HUMAN CELL(Hum Cell)

Vol. 15 No. 3
Printed in Japan

Spotlight

Oncolytic Virus Therapy Using Genetically Engineered Herpes Simplex Viruses

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<Abstract> An increasing number of oncolytic virus vectors has been developed lately for cancer therapy. Herpes simplex virus type 1 (HSV-1) vectors are particularly useful, because they can be genetically engineered to replicate and spread highly selectively in tumor cells and can also express multiple foreign transgenes. These vectors can manifest cytopathic effect in a wide variety of tumor types without damaging normal tissues, provide amplified gene delivery within the tumor, and induce specific antitumor immunity. Multiple recombinant HSV-1 vectors have been tested in patients with brain tumors and other cancers, which showed the feasibility of administering replication-competent HSV-1 vectors safely in human organs including the brain. Different approaches are currently undertaken to improve the efficacy of oncolytic HSV-1 therapy which include development of new generation vectors via further genetic engineering of existing safe vectors, combination with immune gene therapy, and combination with conventional therapies. Oncolytic virus therapy is a promising therapeutic modality that awaits establishing as an important treatment option for cancer patients in the near future.

Key words : Oncolytic Virus Therapy, Herpes Simplex Virus, Gene Therapy, Replication-Competent Vectors, Antitumor Immunity
[HUMAN CELL 15(3) :151 - 159, 2002]

INTRODUCTION

Oncolytic virus therapy is an attractive means of treating tumors. Replication-competent virus vectors are used that can replicate in situ and spread, and, at the same time, exhibit oncolytic activity by a direct cytocidal effect (Fig. 1). In addition, replication-competent virus vectors are capable of transferring and expressing foreign genes in host cells. These virus vectors are either genetically engineered [*e.g.*, herpes simplex virus type 1 (HSV-1), adenovirus, vaccinia virus], naturally attenuated (*e.g.*, Newcastle disease virus), or non-pathogenic in humans (*e.g.*, reovirus), so that they can replicate selectively in tumor cells but do not harm normal tissues.

HSV-1 in particular has many features suitable for cancer therapy[1]: 1) HSV-1 infects most tumor cell types. 2) The life cycle of HSV-1 is well studied. 3) The HSV-1 genome map is available. 4) Functions of the majority of genes are identified. 5) Genes can be manipulated. And, 6) the large size of the viral genome (153 kb) provides space for insertion of large amounts of DNA. Furthermore, HSV-1 has following features particularly suited for clinical applications: 7) A total

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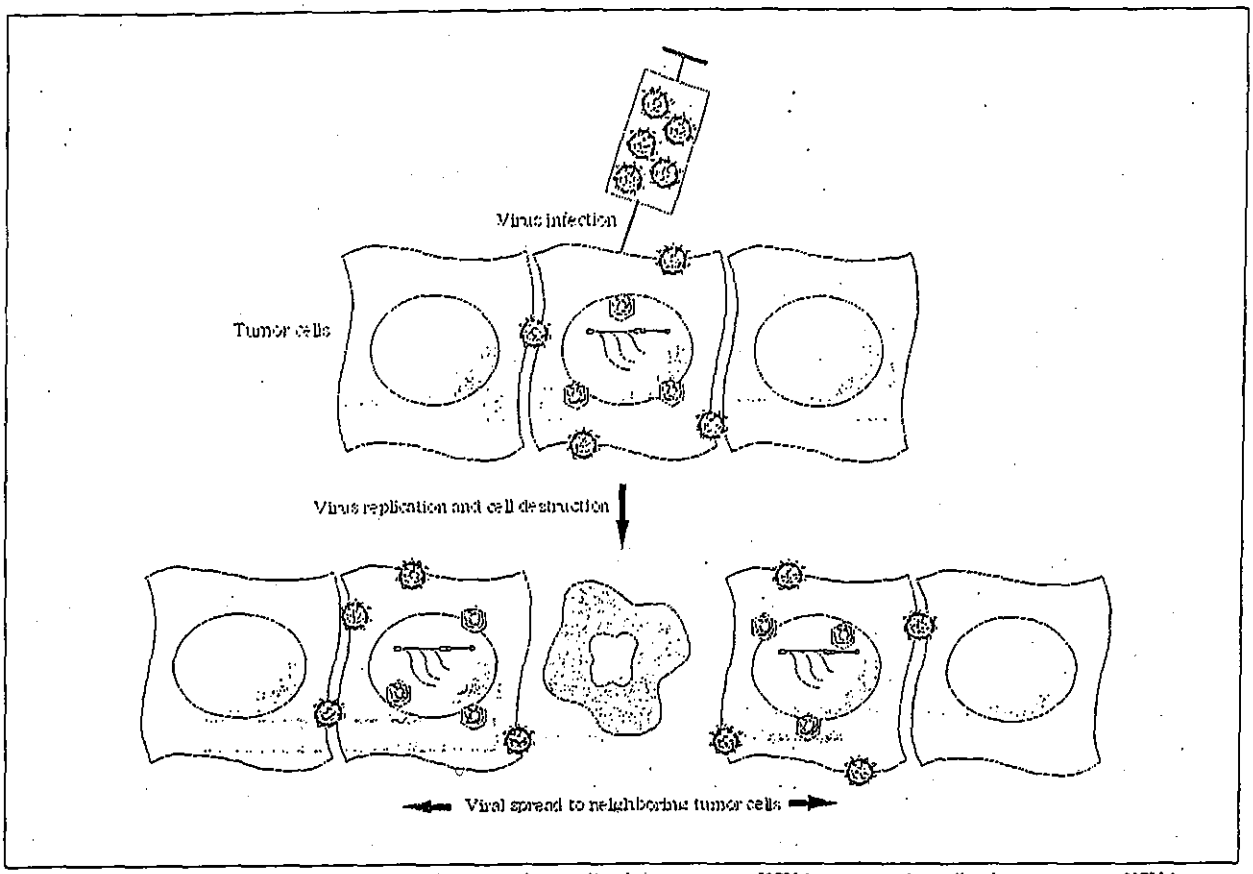

Fig. 1. Schematic diagram of oncolytic virus therapy using replication-competent HSV-1 vectors. A replication-competent HSV-1 vector infects, replicates in and kills tumor cells. Progeny virus vectors spread to neighboring tumor cells and repeat the process until all tumor cells are destroyed. HSV-1 vectors are genetically engineered to replicate selectively in tumor cells, and therefore do not damage the normal tissues surrounding the tumor.

killing of tumor cells can be achieved at a relatively low multiplicity of infection (MOI). 8) Anti-viral drugs are available that enable an optional termination of the therapy. 9) Animal models are available for preclinical evaluation on safety and efficacy. And, 10) the virus DNA does not get integrated into the host cell genome. HSV-1 is neurotropic and genes necessary for neuropathogenicity have been identified and can be mutated, therefore the use of HSV-1 is especially advantageous for brain tumor therapy.

DEVELOPMENT OF G207

In the past decade, the research of oncolytic HSV-1 therapy has drastically advanced from the basic concept to clinical studies. In the early days, replication-competent HSV-1 vectors were genetically engineered to have mutation in one non-essential gene associated with either virulence or viral DNA synthesis, in order to restrict viral replication to transformed cells[1]. These so-called first generation vectors showed that they could in fact efficiently inhibit the growth of tumors without lethally harming the host animal. They also showed that oncolytic HSV-1 therapy could be applied not only to brain tumors but also to various types of neoplasm. There were concerns however regarding the use of the first generation vectors in humans, because the pathogenicity of vectors may not be sufficiently attenuated and a single mutation may not be sufficient to prevent vectors from reverting to wild type HSV-1 via homologous recombination. To address these concerns, so-called second generation vectors were developed that had genetically engineered mutations in two different genes.

Structure

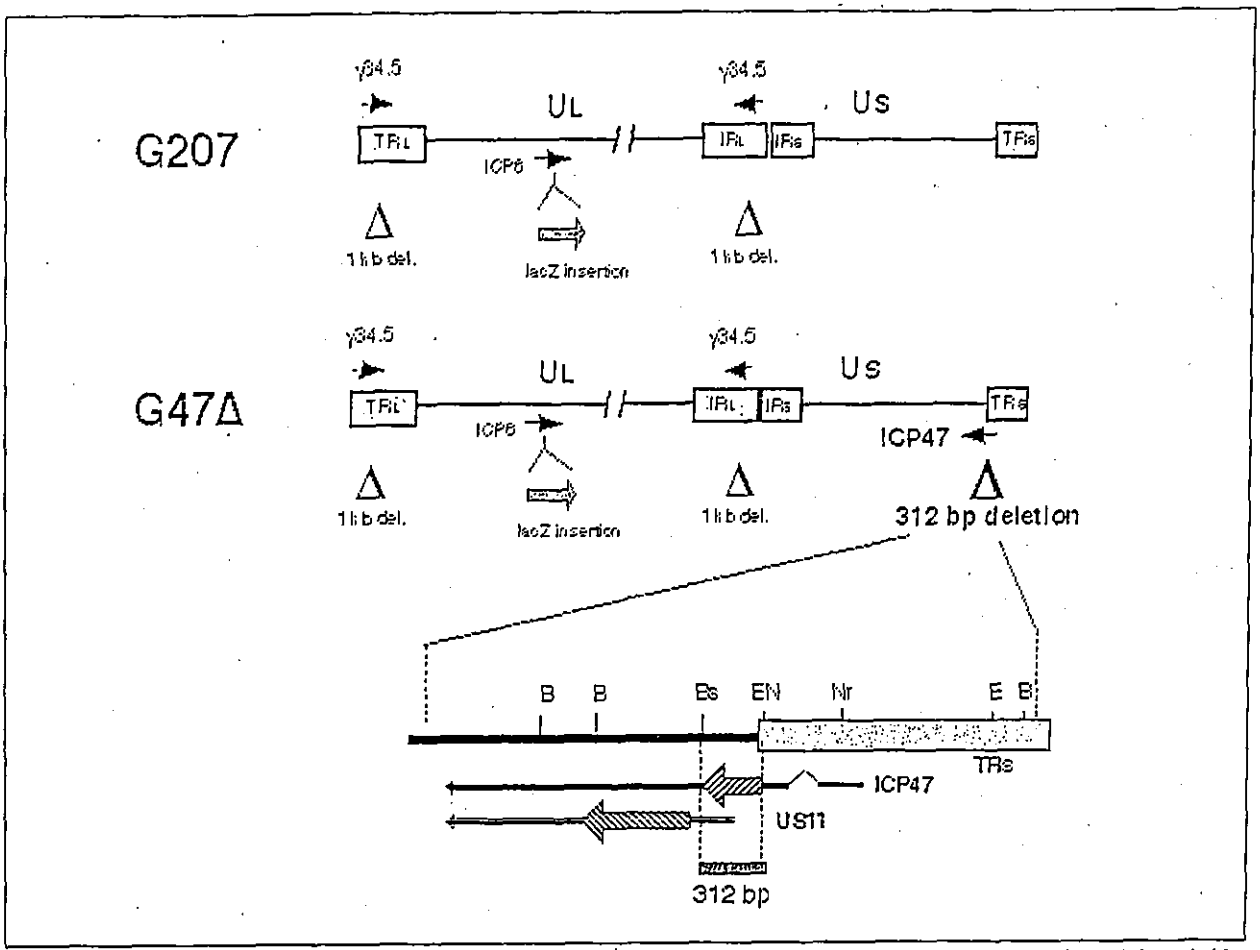

Fig. 2. Structures of G207 and G47Δ. The HSV-1 genome consists of long and short unique regions (UL and US) each bounded by terminal (T) and internal (I) repeat regions (RL and RS). G207 was engineered from wild-type HSV-1 strain F by deleting 1 kb within both copies of the *γ34.5* gene, and inserting the E.coli *lacZ* gene into the ICP6 coding region. G47Δ was derived from G207 by deleting 312 bp from the ICP47 locus. Because of the overlapping 3' co-terminal transcripts of US11 and ICP47, the deletion also places the late gene US11 under control of the ICP47 immediate-early promoter. The ICP47 transcript contains a splicing (indicated by ∧). Restriction site abbreviations: B, BamHI; Bs, BstEII; E, EcoRI, EN, EcoNI, Nr, NruI.

G207 is the first of the second-generation HSV-1 vectors[2]. It was originally designed for the clinical use in brain tumor patients with an emphasis on employing ample safeguards. G207 has deletions in both copies of the *γ34.5* gene, the major determinant of HSV-1 neurovirulence (Fig. 2). *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 R (PKR), which in turn leads to phosphorylation of the α-subunit of eukaryotic initiation factor 2α (eIF-2α) and a subsequent shutdown of host and viral protein synthesis. The product of the *γ34.5* gene antagonizes this PKR activity. However, in tumor cells with an activated Ras signaling pathway, it has been suggested that PKR activity is already inhibited, thereby allowing *γ34.5*-deficient HSV-1 vectors to replicate[3,4]. 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 cells. This double mutation minimizes the chance of G207 to revert to wild-type and confers favorable properties on the virus for treating human cancers: G207 replicates preferentially in tumor cells and is harmless in normal tissue due to attenuated virulence; G207 is about 10 fold more sensitive to ganciclovir/acyclovir than its parent virus R3616; and the reporter gene lacZ allows easy histochemical

detection of G207-infected cells.

Antitumor efficacy

G207 has been tested in more than 60 different cell lines, which revealed that the vast majority, although not all, of human tumor cell lines are susceptible to G207 infection and replication[5]. In human glioma and malignant meningioma cell lines, for example, G207 can achieve cell destruction of the entire cell population in culture within 2 to 6 days at an MOI of 0.1[2,6]. In contrast, at the same MOI, G207 manifests no effect on primary cultures of rat cortical astrocytes or cerebellar granulocytes. 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 athymic mice harboring U87MG glioma or F5 malignant meningioma tumors in the brain or under the skin, a single intraneoplastic inoculation of G207 can significantly inhibit the tumor growth and prolong the animal survival[2,6]. A prominent *lacZ* expression from G207 replication within tumors could still be observed 24 days post-inoculation[6]. Besides brain tumors, G207 has been proven efficacious in a variety of other animal tumor models, in which tumors have been generated in various organs including the liver, peritoneum, urinary bladder, and cheek pouch[5].

Safety

Because HSV-1 is the most common cause of central nervous system viral infections and G207 was the first replication-competent HSV-1 vector to be used in human brains, it was extensively evaluated for its toxicity in the brain. In BALB/c mice, the highest dose of G207 [10^7 plaque forming units (pfu)] caused no symptoms for over 20 weeks when inoculated intracerebrally or intraventricularly[7]. In A/J mice, one of the most susceptible mouse strains to HSV-1 infection, intracerebral inoculation of clinical-grade G207 at 2 x 10^6 pfu caused only a temporary and slight hunching in 2/8 mice[8]. Furthermore, in BALB/c mice that survived an intracerebral inoculation of wild-type HSV-1 (KOS) at an LD50 dose (~10^3 pfu), a subsequent challenge with an intracerebral inoculation of G207 (10^7 pfu) at the same stereotactic coordinates did not result in reactivation of latent HSV-1 infection[7].

Aotus nancymae (New World owl monkeys) are amongst the most sensitive non-human primates to HSV-1 infection. A total of 22 *Aotus* primates have been used for safety evaluation of G207 (intracerebral and/or intraprostatic)[2,9-11]. In *Aotus*, a single intracerebral inoculation of G207, up to 10^9 pfu, or repeat inoculations of 10^7 pfu caused neither virus-related disease nor detectable changes in the brain as assessed by magnetic resonance imaging (MRI) and pathological studies[9]. In contrast, an intracerebral inoculation of 10^3 pfu of wild type HSV-1 (strain F) caused acute viral encephalitis, killing the animal within 5 days of inoculation. Four *Aotus* were used to evaluate the shedding and biodistribution of G207 after intracerebral inoculation of clinical-grade, column-purified G207 (3 x 10^7 pfu)[10]. Using PCR analyses and viral culture, neither infectious virus nor viral DNA was detected from tear, saliva, vaginal secretion, blood or urine samples at any time point up to 1 month post-inoculation. Analyses of tissues obtained at necropsy at 1 month showed G207 DNA distribution restricted to the brain, with no infectious virus being isolated. Histopathology revealed normal brain tissues including the sites of inoculation. All *Aotus* receiving an intracerebral G207 inoculation showed an increase in serum anti-HSV-1 antibody titers as early as 21 days post-inoculation[9,10].

Clinical trial

A phase I clinical trial of G207 for recurrent malignant glioma was performed in 21 patients at 2 institutions in the United States[12]. This dose escalation study started from 10^5 pfu and increased to 3 x 10^9 pfu, with three patients at each dose. G207 was inoculated stereotactically into an enhancing region of the tumor visualized by a computerized tomography (CT) scan with contrast enhancement. No acute, moderate to severe adverse event attributable to G207 was observed. Minor adverse events included seizure (2 cases) and brain edema (1 case). Among 7 biopsied or resected tumor specimens analyzed, specimens from 2

patients were positive for G207 DNA by PCR analysis (56 and 157 days post-inoculation). Five of 19 patients were negative for serum anti-HSV-1 antibody prior to G207 treatment. Despite corticosteroid treatment of these patients, one patient seroconverted after G207 inoculation.

The tools to evaluate the efficacy included Karnofsky performance score and serial MRI. 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. All patients, except one that died from cerebral infarction 10 months after G207 treatment, eventually showed tumor progression. Autopsy was performed in 5 cases, and histology of the brains showed no evidence of encephalitis, white matter degeneration or inflammatory changes, and all were negative for HSV-1 immunoreactivity. In 3 cases, the tumor was localized to one region of the brain without significant tumor cell invasion into the surrounding brain tissue as usually observed with typical glioblastoma cases. One glioblastoma patient that died from cerebral infarction had no evidence of residual tumor at autopsy. Overall, the phase I clinical trial confirmed the safety of G207 inoculated into the brain at doses up to 3×10^9 pfu. Currently, phase Ib/II clinical trials for recurrent malignant glioma are ongoing at the same locations.

Effect of host immune responses

One difficulty in investigating the immune effect had been the lack of suitable animal tumor models that are susceptible to HSV-1 infection. Many mouse strains and a majority of murine cell lines are relatively resistant to HSV-1. It was not recognized until recent development of immunocompetent mouse brain tumor models suitable for HSV-1 evaluation that the host immune response plays an important role in the antitumor action of oncolytic HSV-1 vectors both in the brain and in the periphery. Initially, N18 murine neuroblastoma cells, one of the more susceptible murine cell lines tested for G207 susceptibility, were used in syngeneic A/J mice. In A/J mice harboring established N18 tumors subcutaneously or in the brain, intraneoplastic inoculation with G207 caused a significant reduction of tumor growth or prolongation of survival[13]. Moreover, in A/J mice bearing bilateral subcutaneous N18 tumors, intraneoplastic G207 inoculation into one tumor alone caused growth reduction and/or regression of both the inoculated and the noninoculated contralateral tumor, indicating an induction of systemic antitumor immunity. Animals that were cured of their subcutaneous tumors by G207 were protected against tumor rechallenge, either in the periphery or the brain. The antitumor immunity was associated with cytotoxic T lymphocyte (CTL) activity that was specific to N18 tumor cells and persisted for at least 13 months. In A/J mice bearing subcutaneous and intracerebral N18 tumors simultaneously, G207 inoculation into the subcutaneous tumor alone caused growth suppression of both the subcutaneous and the remote brain tumor, leading to a significant prolongation of survival. G207-induced, systemic antitumor immunity was also observed in BALB/c mice bearing subcutaneous CT26 (colon carcinoma) tumors and DBA/2 mice bearing subcutaneous M3 (melanoma) tumors[14]. In the CT26 model, intraneoplastic inoculation of G207 induced CTL activity that recognized a dominant, tumor-specific, major histocompatibility complex (MHC) class I-restricted epitope (AH1) from CT26 cells. Similar systemic antitumor immunity induction by G207 was observed in Syrian hamsters bearing subcutaneous KIGB-5 (gallbladder carcinoma) tumors[15]. Thus, in an immunocompetent condition, the oncolytic activity of G207 can be augmented by induction of specific and systemic antitumor immunity effective both in the periphery and in the brain.

When high dose dexamethasone was given to A/J mice bearing subcutaneous N18 tumors for an extensive period (16 days), G207 retained the antitumor effect and caused a significant suppression of tumor growth when inoculated into the tumors[16]. However, all immunosuppressed mice treated with G207 showed tumor regrowth despite initial shrinkage, whereas 50% of the G207-treated mice not immunosuppressed were cured. Dexamethasone administration significantly reduced serum neutralizing

antibodies against G207 after intraneoplastic G207 inoculation, but this did not affect the amount of infectious G207 isolated from tumors. The most striking effect of dexamethasone administration was the abolishment of G207-induced CTL activity against N18 cells. These results further support the importance of tumor-specific CTL induction in the course of oncolytic activities by HSV-1 vectors.

The effect of circulating anti-HSV-1 antibodies on the efficacy of oncolytic HSV-1 therapy was investigated. A/J and BALB/c mice were immunized by repeated intraperitoneal inoculations of wild type HSV-1 (KOS) and then the antitumor efficacy of G207 on established subcutaneous N18 and CT26 tumors determined[17]. In both tumor models, the antitumor efficacy of G207 was the same whether the mice were immunized or not for HSV-1. Because HSV-1 predominantly spreads cell-to-cell, circulating antibodies known to neutralize free virus, may have little effect on HSV-1 directly inoculated into tumors.

IMPROVING THE EFFICACY OF ONCOLYTIC HSV-1 THERAPY

While G207 proved safe in glioma patients and efficacious in animal tumor models, G207 is considerably attenuated not only for the pathogenicity but also for the tumor cell killing capability compared to wild type HSV-1. One way to improve the efficacy is to combine the use of G207 with other conventional therapies. For example, a combination with cisplatin was shown to enhance the antitumor effect of G207 against head and neck cancer in mouse models[18]. Others have shown that ionizing radiation amplifies the replication of HSV-1 vector (R3616)[19], leading to improved survival of athymic mice bearing intracerebral U87MG tumors[20]. Although we did not observe such enhancing effect of ionizing radiation with G207, the virus was useful for treating prostate cancers that recurred after radiation therapy[21]. Intraperitoneal administration of cyclophosphamide when combined with HSV-1 vector (hrR3) administered together with RMP-7 (a pharmacological modifier of blood-brain barrier) into the carotid artery significantly enhanced the replication and spread of the virus in tumors formed in rat brains[22].

For improvement of G207 antitumor efficacy, we are currently pursuing two approaches that involve enhancement of antitumor immune responses; 1) further genetic engineering of G207, and 2) combination with immune gene therapy.

New generation recombinant HSV-1

The therapeutic benefits of oncolytic HSV-1 vectors depend on the extent of both intratumoral viral replication and induction of host antitumor immune responses. We are developing new generation HSV-1 vectors by enhancing these properties while retaining the safety features of G207. G47Δ is one of such vectors newly created from G207 by introducing another genetic mutation, i.e., the deletion of the α47 gene and the overlapping US11 promoter region[8] (Fig. 2). Because the α47 gene product inhibits transporter associated with antigen presentation (TAP) which translocates peptides across the endoplasmic reticulum, the deletion results in preclusion of MHC class I down-regulation that normally occurs in human cells infected with HSV-1. G47Δ-infected human cells in fact presented higher levels of MHC class I expression than cells infected with other HSV-1 vectors[8]. Further, human melanoma cells infected with G47Δ caused better stimulation of 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. In the majority of cell lines tested in vitro, G47Δ replicated better than G207, resulting in the generation of higher virus titers, and exhibited greater cytopathic effect. 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[8]. Improved antitumor efficacy of G47Δ has been shown also in other immunocompetent mouse tumor models including prostate cancer and breast cancer. Nevertheless, the

safety of G47Δ remained unchanged from G207 following injection into the brain of HSV-1-sensitive A/J mice. Thus, compared with the parental virus G207, G47Δ showed 1) better induction of human anti-tumor immune cells, 2) better growth properties leading to higher virus yields and increased cytopathic effect in vitro, 3) better antitumor efficacy in both immunocompetent and -incompetent animals, and 4) preserved safety. These features make G47Δ highly attractive for clinical application.

Combination with immune gene therapy

Our preclinical experience using various HSV-1 vectors to treat tumors, including wild type HSV-1, indicates that there is a limit to improving the antitumor efficacy of oncolytic vectors by simply bringing the replication capability closer to that of wild type viruses, putting aside the difficulty of doing so without increasing the pathogenicity. In developing new vectors, therefore, currently more emphasis is placed on enhancing the ability to induce antitumor immunity. Combination of oncolytic HSV-1 vectors with defective vectors expressing immunostimulatory molecules can improve the therapeutic efficacy significantly (Fig. 3).

We have recently developed an immune gene therapy strategy that would work for brain tumors as well as other cancers. The brain is considered an immune-privileged site, and brain tumor patients are often under an immune-suppressed condition due to immunosuppressive factors secreted by the brain tumor and/or corticosteroid administration. On the other hand, a robust, non-specific inflammatory response in the brain can cause undesirable brain edema. To meet these requirements, we created a defective HSV vector (dvB7Ig) expressing a soluble form of B7-1, one of the most potent costimulatory molecules, and used it in combination with G207[20]. The 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. Secreted soluble B7-1 should provide antigen presenting cells increased T-cell stimulatory activity, activate T cells in an anergic

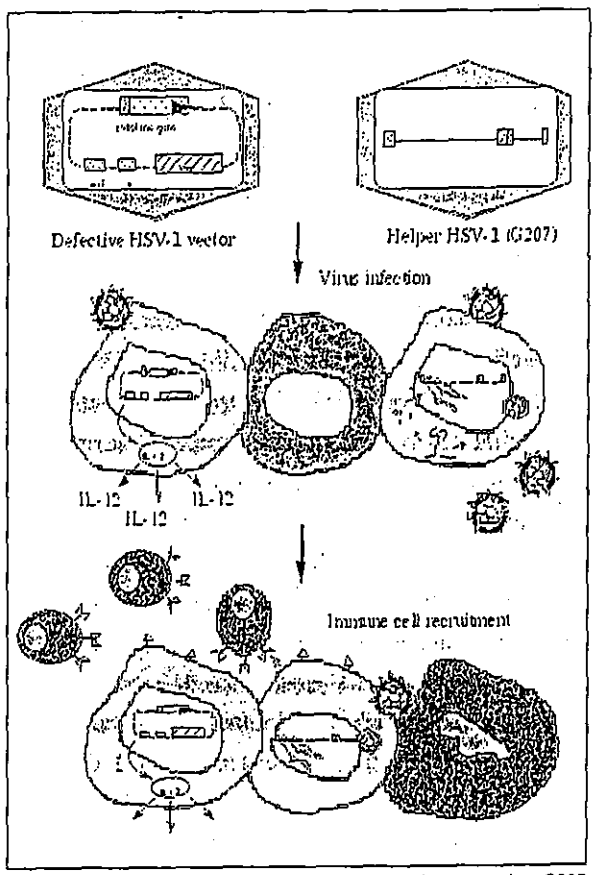

Fig. 3. Schematic diagram of immune gene therapy using G207 in combination with a defective HSV-1 vector expressing an immunostimulatory gene. When a mixture of G207 and a defective vector expressing IL-12 (for example) is inoculated into the tumor, tumor cells infected with G207 allow the virus to replicate and ultimately get 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.

state, and, because it is in a dimeric form, provide a strong stimulation to T cells by cross-linking neighboring CD28. 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 subcutaneous tumors, despite that the expression of B7-1-Ig was detected in only 1% or less of tumor cells at the inoculation site, and prolonged the survival of mice bearing intracerebral tumors[20]. Inoculation of dvB7Ig/G207 induced a significant influx of $CD4^+$ and $CD8^+$ T cells in the tumor. *In vivo* depletion of immune cell subsets further revealed that the antitumor effect required $CD8^+$ T cells

but not CD4+ T cells. The dvB7Ig/G207 treatment conferred tumor-specific protective immunity on cured animals. Thus, the approach proved to be a potent and clinically-applicable means of treating brain tumors and other cancers. With a similar approach, a defective HSV vector expressing murine interleukin (IL)-12 in combination with G207 was tested in BALB/c mice bearing subcutaneous CT26 tumors and was shown effective[39].

More recently, we and others have created replication-competent HSV-1 vectors that contain transgenes for immunostimulatory molecules. In particular, replication-competent HSV-1 vectors that express IL-12 have been shown in several animal tumor models to manifest a direct oncolytic activity and also express a sufficient amount of IL-12 that significantly augments the antitumor effect without increasing the toxicity compared with the parental HSV-1 vectors[25-27].

CONCLUSION

Oncolytic virus therapy is an attractive treatment, because it is based on a new concept that the antitumor reagent can amplify specifically at the tumor site after administration. It also has features practical for clinical application, because it can be1) applied to various types of tumors irrespective of their oncogenetic backgrounds,2) combined with conventional therapies such as surgery, radiation therapy and chemotherapy, 3) combined with immunotherapy for potential synergistic effects, and4) used for obtaining amplified gene delivery *in vivo*. An increasing number of clinical trials using oncolytic viruses has started or being planned in recent years. We anticipate that oncolytic virus therapy will be established as an important modality of cancer treatment in the near future.

Acknowledgments

I thank the past and current members of the Molecular Neurosurgery Laboratory. I especially thank Dr. Robert L. Martuza who first developed the strategy of oncolytic virus therapy using replication-competent HSV-1 vectors and has been instrumental in all aspects of this research.

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悪性神経膠腫に対する death receptor pathway による治療

Treatment of malignant glioma by activation of death receptor pathways

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【Abstract】

TRAIL/Apo2L, a member of TNF family, induces apoptosis preferentially in human tumor cells but not in normal cells, suggesting TRAIL, through its cognate death receptors DR4 or DR5, may serve as a potential therapeutics for intractable malignant gliomas. Here we show that sublethal treatment with DNA-damaging chemotherapeutic drugs, CDDP and VP16, induced increased expression of DR5 in human glioma cells. Exposure of such cells in vitro to soluble human TRAIL in combination with CDDP or VP16 resulted in synergistic cell death characteristic of apoptosis. Moreover, systemic in vivo administration of TRAIL with CDDP synergistically suppressed both tumor formation and growth of established subcutaneous human glioblastoma xenografts in nude mice and also significantly extended the survival of mice bearing intracerebral xenografts without causing significant general toxicity. The combination treatment activated caspase cascades through multiple signaling pathways. Furthermore, combination of TRAIL and X-ray irradiation treatment also induced synergistic cytotoxicity in vitro. These results provide a novel therapeutic strategy for malignant gliomas in which TRAIL could be safely and synergistically combined with conventional DNA damaging chemotherapy or radiotherapy.

Key words: TRAIL, DNA damage, glioma, apoptosis, combination treatment

【はじめに】

代表的悪性脳腫瘍である悪性神経膠腫(malignant glioma)、特に最も悪性型である多形性膠芽腫(glio-blastoma multiforme)は、集学的治療によっても依然予後が極めて不良であり、新規の治療法の開発が望まれて久しい。細胞自体に生来備わっている細胞死のプログラムであるアポトーシス(apoptosis)は、抗癌剤や放射線など殆どの治療法により腫瘍細胞が殺傷される際に関与していることから、apoptosis を直接誘導する治療方法は、高い抗腫瘍効果が期待できるものと考えられる。

Apoptosis では、主として2つのシグナル伝達経路によりapoptosisの実行を担うcaspaseの活性化が生じる。一つは、多くの抗癌剤や放射線治療、成長因子除去などのストレスによる mitochondria の傷害を経由するものである。他方は、mitochondria を介さず、細胞表面に存在する death receptor を介して、直接 caspase を活性化する経路である[1,2](Fig.1)。特に後者の経路は各種治療への耐性機構をバイパスしえる可能性があることから、death ligand(TNF family)であるFasL(CD95L)や TNF-α を用いた悪性腫瘍の治療が検討されてきたが、実験動物への全身投与により急性毒性が著明に認められ[3,4]、臨床応用には至っていない。

1995年に同定されたTRAIL(Apo2L)はTNF familyに属する新たなdeath ligandで、腫瘍細胞に選択的にapoptosis を誘導する一方、正常細胞には殆ど影響がないことから新たな治療法として脚光を浴びてきた[5-8]。TRAIL には細胞表面に発現する特異的な受容体DR4、DR5、DcR1、DcR2が次々と同定され、その内DR4、DR5は細胞内ドメインにdeath domain(DD)が存在し、TRAILがligateすることでreceptor trimeriza

tion が起こり、DD を介して急速な apoptosis を惹起することが出来る。一方、DcR1、DcR2 には機能的な DD が存在せず、TRAIL からの death signal をブロックする decoy receptor の働きがあることが示された[8]。FasL や TNF-α と異なり、TRAIL の担癌実験動物への全身投与では明らかな毒性は認められず、臨床応用への可能性が検討され始めている[9-11]。

DNA 傷害により癌細胞に DR5 の発現が誘導されることが示されたことから[12,13]、DNA 傷害をもたらす化学療法や放射線治療により腫瘍細胞の TRAIL 感受性が亢進し、相乗的殺腫瘍効果が得られる可能性が考えられた。我々はヒト glioma 細胞株を用いてこの仮説を検証し、過半数の細胞株において cisplatin (CDDP)及び etoposide(VP16)治療により DR5 が誘導されるとともに、細胞内の複数の apoptosis の経路が活性化されて相乗的に細胞死が誘導されたこと、また、放射線治療と TRAIL の併用療法においても同様な相乗的殺細胞効果が得られたことを報告する。

【方法・材料】

細胞培養

各ヒト glioma 細胞株は、10% heat-inactivated fetal bovine serum 或いは cosmic calf serum(U87MG、U178 MG のみ)及び L-glutamine、Pen/Strep を加えた DMEM 培地を用いて、5% CO2 下 37℃で培養した。

試薬、抗体

CDDP、VP16 は Sigma(MO)より購入した。使用した抗体は、DR5(R&D Systems)、DR4(BD PharMingen)、β-actin(Sigma)である。TRAIL、DR5-Fc、Fc の精製法は文献参照のこと[14,15]。

in vitro での化学療法、TRAIL による治療

96 穴、12 穴、10-cm dish 等に細胞を播種後、翌日に培地を除去し、CDDP、VP16、TRAIL を含む新鮮な培地を新たに加え、各種アッセイまで培養を継続した。

放射線治療

細胞を適当数播種後、翌日に X 線照射装置(MBR-1505R2)(日立メディコ)を用いて 150kV の条件で X 線照射を行った。放射線照射後に培地を除去し、TRAIL を含む新鮮な培地と交換し各種アッセイまで培養を継続した。

in vivo での治療

nude mice の側腹部或いは脳内に U87MG 細胞を移植し、CDDP(3mg/kg)ip 及び TRAIL(100μg/body/day)一日 2 回 iv を 3 日間連続で投与し、約 1 週間毎に数回繰り返し投与した。対照として同量の生食及び TRAIL の溶解液(PBS+10% Glycerol)を使用した。腫瘍体積は V=a2b/2(a: 短径、b: 長径)で計算した。

MTT アッセイ

96 穴に細胞を播種後、翌日に治療薬を含む 200μl の培地に交換し培養を継続した。アッセイ時に 5mg/ml MTT 10μl を加え 4 時間培養後、培地を除去し 120μl DMSO にて結晶を溶解し、microplate reader (Molecular Devices)にて 562nm の吸光度を計測して、対照との比較により増殖抑制率(%inhibition)を計算した。

Northern blot 法

培養細胞から TRIZOL(Gibco/BRL)を用いて total RNA を抽出し、RNA 量を定量後 15μg を 1% agarose/7% formaldehyde gel にて電気泳動し、Hybond-N 膜に transfer、bake、UV crosslinking の後、methylene blue 染色にて RNA load 量を測定した。その後 32P でラベルした DR5 cDNA probe と hybridization を行い、特異的バンドを autoradiography にて検出した。

Western blot 法

培養細胞を protease inhibitor を加えた lysis buffer (RIPA)にて可溶化[16]、BCA(Pierce)にて蛋白定量後 SDS-PAGE を行い、PVDF 膜に transfer 後、各種抗体と反応させた。対応する HRP 抱合 2 次抗体と反応後 chemiluminescence にて特異的バンドを発光させ、LAS1000(Fuji)を用いて検出・定量した。

【結 果】

DNA 傷害性化学療法による TRAIL receptor DR5 の発現誘導

まず、ヒト glioma 細胞で DNA 傷害性抗癌剤の TRAIL receptor 発現への影響を検討した。各種 glioma 細胞株を致死量以下の容量の CDDP 及び VP16 で治療し、16 時間後に DR5 mRNA 発現量を Northern blot 法により検出した。U87MG、U178MG、A1207 細胞などで明かな DR5 mRNA 発現の誘導が認められた(Fig.2)。一方、U373MG、LNZ308 細胞では発現量に変化は認められなかった。DR5 を含めその他の TRAIL receptor の発現量の変化の有無を multi-probe RNA protection assay で検討すると、一部の細胞株で他の TRAIL receptor(DR4、DcR1、DcR2)の発現誘導も認められたが、その発現量・誘導度ともに DR5 より軽度で、DR5 がこれらの DNA 傷害治療による発現誘導を受ける主たる TRAIL receptor と考えられた(data not shown)。

DNA 傷害性化学療法と TRAIL の併用療法による相乗的殺腫瘍細胞効果

次に、CDDP 及び VP16 と TRAIL の併用効果を glioma 細胞株にて検討した。使用した TRAIL は、soluble FLAG-tagged TRAIL で、殆どの細胞で 10μg/ml の濃度で治療しても細胞死は誘導されなかった。致死量以下の CDDP 或いは VP16 と 0.1μg/ml TRAIL を同時に投与すると、CDDP・VP16 で DR5 の発現が誘導された細胞株において 24 時間後に著明な細胞死が誘導され(Fig.3)、また MTT アッセイにて、この併

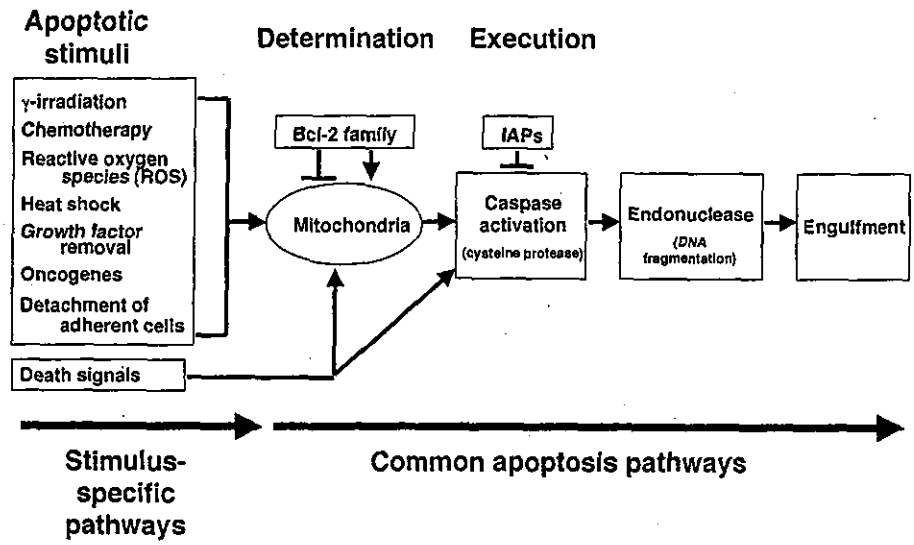

Fig.1
Signal transduction pathways leading to caspase activation and apoptosis execution.

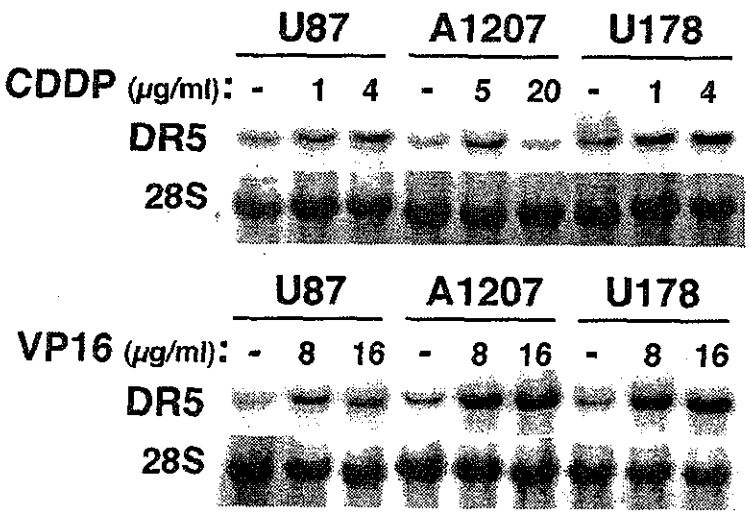

Fig.2
Northern blot analysis showing induction of DR5 mRNA expression by DNA damaging chemotherapeutic agents *CDDP and VP16 in human glioma cell lines.* Cells were treated with drugs at the indicated doses for 16 h and were harvested for preparation of RNA (Ref. 14).

用療法は相乗的殺細胞効果を示すことが明らかになった(Fig.4)。DR5 の細胞外ドメインと免疫グロブリンの Fc 部を fusion させた DR5-Fc の存在下ではこの細胞死は完全に抑制され、相乗的細胞死誘導は TRAIL と TRAIL receptor との interaction により生じることが示された。

相乗的細胞死における細胞内シグナル伝達

glioma 細胞において CDDP 或いは VP16 と TRAIL の併用により誘導される細胞死では、TUNEL アッセイにより apoptosis が生じていることが明らかになり(data not shown)、また apoptosis の実行を担う caspase に対する universal な inhibitor、z-Asp-CH2-DCB の存在下ではこの細胞死は完全に抑制され、caspase 依存性に apoptosis が誘導されることが示唆された(Fig.3)。

CDDP/VP16 と TRAIL の併用療法により、caspase-8、-9、-3 の断片化・活性化が認められ、また direct な death receptor pathway と mitochondria pathway を仲介する Bid、更には下流の実行 caspase である caspase-3 の細胞内基質のひとつ PARP も切断されていた。Mitochondria からの apoptosis シグナルに重要とされる cytochrome c や AIF は、CDDP 単独治療により細胞質への放出が認められたが、CDDP と TRAIL の併用療法ではその放出の増強が認められた(data not shown)。

Nude mouse における CDDP/TRAIL 併用療法による腫瘍増殖抑制

DNA 傷害性抗癌剤と TRAIL による併用療法の動物モデルにおける治療効果を次に検討した。まず、U87MG 細胞を nude mouse の皮下に接種し同日から治療を開始したところ、TRAIL 及び CDDP の単独療法では対照に比べわずかな腫瘍増殖抑制効果を認めるに過ぎなかったが、CDDP/TRAIL 併用治療では、腫瘍形成が著明に抑制され、半数で全く腫瘍の形成が認められなかった(Table 1)。また、腫瘍が皮下に形成してから治療を開始した場合でも、CDDP 単独治療で軽度の腫瘍増殖抑制が認められたが、CDDP/TRAIL の併用治療では有意に増殖が抑制され、約 3 割の腫瘍の消失を認めた(Table 2)。同様の結果は nude mouse での脳内腫瘍モデルでも認められ、CDDP/TRAIL 併用療法群において、有意の生存期間の延長がみられた(Table 3)。これらの治療による明らかな全身の副作用は認められなかった(data not shown)。

治療後の腫瘍標本を用いた TUNEL アッセイにより、この併用療法による腫瘍増殖抑制は腫瘍細胞への apoptosis 誘導効果であることが示された(Fig.5)。

X 線放射線治療と TRAIL 併用療法による glioma 細胞の治療

glioma の標準的治療として用いられている放射線治療も腫瘍細胞に DNA 傷害をきたすことから、放射線治療と TRAIL の併用効果につき検討した。X 線照射 10Gy 単独治療では、短期的細胞傷害は認められなかったが、TRAIL を併用することで治療後 48 時間で著明な細胞死が誘導された(Fig.6)。MTT アッセイにて T98G 細胞では相乗的な殺細胞効果が認められた。相乗的或いは相加的細胞傷害が認められた細胞株では、X 線照射により経時的な DR5 蛋白の発現増強が認められ、放射線照射においても DR5 の発現誘導が TRAIL との併用療法における相乗効果の一因となっていることが示唆された(Fig.7)。

【考 察】

Death ligand/death receptor を介する apoptosis は、抗癌剤などの他の多くの apoptosis を誘導する刺激の際の経路と異なり、apoptosis を実行する casapase の活性化を直接的に導くことから、急速な細胞死を誘導するとともに、抗癌剤に耐性を示す腫瘍細胞にも有効となる可能性が考えられ、有力な抗腫瘍治療法として期待されてきた[8]。ところが、90 年代前半までに death ligand として同定されていた TNF-α や FasL は、動物への全身投与により、急速な肝細胞壊死や急性炎症反応などの重篤な副作用が生じ、その適用には否定的な状況であった[3,4]。それに対して、TRAIL は in vitro で正常細胞には影響を与えず、腫瘍細胞に選択的に apoptosis を誘導し、また、サルや mice を用いた in vivo の毒性試験においても、全身投与によっても明らかな副作用を認めなかったことから[9,10]、現在、悪性腫瘍の治療薬として臨床試験が計画されている。Death signal は death receptor から伝達されることから、腫瘍細胞において death receptor の発現が亢進すれば、TRAIL 治療による殺細胞効果も増強すると考えられる。DNA 損傷をもたらす CDDP 及び VP16 によるヒト glioma 細胞株の治療により、実際に TRAIL receptor の内、主として DR5 の mRNA 発現の亢進が認められた。その結果、単独治療では殆ど効果が認められないのに対し、CDDP 或いは VP16 と TRAIL の併用療法により DR5 の発現亢進がみられた細胞株においては、急速に相乗的殺細胞効果が認められた。TRAIL の作用を中和する DR5-Fc の存在下では無効であるから、この相乗的治療効果には DR5 の発現亢進と TRAIL-receptor の結合が必須と考えられた。更に、nude mice を用いた担癌動物モデルにおいても CDDP と TRAIL の全身投与により腫瘍増殖の著明な抑制と一部腫瘍の消失も認められ、また脳腫瘍モデルでは有意な生存期間の延長がみられ、全身への明らかな副作用も認められなかったことから、DNA 傷害性化学療法と TRAIL の併用療法は、glioma に対する有力な治療法となる可能性が示された。

悪性 glioma の標準的治療のひとつである放射線治療も腫瘍細胞に DNA 損傷を生ずることから、TRAIL

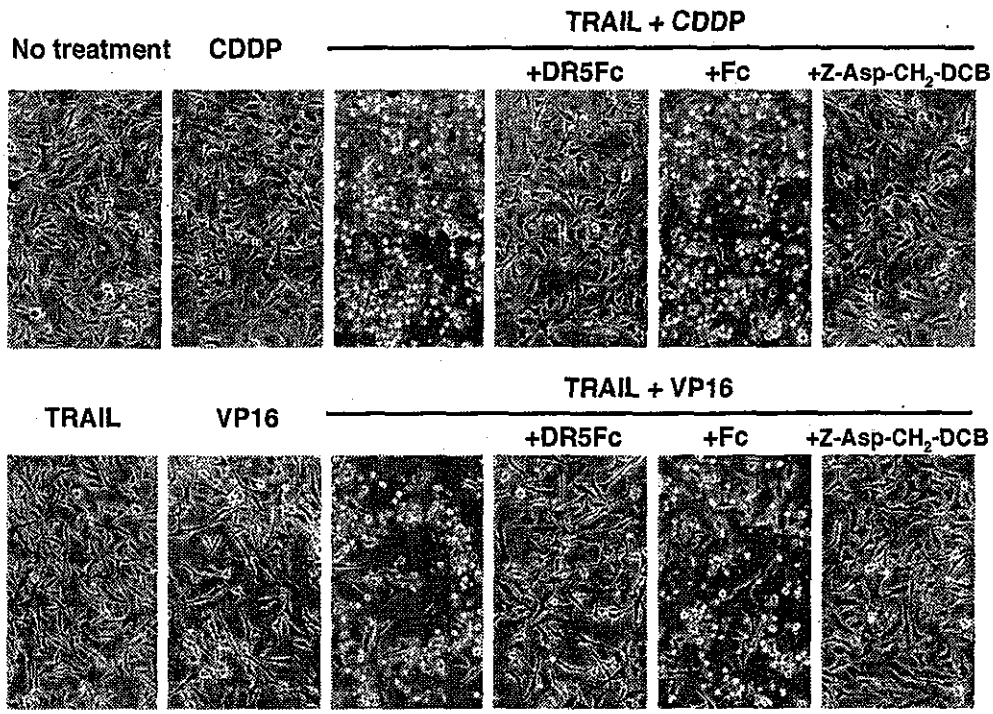

Fig.3

Synergistic cytotoxicity in vitro by combination treatment with TRAIL and DNA damaging agents in U87MG cells. Cells were treated singly or in combination with TRAIL (0.1 μ g/ml), CDDP (4 μ g/ml), VP16 (16 μ g/ml), or Z-Asp-CH_2-DCB (200 μ M) for 24 h (Ref. 14).

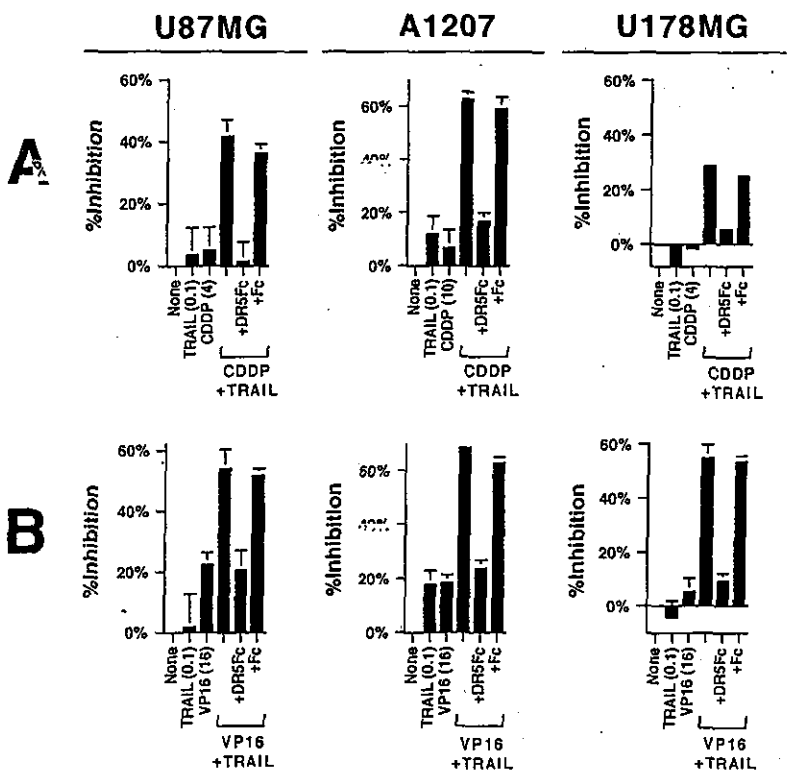

Fig.4

Cytotoxic effects of TRAIL and DNA damaging agents CDDP (A) or VP16 (B) in human glioma cell lines as in Fig.3 (Ref. 14).

Table 1. Synergistic suppression of tumor formation by combination treatment with TRAIL and CDDP *in vivo*.

Treatment	No of mice	Tumor volume (mm^3)*	p value**
Vehicles	4	2243.8 ± 636.5	-
TRAIL (500μg/day)	4	2484.4 ± 473.9	NS
CDDP (3mg/kg)	4	1446.7 ± 370.1	NS
TRAIL + CDDP	4	26.9 ± 23.9	<0.01***

Nude mice received s.c. injections with 2 x 10^6 U87MG cells and were treated with CDDP and TRAIL from the same day for total 4 courses of treatment.
*, tumor volume was measured on Day 35; mean ± SEM.
**, comparison to the Vehcles group.
***, comparison to the CDDP treatment group.

Table 2. Synergistic growth suppression of established U87MG xenografts by combination of TRAIL and CDDP *in vivo*.

Treatment	No of mice	Tumor volume (mm^3)*	p value**
Vehicles	4	2823.3 ± 773.4	-
TRAIL (500μg/day)	4	2868.0 ± 157.6	NS
CDDP (3mg/kg)	4	1159.2 ± 346.6	NS
TRAIL + CDDP	9	240.8 ± 97.9	<0.01***

Nude mice received s.c. injections with 2 x 10^6 U87MG cells and were allowed to establish tumors. From postimplantation day 13, mice were treated with CDDP and TRAIL for total 4 courses of treatment.
*, tumor volume was measured on Day 35; mean ± SEM.
**, comparison to the Vehcles group.
***, comparison to the CDDP treatment group.

Table 3. Survival of nude mice bearing intracerebral U87MG xenografts treated with TRAIL and/or CDDP.

Treatment	No of mice	Lifespan (days)*	p value**
None	6	21.8 ± 0.4	-
Vehicles	6	21.5 ± 0.5	NS
TRAIL (500μg/day)	6	22.7 ± 1.5	NS
CDDP (3mg/kg)	6	23.7 ± 1.5	NS
TRAIL + CDDP	8	28.8 ± 3.2	<0.01***

Nude mice were stereotactically injected with 5 x 10^5 U87MG cells into the brain, and were treated from postimplantation day 7 for three courses of TRAIL/CDDP therapy.
*, mice were sacrificed when became clinically moribund; mean ± SD.
**, comparison to the no treatment group.
***, comparison to the CDDP treatment group.

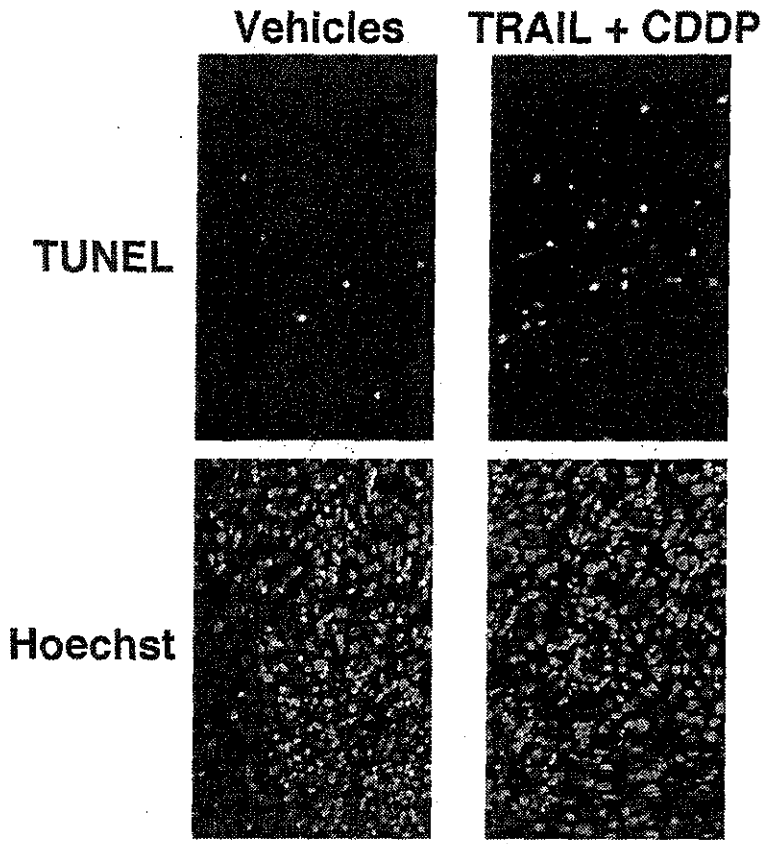

Fig.5
Induction of apoptosis in established U87MG xenografts by the combination treatment with TRAIL and CDDP in vivo.

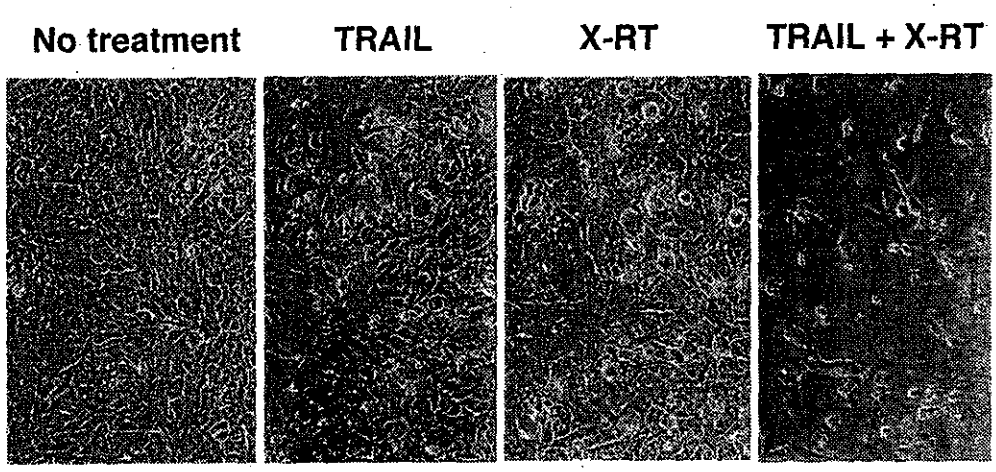

Fig.6
Synergistic cytotoxicity in vitro by combination treatment with X-ray irradiation and TRAIL in T98G cells. Cells were irradiated at 10 Gy followed by TRAIL (0.1 μ g/ml) treatment for 48 h.

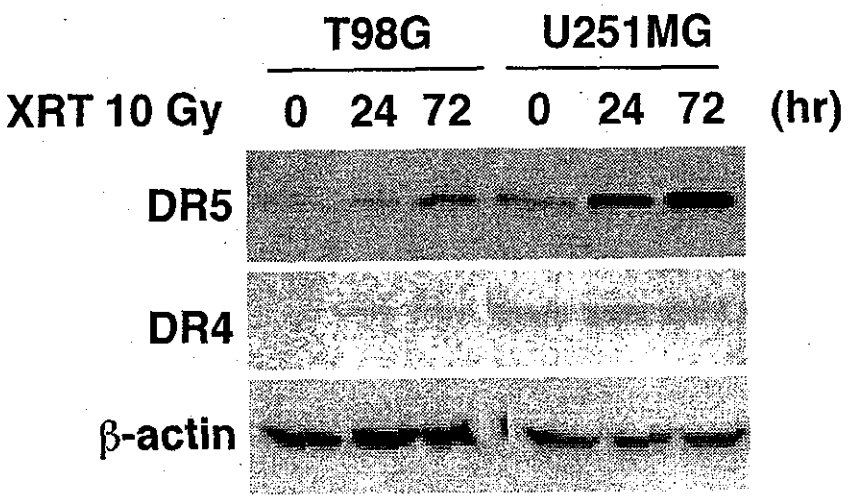

Fig.7
Induction of DR5 protein expression by X-ray irradiation at 10 Gy but not DR4 in human glioma cell lines.

との併用による相乗効果が期待された。Chinnaiyan らは、breast cancer cell に対する ionizing radiation 治療を行い、TRAIL による apoptosis の誘導が亢進し、in vivo においても腫瘍の縮小が認められたと報告している[17]。ヒト glioma 細胞株においては、10Gy 以上の X 線単発照射により DR5 の発現が誘導され、それとともに相乗的に TRAIL による殺細胞効果の増強が認められた。通常の分割照射で使用される 2Gy の線量ではこのような相乗効果は明らかでなかったことから(data not shown)、放射線治療と TRAIL の併用療法は、残存腫瘍或いは再発腫瘍に対する stereotactic radiosurgery による放射線 boost 治療に際する補助療法としての適用が有効である可能性が考えられる。

DNA 損傷による DR5 の発現亢進は、細胞の種類や DNA 損傷のタイプなどにより p53 依存性及び p53 非依存性に制御されていると考えられている[12,13]。我々の結果では、CDDP 及び VP16 治療による DR5 発現誘導は全ての野生型 p53 の細胞株で認められた。しかし、野生型 p53 の細胞株における p53 の不活化や、変異型 p53 の細胞株への野生型 p53 の導入などの実験からは、野生型 p53 が CDDP/VP16 と TRAIL 併用療法における DR5 発現誘導・相乗的殺細胞効果に直接関与している結果は得られなかった(data not shown)。一部の変異型 p53 の細胞株でも DR5 発現誘導・相乗効果がみられたことも併せて、この結果から、この併用療法は p53 の遺伝子変異の有無にかかわらず有効となる可能性が示唆され、より広範な悪性 glioma への適用が考えられる。一方、X 線照射と TRAIL 併用療法においては、野生型 p53 の細胞株では相乗効果が認められず(data not shown)、X 線照射と TRAIL による殺細胞効果は p53 非依存性と考えられた。この結果は、p53 依存性に効果が認められた breast cancer の場合とは異なっており[17]、腫瘍細胞の種類や DNA 傷害のタイプにより TRAIL 感受性や DR5 の発現制御が異なることが示唆された。

Death domain を持つ TRAIL receptor からの death signal 経路では、Fas における signal 伝達経路と同様に receptor と adaptor 分子の FADD 及び上流 caspase である caspase-8 が death-inducing signaling complex (DISC)を形成し、caspase-8 の活性化とともに下流の実行 caspase である caspase-3 が活性化され、apoptosis が進行すると考えられている[18]。CDDP 或いは VP16 と TRAIL の併用療法では、FADD、caspase-8 をそれぞれ阻害する DN-FADD、CrmA により相乗効果が抑制されたことから、この相乗的殺細胞効果には、TRAIL 存在下での CDDP/VP16 による DR5 発現亢進に伴う FADD/caspase-8 による DISC 形成亢進が必須と考えられる。また、mitochondria 経路との cross-talk に関与する Bid の切断・活性化とともに mitochondria 経路に関わる cytochrome c の細胞質内への放出、caspase-9 の切断・活性化が認められたことから、本併用療法では、直接的 caspase 活性化の経路のみならず、mitochondria を介する apoptosis 経路(apoptosome)も重要な役割を果たしている可能性が考えられる。更に、apoptosome を介さず DNA の断片化を促進する AIF の細胞質内放出も増加していたことから、多数の経路を通じて強力に apoptosis を誘導していることが示唆された。

TRAIL に感受性のある glioma に対する TRAIL の局所・或いは全身投与による単独治療の有効性は Roth、Pollack らにより報告されている[19,20]。一方、今回使用した soluble FLAG-TRAIL の単独治療では、何れの glioma 細胞株でも無効であったが、化学療法或いは放射線治療との併用により著明な感受性化が認められた。即ち、TRAIL に耐性を示す悪性 glioma に対して、DNA 傷害性治療の併用による TRAIL 耐性克服の可能性が示された。TRAIL 感受性に関与する因子として、DR5 や DR4 の mutation[21]、TRAIL decoy receptors(DcR1、DcR2)の発現度、caspase-8、FADD の発現量[22]、細胞内の c-FLIP[23]、PED/PEA-15 の高発現[24]などが報告されている。しかし、DNA 傷害性治療／TRAIL の併用療法へ耐性を示した細胞株における原因は未だ不明であり、今後更に glioma における TRAIL 耐性機序の究明及び臨床試験の検討が期待される。

【謝 辞】

本研究を進めるに当たり、直接指導を頂いた Ludwig Insititute for Cancer Research、San Diego の所長である Dr. W. K. Cavenee 及び Dr. Su Huang に深謝いたします。TRAIL 等の construct を供与頂いた Genen -tech の Dr. G. Pan 及び Dr. V. M. Dixit に感謝いたします。又、実験を補助してくれた Ms. Hong Lin 及び岡本郁子嬢に御礼申し上げます。

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杏林大学における悪性神経膠腫の治療戦略

Treatment strategy of malignant glioma in Kyorin University Hospital

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【はじめに】

多形性膠芽腫(glioblastoma multiforme:GBM)を主とする悪性神経膠腫(malignant glioma)は依然致死的疾患であり、現代医学を以てしても未だ腫瘍死を免れえない状況にある。今回、われわれの施設において施行している悪性神経膠腫に対する治療法につき、特に術中の運動機能モニタリング、蛍光診断法、および個別化化学療法の試みにつき報告し検討を加えた。

【悪性神経膠腫に対する基本的治療方針】

①術前評価：

2000年版の日本脳腫瘍統計によれば、手術による腫瘍摘出率と悪性gliomaの予後には相関が認められ[1]、また米国からも同様の報告が出されており[2]、手術での腫瘍摘出率の向上は悪性gliomaの予後改善に重要な因子であることは明らかである。そのためには、術前に画像診断による正確な腫瘍及び周辺正常脳の解剖学的・生理学的情報を収集する必要がある。画像診断はMRIが主体であり、通常の条件下での撮像(T1強調、T2強調、FLAIR、Gadoliniumによる造影、diffusion強調画像(DWI))に加え、functional MRI(fMRI)によるeloquent areaの同定、tensor diffusion画像(TDI)による白質・錐体路と腫瘍との位置関係、腫瘍と脳表静脈をsuperimposeしたsurface anatomy scanning(SAS)による術野viewの術前検討を行っている。Proton MR spectros-copy(MRS)による病巣の生化学的解析も腫瘍と他病変との鑑別に有用と考えられる。

②術中評価：

1）腫瘍の局在同定：

手術の際に腫瘍の局在を術野で同定するため、脳表からの超音波断層画像をroutineに施行し、良好な脳内腫瘍の描出を得ている(Fig.1B)。しかし腫瘍摘出後の切除面に生じるartifactのため、手術後半での残存腫瘍の評価には難がある。深部あるいは比較的小さい病巣の同定には、CT-basedのneuro-navigation systemであるBrain Pointer(三鷹光機製)を使用し、正確な術中orien-tationの確保を試みている。Open CTあるいはMRIは設置されていないため、術中のbrain shiftに対して対応できない弱点は解決できていない。

2）脳機能モニタリング：

腫瘍摘出に際し、重要な脳機能の温存を図るため、腫瘍がeloquent area近傍にある際には術中神経モニタリングを施行している。

(1)Short latency somatosensory evoked potential (SSEP)：

全身麻酔導入後、手関節の正中神経上に針電極を留置後15mAにて刺激し、刺激反対側の頭皮(C3'あるいはC4'部)からN20を導出し、術中持続的SEPモニタリングを行っている(加算回数200回)。また、開頭・硬膜切開後、中心溝付近の脳表に4連電極を矢状方向に設置し、SEP上のN20の位相逆転をもとに中心溝を同定している。

(2)Motor evoked potential(MEP)：

腫瘍の局在が運動領あるいは錐体路近傍にある症例では、術中にSEPを用いて中心溝を同定した後、脳表の運動領皮質周辺および腫瘍の直上などを直接電気刺激し、対側の顔面・肩・上下肢に計8ヶ所の筋電図をモニターすることでMEPを検出し、脳表の運動領マッピングを行っている(Fig.1C,D)。MEP施行時は、fentanylとpropofolによる全身麻酔とし、筋弛緩剤のbecroniumは麻酔導入期のみに使用している。現在は刺激は単電極を用いており、刺激強度を25~35mA、持続時間0.2ms、インターバル1ms, train number 5, High cut 2 kHz, Low cut 50Hzの測定条件を使用している(ニュー