) interfere with viral replication.

Recently, several replication-competent HSV-1 vectors have been created that contain transgenes for immunostimulatory molecules. *γ34.5*-Deficient HSV-1 containing the murine IL-4 gene displayed significantly higher antitumor activity and prolonged survival of mice with intracranial tumors as compared to its parent virus or to the virus expressing IL-10 (43). Recombinant HSV-1 expressing IL-12 (M002 and NV1042) showed improved *in vivo* efficacy against murine neuroblastoma (44), murine squamous cell carcinoma (45) and murine colorectal tumor (46). Immunohistochemical analyses of tumors treated with these HSV-1 mutants revealed a significant influx of $CD4^+$, $CD8^+$ T cells and macrophages. The replication-competent HSV-1 vector expressing IL-12 (NV1042) was more effective than the vector expressing GM-CSF in the same backbone (NV1034) in mice with s.c. squamous cell carcinoma (45). The mice cured by NV1042 had a higher rate of rejecting rechallenged tumor cells than those cured by NV1034 (45).

G47Δ

Another way to improve the antitumor action of G207 while retaining its safety is to construct a new HSV-1 vector by adding further mutations to the genome of G207. The *α47* gene product (ICP47) of HSV-1 inhibits the transporter associated with antigen presentation (TAP) that mediates antigen presentation in the context of MHC class I by translocating peptides across the endoplasmic reticulum (47-50). Upon infection, ICP47 therefore causes downregulation of MHC class I expression on the cell surface, allowing the HSV-1-infected host cells to escape the host immune surveillance (51). An *α47*-deleted

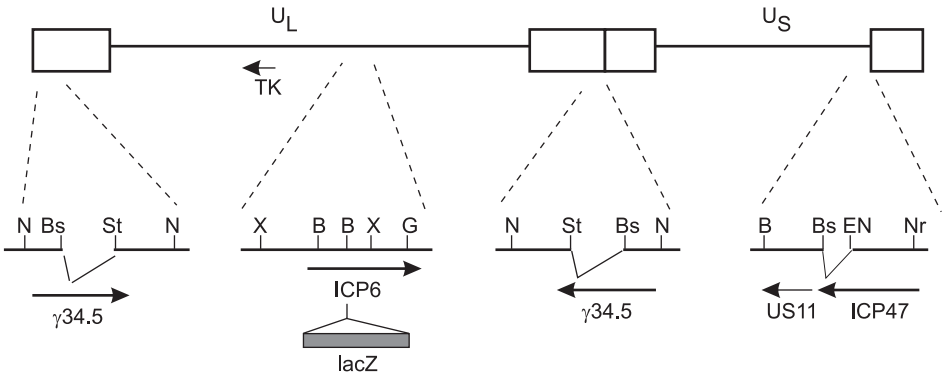

Fig. 1. Structure of G47Δ. The boxes (top line) represent inverted repeat sequences flanking the long (U_L) and short (U_S) unique sequences of HSV-1 DNA. The *E. coli LacZ* coding sequence is inserted into the indicated BamHI site in the ICP6 coding region. There is a 1.0-kb deletion in both copies of the *γ34.5* gene located in the expanded domain of the long inverted repeat sequences. G47Δ was created from G207 by further deleting 312 bp between the indicated BstEII and EcoNI sites in the ICP47 locus. The deletion also places the *US11* gene under control of the ICP47 immediate-early promoter. Thick arrows indicate transcribed region. (N, NcoI; Bs, BstEII; St, StuI; X, XhoI; B, BamHI; G, BglII; EN, EcoNI; Nr, NruI)

HSV-1 vector did not downregulate MHC class I expression in human melanoma cells (52). Also, *α47*-deleted replication-competent HSV-1 was less virulent than the parent wild-type virus in the brains of HSV-1-sensitive A/J mice, and the attenuation of neurovirulence was dependent on $CD8^+$ T cells (53). In order to confer an enhanced MHC class I presentation, we have constructed a new generation, replication-competent HSV-1 vector, G47Δ, by further creating a 312 bp deletion within the *α47* gene of G207 (Fig. 1). G47Δ-infected human melanoma cells which showed the greatest enhancement of MHC class I expression among cells tested, caused a significantly better stimulation of tumor infiltrating lymphocytes (TIL) as compared to G207-infected cells, resulting in 41% more IFN-γ secretion (6). The results showed that higher MHC class I expression in cells infected with G47Δ can indeed enhance the antitumor T cell stimulation.

Because of the overlap of the *US11* promoter region and the *α47* gene, the 312 bp deletion in G47Δ also results in deletion of the *US11* promoter region, placing the late *US11* gene under control of the immediate-early *α47* promoter (6). The altered expression of the *US11* gene functions as a second-site suppressor of the *γ34.5* mutation (54-56) and recovers the impaired growth properties of *γ34.5*-deficient HSV-1 mutants by precluding the shutoff of protein synthesis. G47Δ gave higher virus yields than G207 in all cell lines tested, resulting in an approximately 4- to 1000-fold increase in titer (6). The improved replication capability of G47Δ also led to an enhanced cytopathic effect in a variety of tumor cells tested, including glioma, neuroblastoma and prostate cancer (6; Y. Ino, H. Fukuhara, T. Todo, unpublished data).

The enhanced tumor cell killing of G47Δ shown *in vitro* was reflected in the improved antitumor efficacy *in vivo*. In athymic mice with s.c. U87MG human glioma, intraneoplastic inoculation of G47Δ caused a significantly greater reduction in tumor growth, leading to higher numbers of "cures" as compared G207-treated animals. In the same model, tumors were harvested periodically to measure the recovery of infectious viruses, and the peak recovery of G47Δ was 20-fold higher than the peak obtained for G207 (57). G47Δ was also significantly more efficacious than G207 in immunocompetent A/J mice with s.c., poorly immunogenic Neuro2a tumors, prolonging the survival of animals compared with mock or G207 (6). Moreover, in A/J mice bearing intracerebral Neuro2a tumors, intraneoplastic inoculations of G47Δ at a low dose caused moderate extension of survival compared with G207-treated animals (58). Most importantly, G47Δ was as safe as G207 when inoculated into the brains of A/J mice at 2×10^6 pfu (6).

Thus, G47Δ has attractive features for human cancer therapy, including potent stimulation of antitumor immune cells, high yields of virus, improved oncolytic activity, and safety in an HSV-1-sensitive rodent model. The preserving of the host TAP function also make G47Δ useful as a backbone vector for expressing foreign antigens in the context of vaccination. While the use of replication-competent HSV-1 vectors for expressing immunostimulatory cytokines has proven useful (44-46), the backbone vectors used by others to test the strategy were likely less attenuated compared with G207, leaving concerns for clinical applications. The safety and preclusion of the shutoff of protein synthesis make G47Δ an ideal replication-competent vector to express any foreign protein molecules. We are currently generating multiple replication-competent vectors with different transgenes using G47Δ as the backbone.

Conclusions

The development of G47Δ was built upon accumulated experience of over a decade by us as well as by

others with so-called first- and second-generation replication-competent HSV-1 vectors. This new generation HSV-1 vector is particularly attractive for clinical application because it may be the safest oncolytic HSV-1 vector described to date while retaining its ability to replicate in tumor cells which leads to enhanced antitumor activity. The capability of obtaining high yields of G47Δ is also beneficial from a manufacturing standpoint. Furthermore, G47Δ is an ideal vector for expressing foreign proteins, especially immunostimulatory molecules, due to the preservation of the host TAP function, leading to enhanced MHC class I expression. We believe G47Δ will contribute to the progress of oncolytic virus therapy.

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