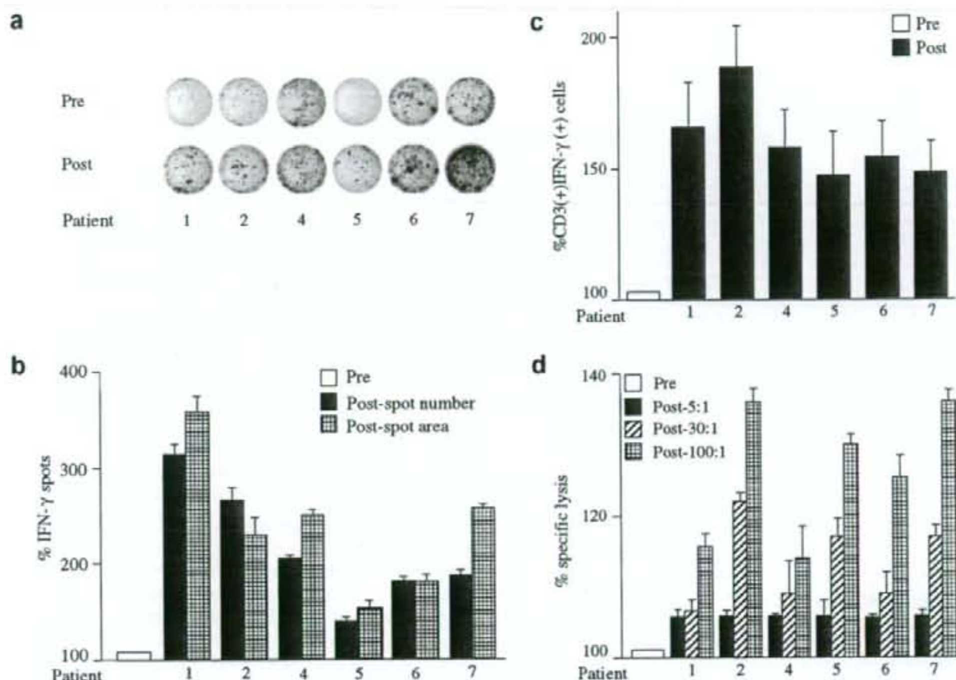

Fig. 3 – Specific cellular response. (a) IFN-γ secretion by patients' PBMC in the presence of HUVECs measured by enzyme-linked immunospot assay. Representative wells with stained immunospots are shown. (b) Relative numbers and areas of IFN-γ immunospots (Post/Pre) obtained by image analysis. (c) Relative numbers of lymphocytes secreting IFN-γ (Post/Pre) detected by intracellular cytokine flow cytometry assay. (d) Relative specific lysis of HUVECs by patients' cellular effectors in an effectors:targets ratio-dependent manner (at E:T ratios of 5:1, 30:1 and 100:1; Post/Pre), detected by a chromium-release cytotoxicity assay.

4. Discussion

In this study, we tested the clinical utility of vaccination using glutaraldehyde-fixed human umbilical vein endothelial cells (HUVECs) in patients with a progressive malignancy. HUVECs share specific angiogenic properties with tumour endothelium such as the overexpression of angiogenic antigens CD51[19] and CD105,[20] and inhibit tumour growth in pre-clinical mouse models.[14,21,22] Therefore, we hypothesised that HUVECs could also be effective in clinical settings, especially in the patients with malignant brain tumours, which are known to be among the most vascularised tumours.[23] In addition, we were interested in whether HUVECs could work in the patients with colorectal cancer, which on the one hand still remains a difficult target for cancer immunotherapy,[24] but on the other hand was recently shown to respond to anti-angiogenic therapy.[14,25]

The vaccination protocol in this study was designed to provide strong antigenic stimulation to overcome the peripheral tolerance of angiogenesis. Therefore, the number of endothelial cells administered in one dose was 5×10^7, i.e. five times more than commonly used in the vaccines based on tumour cells, to ensure that the amount of administered antigens is sufficient to induce a specific immune response. In addition, here, we decided to continue the vaccination protocol in a limited number of patients for long periods of time rather than to treat many patients with a limited number of doses; first, to reveal potential toxicity of the endothelial vaccine, and second, to ensure that the antigenic stimulation is sufficient for inducing a long lasting immune response. We found that except for a DTH-like skin reaction at the injection site, the endothelial vaccine caused no adverse effects to the recipients during a long term administration. In future studies, a minimum effective dose will be evaluated to improve the cost-to-performance ratio of the vaccination protocol.

One month after starting the vaccination protocol, specific antibodies and cellular effectors against HUVECs' membrane antigens were detected in the patients with recurrent malignant brain tumours as well as metastatic colorectal cancer. Analysis of candidate target antigens has recently been ongoing so as to get better insight into the mechanisms controlling immune tolerance of angiogenesis, and to develop more effective protocols for anti-angiogenic endothelial vaccination.

In three patients with recurrent malignant brain tumours, marked tumour shrinkage could be observed over an

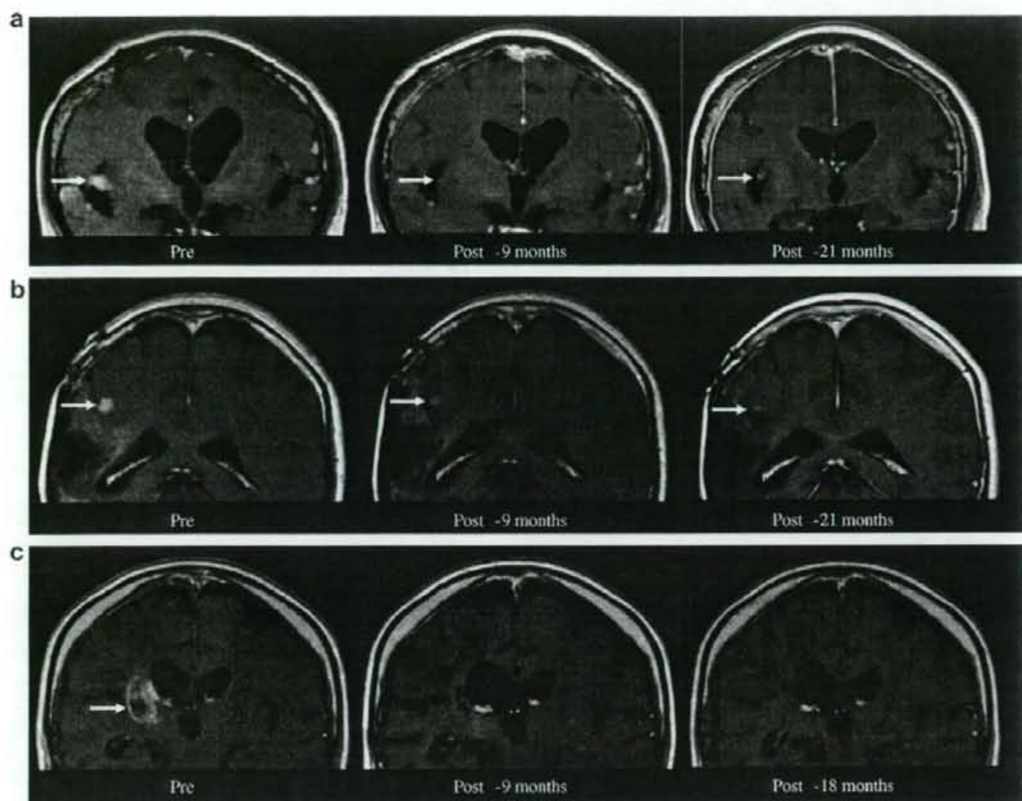

Fig. 4 – Clinical tumour response. Partial tumour responses were observed in patient #1 with pinealoblastoma (a) and patient #2 with glioblastoma (b), whereas a complete tumour response was observed in patient #3 with anaplastic oligodendroglioma (c). Representative gadolinium contrasted magnetic resonance imaging scans taken before vaccination (Pre), after 9 months of vaccination (Post-9 months), and after an extended vaccination period (Post-21 months and Post-18 months) are shown. Main target lesions are indicated by arrows.

extended period of vaccination. Recently, it has been reported that in some patients with malignant brain tumours, immediate post-radiotherapy changes that mimic tumor progression on MRI, the so called 'pseudo-progression', can cause an overestimation of therapeutic results, and therefore the patients enrolled in a clinical study should be at least 3 months after completed radiation therapy.[26–29] In the present study, recurrent disease was first observed in our patients more than 9 months after completed post-operative radiation therapy of the original tumour, some of the recurrent tumours being localised outside the radiation therapy field, and therefore suggested to be true progression. Given that neither recurrent tumour was treated by radiation therapy, and chemotherapy with temozolomide was ineffective, we concluded that the tumour responses observed in this study should be attributed to the effects of endothelial vaccination.

Human tumour cells share some properties with the angiogenic endothelium, such as expression of CD51 and CD105, and therefore it can be hypothesised that, theoretically, tumour cells might be potential co-targets of antiangiogenic endothelial vaccination. Although our preliminary data of *in vitro* immunological assays supported this hypothesis (data not shown), we suppose that under *in vivo* conditions, tumour endothelium should be a primary target. It is due to the fact that tumour endothelium is lining the intra-luminal surface of tumour vasculature, and consequently is first reached by immune effectors induced by endothelial vaccination. As mentioned in the Introduction section, therapeutic damage of tumour endothelium activates the coagulation cascade, and consequently results in the obstruction of tumour vasculature that makes the immune effectors unable to sufficiently reach tumour cells.

In contrast, with the three responding malignant brain tumour patients, neither tumour response nor other improvement of the clinical outcome could be observed in the other patients. The reason for the discrepancy between the immune response to vaccination and tumour response

in these patients is not yet known, but we can speculate that it might be caused by either strong immunosuppression in the tumour microenvironment ,[30–32] or by possible adaptation of some tumour cells to the consequences of anti-angiogenic therapy, as was recently described by others.[33] Discrepancy between the immunological and anti-tumour effects was also reported by many other authors clinically investigating cancer vaccines,[24,34] and therefore we suppose that there is a strong need for studies searching for factors that make cancer patients responsive or resistant to active immunotherapy.

In summary, the present pilot study showed the safety and potential clinical utility of the anti-angiogenic vaccine using fixed whole endothelium. Immune response, involving activation of both specific humoral and cellular immunity, was observed in eight of nine patients. Partial or complete tumour responses were observed in three of six malignant brain tumour patients, but not in three colorectal cancer patients. To obtain further insight into the possibilities and limitations of this novel approach, another study employing different dose levels, adjuvants and combination with conventional therapy modalities against various tumour types is now ongoing.

Conflict of interest statement

None declared.

Acknowledgement

The authors thank Ms. Michiru Kawabata and Ms. Madoka Nishimori from the Department of Transfusion Medicine (The University of Tokyo), and Dr. Tomoyuki Fujii from the Department of Gynaecology and Obstetrics (The University of Tokyo) for their kind advisory and technical assistance. This study was supported in part by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; in part by the Ministry of Health, Labor and Welfare of Japan; and in part by the Japan Society for the Promotion of Science.

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[Frontiers in Bioscience 13, 2060-2064, January 1, 2008]

Oncolytic virus therapy using genetically engineered herpes simplex viruses

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. G207 – a second-generation oncolytic HSV-1
4. G207 clinical trial
5. G47Δ - a third-generation oncolytic HSV-1
6. "Armed" oncolytic HSV-1
7. Summary
8. Acknowledgments
9. References

1. ABSTRACT

Genetically engineered, conditionally replicating herpes simplex viruses type 1 (HSV-1) are promising therapeutic agents for cancer. They can replicate *in situ*, spread, and exhibit oncolytic activity via a direct cytocidal effect. In addition, oncolytic HSV-1 can transfer and express foreign genes in host cells. The phase I clinical study with G207, a double-mutated HSV-1, in recurrent malignant glioma patients has shown that oncolytic HSV-1 can be safely administered into human brains. The therapeutic benefits of oncolytic HSV-1 depend on the extent of both intratumoral viral replication and induction of host antitumor immune responses. We develop new-generation oncolytic HSV-1 by enhancing these properties while retaining the safety features. G47Δ was created from G207 by introducing another genetic mutation. Compared with G207, G47Δ showed 1) better stimulation of human antitumor immune cells, 2) better growth properties leading to higher virus yields and increased cytopathic effect *in vitro*, 3) better antitumor efficacy in both immuno-competent and -incompetent animals, and 4) preserved safety in the brain of HSV-1-sensitive mice. Preparation is under way for a clinical trial using G47Δ in progressive glioblastoma patients. G47Δ is also suited as a backbone vector for expressing foreign molecules. Using bacterial artificial chromosome and two DNA recombinases, we have created an "armed" oncolytic HSV-1 generation system that allows insertion of transgene(s) into the genome of G47Δ in a rapid and accurate manner. We found that expression of immunostimulatory molecules can significantly enhance the antitumor efficacy of G47Δ. Based on these advances, we anticipate that oncolytic virus therapy using oncolytic HSV-1 will soon be established as an important modality of cancer treatment.

2. INTRODUCTION

Oncolytic virus therapy is an attractive means of treating cancer (1). Viruses, especially herpes simplex virus type 1 (HSV-1) and adenoviruses, are genetically engineered to restrict virus replication to tumor cells and to widen the therapeutic window. Infected tumor cells are destroyed by a direct oncolytic activity of the viruses, and the recombinant viruses do not harm normal tissues. Oncolytic virus vectors can also be used for transgene delivery.

HSV-1, especially in comparison with adenovirus, has suitable features for cancer therapy: (i) HSV-1 infects most tumor cell types. (ii) A relatively low multiplicity of infection (MOI) is needed for total cell killing. (iii) Anti-viral drugs are available. (iv) A large genome (~152 kb) allows the insertion of large and/or multiple transgenes. (v) The host immune reactions enhance antitumor effects. (vi) Circulating anti-HSV-1 antibody does not affect cell-to-cell spread of the virus. (vii) There are HSV-1 sensitive mouse and nonhuman primate models for preclinical evaluation. (viii) Viral DNA is not integrated into the host genome.

3. G207 – A SECOND-GENERATION ONCOLYTIC HSV-1

For HSV-1, the principle of how to target viral replication to tumor cells is to inactivate or delete viral genes that are essential for viral replication in normal cells but dispensable in tumor cells, using features common for all types of cancer. Oncolytic HSV-1 therefore can be applied to a wide variety of cancer. The key to successful and practical development of oncolytic HSV-1 is the safety, *i.e.*, a wide therapeutic window, which can be achieved only via genetic engineering.

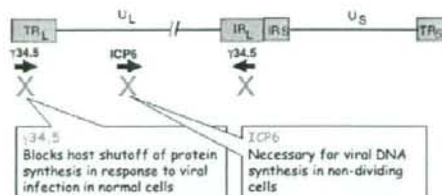

Figure 1. Structures of G207. The HSV-1 genome consists of long and short unique regions (U_L and U_S) each bounded by terminal (T) and internal (I) repeat regions (R_L and R_S). G207 was engineered from wild-type HSV-1 strain F by deleting 1 kb within both copies of the *γ34.5* gene, and inserting the E.coli *lacZ* gene into the ICP6 coding region.

G207 is the first oncolytic HSV-1 used in a clinical trial in the United States (2). This second-generation oncolytic HSV-1 has double mutations created in the HSV-1 genome (Figure 1). G207 has deletions in both copies of the *γ34.5* gene, the major determinant of HSV-1 neurovirulence (3). *γ34.5*-deficient HSV-1 vectors are considerably attenuated in normal cells, but retain their ability to replicate within neoplastic cells. In normal cells, HSV-1 infection induces activation of double-stranded RNA-dependent protein kinase (PKR), which in turn leads to phosphorylation of the alpha-subunit of eukaryotic initiation factor 2 (eIF-2α) and a subsequent shutdown of host and viral protein synthesis (4). The product of the *γ34.5* gene antagonizes this PKR activity. However, tumor cells have low PKR activities, thereby allowing *γ34.5*-deficient HSV-1 vectors to replicate (5, 6). 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 non-dividing cells but not in dividing cell (7).

G207 has been tested in more than 60 different cell lines and proved effective in all human tumor cell lines except for those derived from bone marrow (8). In human glioma and malignant meningioma cell lines, for example, G207 can achieve cell destruction of the entire cell population within 2 to 6 days at an MOI of 0.1 (2, 9). Whereas rodent cells are generally less susceptible to HSV-1 infection than human cells, G207 can destroy the entire cell population of N18 murine neuroblastoma cells within 3 days at an MOI of 1 (10). G207 manifests no effect on primary cultures of rat cortical astrocytes or cerebellar neurons even at 9 days post-infection, whereas, at the same MOI, the wild type HSV-1, the parental strain F, kills these normal cells (2). This difference in G207 cytopathic effect observed *in vitro* between tumor cells and normal cells is directly reflected in the results of *in vivo* studies.

In A/J mice harboring established syngeneic N18 tumors subcutaneously or in the brain, a single intraneoplastic inoculation with G207 caused a significant reduction in tumor growth or prolongation of survival (10). The most remarkable finding with G207 when tested in immuno-competent animals was that it induced systemic antitumor immunity (10, 11). In A/J mice bearing bilateral subcutaneous N18 tumors, intraneoplastic G207 inoculation into the left tumor alone caused growth reduction not only of the inoculated tumors but also of the non-inoculated contralateral tumors. The antitumor immunity was associated with an elevated cytotoxic T lymphocyte (CTL) activity specific to N18 tumor cells that persisted for at least 13 months.

An extensive *in vivo* safety evaluation was performed with G207 (12-15). The results are summarized as follows: 1) G207 does not cause disease in HSV-1-susceptible mice after intracerebral, intra-ventricular, intravenous, intraprostatic or intrahepatic injection at 10^7 plaque-forming units (pfu). 2) G207 does not cause disease in HSV-susceptible primates (Owl monkeys; *Aotus nancymae*) after intracerebral or intraprostatic injections of 1-3 x 10^7 or 10^9 pfu. 3) No elicitation of severe inflammatory response was observed with multiple inoculations or in animals with prior HSV-1 exposure. 4) No detectable reactivation of 'latent' HSV-1 in the brain was observed in Balb/c mice. 5) No detectable shedding of virus was observed after intracerebral inoculation in Owl monkeys. 6) Induction of anti-HSV antibodies was observed after intracerebral or intraprostatic injection in Owl monkeys. 7) G207 DNA was detectable in the brain up to 2 years after intracerebral injection and in the urethra, spleen and lymph nodes after intraprostatic injection in Owl monkeys.

4. G207 CLINICAL TRIAL

The G207 phase I clinical trial was performed between 1998 and 2000 at two institutions in the United States, Georgetown University Medical Center and University of Alabama at Birmingham (16). We treated 21 patients with recurrent malignant glioma: 16 patients with glioblastoma and 5 patients with anaplastic astrocytoma. G207 was administered directly into the tumor via stereotactic inoculation. This dose escalation study started from 10^6 pfu and increased to 3 x 10^9 pfu, with three patients at each dose. No acute, moderate to severe adverse events attributable to G207 were observed. Minor adverse events included seizure (2 cases) and brain edema (1 case). An improvement in Karnofsky score was observed in 6 of 21 patients (29%) at some time after G207 inoculation. Eight of 20 patients that had serial MRI evaluations had a decrease in tumor volume (enhancing area) between 4 days and one month post-inoculation. Patient #4 that received 10^7 pfu showed continuous decrease in tumor size after G207 inoculation, but died from irrelevant cerebral infarction 10 months after treatment. The trial proved the safety of G207 inoculated intratumorally in the brain up to 3 x 10^9 pfu.

5. G47Δ - A THIRD-GENERATION ONCOLYTIC HSV-1

We further developed a third-generation oncolytic HSV-1 termed G47Δ (Figure 2). G47Δ was newly created from G207 by introducing another genetic alterarion, *i.e.*,

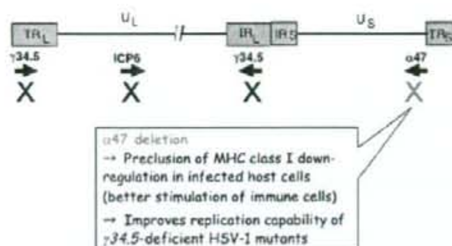

Figure 2. Structure of G47Δ. G47Δ was created from G207 by further deleting 312 bp within the *α47* gene. The deletion also places the *US11* gene under control of the *α47* immediate-early promoter.

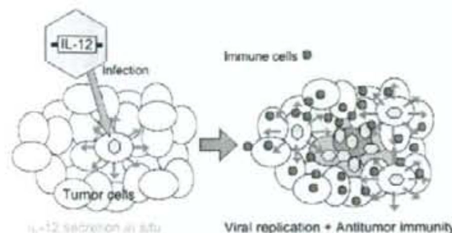

Figure 3. Concept of cancer therapy using oncolytic HSV-1 armed with an immunostimulatory gene. When oncolytic HSV-1 armed with the interleukin 12 (IL-12) gene infects tumor cells, IL-12 is secreted in the course of viral replication and stimulates the immune cells. In addition to direct tumor cell killing via viral replication and spread, tumor cells are destroyed by augmented antitumor immune responses, resulting in enhanced antitumor activities.

the deletion of the *α47* gene and the overlapping *US11* promoter region, in the G207 genome (17). Because the *α47* gene product (ICP47) inhibits transporter associated with antigen presentation (TAP), which translocates peptides across the endoplasmic reticulum, the down-regulation of MHC class I that normally occurs in human cells after infection with HSV-1 does not occur when the *α47* gene is deleted (18). G47Δ-infected human cells in fact presented higher levels of MHC class I expression than cells infected with other HSV-1 vectors (17). Further, human melanoma cells infected with G47Δ were better at stimulating their matched tumor-infiltrating lymphocytes *in vitro* than those infected with G207. The deletion also places the late *US11* gene under control of the immediate-early *α47* promoter, which results in suppression of the reduced growth phenotype of *γ34.5*-deficient HSV-1 mutants including G207 (19). In the majority of cell lines tested, G47Δ replicated better than G207, resulting in the generation of higher virus titers, and exhibiting greater cytopathic effect (17). In athymic mice bearing subcutaneous U87MG human glioma and A/J mice bearing subcutaneous Neuro2a neuroblastoma, G47Δ was significantly more efficacious than G207 at inhibiting the tumor growth when inoculated intraneoplastically (17). G47Δ was also more efficacious than G207 in athymic mice bearing intracerebral U87MG tumors (Ino Y, *et al.* unpublished data). Nevertheless, the safety of G47Δ remained unchanged from G207 following injection into the brain of HSV-1-sensitive A/J mice (17). Thus, with G47Δ, by creating the third engineered mutation within the G207 genome, we improved the efficacy of G207 without compromising its safety. G47Δ has been shown efficacious in animal tumor models of a variety of cancers including brain tumors, prostate cancer, breast cancer and schwannoma (17, 20-22). A phase I-II clinical trial using G47Δ in patients with progressive glioblastoma is underway at the University of Tokyo.

6. "ARMED" ONCOLYTIC HSV-1

One of the advantages of HSV-1 is the capacity to incorporate large and/or multiple transgenes within the viral genome. Certain antitumor functions may be added to oncolytic activities of recombinant HSV-1. Conventional homologous recombination techniques had required time-consuming processes to create "armed" oncolytic HSV-1. We recently established an innovative "armed" oncolytic HSV-1 construction system utilizing bacterial artificial chromosome (BAC) and two DNA recombinase systems (Cre/loxP and FLP/FRT) (23). Using G47Δ as the backbone, this system allows a rapid generation of multiple vectors with desired transgenes inserted in the deleted *ICP6* locus.

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 (10, 11). Therefore, while any transgene that does not interfere with HSV-1 replication may be used (24), the genes of immunomodulatory molecules would be reasonable candidates for "arming" oncolytic HSV-1. Using this construction system, we first created oncolytic HSV-1 armed with immunostimulatory genes such as interleukin 12 (IL-12), IL-18, or soluble B7-1. Immunostimulatory functions should augment the antitumor immunity induction that adds to direct oncolytic activity of the virus, resulting in enhanced antitumor activities (Figure 3). Using A/J mice bearing bilateral subcutaneous Neuro2a tumors, known to be poorly immunogenic, we tested the efficacy of a third-generation oncolytic HSV-1 armed with mouse fusion-type IL-12, termed T-mfIL12. When the viruses were inoculated into the left tumor only, T-mfIL12 showed a significantly better antitumor activity than the unarmed control virus, T-01, not only in the inoculated left tumors but also in the non-inoculated remote tumors (Miyamoto S, *et al.* unpublished data). When three oncolytic HSV-1 expressing IL-12, IL-18 or soluble B7-1 were tested together using the same Neuro2a model, the triple combination of the three armed viruses exhibited the highest efficacy amongst all single virus or combinations of two viruses (25). Combining 1×10^5 pfu each of the three "armed" viruses showed stronger antitumor activities than any single "armed" virus at 3×10^5 pfu in inoculated tumors as well as non-inoculated remote tumors. We have also created a G47Δ-backbone HSV-1 double-armed with

IL-18 and soluble B7-1 (23). This double-armed oncolytic HSV-1 showed a significant enhancement of antitumor efficacy via T-cell mediated immune responses in A/J mice with subcutaneous Neuro2a tumors as well as in C57BL/6 mice bearing subcutaneous TRAMP-C2 prostate cancer.

Whereas the most common route of delivery of oncolytic HSV-1 has been a direct intratumoral inoculation, an intravenous delivery would further broaden the clinical application of oncolytic HSV-1 vectors if proven effective. One of approaches to generate therapeutic efficacy via intravenous administration is by "arming" of oncolytic HSV-1. We observed that intravenous delivery of T-mfIL12 caused a significant inhibition of tumor growth compared with mock and T-01 treatments in A/J mice bearing subcutaneous Neuro2a tumors (Guan Y, *et al.* unpublished data). Also, in a renal cancer lung metastases model using BALB/c mice and syngeneic RenCa cells, intravenous administrations of T-mfIL12 significantly inhibited the number of metastases compared with mock and T-01 treatments (Tsurumaki Y, *et al.* unpublished data).

7. SUMMARY

In summary, oncolytic HSV-1 has high potential as a new drug for cancer therapy for following reasons. 1) It can be applied to all types of solid tumor: Oncolytic HSV-1 therapy has a wide variety of application in cancer therapy. 2) It can be combined with conventional therapies, *i.e.* surgery, radiotherapy and chemotherapy. 3) It can be administered repeatedly: Neither myelosuppression nor accumulating toxicity has been observed so far. And, 4) it may work synergistically with immunotherapy.

"Arming" of oncolytic HSV-1 with transgenes leads to development of a variety of oncolytic HSV-1 with certain antitumor functions resulting in enhancement of antitumor efficacy, which in turn leads to development of a series of oncolytic HSV-1 suited for certain tumor types and different administration routes.

8. ACKNOWLEDGMENTS

I thank current members of my laboratory, especially Drs. Yasushi Ino and Hiroshi Fukuhara, and all current research collaborators. I also thank the researchers whom I worked with in the past, especially Dr. Robert L. Martuza, Massachusetts General Hospital, who first developed the strategy of oncolytic virus therapy using replication-competent recombinant HSV-1. This research has been supported in part by grants to T. Todo from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Abbreviations: HSV-1: herpes simplex viruses type 1

Key Words: Oncolytic Virus Therapy, Replication-Competent Virus Vectors, Herpes Simplex Virus Type I, Antitumor Immunity, Review

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http://www.bioscience.org/current/vol13.htm

[Cell Adhesion & Migration 2:3, 208-213; July/August/September 2008]; ©2008 Landes Bioscience

Special Focus: Glioma Therapy

"Armed" oncolytic herpes simplex viruses for brain tumor therapy

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Abbreviations: HSV-1, herpes simplex virus type 1; PKR, double-stranded RNA-dependent protein kinase; ICP6, infected-cell protein 6; pfu, plaque-forming units; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; GALV.fus, gibbon ape leukemia virus envelope fusogenic membrane glycoprotein; yCD, yeast cytosine deaminase; TNFα, tumor necrosis factor alpha

Key words: oncolytic virus therapy, gene therapy, herpes simplex virus, viral vectors, G47Δ, G207, antitumor immunity

Genetically engineered, conditionally replicating herpes simplex viruses type 1 (HSV-1) are promising therapeutic agents for brain tumors and other solid cancers. They can replicate in situ, spread and exhibit oncolytic activity via a direct cytocidal effect. One of the advantages of HSV-1 is the capacity to incorporate large and/or multiple transgenes within the viral genome. Oncolytic HSV-1 can therefore be "armed" to add certain functions. Recently, the field of armed oncolytic HSV-1 has drastically advanced, due to development of recombinant HSV-1 generation systems that utilize bacterial artificial chromosome and multiple DNA recombinases. Because antitumor immunity is induced in the course of oncolytic activities of HSV-1, transgenes encoding immunomodulatory molecules have been most frequently used for arming. Other armed oncolytic HSV-1 include those that express antiangiogenic factors, fusogenic membrane glycoproteins, suicide gene products, and proapoptotic proteins. Provided that the transgene product does not interfere with viral replication, such arming of oncolytic HSV-1 results in augmentation of antitumor efficacy. Immediate-early viral promoters are often used to control the arming transgenes, but strict-late viral promoters have been shown useful to restrict the expression in the late stage of viral replication when desirable. Some armed oncolytic HSV-1 have been created for the purpose of noninvasive in vivo imaging of viral infection and replication. Development of a wide variety of armed oncolytic HSV-1 will lead to an establishment of a new genre of therapy for brain tumors as well as other cancers.

Introduction

Oncolytic virus therapy is an attractive and rapidly developing means for treating cancer.[1] Genetically engineered viruses, such as herpes simplex virus type 1 (HSV-1) and adenovirus, are designed so that virus replication is restricted to tumor cells and therefore infection causes no harm to normal tissues. In principle, infected tumor cells are destroyed by a direct oncolytic activity of the viruses. Importantly, oncolytic viruses can also act as vectors that provide amplified transgene delivery.

HSV-1, especially in comparison with adenovirus, has suitable features for cancer therapy: (1) HSV-1 infects most tumor cell types. (2) A relatively low multiplicity of infection is needed for total cell killing. (3) Anti-viral drugs are available. (4) A large genome (~152 kb) allows the insertion of large and/or multiple transgenes. (5) The host immune reactions enhance antitumor effects. (6) Circulating anti-HSV-1 antibodies do not affect cell-to-cell spread of the virus. (7) There are HSV-1 sensitive mouse and nonhuman primate models for preclinical evaluation. (8) Viral DNA is not integrated into the host genome. HSV-1 is neurotropic and the genes necessary for neuropathogenicity have been identified and can be mutated. Therefore, the use of HSV-1 is especially advantageous for brain tumor therapy.

In order to target HSV-1 replication to tumor cells, viral genes that are essential for viral replication in normal cells but dispensable in tumor cells are inactivated or deleted.[2] This principle uses features common for all types of cancer, therefore the application of oncolytic HSV-1 is not restricted to brain tumors, but also includes a wide variety of cancer. The key for successful and practical development of oncolytic HSV-1 is to achieve a wide therapeutic window by the use of genetic engineering technology.

Second-Generation Oncolytic HSV-1

G207 was the first oncolytic HSV-1 used in a clinical trial in the United States.[3] This second-generation oncolytic HSV-1 has double mutations created in the HSV-1 genome.[4] G207 has deletions in both copies of the *γ34.5* gene, the major determinant of HSV-1 neurovirulence.[5] *γ34.5*-deficient HSV-1 vectors are considerably attenuated in normal cells, but retain their ability to replicate within neoplastic cells. In normal cells, HSV-1 infection induces activation of double-stranded RNA-dependent protein kinase (PKR), which in turn leads to phosphorylation of the α-subunit of eukaryotic initiation factor 2 and a subsequent shutdown of host and viral protein synthesis.[6] The product of the *γ34.5* gene antagonizes this PKR activity. However, tumor cells have low PKR activities, thereby allowing *γ34.5*-deficient HSV-1 vectors to replicate.[7,8] G207 also has an insertion of the *E. coli lacZ* gene in the infected-cell protein 6

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Submitted: 04/30/08; Accepted: 05/28/08

Previously published online as a *Cell Adhesion & Migration* E-publication: http://www.landesbioscience.com/journals/celladhesion/article/6353

(*ICP6*) coding region (UL39), inactivating ribonucleotide reductase, a key enzyme for viral DNA synthesis in non-dividing cells but not in dividing cell.[9]

In preclinical studies using immunocompetent animals, the most remarkable finding with G207 was that it induced systemic antitumor immunity in the course of oncolytic activity.[10,11] For example, in A/J mice bearing bilateral subcutaneous N18 (syngeneic neuroblastoma) tumors, intraneoplastic G207 inoculation into the left tumor alone caused growth reduction not only of the inoculated tumors but also of the non-inoculated contralateral tumors. The antitumor immunity was associated with an elevated cytotoxic T lymphocyte activity specific to N18 tumor cells that persisted for at least 13 months.

After an extensive in vivo safety evaluation using HSV-1-susceptible mice and non-human primates, the G207 phase I clinical trial was performed between 1998 and 2000 at two institutions.[3] Twenty-one patients with recurrent malignant glioma were treated, and G207 was administered directly into the tumor via stereotactic inoculation. This dose escalation study started from 10^6 plaque-forming units (pfu) and increased to 3×10^9 pfu, with three patients at each dose. As a result, no acute, moderate to severe adverse events attributable to G207 were observed. Eight of 20 patients that had serial MRI evaluations had a decrease in tumor volume between four days and one month post-inoculation and two patients survived for more than five years.

Third-Generation Oncolytic HSV-1

The phase I clinical trial proved the safety of G207 and hinted its efficacy for human brain tumors. However, in order to further improve the efficacy without compromising its safety, a third-generation oncolytic HSV-1 termed G47Δ was newly created from G207 by introducing another genetic alteration, i.e., the deletion of the *α47* gene and the overlapping *US11* promoter region, in the G207 genome.[12] Because the *α47* gene product inhibits transporter associated with antigen presentation, which translocates peptides across the endoplasmic reticulum, the downregulation of MHC class I that normally occurs in human cells after infection with HSV-1 does not occur when the *α47* gene is deleted.[13] G47Δ-infected human cells in fact presented higher levels of MHC class I expression than cells infected with other HSV-1 vectors.[12] Further, human melanoma cells infected with G47Δ were better at stimulating their matched tumor-infiltrating lymphocytes in vitro than those infected with G207. The deletion also places the late *US11* gene under control of the immediate-early *α47* promoter, which results in suppression of the reduced growth phenotype of *γ34.5*-deficient HSV-1 mutants including G207.[14] In the majority of cell lines tested, G47Δ replicated better than G207, resulting in the generation of higher virus titers, and exhibiting greater cytopathic effect.[12] In athymic mice bearing subcutaneous U87MG human glioma and A/J mice bearing subcutaneous Neuro2a neuroblastoma, G47Δ was significantly more efficacious than G207 at inhibiting the tumor growth when inoculated intraneoplastically.[12] G47Δ was also more efficacious than G207 in athymic mice bearing intracerebral U87MG tumors (Ino Y et al., manuscript in preparation). Nevertheless, the safety of G47Δ remained unchanged from G207 following injection into the brain of HSV-1-sensitive A/J mice.[12] In Japan, a clinical trial of G47Δ in recurrent glioblastoma patients is underway. G47Δ has been shown efficacious in animal tumor models of a variety of cancers including brain tumors, prostate cancer, breast cancer and neurofibroma.[12,15-17]

Construction of "Armed" Oncolytic HSV-1

One of the advantages of HSV-1 is the capacity to incorporate large and/or multiple transgenes within the viral genome. Certain antitumor functions may be added to oncolytic activities of HSV-1. The use of replication-competent HSV-1 for transgene expression has multiple attractive advantages over replication-incompetent or defective HSV-1 vectors: (1) A continuous generation of a high-titer, homogenous vector stock is possible, which allows manufacturing of a large amount with a better quality control. (2) An amplified gene delivery can be obtained in vivo. And, (3) transgene expression may lower administering doses required, therefore decrease toxicity. On the other hand, potential demerits of using replication-competent viruses for expressing foreign proteins are that the transgene expression may increase the toxicity of the vector, and may also interfere with viral replication. Transgene expression by armed oncolytic HSV-1 could be shorter in duration than replication-incompetent vectors due to destruction of the host cell by viral replication. However, we have observed that, because continuous viral spread and infection occur within the tumor, a larger number of tumor cells consequently express the transgene, therefore a much higher total amount of transgene product is achieved compared with non-replicating vectors.

In the past, a recombinant HSV-1 was constructed by conventional homologous recombination techniques that required selection of a correctly structured clone from millions of candidates. It often took a few years until the intended HSV-1 was obtained. In order to circumvent the time-consuming processes, we have developed an innovative "armed" oncolytic HSV-1 construction system using G47Δ as the backbone.[18] Besides its favorable features for human cancer therapy, including the safety, high yields of virus, improved oncolytic activity and potent stimulation of antitumor immune cells, G47Δ is especially suited as a replication-competent backbone for expressing any foreign protein molecules, because of the wide therapeutic window and preclusion of the shutoff of protein synthesis in the infected host cells. The system, termed T-BAC system, utilizes bacterial artificial chromosome and two DNA recombinase systems (Cre/loxP and FLP/FRT) (Fig. 1). It allows (1) a construction of armed oncolytic HSV-1 in a short period (usually 3–4 months), (2) a simultaneous construction of multiple vectors, (3) an accurate insertion of a desired transgene into the deleted *ICP6* locus, (4) an insertion of multiple transgenes using the same effort as inserting a single transgene, and (5) a direct comparison of multiple "armed" oncolytic HSV-1 with the same backbone. A similar system, termed HSVQuik system, has been also developed using a G207-like backbone.[19,20]

Oncolytic HSV-1 Armed with Immunostimulatory Genes

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.[10,11] Therefore, while any transgene that does not interfere with HSV-1 replication may be used, the genes encoding immunomodulatory molecules would be reasonable candidates for arming oncolytic HSV-1. Immunostimulatory functions should augment the antitumor immunity induction that adds to

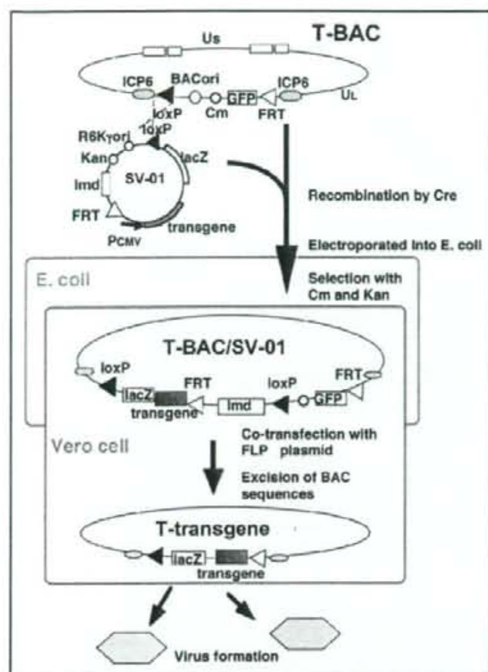

Figure 1. A schema describing the T-BAC system for constructing "armed" oncolytic HSV-1 with the G47Δ backbone. The desired transgene for "arming" is inserted into the multiple cloning site of the shuttle vector (SV-01). The first step is to insert the entire sequence of the shuttle vector into the loxP site of T-BAC by a Cre-mediated recombination, followed by an electroporation into *E. coli*. The second step is to co-transfect the co-integrate with a plasmid expressing FLP onto Vero cells to excise the BAC sequence flanked by the FRT sites. The objective armed oncolytic HSV-1 appear as GFP-negative and lacZ-positive virus plaques. Non-recombined viruses do not appear, due to the presence of the lambda stuffer sequence (lmd) causing an oversize of the genome.[18]

direct oncolytic activity of the virus, resulting in enhanced antitumor activities (Fig. 2).[2] *γ34.5* -deficient HSV-1 containing the murine interleukin 4 (IL-4) gene displayed a significantly higher antitumor activity and prolonged survival of mice with intracranial tumors compared with its parental virus or the one expressing IL-10.[21] First-generation oncolytic HSV-1 expressing IL-12 (M002 and NV1042) showed improved in vivo efficacy against 4C8 glioma in syngeneic B6D2F mice[22] and brain tumors of Neuro2a neuroblastoma in syngeneic A/J mice,[23] and also against murine squamous cell carcinoma[24] and murine colorectal tumor.[25] Immunohistochemical analyses of tumors treated with these IL-12-expressing HSV-1 revealed a significant influx of $CD4^+$, $CD8^+$ T cells and macrophages. The oncolytic HSV-1 expressing IL-12 (NV1042) was more efficacious than the one expressing granulocyte macrophage colony-stimulating factor (GM-CSF) in the same backbone (NV1034) in mice with subcutaneous squamous cell carcinoma.[24] The mice cured by NV1042 had a higher rate of rejecting rechallenged tumor cells than those cured by NV1034.[24]

The HSVQuik was used to create G207-like second-generation oncolytic HSV-1 armed with murine IL-4, CD40 ligand or 6CK (Fig. 3).[20] In BALB/c mice bearing 4T1 breast cancer in the brain, all of these armed HSV-1 showed better antitumor efficacy than the control virus. Using the HSVQuik system, we also created oncolytic HSV-1 armed with IL-12, IL-18 or soluble B7-1.[19] All of these armed HSV-1 demonstrated replicative capabilities similar to the parental virus in vitro. The in vivo efficacy was tested in A/J mice harboring subcutaneous tumors of syngeneic and poorly immunogenic Neuro2a neuroblastoma. IL-12 was the most efficacious among the immunostimulatory molecules investigated when expressed by the G207-like HSV-1. The triple combination of the three armed viruses exhibited the highest efficacy amongst all single viruses or combinations of two viruses. Combining 1×10^5 pfu each of the three armed viruses showed stronger antitumor activities than any single armed virus at 3×10^5 pfu in inoculated tumors as well as non-inoculated remote tumors.

Using the Neuro2a subcutaneous tumor model, another research group demonstrated that the antitumor efficacy of M002, a first-generation *γ34.5*-deficient HSV-1 that expresses IL-12, could be augmented when used in combination with M010, the same backbone HSV-1 that expresses chemokine CCL2.[26] The group also demonstrated that the virus selected after in vivo serial passage of M002 in tumors of a D54-MG human malignant glioma cell line improved survival in two independent murine brain tumor models compared to the parent M002.[27] This enhanced antitumor efficacy was not due to restoration of protein synthesis or early *US11* expression.

Recently, using the T-BAC system, we generated a G47Δ-backbone oncolytic HSV-1 armed with mouse fusion-type IL-12, termed T-mfIL12 (Fig. 3). In A/J mice bearing bilateral subcutaneous Neuro2a tumors, intraneoplastic inoculation with T-mfIL12 into the left tumor alone led to a significantly better antitumor activity than the unarmed control virus, T-01, not only in the inoculated left tumors but also in the non-inoculated remote tumors (Miyamoto S, et al., manuscript in preparation). We also created a G47Δ-backbone HSV-1 armed with both IL-18 and soluble B7-1.[18] This double-armed oncolytic HSV-1 showed a significant enhancement of antitumor efficacy via T-cell mediated immune responses in A/J mice with subcutaneous Neuro2a tumors as well as in C57BL/6 mice bearing subcutaneous TRAMP-C2 prostate cancer.

An armed oncolytic HSV-1 has not been tested in patients with brain tumors, however a phase I clinical trial with a second-generation oncolytic HSV-1 expressing GM-CSF was conducted in patients with cutaneous or subcutaneous deposits of breast, head and neck and gastrointestinal cancers and recurrent malignant melanoma.[28] OncoVEX$^{GM\text{-}CSF}$ has a deletion in the *α47* gene and the *γ34.5* gene replaced with the *GM-CSF* gene driven by a CMV promoter (Fig. 3). A single dose (13 patients) or multiple doses (17 patients), ranging from 10^6 to 10^8 pfu/ml/dose, were injected intratumorally. Local inflammation, erythema and febrile responses were the main side effects, and the local reaction to injection was dose limiting in HSV-1-seronegative patients at 10^7 pfu/ml. Some of biopsy specimens after treatment showed areas of necrosis that strongly stained for HSV-1. Three patients had stable disease, six patients showed flattened injected and/or uninjected tumors, and four patients showed inflammation of uninjected tumors.

Armed Oncolytic HSV-1 with Other Antitumor Functions

Various types of transgenes other than immunomodulatory genes have been used to arm oncolytic HSV-1. Theoretically, antiangiogenic factors can augment the antitumor activities of oncolytic HSV-1 without compromising the viral replication and antitumor immunity induction. Early generation oncolytic HSV-1, such as G207, was shown to retain the ability of wild type HSV-1 to increase infected tissue vascularity, whereas third-generation G47Δ showed suppressed vascularity in infected tumors.[29] By using the G47Δ-BAC system, a preliminary version of the T-BAC system, G47Δ-backbone oncolytic HSV-1 armed with Platelet Factor 4 or dominant negative fibroblast growth factor receptor have been created.[30,31] Both of these armed oncolytic HSV-1 were more efficacious in inhibiting the tumor growth and angiogenesis than the control virus in both human U87MG glioma and mouse 37-3-18-4 malignant peripheral nerve sheath tumor models. By using the HSVQuik system, an oncolytic HSV-1 armed with tissue inhibitor of metalloproteinases 3, termed rQT3, has been created.[32] In athymic mice bearing human neuroblastoma or malignant peripheral nerve sheath tumor, treatment with rQT3 caused delayed tumor growth, increased peak levels of infectious virus, and immature collagen extracellular matrix. Remarkably, rQT3 treatment caused reduced tumor vascular density, which was associated with reduced circulating endothelial progenitors.

Another approach for arming oncolytic HSV-1 is the use of fusogenic membrane glycoproteins. Expression of fusogenic proteins by infected tumor cells could cause involvement of surrounding uninfected cells to form syncytium and facilitate tumor cell killing, but might also increase toxicity in the normal tissue. Fu et al. constructed an oncolytic HSV-1 armed with a truncated form of the gibbon ape leukemia virus envelope fusogenic membrane glycoprotein (*GALV.fus*).[33] In athymic mice bearing human Hep 3B hepatocellular carcinoma xenografts, the expression of *GALV.fus* significantly enhanced the antitumor effect of the virus. Furthermore, by using a strict late viral promoter instead of a CMV promoter, *GALV.fus* glycoprotein could be expressed only in tumor cells and not in normal non-dividing cells.

So-called suicide genes have been used from early stages of armed oncolytic HSV-1 development. Expression of a suicide gene by an infected tumor cell should elicit bystander killing of surrounding uninfected tumor cells via extracellular diffusion of activated prodrug, but premature killing of the host cell could also suppress viral replication. HSV-1 naturally expresses thymidine kinase that activates the prodrug ganciclovir. However, a combination with systemic ganciclovir administration did not significantly enhance the efficacy of G207 in A/J mice with intracerebral N18 neuroblastoma.[34] rRp450 was engineered by replacing the *lacZ* gene within the *ICP6* locus of the first-generation oncolytic HSV-1, hrR3, with the gene encoding rat cytochrome P450 2B1 (CYP2B1), a member of the cytochrome P450 family responsible for activating the prodrug cyclophosphamide.[35] In rat 9L and human U87ΔEGFR glioma models, systemic administration of both cyclophosphamide and ganciclovir in combination with rRp450 showed the most efficacy compared with any other combinations.[36] By using the HSVQuik system, an oncolytic HSV-1, termed MGH2, was created that expressed both CYP2B1 and secreted human intestinal carboxylesterase.[37] The latter enzyme converts irinotecan into an active metabolite. In athymic mice bearing Gli36ΔEFGR glioma in the brain, MGH2 displayed increased antitumor efficacy when combined with cyclophosphamide and irinotecan. The researchers found that, unlike ganciclovir, cyclophosphamide, irinotecan or the combination of both did not significantly affect virus replication. HSV1yCD was created by replacing the *ICP6* gene of HSV-1 with the gene encoding yeast cytosine deaminase (yCD).[38] yCD converts the prodrug 5-fluorocytosine (5-FC) to a cytotoxic agent, 5-fluorouracil. This research group also observed that the approach enhanced cytotoxicity without significantly reducing viral replication and oncolysis. In BALB/c mice bearing subcutaneous tumors or diffuse liver metastases of MC26 colon cancer, anti-neoplastic activity of HSV1yCD combined with systemic 5-FC administration was greater than HSV1yCD alone. By utilizing the same backbone as OncoVEX^{GM-CSF} (Fig. 3), an oncolytic HSV-1 termed OncoVEX$^{GALV/CD}$ double-armed with yCD/uracil phosphoribosyltransferase fusion and GALV.fus has been created.[39] In Fischer f344 rats bearing subcutaneous 9L glioma, OncoVEX$^{GALV/CD}$ proved most efficacious compared with the control viruses (OncoVEX, OncoVEX^{GALV} or OncoVEX^{CD}) when combined with systemic 5-FC administration.

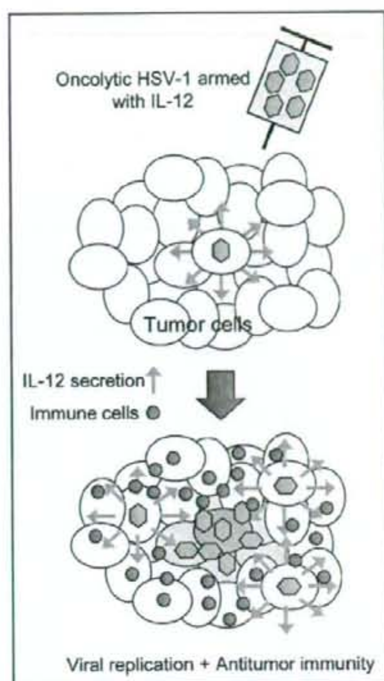

Figure 2. Concept of antitumor efficacy augmentation using oncolytic HSV-1 armed with an immunostimulatory gene. When oncolytic HSV-1 armed with the *IL-12* gene infects tumor cells, IL-12 is secreted in the course of viral replication and stimulates the immune cells. In addition to direct tumor cell killing via viral replication and spread, tumor cells are destroyed by augmented antitumor immune responses, resulting in enhanced antitumor activities.

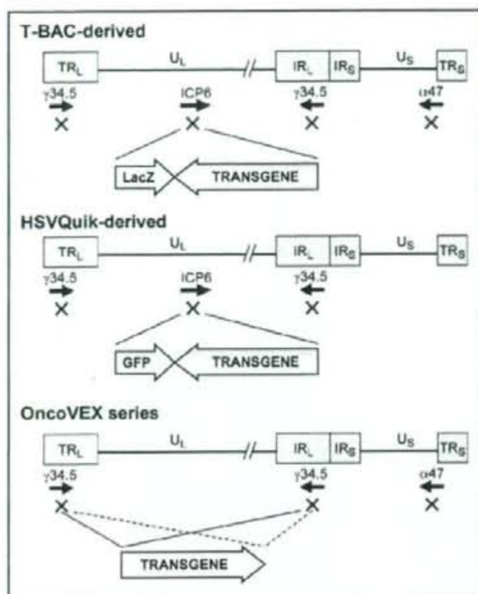

Figure 3. Structures of representative armed oncolytic HSV-1. The HSV-1 genome consists of long and short unique regions (U_L and U_S) each bounded by terminal (T) and internal (I) repeat regions (R_L and R_S). Armed oncolytic HSV-1 created by using the T-BAC (or G47Δ-BAC) system has the backbone structure of G47Δ, a third-generation oncolytic HSV-1. It has triple deletions in the *γ34.5*, *ICP6* and *α47* genes. The transgene is inserted into the deleted *ICP6* locus. As a marker, it also expresses the *LacZ* gene driven by the *ICP6* promoter. Armed oncolytic HSV-1 created by using the HSVQuik system has the backbone structure similar to G207 or MGH1, second-generation oncolytic HSV-1. It has double deletions in the *γ34.5* and *ICP6* genes. The transgene is inserted into the deleted *ICP6* locus. As a marker, it also expresses the *GFP* gene driven by the *ICP6* promoter. The OncoVEX series has the backbone structure of a second-generation oncolytic HSV-1 with double deletions in the *γ34.5* and *α47* genes. The transgene is inserted into the deleted *γ34.5* loci.

Han et al. recently created an oncolytic HSV-1, with double deletions in the *γ34.5* and *α47* genes, armed with tumor necrosis factor alpha (TNFα).[40] TNFα is a cytokine with a potent antitumor activity, but a local delivery of TNFα is known to cause toxicity, and its ability to induce tumor cell apoptosis could interfere with viral replication. To avoid these problems, they used the *US11* true late HSV-1 promoter to drive the *TNFα* gene. Whereas the virus armed with *US11*-driven TNFα expressed lower amounts of TNFα, it exhibited higher antitumor effects and less toxicity than the virus that used the immediate-early CMV promoter.

Armed Oncolytic HSV-1 for in vivo Imaging

With the advancement of oncolytic virus therapy development, there has been an increasing need for non-invasive methods of imaging or monitoring of viral infection and replication. Oncolytic HSV-1 can be armed not only for the purpose of augmenting the therapeutic efficacy but also for realizing such non-invasive in vivo imaging. In preclinical settings, one approach is to utilize a luciferase-based bioluminescent system. Two HSVQuik-based oncolytic HSV-1 were generated that express firefly luciferase under the control of the *immediate-early (IE) 4/5* promoter or *gC* promoter.[41] The *IE4/5* promoter acts immediately after viral infection, whereas the strict late *gC* promoter acts in the late stage of the replication cycle. When athymic mice bearing subcutaneous tumors of Gli36ΔEGFR glioma were observed under a supersensitive charged coupled device camera, the expression of luciferase controlled by the *IE4/5* promoter correlated with viral infection and that by the *gC* promoter with viral replication.

Systemic Delivery of Armed Oncolytic HSV-1

Whereas the most common route of delivery of oncolytic HSV-1 has been a direct intratumoral inoculation, an intravenous delivery would further broaden the clinical application of oncolytic HSV-1 if proven effective. The main hurdle for intravenous delivery is that only a small percentage of the administered virus reaches the tumor. By arming of oncolytic HSV-1, a large antitumor effect can be induced from a small number of virus that initiates replication at the tumor. We observed that intravenous delivery of IL-12-expressing T-mfIL12 caused a significant inhibition of tumor growth compared with mock and the unarmed control virus (T-01) treatments in A/J mice bearing subcutaneous Neuro2a tumors (Guan et al., manuscript in preparation). When A/J mice bearing intracerebral tumors were treated by repeated intravenous injections, T-mfIL12, but not T-01, significantly prolonged the survival compared with mock. Also, in a renal cancer lung metastases model using BALB/c mice and syngeneic RenCa cells, intravenous administrations of T-mfIL12 significantly inhibited the number of metastases compared with mock and T-01 treatments (Tsurumaki et al., manuscript in preparation).

Summary

In summary, "arming" of oncolytic HSV-1 with transgenes leads to development of a variety of oncolytic HSV-1 with certain functions resulting in enhancement of antitumor efficacy and/or in vivo imaging capability. In the future, a series of armed oncolytic HSV-1 suited for certain tumor types or certain administration routes may be used differentially or in combination according to conditions of patients. Armed oncolytic HSV-1 has high potential as a new genre of therapy for brain tumors as well as other cancers.

Acknowledgements

I thank the current members of my laboratory, especially Drs. Yasushi Ino and Hiroshi Fukuhara. Our research has been supported in part by grants to T.T. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Current Organ Topics:	Central Nervous System Tumor 脳腫瘍 グリオーマ Ⅲ. 悪性グリオーマ治療における薬剤耐性機構の最近の知見—temozolomide 耐性・分子標的薬・脳腫瘍幹細胞— 永根　基雄（杏林大学医学部脳神経外科）

〔*Jpn J Cancer Chemother* 35(6): 918-925, *June*, 2008〕

はじめに

神経膠腫（glioma）は悪性脳腫瘍を代表する疾患であるが，近年の医学・医療機器の発展・進歩にもかかわらず，集学的治療による悪性 glioma の治療予後は依然極めて不良である。悪性 glioma 治療の柱の一つである化学療法は効果が不十分であることが多く，その原因として，脳に特異的な血液脳関門の存在により腫瘍組織への薬剤到達性が低く抑えられる点に加え，腫瘍細胞自体のもつ薬剤耐性の存在が重要な原因として考えられている[1]。悪性脳腫瘍の中には，胚芽腫や悪性リンパ腫等薬剤感受性が高く，予後の延長が得られてきた腫瘍型も含まれるが，悪性 glioma では薬剤耐性の機序の解明と対策が予後改善に向けての重要な課題である。

悪性 glioma に対する化学療法は，2005 年に発表された Stupp らの第Ⅲ相無作為臨床試験の結果，過去 30 年来で初めてともいえる大きな変革がみられた。最も悪性である WHO grade Ⅳの膠芽腫（glioblastoma: GBM）に対しては，可及的摘出の後，放射線治療に併用して temozolomide（TMZ）の連日投与を行い，その後引き続き TMZ による維持療法を行っていくことが世界標準治療となったからである[2]。確かに TMZ 治療により劇的な腫瘍縮小がみられる症例も少なからず認められるが，一方で TMZ が無効な症例も多く，膠芽腫では放射線治療単独に比べ，TMZ 併用により全生存期間（overall survival: OS）の延長効果は高々 2.5 か月に過ぎない[2]。TMZ などの化学療法剤への感受性を規定する因子を明らかにしていくことで，治療効果を予測し，また感受性を高める個別化治療の道も開けてくるものと期待される。

本稿では，主として glioma における TMZ や分子標的治療などに対する薬剤耐性機序と脳腫瘍幹細胞仮説につき最近の知見を概説する。

1．TMZ などのアルキル化剤による抗腫瘍作用と O^6-methylguanine-DNA methyltransferase（MGMT/AGT）発現の関与

TMZ や procarbazine（PCZ）は主として核 DNA 中の guanine 残基内 O^6 部位に methyl 基を付加し，O^6-methylguanine（MG）を形成する。また，BCNU や ACNU などのニトロソウレア系抗癌剤は，同部に chloroethyl 基を付加することで O^6-alkylguanine（AG）を形成する[3,4]。O^6-AG は更に相補的な DNA 鎖の cytosine と不可逆的で致死的な DNA 鎖間架橋を形成するが，TMZ により誘導される O^6-MG は DNA 複製の際に thymine とミスマッチし（O^6-MG: T），DNA の変異（G: T）が生じる。この O^6-MG: T 病巣に対してミスマッチ修復（mismatch repair, MMR）機構による修復機転が働くが，異常 DNA 塩基対は O^6-MG が残存するために正常に修復されず，不毛の修復機転が繰り返され（futile repair），最終的に DNA breaks が生じ，細胞死（apoptosis）が誘導されると考えられている[5]。Glioma 細胞においては更に TMZ による細胞周期障害の重要性が指摘されている[6]。DNA 修復酵素である O^6-methylguanine-DNA methyltransferase（MGMT）は，O^6-MG/O^6-AG を正常な guanine に修復する機能を持つ[7]。MGMT は単独で O^6 部位のアルキル基を除去し，DNA を正常に復すと同時に MGMT 自身は不活化される自殺酵素であり，他の DNA 修復機構とは異なる特徴を有している[4,8]（図 1）。したがって MGMT が発現している場合や[9]，MGMT 発現が欠落していても MMR 機能が欠如している場合に腫瘍細胞は TMZ やニトロソウレア剤に耐性を示すと考えられる[10,11]。

MGMT 遺伝子の発現調節は主として遺伝子プロモーター領域の epigenetic なメチル化によると考えられ[12]，methylation-specific PCR 法（MSP）を用いたこの領域のメチル化解析や，mRNA あるいは蛋白発現量，MGMT 酵素活性などが MGMT 解析の方法として使用されてきている[13-15]。Glioma 手術標本では 70％以上に

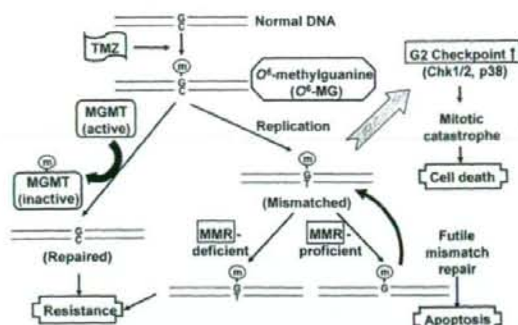

図 1　Temozolomide による DNA メチル化と抗腫瘍細胞効果

Temozolomide（TMZ）は腫瘍 DNA 中の guanine 残基（G）の O^6位を中心にメチル化し（ⓜ），O^6-methylguanine（O^6-MG）が形成される。腫瘍細胞の DNA 複製時に O^6-MG は cytosine（C）ではなく thymine（T）とミスマッチし，mismatch repair（MMR）機構により thymine が除去修復されるが，O^6-MG は残存するため MMR 作用が繰り返され，最終的に DNA 切断が生じ細胞死に至る。Glioma 細胞においては，TMZ による DNA 傷害は細胞周期での G2 チェックポイント機構を活性化し，その結果 mitotic catastrophe が誘導され細胞死が生じる（文献 6）。DNA 修復酵素の O^6-methylguanine-DNA methyltransferase（MGMT）は O^6-MG からメチル基を除去し，DNA を正常に修復すると同時に自ら失活する。

MGMT 活性が検出され[16]，一方 MGMT 遺伝子プロモーター領域のメチル化（発現抑制）は約半数（40～76%）の症例で認められている[15]。Glioma を含め各臓器癌由来の細胞株を用いた実験で，各細胞株の MGMT 活性あるいは発現量が ACNU 耐性度と良く相関することが報告されており[17]，また MGMT を高発現している glioma 症例では，有意に早期の腫瘍再発が認められている[18]。Stupp らの初発膠芽腫に対する放射線治療併用 TMZ 療法の第Ⅲ相試験の症例で，Hegi らは TMZ 治療後の生存率は MGMT プロモーターのメチル化が認められる群で有意に延長することを示し[19]，われわれも再発膠芽腫に対し TMZ 単独療法を行った症例で，腫瘍 MGMT 蛋白の発現量と無再発生存期間（progression-free survival: PFS），OS がともに有意に相関することを報告した[14]。これらの結果は，MGMT 発現が TMZ やニトロソウレア剤の治療反応性を規定する独立した予後因子となることを強く示唆しており，治療効果を高めるためには，MGMT が高発現或いは非メチル化 MGMT プロモーターの腫瘍に対し，アルキル化剤以外の治療薬を選択するか，MGMT 機能の抑制を図ることが必要となってくる。しかし，現時点で Stupp らの成績を凌駕する臨床試験結果はこの 30 年来ほとんどなく[2]，非メチル化 MGMT プロモーターであったとしても毒性が少なく経口薬である TMZ を投与しない治療法は現実的ではないと考えられる[20]。

一方，glioma を含む脳腫瘍では，小児例を除き成人症例での MMR 欠損や microsatellite instability（MSI）の検出は少なく[16,21-24]，自験例でもほとんどの症例で MMR 関連因子の MLH1，MSH2，MSH6 の発現が検出されており[25]，成人悪性 glioma では MMR 機能異常の耐性への関与は少ないと予想される。しかし，最近アルキル化剤使用後の再発悪性 glioma で MSH6 の異常が報告され[26]，また小児 glioma では MSI の頻度が成人症例より高く[21]，Children's Oncology Group（COG）の TMZ 療法の第Ⅱ相試験（ACNS0126）でも有効性が認められなかったことなどからも[27]（http://www.cancer.gov/clinicaltrials/COG-ACNS0126），小児や一部の症例では MMR 異常が TMZ 耐性に関与している可能性もある。

先述のごとく，glioma 細胞では TMZ 治療により細胞周期の異常，主として G2 期での停止が誘導される（G2 チェックポイント機構）[6]。Hirose らは，がん抑制遺伝子の TP53 が正常な glioma 細胞ではこの作用が強く，1 週間以上継続した後細胞老化状態に至り，その結果増殖能が低下するが細胞死はむしろ抑制されることを報告した[28]。一方，TP53 異常がある glioma 細胞ではこの期間が短く，DNA 傷害を維持したまま分裂期へ至り，mitotic catastrophe と呼ばれる細胞死をきたした。TMZ による G2 チェックポイントの活性化には，G2 期に作用する Chk1，Chk2，p38 などのリン酸化酵素が関与しており，これらの酵素機能を阻害することで，TP53 遺伝子異常と関係なく TMZ による細胞毒性が増強したことから[28-30]，G2 チェックポイントが TMZ 増感のための標的となる可能性も考えられている。

2．MGMT を標的とした TMZ 耐性克服

TMZ 耐性の主因となる MGMT 活性を抑制することは，TMZ への感受性化を図るアプローチとして合理的である。MGMT は DNA からアルキル基を除去する際に失活する自殺酵素である特徴を利用して，MGMT の基質となる分子・薬剤により前処理を行う MGMT 枯渇法が試みられている[31]。これまで臨床試験も含め最も用いられている薬剤は O^6-benzylguanine（BG）である。O^6-BG は自身のベンジル環を MGMT の活性部位である cysteine と共有結合させることで MGMT を不可逆的に失活させ，腫瘍細胞のアルキル化剤への感受性を増強することが示されている。悪性 glioma に対しては BCNU に対する併用第Ⅱ相試験が行われているが[32]，TMZ に対しては第Ⅰ相が行われ[33]，現在第Ⅱ相試験が施行されている。この際に重要な点は O^6-BG の作用持続時間である。MGMT は枯渇剤が取り除かれると速やかに再合成されるため[4]，TMZ の細胞傷害効果が現れる 2 日間以

上の O^6-BG 曝露が望ましく[29]，上記試験では 48 時間の持続点滴投与を行っている[33]。また新規の MGMT 不活剤として O^6-(4-bromothenyl) guanine (PaTrin-2) も臨床試験が計画されている[34]。

TMZ 自身も O^6-MG の誘導により MGMT を消耗させる機能を持つ。低容量での連日投与にて MGMT 不活化効果が高いことが報告され[35]，21 日間の連日投与や 7 日間投与-7 日間休薬を繰り返す投与法が，TMZ 不応性悪性 glioma 症例に対して，現在臨床試験として行われている。

MGMT 遺伝子の mRNA 転写を抑制することによる耐性克服も試みられている。Natsume らは，glioma 細胞を interferon-β で処理すると TMZ 感受性が増強され，その効果は p53 を介した MGMT 遺伝子発現の抑制によることを報告した[36,37]。Interferon-β と TMZ 併用療法の第 I 相試験が初発・再発悪性 glioma に対し施行され，現在第 II 相試験が計画されている。Cisplatin も MGMT mRNA 発現を抑制し，活性を低下させる作用があることが知られており，TMZ と併用した再発膠芽腫に対する第 II 相試験が行われた[38,39]。いずれの試験でも奏効率は 20%，PFS-6 m は 35%前後，中間 PFS が 20 週前後であり，再発膠芽腫に対する TMZ 単独療法での治療効果（奏効率 8%，6-m PFS 21%，中間 PFS 9 週）に比べ良好な成績が報告されており[40]，TMZ 無効例に対しての効果が今後検証されることが期待される。

3．染色体欠失と化学療法反応性

退形成性乏突起膠腫（anaplastic oligodendroglioma; AO）或いは退形成性乏突起星細胞腫（anaplastic oligoastrocytoma; AOA）では，高頻度（60〜80%）に染色体 1 番短腕（1p）および 19 番長腕（19q）に遺伝子欠失（1p/19q deletions）が認められる。Cairncross らが 1998 年に AO の症例で 1p/19q 欠失が PCV（PCZ＋CCNU＋vincristine）療法への高感受性と生存期間の延長と相関することを報告して以来[41]，本遺伝子異常と薬剤感受性，組織診断との関連が精力的に検討されてきた。これまでのところ，これらの染色体領域に明らかな薬剤感受性を規定する遺伝子は見出されておらず，むしろ 1p/19q 共欠失は染色体 1 番と 19 番の centromer での転座の結果である可能性が示唆されている[42]。最近の報告では，1p/19q 共欠失は PCV 療法への反応性のみならず，TMZ への反応性の予測因子であることも示された[43-45]。しかしこれらの臨床試験では放射線治療単独群内でも 1p/19q 共欠失群の方が予後良好であり，この遺伝子異常の存在は広く治療全体への予測因子，更には本腫瘍自体の予後因子である可能性も指摘されている[20]。現時点では，乏突起膠腫系腫瘍では 1p/19q 共欠失の有無により層別化し，欠失のない AO/AOA に対しては悪性 glioma としての治療（放射線治療併用 TMZ）或いは新規の治療を目指した臨床試験を検討することが望ましい。

4．分子標的治療薬における耐性

悪性 glioma における遺伝子異常の解析が進む中で，細胞増殖亢進・細胞死抑制など悪性腫瘍としての基盤をなす生物学的特徴に直接的に関与する因子が明らかとなってきた[46]。腫瘍細胞では増殖・生存のためにそのような促進因子への依存度が増大すると考えられることから（pathway addiction），これらの因子を新規標的として阻害する分子標的治療が，腫瘍細胞に対する特異的な治療法として近年脚光を浴びている。分子標的治療薬としては，既に慢性骨髄性白血病における Ph1 染色体上の Bcr-Abl 遺伝子を標的とした imatinib (Glivec) や，乳癌における Her2/ErbB2 に対する trastuzumab（Herceptin）など，飛躍的に治療成績の向上に貢献してきており，悪性 glioma においても，欧米を中心に精力的に検討されている。特に細胞内のシグナル伝達を亢進させる機能を発揮する EGFR 遺伝子の遺伝子増幅や発現亢進は膠芽腫を中心に悪性 glioma で高頻度に検出され，その tyrosine kinase domain を標的とする小分子阻害剤（tyrosine kinase inhibitor; TKI）である gefitinib（Iressa），erlotinib（Tarceva）などが既に臨床試験で試みられている。

Glioma に対する分子治療薬では，主として再発膠芽腫を対象とした第 II 相試験が報告されているが[47]，これまでのところ EGFR TKIs の効果は期待通りとは言えず，腫瘍縮小効果が 0〜25%，PFS が 2〜3 か月，6 か月後の PFS（6 m-PFS）が 0〜26%と，再発膠芽腫に対する化学療法剤を使用した第 II 相試験の結果を凌駕するものではなかった。その要因として幾つかの因子が考えられている。

1）分子標的治療薬においても CPT-11 などの抗癌剤と同様，酵素誘導性薬剤，特に抗てんかん薬（enzyme-inducing antiepilepsy drugs; EIAED）服用下での薬剤活性の低下が認められ，そのため他臓器癌での至適投与量では治療効果が得られず，投薬量の増量が必要となることが多い[47,48]。

2）EGFR 遺伝子異常が高頻度にみられる肺癌では，EGFR の kinase 領域での mutation が gefinitib 感受性に重要な規定因子であることが明らかにされたが，glioma では EGFR 遺伝子の mutation は主として細胞外ドメインに生じる vIII タイプであり，kinase ドメインには肺癌と同様の mutation は検出されず，従って EGFR TKI への感受性が高い腫瘍を予見することができない[49,50]。

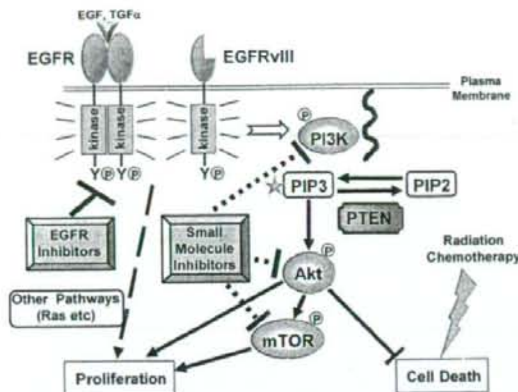

図 2 PI3K/Akt シグナル経路と分子標的治療
細胞膜に存在するEGFRはリガンドであるEGFと結合すると活性化され，引き続きPI3Kを細胞膜へ誘導し，PIP2がセカンド・メッセンジャーであるPIP3へ変換されるが，この反応はPTENの存在下では抑制されている。PIP3により下流のAkt，mTORなどのserine/threonineリン酸化酵素が活性化され，細胞増殖促進および細胞死抑制のシグナルが伝達される。変異型EGFR（vⅢ）はEGFに非依存的に恒常的に活性化している。悪性gliomaでは高頻度でPTENが欠失しており，PIP3，Aktのシグナルが活性化しているため，EGFR阻害に加えてPI3K/Aktシグナルの同時阻害により相乗的効果が期待される。

3）Gliomaでは，遺伝子異常に伴い活性化されるさまざまなシグナル伝達経路により，腫瘍細胞の悪性化という生物学的変化がもたらされる。EGFRなどの成長因子受容体，PI3 kinase（PI3K）/Akt/PTEN/mTORからの経路，Ras/Raf/MEK/MAPK系の経路，sonic hedgehog/PTCHの経路など，それぞれが独立に或いは協調しながら複雑に活性化・抑制されていると考えられ（図2），その一部の機能を阻害しても，他の経路のシグナル活性によりその効果が発揮されない可能性がある。Mellinghoffらは，膠芽腫においてEGFRvⅢとPTENの両者が発現していることが，EGFR TKIの治療効果を*in vitro*で向上させ，臨床上も有意に治療奏効率と相関することを示した[51]。即ち，PTENの異常・欠失によりPI3K/Aktのシグナル経路が活性化した状態では，EGFRを阻害するのみでは抗腫瘍効果は得られず，同時にAktによる生存促進シグナルを阻害することがEGFR TKIへの感受性を高めるため戦略となる可能性を示している。実際にWangらは，PTENを欠失しEGFR或いはEGFRvⅢを高発現している膠芽腫細胞では，PTEN/PI3Kシグナルの下流に位置するmTORの阻害剤rapamycinで治療することで，EGFR TKIによる増殖抑制・細胞死誘導能が高まることを報告しており，PTENからの経路が治療耐性の一因となることが示唆された[52]。このように，複数の細胞内シグナル経路が互いに影響しながら腫瘍増殖に関与していると考えられ，多段階シグナル阻害による相乗効果が期待される。

4）標的分子によるシグナル経路のnegative feedbackの存在。PTEN経路が野生型の細胞では，Aktを介してmTORが活性化すると，mTORはRaptorとrapamycin感受性の複合体を形成し，その結果negative feedbackによりAkt活性が抑制される[53]。したがって，mTORを阻害するとこのfeedbackが抑制され，逆に上流のAkt活性が増加し，標的治療効果が減衰する可能性が考えられる。O'ReillyらはmTORの標的薬であるrapamycin誘導体のRAD001を用いた乳癌・大腸癌の臨床試験や*in vitro*での癌細胞への治療において，結果的にAktが活性化されることを報告し，Akt経路の上流を同時に阻害することでmTOR阻害剤への増感作用が認められることを示した[54]。同様な関係はgliomaにおけるEGFR/MAPK系シグナルでもみられ[55]，標的分子のもつより詳細な分子ネットワークの解明が今後より効果的な治療戦略の確立に必要となろう。

5．脳腫瘍幹細胞（Brain tumor stem cells; BTSC）

近年，自己複製能と多分化能を併せ持つ幹細胞の存在が明らかになるとともに，幹細胞を用いた再生医学への応用に関する研究が広く行われるようになってきた。幹細胞は造血器系の分野で先駆的に研究が進められてきたが，その他生体内の様々な組織中にも体性幹細胞の存在が明らかにされており，神経系では神経幹細胞が同定されている。腫瘍細胞の中にも，一部に自己複製能を保ち，造腫瘍形成能の高い幹細胞の性格をもつ細胞群の存在が示唆され，このような無制限に分裂可能な能力をもつ"癌幹細胞（cancer stem cells; CSC）"の概念が注目を集めている[56]。

脳腫瘍においても，glioma細胞株や膠芽腫・髄芽腫などの摘出腫瘍から脳腫瘍幹細胞（brain tumor stem cell; BTSC或いはbrain tumor stem-like cells）が分離培養可能であることが報告された[57-59]（図3）。BTSCは，マウスなどに移植すると腫瘍を形成する造腫瘍性があり，ニューロン，星細胞，あるいは乏突起膠細胞などのグリア細胞への多分化能を維持するとともに，高度の自己複製能を持ち，腫瘍化に関わる遺伝子異常を伴う性質を持つ細胞と考えられている[59]。このような細胞は，成長因子のEGFとFGF2を添加した培地で培養すると神経幹細胞と同様にneurosphereを形成し，神経前駆細胞のマーカーであるCD133陽性細胞という特徴を有する[58]。BTSCは核染色剤であるHoechst 33342を排泄する機能が亢進している細胞群（side population，SP細胞）としてもrat glioma C6細胞から分離されたが[57]，最近はCD133陰性，非SP細胞にもBTSCが存在することも報告されている[60]。

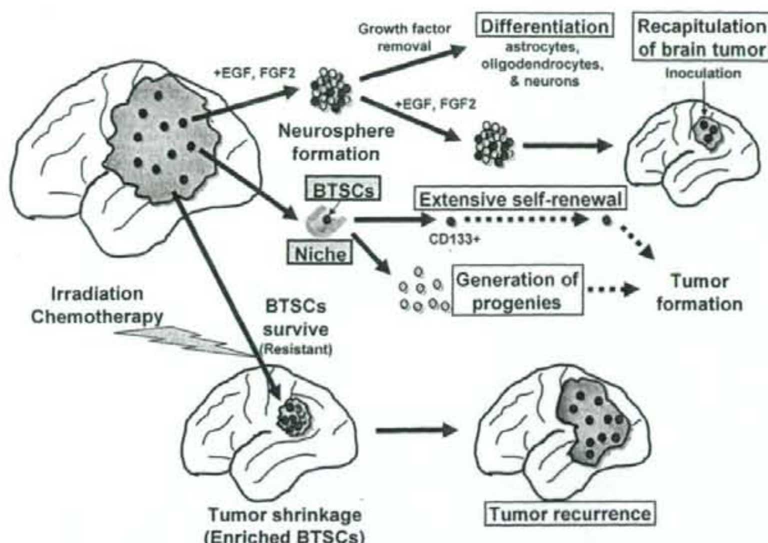

図 3　脳腫瘍幹細胞（brain tumor stem cell: BTSC）仮説と BTSC による薬剤耐性
Glioma などの悪性脳腫瘍組織内には少数の BTSC が生存維持に適する niche に接して存在すると考えられ，不均等分裂により自己再生と子孫となる腫瘍細胞が産生される。腫瘍細胞は増殖し腫瘍を形成するが，放射線や抗癌剤治療の影響を受けやすいのに対し，BTSC は様々な機序（未解決な点が多い）により治療耐性の形質をもつと考えられており，腫瘍縮小後も残存し，引き続き腫瘍再発・形成の母体となる。（現在提唱されている BTSC に関する仮説を模式的に提示した。）

重要な点は，BTSC は放射線や抗癌剤等の治療への抵抗性を示す形質をもち，悪性脳腫瘍の治療耐性の一因となる可能性である。癌幹細胞は，その子孫である腫瘍に分化していく細胞に比べると一般に分裂速度が遅く，抗癌剤の作用が及びにくい G0 期の分画が多いこと[61]，薬剤排泄機能を担う ABC カセットを含む細胞膜トランスポーターの発現，DNA 修復機構の亢進や apoptosis への耐性などを示すと考えられている。

癌幹細胞における薬剤耐性能を示す一例としては，Michor らの慢性骨髄性白血病細胞に対するイマチニブ感受性の細胞特異的効果を検討した報告がある[62]。白血病細胞のうち，イマチニブの標的分子である Bcr-Abl 融合蛋白の発現の多い成熟あるいは分化白血病細胞はイマチニブ治療により早期に死滅するが，絶対数の少ない増殖速度の遅い癌幹細胞は生存し，最終的に耐性細胞として再増殖をきたすことが示された。Glioma においても同様に幹細胞が耐性を示す報告がなされている。Kang らは glioma 細胞株の A172 と膠芽腫から BCNU に耐性を示す細胞群を分離樹立したところ，これらの細胞の中に CD133，CD45 などの細胞表面マーカーを発現し，多分化能をもち，SCID マウスの脳内に腫瘍を形成する BTSC が含まれていることを示した[63]。Liu らは膠芽腫の初期培養細胞から CD133 陽性細胞を分離したところ，これらの細胞は glioma 治療に使用される抗癌剤の TMZ，carboplatin，etoposide や paclitaxel に耐性を示し，薬剤耐性関連遺伝子である MGMT や BCRP1，更に FLIP，Bcl-2 などの apoptosis 抑制遺伝子の mRNA 発現が有意に亢進していたと報告している[64]。さらに BCRP1 に加え，薬剤排泄機能に関わる ABC トランスポーター・スーパーファミリーに属する MRP1，MRP3 などの発現亢進が CD133 陽性の BTSC で示されている[65]。また，多くの抗癌剤と同様に DNA 傷害をきたす放射線治療に耐性を示す膠芽腫細胞には CD133 陽性細胞が高率に含まれており，これらの細胞では DNA 傷害修復機構の反応亢進が認められ，その機能は主として Chk1，Chk2 活性に依存していたとの報告もある[66]。

放射線治療や化学療法に対し BTSC から分化し形成された腫瘍細胞が感受性を示す場合でも，BTSC は治療後も生存し，再び腫瘍細胞を産生することで腫瘍塊を形成し再発をきたす[67]。即ち，治療上 BTSC を有効に傷害できる治療でなければ，腫瘍の再発・再増大は免れないことになるため，BTSC，或いはこの細胞における耐性規定因子そのものが薬剤耐性を克服する重要な細胞・分子標的となりえると考えられる。最近，神経幹細胞の維持・分化に重要な役割を果たす BMP（bone morphogenetic proteins）-BMPR1B の発現と STAT を介したシグナルが，BTSC の腫瘍形成能と関連するとの報告や[68]，Hedgehog-Gli シグナル経路による BTSC 機能の制御な