region with the highest tumor cell density, or the most highly proliferative portion of the tumor.

Several tumor-specific factors such as necrosis and neovascularity may affect prognosis (2). Results of many studies have indicated the usefulness of MR spectroscopy and MR perfusion imaging, which can depict necrosis or neovascularity, in addition to ADC measurements for the prediction of grade or malignancy of brain tumors (18, 20). Some investigators suggested the benefits of a combination of these methods (10,21). Among the various noninvasive techniques, however, DW imaging should be available in many hospitals and is the easiest to use and the least time consuming of them. The postprocessing of the data also is simple, and variation in the analyzed results should be minimum.

One of the limitations of this study was that there was no accordance of the areas for the minimum ADC measurement with those for the Ki-67 LI, which was already discussed previously. A second limitation was that we used only the minimum ADC values of tumors for estimation of the prognosis. A patient's prognosis is believed to depend on the most malignant site within a heterogeneous tumor, and it is on the basis of this site that the assignment of a histologic tumor grade usually is determined. As we have shown before, tumoral ADC should correlate well with the cell density and Ki-67 LI. Thus, it may be reasonable to think the site with minimum ADC value should represent one of the most malignant portions of the tumor. When the postoperative prognosis is considered, however, aggressiveness of the peripheral regions of a tumor might be more important than the central portions because the peripheral portions, where tumor recurrence usually occurs, tend to remain after surgery. With this view in mind, the minimum ADC values of the peripheral portion might correlate better with the prognosis.

Another limitation was the sample size of this study. The number of subjects was not large, and the performance of surgery was not uniform, and this nonuniformity led to a mixture of patients who underwent surgery with those who underwent biopsy, as well as a mixture of patients who underwent subtotal removal of tumor with those who underwent total removal. We found a significant difference in minimum ADC values between the stable and progressive groups and tentatively determined the cutoff value of minimum ADC for differentiating these two groups. Substantial overlap, however, was noted between these groups. A similar analysis in a larger group of subjects with uniform treatments may be required to determine whether the cutoff point of minimum ADC would really be reliable for this purpose.

In conclusion, minimum ADC values of the tumor were well correlated with the Ki-67 LI and were related to tumoral prognosis. We believe that ADC analysis should be one of the clinically feasible techniques used for prediction of prognosis of malignant astrocytic tumors, and it might be useful for planning initial treatment strategy in patients with these malignant tumors.

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増殖型ヘルペスウイルスを用いた脳腫瘍治療

宮 本 伸 哉　　藤 堂 具 紀

Brain Tumor Therapy using Oncolytic Herpes Simplex Virus Vectors

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Despite recent advances in surgical techniques, chemotherapy and radiotherapy, the poor prognoses of malignant glioma patients have not changed in the past decades. The use of oncolytic viruses is a promising new strategy for treating cancer including malignant glioma. The basic concept of oncolytic virus therapy is to kill the tumor cells *via* infection with replication-competent viruses such as herpes simplex virus. This concept, however, was not practical until the recent progress in genetic engineering enabled a manipulation of the viral genome to restrict the virus replication to tumor cells. Oncolytic viruses can also serve as vectors that can provide an amplified transgene delivery within the tumor. Multiple oncolytic virus vectors have been tested in clinical trials for malignant gliomas, most by direct intratumoral administration, the results of which have demonstrated the feasibility and potential of this therapeutic approach. The efficacy of oncolytic virus therapy may be enhanced by combining it with other adjuvant therapies. There remain problems to be solved which include how to obtain maximum delivery of the virus to tumor cells *via* systemic administration without losing a substantial amount by attachment to the vessel wall, hemodilution, filtration in the liver, and inability to pass the blood-brain barrier. Nonetheless, the development of oncolytic virus therapy may lead to a breakthrough in the treatment for malignant gliomas.

はじめに

悪性神経膠腫は，数多い悪性新生物の中において，有病率は低いもののきわめて予後が悪く，しかも近年の画像診断，放射線療法，化学療法等の目覚ましい進歩にもかかわらず，治療成績の向上がほとんどみられていない．例えば，悪性神経膠腫の代表である膠芽腫においては，生存期間中央値 (median survival time ; MST) が約 12〜14 カ月，5 年生存率が 10％以下できわめて予後が悪い．

現在，悪性脳腫瘍に対する治療は，手術に加えて，化学療法，放射線療法を適宜併用する集学的治療が一般的である．しかしながら，放射線または化学療法が実際奏効するのは悪性脳腫瘍患者のごく一部であり，多くの場合効果は限定的である．ウイルス療法は，脳腫瘍に限らず多くの悪性腫瘍において有効性が報告されており，新たな補助療法として期待されている．

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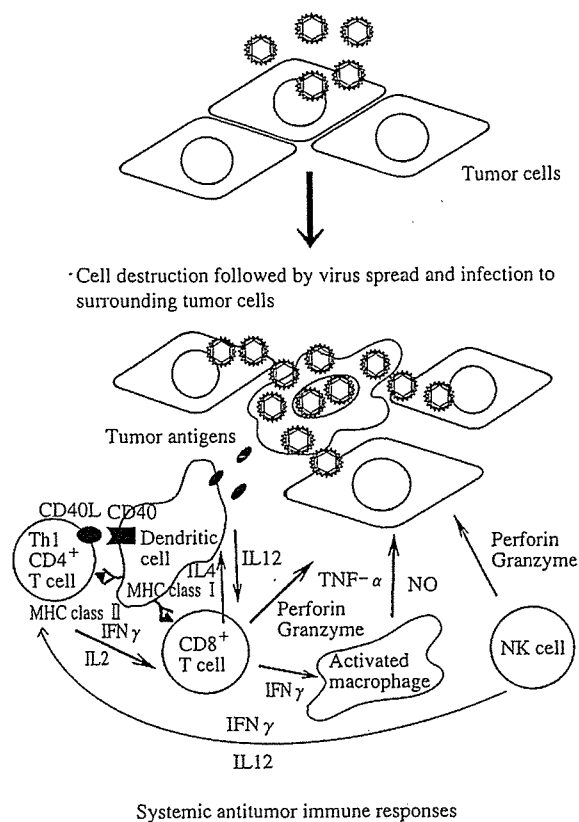
Oncolytic viruses

Tumor cells

·Cell destruction followed by virus spread and infection to surrounding tumor cells

Tumor antigens

CD40L CD40
Th1 CD4+ T cell
Dendritic cell
MHC class II
IFNγ
IL2
IL4
IL12
MHC class I
CD8+ T cell
IFNγ
Perforin Granzyme
TNF-α
NO
Activated macrophage
Perforin Granzyme
NK cell
IFNγ
IL12

Systemic antitumor immune responses

Fig. 1 Schematic demonstrating the concept of oncolytic virus therapy

ウイルス療法開発の背景

ウイルスを悪性腫瘍の治療に応用するという発想自体は，決して新しいものではない．すでに前世紀初頭には急性のウイルス感染を起こした白血病患者が緩解した例が観察されたことが契機となり[7]，1950年までにウイルスを用いた数多くの動物実験が行われた[22]．しかし，著明な腫瘍縮小効果が認められたものの，当時は野生型ウイルスや継代による弱毒化ウイルス，自然に発生した変異ウイルスを用いたため，ウイルスによる毒性が問題となり，結局広く臨床応用されるに至らなかった．

その後，近年のウイルスゲノムの解明や遺伝子組換え技術等の目覚ましい進歩により，ウイルスの病原性を人為的に制御することが可能となった．また，癌は，癌遺伝子の発現，癌抑制遺伝子の不活化などの遺伝子異常により，多くの因子が関与する細胞増殖シグナルの伝達，制御系が破綻し，結果的に無秩序な細胞増殖が生ずることから発生すると考えられ，1つの因子をターゲットとしても，汎用される治療法とはなりにくい．ウイルス療法は，基本的には分子標的治療薬のような特定の因子を

攻撃する治療とは異なり，癌細胞に共通した性質を利用するため，一種類のウイルスがまったく成因の異なる種々の腫瘍に有効であり，汎用性が高い．以上のことから，正常組織を傷害せずに腫瘍細胞に対して直接溶解作用を持つ増殖型ウイルスベクターの開発の研究が再び活発となった[18]．実際，さまざまな遺伝子改変型の増殖型ウイルスが多くの研究者により開発され，一部ではすでに臨床試験が行われ，その有効性かつ安全性が報告されている．現在では，さらに外来治療遺伝子をウイルスゲノムに直接組み込むことにより，増殖型の遺伝子治療ベクターとしての機能も持たせ，複合的な抗腫瘍効果を目指した研究が精力的に行われている．

ウイルス療法の基本理論と抗腫瘍免疫

元来ウイルス療法とは，増殖型ウイルスを腫瘍細胞に感染させ，ウイルス複製に伴うウイルスそのものの直接的な殺細胞作用により，腫瘍を縮小させる治療法である．すなわち，ウイルス本来の複製能力を最大限に利用することが基本となる．

腫瘍治療用の増殖型ウイルスは，腫瘍細胞内にて複製能力を保ちつつ，正常組織での病原性を最小限に抑える必要がある．ヒトを自然宿主とするウイルスを用いる場合，弱毒化野生株や自然変異株は，腫瘍細胞と正常細胞に対する効果の差，いわゆる therapeutic window が小さいことから，現在では遺伝子組換えウイルスが主に使用されている．

腫瘍細胞に感染したウイルスは細胞内で複製し，その過程で感染細胞を死滅させる．増えたウイルスは，周囲に散らばり，再び他の腫瘍細胞に感染し，同様なサイクルを繰り返す（**Fig. 1**）．正常組織を傷害しない機序は，ウイルスの種類により異なる．遺伝子組換え単純ヘルペスウイルスⅠ型（HSV-1）の場合は，正常細胞にも感染するが，正常細胞では複製できないようにウイルスゲノムに遺伝子操作が加えてある[19]．

HSV-1はまた，腫瘍特異的な細胞傷害性T細胞活性の増加を伴う抗腫瘍免疫も誘導する．抗腫瘍免疫が誘導される機序は一般に次のように考えられている[8]．腫瘍細胞がウイルス感染と複製によって破壊されると，樹状細胞（dendritic cell；DC）を主とするプロフェッショナル抗原提示細胞（professional antigen presenting cell；professional APC）により外来性蛋白として取り込まれる．癌抗原蛋白は，蛋白分解酵素によりペプチドの断片となり，大部分は endosome に入り，MHC クラスⅡ蛋白との複合体をつくる．しかし，一部は，細胞質に漏出し，MHC

クラス I 蛋白との複合体を作る．これらの複合蛋白は
DC の細胞膜上に発現される．従来，外来性抗原は MHC
クラス II 抗原提示経路に入るとされていたが，この DC
によるクラス I MHC 抗原提示経路はクロスプリゼン
テーションと呼ばれ，近年注目されている．DC はリン
パ組織（リンパ節，脾臓など）に移動して，MHC クラ
ス II 蛋白複合体を CD4$^+$ヘルパー T リンパ球に，MHC
クラス I 蛋白との複合体を感作 CD8$^+$T リンパ球に提示
する．これらのリンパ球は細胞膜上の T 細胞レセプター
(TCR) を介してこれらを認識し，同時に活性化される．活
性化 CD4$^+$ヘルパー T リンパ球 (Th1) は主に IL-2 およ
び IFN-γ を分泌し，感作 CD8$^+$T リンパ球の活性化に寄
与する．活性化した CD8$^+$ の細胞傷害性 T リンパ球
(Cytotoxic T lymphocyte；CTL) は，腫瘍組織に移行して，
膜穿孔性蛋白であるパーフォリンと蛋白分解酵素グラン
ザイムを用いる方法と Fas 分子などの death receptor を
刺激する方法の 2 つの方法で腫瘍細胞を攻撃する．ウイ
ルス療法において腫瘍細胞の膜表面に発現されたウイル
ス蛋白は，APC によるプロセシングを促進し，またア
ジュバント効果を呈すると推測される．

　一方，ナチュラルキラー (NK) 細胞は，腫瘍抗原を認
識することなく，腫瘍細胞を攻撃することができる．NK
細胞は多くの活性化受容体と抑制性受容体を持ち，その
両者のシグナルのバランスにより，細胞傷害性が決定さ
れる．抑制性受容体が MHC クラス I を認識するため，
MHC クラス I の発現が低下している腫瘍細胞をパー
フォリン，グランザイムにて攻撃する．NK 細胞は CTL
と相補う形で抗腫瘍免疫を支えている．また，周囲組織
より産生されるタイプ I IFN (IFNα, β)，Th1 の産生す
る IL-2，DC 等の産生する IL-12 は，NK 細胞を活性化
する．活性化 NK 細胞自身も IL-12，タイプ II IFN (IFN-
γ) を分泌し，autocrine 的に自身の活性化を維持する．
さらに，IFN-γ は，マクロファージを活性化し，活性化
マクロファージは NO，TNF-α により，腫瘍細胞を攻撃
する．NO は腫瘍細胞中の活性酸素 reactive oxygen inter-
mediate と結合し，peroxynitrite を生成して細胞傷害性に
働くことが知られている．

増殖型単純ヘルペスウイルス I 型
ベクターの特徴

　HSV-1 やアデノウイルスは，腫瘍治療用として精力的
に研究されてきた代表的な増殖型ウイルスベクターであ
る．いずれもウイルス遺伝子機能の解明が進み，ウイル
スゲノムの操作が可能であり，増殖型遺伝子導入ベク

ターとしても使用できる．

　HSV-1 はエンベロープを有する二重鎖 DNA ウイルス
に分類され，ウイルス療法に有利な以下の特徴を備えて
いる．①強い殺細胞効果を呈する．②ヒトのあらゆる細
胞に感染可能である．③ウイルスの生活環とゲノム配列
が解明されている．④アシクロビルなどの抗ウイルス薬
が存在する．⑤病原性に関連した遺伝子が解明されてお
り，遺伝子操作が可能である．⑥ウイルスゲノムが大き
い（約 152 kb）ので，大きな，あるいは複数の外来遺伝
子を組み込むことができる．⑦HSV-1 に感受性を示すマ
ウスやサルが存在するために，動物で安全性や効果の前
臨床的評価を行える．⑧ヒトのゲノムに取り込まれない．

　一方，不利な点としては，ゲノムサイズが大きいこと
から，従来の相同組換法で行う遺伝子操作では目的の遺
伝子組換え HSV-1 を得るのにかなりの時間と労力を要
することである．しかし，近年の bacterial artificial chro-
mosome (BAC) を用いた技術の開発により，HSV-1 ゲ
ノムを丸ごとプラスミドに組み込んで扱うことが可能と
なり，遺伝子の組換えが格段に容易となった[9)12)]．

遺伝子組換え HSV-1 による
腫瘍細胞のターゲッティング

　HSV-1 の複製能を腫瘍細胞に限定するためには，ゲノ
ムを操作して非必須遺伝子を 1〜複数個除去あるいは不
活化すればよい[19)]．$\gamma34.5$ 遺伝子は，病原性 (virulence)
に関連した遺伝子である．正常細胞では通常，ウイルス
感染の拡大を防ぐために，ウイルスの侵入に呼応して，
二本鎖 RNA 依存性プロテインキナーゼ (double-
stranded RNA-activated protein kinase；PKR) がリン酸化
され，最終的にウイルス蛋白の合成が遮断される．$\gamma34.5$
遺伝子産物は，この PKR に拮抗し，ウイルス蛋白の合
成を可能とする．$\gamma34.5$ 遺伝子は HSV-1 ゲノムに 2 コ
ピーあり，その双方とも欠失させた遺伝子組換え HSV-
1 は，正常細胞内では細胞の PKR を制御できないためウ
イルス複製が不可能となるが，腫瘍細胞内では腫瘍細胞
の PKR が元来低下しているため，$\gamma34.5$ 遺伝子がなくて
も複製可能となる．

　また，チミジンキナーゼ (thymidine kinase；tk) やリ
ボヌクレオチド還元酵素 (ribonucleotide reductase；RR)
はウイルス DNA 合成に必須で，これらをコードするウ
イルス遺伝子を除去・不活化すると，変異 HSV-1 は分
裂が盛んで，tk 活性や RR 活性の上昇した腫瘍細胞での
みウイルスの欠落酵素が補われてウイルス複製が可能と
なる．

Recombinant HSV-1	Generation	Parental HSV-1	Mutation	Inserted gene
1716	1st	17$^+$	759 bp deletion in both copies of γ34.5	
R3616	1st	strain F	1000 bp deletion in both copies of γ34.5	
R4009	1st	strain F	Stop codons in both copies of γ34.5	Stop codon
NV1020 (R7020)	1st	strain F	Deletion of one copy of γ34.5, tk, UL24. UL55, UL56, and IR	α4 (p)-TK, HSV-2gG, gJ, gD, gI
NV1023	2nd	F/NV1020	Deletion of IR & α47	lacZ, HSV-2gG, gJ, gD, gI
NV1034	2nd	F/NV1023	Deletion of IR & α47	mGM-CSF, lacZ, HSV-2gG, gJ, gD, gI
NV1042	2nd	F/NV1023	Deletion of IR & α47	mIL12, lacZ, HSV-2gG, gJ, gD, gI
G207	2nd	F/R3616	1 kb deletion in both copies of γ34.5, lacZ insertion in ICP6/UL39	lacZ
G47Δ	3rd	F/G207	Deletion in both copies of γ34.5 & α47 lacZ insertion in ICP6	lacZ
T-01	3rd	G47Δ	Deletion in both copies of γ34.5, α47, & ICP6 lacZ insertion in ICP6	lacZ

$\alpha47$ 遺伝子産物は，抗原提示関連トランスポーター (transporter associated with antigen presentation；TAP) を阻害することによって，ウイルス蛋白が細胞表面に提示されることを防ぎ，ウイルスが宿主の免疫サーベイランスから逃れる作用を有する．HSV-1 が感染すると $\alpha47$ 遺伝子産物により宿主細胞の MHC クラス I 発現が低下するが，$\alpha47$ 遺伝子を欠失させると宿主細胞の MHC クラス I 発現レベルが維持されるため，抗腫瘍効果惹起に有利に働くことが期待される．また，$\alpha47$ 遺伝子と重なる US11 遺伝子プロモーターが同時に欠失すると，US11 遺伝子発現時期が変化することに伴い，$\gamma34.5$ 欠失 HSV-1 の減弱したウイルス複製能を腫瘍細胞に限って回復させ，殺腫瘍細胞効果を向上させる．

各種遺伝子組換え HSV-1 の分類と概要 (Table 1)

① 第一世代遺伝子組換え HSV-1

ウイルスの非必須遺伝子を1つだけ変異させた増殖型遺伝子組換え HSV-1，または単一の機序により腫瘍細胞特異性を得ているものを第一世代と呼ぶ．第一世代は，一般に第二世代より複製能力を維持するが，病原性減弱の不徹底や，変異が一箇所だけであるため相同組換えによって野生型 HSV-1 へ自然に戻ってしまう可能性などがあり，安全性の面で問題が残る．HSV1716 や NV1020 (R7020) がこれに該当する．

② 第二世代遺伝子組換え HSV-1

$\gamma34.5$ 遺伝子と DNA 合成酵素遺伝子など，2つの異なる機序を介して腫瘍特異的なウイルス複製を得た増殖型遺伝子組換え HSV-1 を第二世代と呼ぶ．病原性が一層弱められ，野生型に戻る可能性がきわめて少なく，安全性が著明に改善した．G207 は第二世代の代表で，米国で再発悪性グリオーマを対象とした臨床試験が行われた[17][19]．

③ 第三世代遺伝子組換え HSV-1

3つの異なる機序を介して腫瘍特異性を持たせた増殖型遺伝子組換え HSV-1 を第三世代と呼ぶ．G47Δ は，G207 と同じ $\gamma34.5$ 遺伝子欠失と ICP6 遺伝子 (RR) 変異に加えて，さらに $\alpha47$ 遺伝子およびそれと重なる US11 プロモーター領域を欠失する[26]．最近，G47Δ の ICP6 遺伝子の不活化変異を欠失変異に改良した T-01 も開発された．第三世代は，第二世代の安全性を保ちつつ，ウイルス複製能と抗腫瘍効果が著しく向上した．

④ 「武装」遺伝子組換え HSV-1

増殖型 HSV-1 のゲノムに外来治療遺伝子 (transgene) を直接組み込むと，増殖型 HSV-1 そのものを増殖型の治療遺伝子発現ベクターとして機能させることができる．現在，治療効果をさらに高めるための外来治療遺伝子を組み込んだウイルスの開発が精力的に進められてい
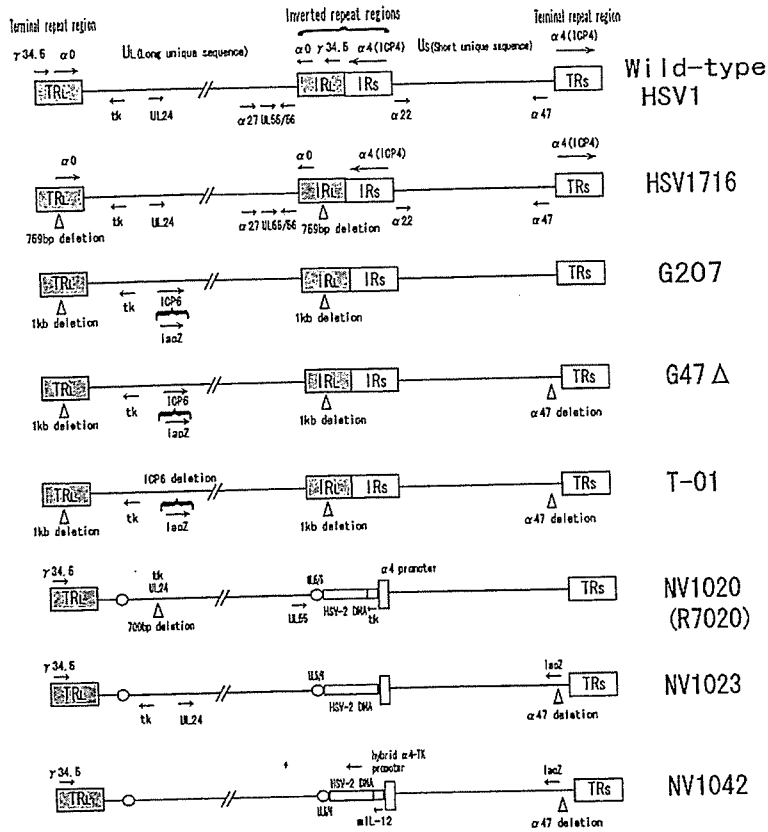
Fig. 2   HSV-1 structures and their representative recombinant oncolytic HSV-1 vectors

る．例えば，NV1020（R7020）を少し修飾した NV1023 の基本骨格でマウス IL-12 を発現する NV1042（Fig. 2）は，ウイルスによる直接的な殺細胞作用に加えて，抗腫瘍免疫の惹起と腫瘍血管新生の抑制効果を呈し，マウス IL-12 を発現しない NV1023 に比べ抗腫瘍効果を有意に増強することが，複数のマウス腫瘍モデルにおいて示された[3)28)]．

⑤ 臓器・腫瘍特異的プロモーターを利用した遺伝子組換え HSV-1

ある臓器，腫瘍に特異的に発現される蛋白質の遺伝子のプロモーターでウイルスの遺伝子を制御することにより，その臓器や腫瘍においてのみウイルスが複製可能になるか複製能が向上するように遺伝子操作した増殖型 HSV-1 である．肝細胞に特異的なアルブミン[21)]や未分化神経細胞に特異的なネスチンのプロモーター[14)]などを利用したウイルスベクターが開発されている．

中間径フィラメントを構成する蛋白であるネスチンはグリオーマのマーカーの一つであるとされる．ネスチンは，神経細胞の発生の段階で発現されるが，成熟脳では発現が抑制されており，腫瘍細胞などの増殖細胞中のみ発現している．rQNestin34.5 は，ネスチンプロモーターにより γ34.5 をコントロールし，γ34.5 を腫瘍細胞の中でのみ発現させ，腫瘍細胞のみでウイルスの複製能を向上して抗腫瘍効果を発揮させるべくデザインされた HSV-1 ベクターである[14)]．

脳腫瘍に対する増殖型遺伝子組換え HSV-1 の臨床試験

臨床に応用された増殖型遺伝子組換え HSV-1 の代表として，HSV1716，G207 が挙げられる．HSV1716 は，γ34.5 遺伝子のみを欠失した第一世代の増殖型 HSV-1 ベクターである（Fig. 2）．1990 年代後半，英国のグループにより，再発悪性グリオーマに対する HSV1716 の第 I 相臨床試験が行われた．腫瘍内に定位的に 10^3，10^4，10^5 プラーク形成単位（plaque-forming units；pfu）の HSV1716 を 3 人ずつ投与が行われたが，どの患者においても脳炎などの副作用や潜在性 HSV-1 の再活性化などは認められず，安全性が確認された．またその 9 人の患者のうち，投与 14～24 カ月後において 4 人の生存が確認され，HSV1716 の有効性も示唆された[24)]．

また同グループは，あらかじめ HSV-1 の抗体値を測定した 12 人の悪性グリオーマの患者に対して生検を兼ねて，まず 10^5pfu の HSV1716 を定位的に投与した．その 4〜9 日後に腫瘍を切除し，その検体を調べたところ，10 人から HSV-1 DNA，2 人より抗 HSV-1 免疫染色で HSV1716 の複製が確認された．また，5 人に血清 HSV-1 抗体の陽転を認め，HSV-1 に対する免疫反応が認められた．この試験において HSV-1 抗体保有の有無にかかわらず，毒性を惹起することなく，悪性脳腫瘍内で HSV1716 が複製することが示された[23]．

さらに同グループにより，12 人の悪性グリオーマの患者に対して，今度は腫瘍切除後の腫瘍腔壁に HSV1716 を直接投与する臨床試験が行われた．術後，適応があれば放射線療法，化学療法も行われた．術後 14 カ月後の時点で，画像上 10 人の患者において腫瘍の増大を認め，術後 15 カ月の時点で 3 人の生存が確認された[10]．

HSV1716 は，弱毒化の程度が低く複製能を維持するため，少ないウイルス量で高い殺細胞効果が得られる可能性がある一方で，毒性や野生型への復元（spontaneous reversion）の危険性が第二世代などに比べて高いため，高い投与量を実施し得ない．その点，第二世代の G207 は元来脳腫瘍治療用に開発され，*γ34.5* 遺伝子の欠失と *lacZ* 遺伝子挿入による *ICP6* 遺伝子（RR）の不活化の二重変異が施されて，安全性が格段に改善されている（Fig. 2）[20]．再発悪性グリオーマ患者 21 名を対象に米国で第 I 相臨床試験が行われた．ウイルス投与量は，1×10^6 から 3×10^9pfu まで 3 名ずつ段階的に増量した．造影 CT の増強部位に定位的に腫瘍内投与されたところ，どの患者においてもグレード 3，4 相当の重篤な有害事象はみられなかった[17]．この最高投与量は，英国で臨床試験が行われた HSV1716 の最高 10^5pfu と比べ 3 万倍で，G207 の安全性の高さが推測される．効果については，6 例（29%）に Karnofsky スコアの一時的な改善がみられた．MRI にて腫瘍体積の経時的評価を行ったところ，投与前と比較し投与 1 カ月後において 20 例中 6 例に腫瘍の縮小を認めたが，死亡した 1 例を除いた全例にて再増大を認めた．生検あるいは再摘出術で得られた腫瘍組織 7 例中 2 例で，PCR にて G207 の DNA が検出された．剖検 5 例中 3 例にて腫瘍が脳の一領域に限局し，神経膠芽腫に通常みられるような腫瘍細胞の周囲脳組織への著明な浸潤を認めなかった[17]．第 Ib 相試験では，脳腫瘍摘出後，摘出腔壁に G207 を直接投与する方法が採られた．放射線治療や化学療法と G207 を併用した臨床試験が米国で計画されている．

ウイルス療法の展望

ウイルス療法の臨床試験はまだ黎明期であり，抗腫瘍効果を最適化するプロトコールの開発や腫瘍内のウイルス複製のイメージング評価の確立などまだ課題も多い．
In vivo におけるウイルス複製の評価は，非侵襲的にルシフェラーゼを用いてイメージングすることがすでにマウスでは実用化されている．さらに，最近 tk の選択的基質である

5-iodo-2'-fluoro-1-β-D-arabinofuranosyl-uracil (FIAU) を用いた PET imaging において，HSV-1 の tk 遺伝子の発現レベルを非侵襲的にモニターすることが可能であることが報告された．Bennett ら[2]は，大腸癌細胞株 HCT8 のラット側腹部 0.5 cm 径の皮下腫瘍内に HSV-1 ベクターを投与し，その 8 時間，48 時間後に ^{124}I-labeled FIAU を投与し，さらにそれぞれその 3 日後に PET を施行した．その結果，1×10^7pfu の低投与量でも FIAU の集積を検出可能であり，0.5log のウイルス投与量の差を集積度の違いにより検出できた．またウイルス投与 8 時間後よりも 48 時間後に FIAU を投与したほうが集積が強かった．このことから，HSV-1 の tk 遺伝子発現レベルを PET で検出することにより，増殖型 HSV-1 ベクターの複製の程度と分布を非侵襲的に体外から確認できることを示した．ヒトにおいても，ウイルス療法の効果を PET にて判定できることが今後期待される．

また，ウイルス療法と他の補助療法との併用の有効性が指摘されている[6]．Advani ら[1]は，ヌードマウスにおける U87 MG の皮下腫瘍モデルにおいて，R3616 ウイルス治療および放射線の併用療法とそれぞれの単独治療との抗腫瘍効果の比較検討を行った．ウイルス投与の 1 日後に 20 Gy，そして 2 日後に 25 Gy という分割照射放射線の併用療法は，R3616 ウイルス単独治療と比べ，ウイルスの複製を 2〜5 倍に増加させることにより抗腫瘍効果を相乗的に増強し，著明な延命効果がみられた．また，ヌードマウスの扁平上皮癌細胞株皮下腫瘍モデルにおける G207 ウイルス治療と放射線療法（5 Gy の 1 回照射）の併用療法[15]やヌードマウスの U87 MG の脳腫瘍モデルにおける R3616 ウイルス治療と分割放射線療法（5 Gy を 1 日おきに計 6 回，Total 30 Gy）の併用療法[5]を検証した異なる腫瘍モデルにおいても，同様の結果が観察されている．G207 に関しては，放射線による DNA の損傷により，腫瘍細胞の ribonucleotide reductase（RR）およびウイルスの複製に直接関与していると考えられている GADD34 蛋白の up-regulation がウイルス複製増強の機構ではないかと推察されている[15][16][25]．その一方でヒ

ト前立腺癌細胞株 LNCap をヌードマウスに，またマウス前立腺癌細胞株 TRAMP を C57BL/6 マウスに植えたそれぞれの皮下腫瘍モデルにおいて，G207 と放射線の同時投与が行われたが，その相乗効果はみられなかった[13]．このように，放射線療法との併用の相乗効果に関しては，依然 controversial であるが，放射線照射のタイミング，線量などの条件が最適化できれば，将来臨床においても，ウイルス治療と放射線療法の併用による治療成績の向上が期待できる．

化学療法についても，マウスの非小細胞癌細胞株 NCI-H460 の皮下腫瘍に対して，HSV-1716 の投与 1 日後にマイトマイシン C の静注を施行したところ，単独治療と比べ，有意に腫瘍縮小効果を認めたという[27]．また，マウスの胃癌モデルにおいて，マイトマイシン C の併用が腫瘍細胞の GADD34 を誘導し，G207 の抗腫瘍効果を著明に増強したと報告されている[4]．

これら従来の補助療法が，ウイルス療法と併用できるのみでなく，相乗的抗腫瘍効果が期待できることは，実際の臨床においては特に重要な点であるが，現時点ではまだ報告は少数であり，今後の研究課題となっている．動物実験と臨床試験を経て，適切なプロトコールが確立されることが望まれる．

おわりに

ウイルス療法は，ウイルスが腫瘍細胞内で選択的に複製して直接細胞を破壊することが基本であり，腫瘍細胞に共通した性質を利用するため腫瘍の種類を問わないこと，患者の年齢，性別などによる適用制限が少ないこと，放射線療法，化学療法，免疫療法など他の補助療法との併用で相乗効果が期待できること，増殖型治療遺伝子発現ベクターとして遺伝子分配も可能であることなど多くの優れた点を有する．

一方で，増殖型ウイルスとはいえ，臨床においてすべての腫瘍細胞に感染をいき渡らせることは困難であり，より効果的なウイルス投与法と抗腫瘍効果の増強法を開発していく必要がある．静脈内投与など非観血的投与法で効果的なウイルス療法を行うのが理想だが，血液による希釈や，血管壁への吸着，肝臓での補足などの問題に加え，特に脳腫瘍治療においては脳血液関門（blood brain barrier；BBB）という障壁が存在し，血管内投与法の実用化にはなお工夫を要する．ラットでは増殖型 HSV-1 を頸動脈内に投与する際に，cyclophosphamide やマニトールを予め投与しておくと脳腫瘍へのウイルス分配が格段に向上することが観察されている[11]．

ウイルス療法は，より効果的で安全な治療遺伝子発現型ウイルスベクターの開発や他の補助療法との併用など今後大きな発展性を有している．新たな delivery system の開発により静脈内投与法や頻回反復投与などが確立されれば，ウイルス療法が近い将来脳腫瘍補助療法の first line therapy となることが期待される．

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─────────────── 要　旨 ───────────────

増殖型ヘルペスウイルスを用いた脳腫瘍治療

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　悪性神経膠腫の治療成績がこの数十年間改善されない中，ウイルス療法は新しい治療法として注目される．増殖型ウイルスを腫瘍細胞に感染させ，直接的な殺細胞作用で死滅させる．遺伝子工学の発達により，ウイルス複製を腫瘍細胞に限定し，さらに治療遺伝子発現型ベクターとして利用することも可能となった．悪性神経膠腫を対象に，すでに複数の増殖型ウイルスを用いた臨床試験が行われ，安全性と有効性が示された．免疫療法や化学療法，放射線治療の併用により相乗効果が期待される．血管内投与法などに改善の余地が残る一方で，実用性は着実に向上しており，悪性神経膠腫治療に革新をもたらす治療法としてその発展が期待される．

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

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Abstract   Conditionally replicating herpes simplex virus-1 (HSV-1) vectors are promising therapeutic agents for cancer. Insertion of therapeutic transgenes into the viral genome should confer desired anticancer functions in addition to oncolytic activities. Herein, using bacterial artificial chromosome and two recombinase-mediated recombinations, we simultaneously created four "armed" oncolytic HSV-1, designated vHsv-B7.1-Ig, vHsv-interleukin (IL)-12, vHsv-IL-18, and vHsv-null, which express murine soluble B7.1 (B7.1-Ig), murine IL-12, murine IL-18, and no transgene, respectively. These vHsv vectors possess deletions in the $\gamma 34.5$ genes and contain the green fluorescent protein gene as a histochemical marker and the immunostimulatory transgene inserted in the deleted *ICP6* locus. The vHsv showed similar replicative capabilities *in vitro*. The *in vivo* efficacy was tested in A/J mice harboring s.c. tumors of syngeneic and poorly immunogenic Neuro2a neuroblastoma. The triple combination of vHsv-B7.1-Ig, vHsv-IL-12, and vHsv-IL-18 exhibited the highest efficacy among all single vHsv or combinations of two viruses. Combining 1×10^5 plaque-forming units each of the three armed viruses showed stronger antitumor activities than any single armed virus at 3×10^5 plaque-forming units in inoculated tumors as well as in noninoculated remote tumors. Studies using athymic mice indicated that this enhancement of antitumor efficacy was likely mediated by T-cell immune responses. The combined use of multiple oncolytic HSV-1 armed with different immunostimulatory genes may be a useful strategy for cancer therapy.

Replication-competent viral vectors are useful tools for the treatment of malignant tumors, because they can serve as oncolytic bioreagents as well as vectors that provide amplified gene delivery within the tumor. Herpes simplex virus-1 (HSV-1) is suited for clinical application, because it infects a wide variety of cell types, it exhibits strong cytotoxicity, circulating antibodies do not affect the cell-to-cell spread of the virus, and antiviral drugs are available (1). Oncolytic HSV-1 vectors have one or more genetic mutations in the viral genome, which

restricts the viral replication to tumor cells, and therefore kill the host tumor cells without harming the normal tissue (2).

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

Engineering new recombinant HSV-1 vectors using conventional homologous recombination techniques had been a laborious task that required time-consuming processes of selection and structure confirmation. Bacterial artificial chromosome (BAC) is a single-copy plasmid that can stably retain a large size (~300 kb) DNA as an insert (12). BAC plasmids have been used to propagate the entire HSV-1 genome in *Escherichia coli*, allowing an easy genetic manipulation (13, 14). In this article, we use a new BAC-using method for generating "armed" oncolytic HSV-1 vectors with the backbone of MGH-1, an oncolytic HSV-1 vector with the genome structure identical to G207 (i.e., deletions in both copies of the $\gamma 34.5$ gene and a *lacZ* insertion inactivating the *ICP6* gene; ref. 2). The method also uses two recombinase systems (FLP/FRT and Cre/loxP) to allow

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

Materials and Methods

Cells and viruses. Vero (African green monkey kidney) and Neuro2a (murine neuroblastoma) cell lines were purchased and maintained as described previously (6). MGH-1 has the structure identical to G207 (2) and was constructed from strain F as described (15). Virus titers were determined by standard methodology (16).

Vector construction. The vector construction method using HsvQuik system is described (17). Briefly, the pT IE4/5 shuttle plasmid has a HSV immediate-early promoter IE4/5, a multiple cloning site, a bovine growth hormone gene polyadenylate sequence, the ampicillin resistance gene (Amp), a loxP site, a R6Kγ ori sequence, and a FRT site (Supplementary Fig. S1). The fHsvQuik1 is a BAC plasmid and was created by a homologous recombination replacing the *lacZ* gene and adjacent 764-bp sequence within the *ICP6* gene of MGH-1 with a 9.4-kb sequence consisting of BAC sequences [F ori and the chloramphenicol resistance gene (Cmr)], a loxP site, a FRT site, a red fluorescent protein cDNA, and an enhanced green fluorescent protein (EGFP) cDNA.

The 2.4-kb *Eco*RV/*Not*I fragment from pB7.Ig (6) was inserted to the *Eco*RV/*Not*I site within the multiple cloning site of pT IE4/5 shuttle plasmid. The 2.3-kb *Spe*I-*Afl*II fragment from pIL-12 p40-IRES-p35 (5) was inserted into the *Nhe*I/*Afl*II site within the multiple cloning site of pT IE4/5 shuttle plasmid. The 0.55-kb *Eco*RI fragment from pCEXV3-IFN-β-IL-18 (ref. 18; gift from Dr. Isao Hara, Department of Urology, Kobe University, Kobe, Japan) was inserted into the *Eco*RI site within the multiple cloning site of pT IE4/5 shuttle plasmid. The entire sequence of each shuttle plasmid with the transgene (B7.1-Ig, IL-12, or IL-18) or the control shuttle plasmid without a transgene was inserted into the FRT site of fHsvQuik1 by coelectroporation with pFTP-T, a plasmid that expresses tetracycline (Tc)–inducible FLP and a tetracycline selection marker. Ampicillin and chloramphenicol double-resistant colonies were selected and the genomic structures of the fHsvQuik1 transgenes were confirmed by restriction enzyme digestion.

Each fHsvQuik1 transgene DNA was purified from *E. coli* and cotransfected with the pc-nCre plasmid that expresses Cre recombinase into Vero cells using LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. A transient expression of Cre recombinase results in excision of the sequence between the two loxP sites (containing F ori, γ ori, Amp gene, Cmr gene, and red fluorescent protein). Five days later, the viruses were harvested and passaged on Vero cells, and virus plaques negative for red fluorescence were further selected and purified through three-time limiting dilutions on Vero cells. The structures of the recombinant viruses were confirmed by Southern blot analyses: Virus DNA was digested with *Hin*dIII, transferred to a nylon membrane, and probed with cDNA for murine IL-12, murine IL-18, or murine B7.1-Ig from the shuttle plasmids. The hybridized DNA bands were visualized using AlkPhos Direct Labeling and Detection System with CDP-Star (Amersham, Piscataway, NJ). The resultant recombinant virus contains deletions in both copies of the γ34.5 gene, a deletion within the *ICP6* gene, the ICP6-GFP fusion driven by the endogenous ICP6 promoter, and a therapeutic transgene driven by the IE4/5 promoter. The vectors expressing soluble murine B7-1, murine IL-12 p35 and p40, murine IL-18, or no transgene were designated as vHsv-B7.1-Ig, vHsv-IL-12, vHsv-IL-18, and vHsv-null, respectively (Fig. 1). Virus stocks were purified and concentrated as described (19).

Virus yield studies. Vero or Neuro2a cells (4 × 10⁵) were seeded on six-well plates. After a 24-hour incubation at 37°C, cells were infected with vHsv-B7.1-Ig, vHsv-IL-12, vHsv-IL-18, vHsv-null, or MGH-1 at a multiplicity of infection (MOI) of 0.01 (for Vero) or 0.1 (for Neuro2a) and further incubated at 37°C for 48 hours (20). Progeny viruses were titered on Vero cells.

In vitro *cytotoxicity studies.* In vitro cytotoxicity studies were done as described (4). Neuro2a cells (2 × 10⁵) were seeded on six-well plates and incubated at 37°C overnight. Virus or mock was inoculated onto cells in a volume of 0.7 mL for 1 hour. The inoculum was then removed and cells were incubated at 34.5°C in DMEM supplemented with 1% heat-inactivated FCS. The number of surviving cells was counted daily with a Coulter counter (Beckman Coulter, Miami, FL) and expressed as a percentage of mock-infected controls. Our basic studies indicated that the viable cell count obtained by the above method is consistent with that 'determined by trypan blue exclusion.

In vitro *detection of transgene expression.* Vero cells (1 × 10⁵) were seeded onto 24-well plates and incubated at 37°C overnight. The pT IE4/5 shuttle plasmids (pT IE4/5 B7.1-Ig, pT IE4/5 IL-12, and pT IE4/5 IL-18; 0.5 μg each) were transfected to cells using LipofectAMINE Plus reagent according to the manufacturer's instruction. The cells were further incubated at 37°C in 0.6 mL DMEM supplemented with 1% heat-inactivated FCS for 48 hours. For the recombinant viruses, Vero cells were inoculated with the virus (MOI = 1) or mock extract and incubated at a nonpermissive temperature of 39.5°C in 0.6 mL DMEM supplemented with 1% heat-inactivated FCS. We observed that the cytopathic activities of the viruses were remarkably attenuated at 39.5°C. The supernatant was collected daily and cytokine concentrations were
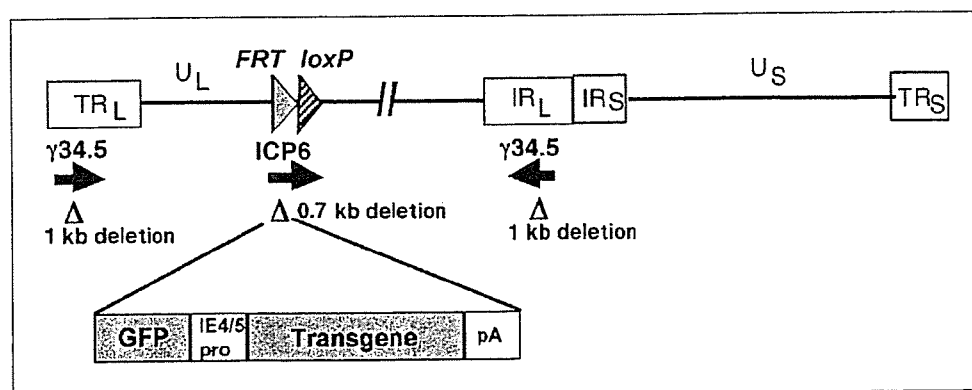

Fig. 1. Structures of vHsv vectors constructed by the HsvQuik system. The armed oncolytic HSV-1 vectors (vHsv vectors) constructed using the HsvQuik system have 1-kb deletions in both copies of the γ*34.5* gene, a 0.7-kb deletion in the *ICP6* gene, the *GFP* gene driven by the ICP6 promoter, and the transgene driven by the IE4/5 promoter, both cassettes inserted in the *ICP6* locus.
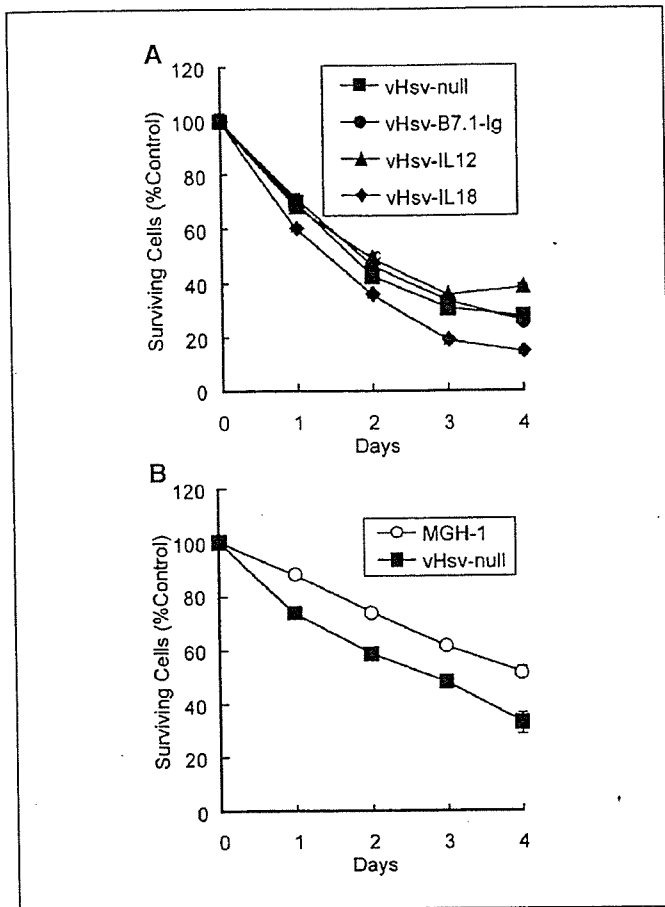
Fig. 2. Cytopathic effect of vHsv vectors and MGH-1 on Neuro2a murine neuroblastoma cells *in vitro*. Neuro2a cells were plated into six-well plates at 2 × 10⁵ per well. After a 24-hour incubation, cells were infected with virus at MOI = 0.1 or virus buffer (mock). The number of surviving cells was counted daily and expressed as a percentage of mock-infected control. *A*, vHsv-null and three armed vHsv vectors showed comparable cytopathic effects *in vitro*. *B*, MGH-1 killed Neuro2a cells less rapidly than vHsv-null (*P* < 0.001, days 1-4). Points, mean of triplicates; bars, SD.

measured (for IL-12 and IL-18) or cells were fixed with 4% paraformaldehyde in PBS (for B7.1-Ig). IL-12 concentration was measured by mouse IL-12 p70 Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN), and IL-18 concentration was measured by mouse IL-18 ELISA kit (MBL, Nagoya, Japan). B7.1-Ig expression was determined by immunohistochemical staining using a biotin-conjugated antihuman IgG Fc antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:50 dilution) as described (6).

In vitro IFN-γ stimulation studies. Spleen cells were harvested from a naive 6-week-old female A/J mouse. In 96-well plates, RBC-depleted spleen cell suspension (1.5 × 10⁵ cells per well) were cultured with conditioned medium of Vero cells transfected with a shuttle plasmid (pT IE4/5 IL-12 or pT IE4/5 IL-18) or mock in 200 µL complete medium (RPMI 1640 supplemented with 10% FCS, 100 units/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL fungizone, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, 50 µmol/L 2-mercaptoethanol) for 24 hours at 37°C. Recombinant mouse IL-18 (MBL) was used as positive control. After centrifugation at 2,000 rpm for 10 minutes, the IFN-γ concentration in the medium was measured by mouse IFN-γ ELISA kit (Endogen, Inc., Woburn, MA).

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

Subcutaneous tumor therapy. Subcutaneous tumors were generated by injecting 5 × 10⁶ Neuro2a cells s.c. into the bilateral or left flank(s) of 6-week-old female A/J mice or athymic mice (BALB/c *nu/nu*). When s.c. tumors reached ~5 mm in diameter 5 to 6 days after implantation, a standard size used in our previous studies using this model (6, 20), animals were randomized, and mock or virus [8 × 10³-2 × 10⁶ plaque-forming units (pfu)] in 20 µL PBS containing 10% glycerol was inoculated into left tumors. Viral administration was repeated 3 days later. Mock-infected extract (mock) was prepared from virus buffer-infected cells using the same procedures as those used for virus inoculum. Tumor growth was determined by measuring the tumor volume (length × width × height) thrice weekly (4). Animals were sacrificed when the maximum diameter of the tumor on either side reached 20 mm.

In vivo measurement of IL-12 and IL-18. S.c. Neuro2a tumors were generated in the left flank of female A/J mice. When tumors reached ~5 mm in diameter, vHsv-IL-12, vHsv-IL-18 (2 × 10⁶ pfu), or mock virus extract in 20 µL PBS containing 10% glycerol was inoculated into the tumor. On days 1, 4, and 7, blood was collected by retro-orbital bleeding from two mice per group, after which the mice were euthanized and tumors were harvested. Tumors were homogenized in ice-cold PBS, sonicated, and centrifuged, and the cytokine concentration in the supernatant was measured by ELISA. Samples from each mouse were measured in triplicates. The detection limits for IL-12 were 12.5 pg/mL and 0.5 pg/mg for serum and tumor homogenates, respectively, and those for IL-18 were 125 pg/mL and 5 pg/mg, respectively.

Rechallenge studies. Mice whose established s.c. Neuro2a tumors regressed after vHsv treatment as well as age-matched naive female A/J mice were used. Neuro2a cells (5 × 10⁶) were injected s.c. into the right flank, and tumor growth was observed as described above. The animals were followed for 60 days.

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

Results

Generation and in vitro characterization of vHsv vectors. Using the HsvQuik system, we generated four oncolytic HSV-1 vectors unarmed or armed with soluble murine B7-1, murine IL-12, or murine IL-18, termed vHsv-null, vHsv-B7.1-Ig, vHsv-IL-12, and vHsv-IL-18, respectively. These vHsv vectors had a 1-kb deletion in both copies of the γ34.5 gene and a 764-bp deletion within the ICP6 gene (Fig. 1). The transgene driven by the IE4/5 promoter and the GFP gene as a marker were inserted in the deleted ICP6 locus. The four vectors were constructed in parallel, and the final products were obtained within 6 months, after which the structures were confirmed by Southern blot analyses (Supplementary Fig. S2).

The virus yield obtained 48 hours after infection of Vero cells at a MOI of 0.01 for vHsv-null, vHsv-B7.1-Ig, vHsv-IL-12, and vHsv-IL-18 were 5.6 × 10⁵, 4.2 × 10⁵, 6.4 × 10⁵, and 2.8 × 10⁵ pfu, respectively. The results were considered to reflect both replicative and infective capabilities, and there was no significant difference among the vHsv vectors. The virus yield of the parental MGH-1 in Vero cells was 2.9 × 10⁶ pfu, 5- to 10-fold greater than vHsv vectors. In Neuro2a murine neuroblastoma cells, the ratio of progeny virus recovery (recovery/input) 48 hours after infection was 0.10 for vHsv-null and 0.021 for MGH-1, both considerably lower than the yields obtained in Vero cells. We further tested the *in vitro* cytopathic activity of vHsv vectors in Neuro2a cells. At a MOI of 0.1, all vHsv vectors showed comparable cytopathic activities, killing ~70% to 80% of the cells by day 4 (Fig. 2A). In contrast to the results from virus yield studies, vHsv-null (MOI = 0.1) killed Neuro2a cells more rapidly than MGH-1 (*P* < 0.001, days 1-4; Fig. 2B).

The *in vitro* expression of the transgenes arming the vHsv vectors was checked in Vero cells. After infection with vHsv-IL-12 at a MOI of 1, the IL-12 concentration of the conditioned medium at 24, 48, and 72 hours was 7.84, 21.2, and 23.7 ng/mL, respectively. Similarly with vHsv-IL-18, the IL-18 concentration at 24, 48, and 72 hours was 0.649, 1.32,

and 1.45 ng/mL, respectively. The IL-12 or IL-18 expressed by the transgenes was confirmed to have an intact bioactivity by the ability of conditioned medium of Vero cells transfected with pT IE4/5 IL-12 or pT IE4/5 IL-18 to stimulate IFN-γ secretion from mouse spleen cells (data not shown). The expression of soluble murine B7-1 by vHsv-B7.1-Ig was
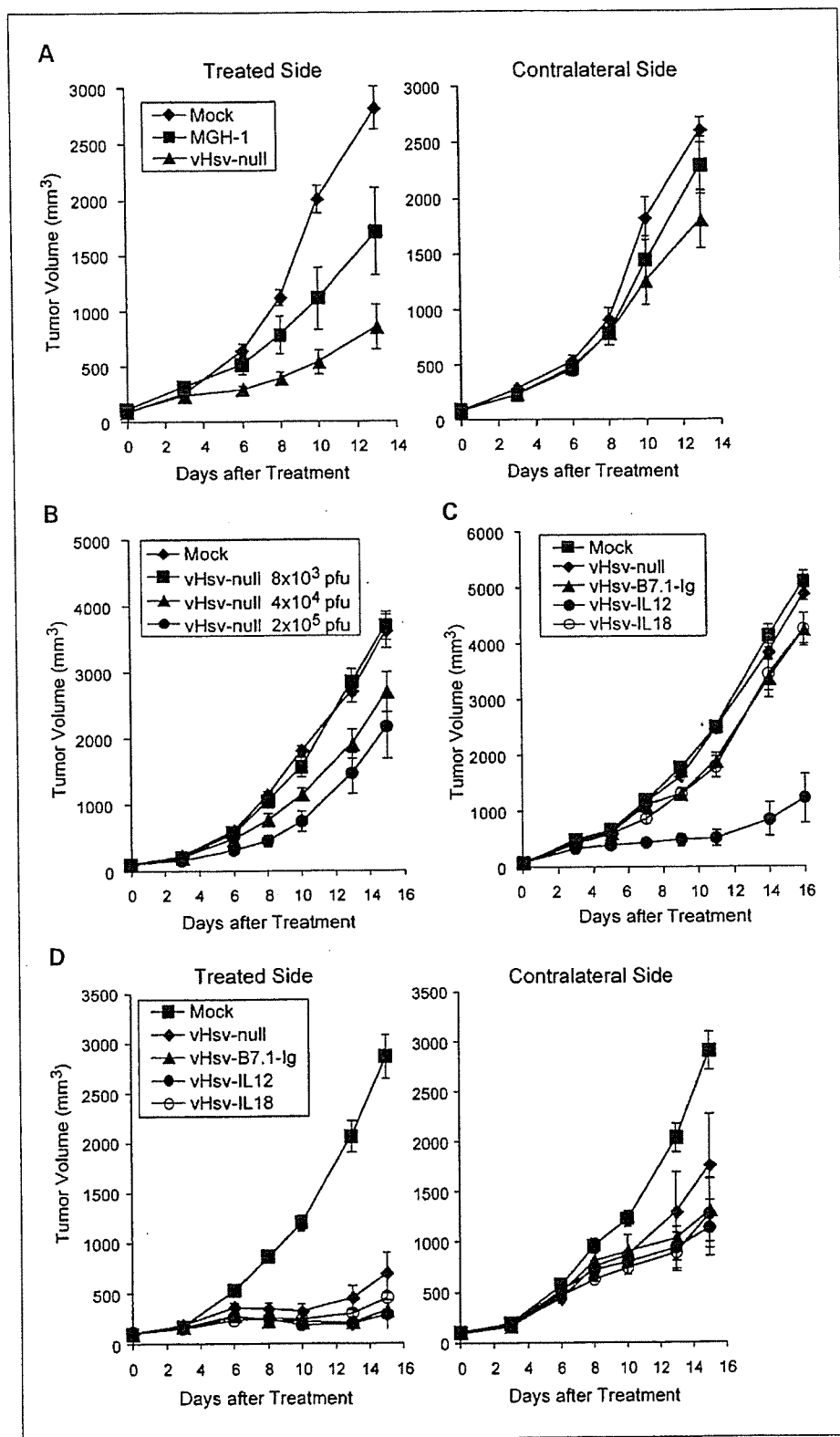

Fig. 3. *In vivo* efficacy of vHsv vectors in A/J mice harboring established s.c. Neuro2a tumors. S.c. Neuro2a tumors were generated in the bilateral or left flank(s) of 6-week-old female A/J mice, and the left tumors alone were inoculated with mock-infected extract (mock) or virus on days 0 and 3. *A*, left tumors were inoculated with mock, MGH-1, or vHsv-null at 2×10^6 pfu ($n = 9$ per group). In the inoculated side, vHsv-null exhibited a significantly greater efficacy than MGH-1 ($P < 0.05$, days 6-13). The vHsv-null treatment also caused a growth inhibition of the contralateral noninoculated tumors, whereas mock or MGH-1 treatments showed no effect ($P < 0.05$, days 10-13). *B*, unilateral tumors were inoculated with mock or vHsv-null at 2×10^5, 4×10^4, or 8×10^3 pfu ($n = 9$ per group). Inoculations with vHsv-null at doses 4×10^4 pfu and higher significantly inhibited the tumor growth in a dose-dependent manner. *C*, unilateral tumors were treated with mock, vHsv-null, vHsv-B7.1-Ig, vHsv-IL-12, or vHsv-IL-18 at 1×10^4 pfu ($n = 9$ per group). vHsv-IL-12 caused a significantly greater tumor growth inhibition than any other treatment ($P < 0.05$ versus each of other groups, days 5-14). Both vHsv-B7.1-Ig and vHsv-IL-18 showed a mild tumor growth inhibition compared with mock ($P < 0.05$, days 9-14) but not with vHsv-null. *D*, left tumors were treated with mock, vHsv-null, vHsv-B7.1-Ig, vHsv-IL-12, or vHsv-IL-18 at 2×10^6 pfu ($n = 6$ per group). All vHsv vectors inhibited the growth of inoculated tumors. Also in the contralateral side, all armed vHsv vectors significantly suppressed the tumor growth compared with mock ($P < 0.01$, days 10-15 for vHsv-IL-12 and vHsv-IL-18 and days 13-15 for vHsv-B7.1-Ig; $P < 0.05$, days 13-15 for vHsv-null). Tumor volume = length × width × height (mm). Bars, SE.

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confirmed by immunostaining for human IgG Fc in Vero cells 48 hours after infection (data not shown).

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

To determine the effective dose range for vHsv vectors, A/J mice with unilateral s.c. Neuro2a tumors were treated with intraneoplastic inoculations of vHsv-null at three different doses (2 × 10⁵, 4 × 10⁴, and 8 × 10³ pfu) on days 0 and 3 (n = 9 per group). Inoculations with vHsv-null at 4 × 10⁴ pfu or higher significantly inhibited the growth of Neuro2a tumors in a dose-dependent manner (Fig. 3B). Therefore, to investigate the effect of transgenes expressed by the vHsv vectors, we initially selected 1 × 10⁴ pfu, a dose at which vHsv-null would exhibit a minimal effect. A/J mice with unilateral s.c. Neuro2a tumors were treated with intraneoplastic inoculations of mock, vHsv-null, vHsv-B7.1-Ig, vHsv-IL-12, or vHsv-IL-18 on days 0 and 3 (n = 9 per group). The treatment with vHsv-IL-12 resulted in a significantly greater tumor growth inhibition than other treatments ($P < 0.05$ versus each group, days 5-14; Fig. 3C). Both vHsv-B7.1-Ig and vHsv-IL-18 showed a marginal but significant tumor growth inhibition compared with mock ($P < 0.05$, days 9-14). Next, we chose a higher dose (2 × 10⁶ pfu) and did a study using A/J mice with bilateral s.c. Neuro2a tumors, mainly focusing on the antitumor effect in the contralateral noninoculated tumors. All vHsv vectors, inoculated intraneoplastically on days 0 and 3, significantly and equally inhibited the growth of inoculated tumors (n = 6 per group). Also in the contralateral side, all vHsv
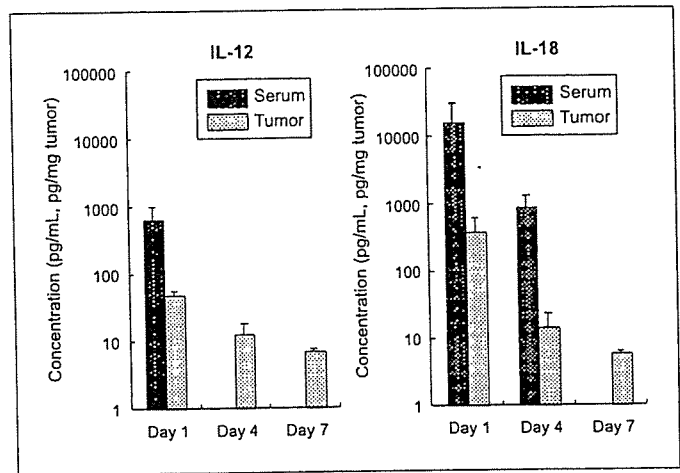

Fig. 4. *In vivo* transgene expression of vHsv vectors in A/J mice harboring established s.c. Neuro2a tumors. A/J mice harboring s.c. Neuro2a tumors were treated with a single intraneoplastic inoculation with vHsv-IL-12 (*left*) or vHsv-IL-18 (*right*) at 2 × 10⁶ pfu, and serum and intratumoral concentrations of IL-12 or IL-18 were determined on days 1, 4, and 7. Neither IL-12 nor IL-18 was detected in serum and tumor in mock-treated or vHsv-null-treated mice. Columns, average of two animals; bars, SD.

vectors significantly suppressed the tumor growth compared with mock ($P < 0.01$, days 10-15 for vHsv-IL-12 and vHsv-IL-18 and days 13-15 for vHsv-B7.1-Ig; $P < 0.05$, days 13-15 for vHsv-null), and there was no significant difference among the four vHsv vectors (Fig. 3D).

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

Enhancement of in vivo efficacy by combinations of vHsv vectors. Certain combinations of immunostimulatory molecules, such as IL-12/B7-1 and IL-12/IL-18, have been reported to act synergistically (24-26); therefore, we further investigated whether combining vHsv vectors could enhance the antitumor efficacy. First, each armed vHsv vector (1 × 10⁵ pfu) was administered alone or in combination with others, and the total administered virus amount was adjusted to 3 × 10⁵ pfu (except mock) by adding necessary amounts of vHsv-null. The left tumors of A/J mice bearing bilateral s.c. Neuro2a tumors were treated with intraneoplastic inoculations on days 0 and 3 (n = 8 per group; Fig. 5A). In the inoculated side, all armed vHsv vectors, alone or combined, showed significant tumor growth inhibition ($P < 0.05$, days 6-13). The combination of vHsv-IL-12 and vHsv-IL-18 showed a significantly greater antitumor efficacy than other combinations of two armed vectors ($P < 0.05$, days 6-13). In the contralateral noninoculated tumors, an enhancement of antitumor efficacy was observed when the left tumors were
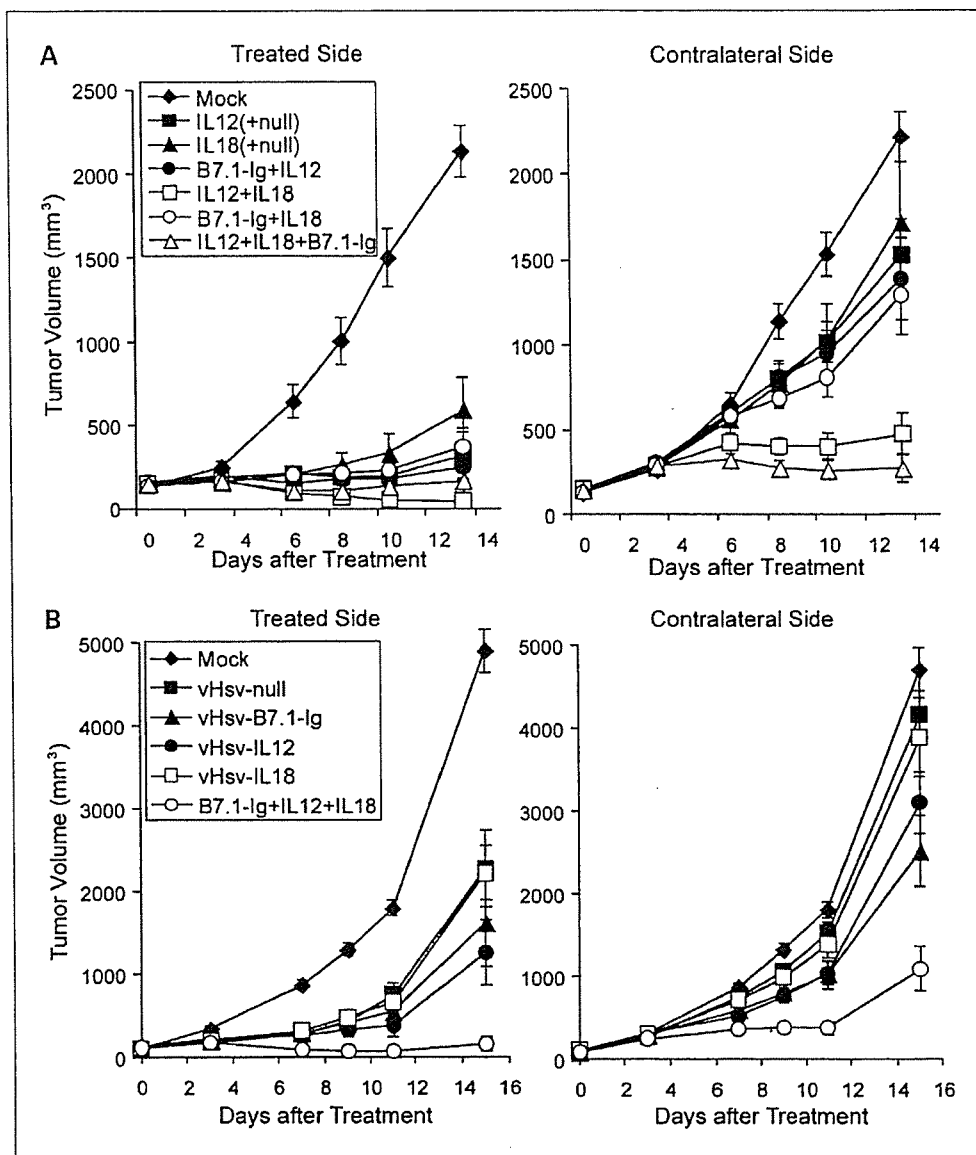
Fig. 5. Enhancement of *in vivo* efficacy by combinations of vHsv vectors. The left tumors of A/J mice bearing bilateral s.c. Neuro2a tumors were treated with intraneoplastic inoculations of mock or vHsv vectors on days 0 and 3 (n = 8 per group). *A*, each armed vHsv vector (1×10^5 pfu) was administered alone or in combination with others. The total administered virus amount was adjusted to 3×10^5 pfu by adding necessary amounts of vHsv-null. In the inoculated side, all armed vHsv vectors, alone or combined, showed a significant and strong tumor growth inhibition ($P < 0.05$, days 6-13). The combination of vHsv-IL-12 and vHsv-IL-18 showed a significantly greater antitumor efficacy than other combinations of two armed vectors ($P < 0.05$, days 6-13). In the contralateral tumors, an enhancement of antitumor efficacy was observed when the left tumors were treated with vHsv-IL-12 plus vHsv-IL-18 or all three vectors combined, the latter treatment being more efficacious than the former treatment ($P < 0.05$, days 8-13). *B*, efficacy of the combination of the three armed vHsv vectors (1×10^5 pfu each) was compared with that of a single vector given at 3×10^5 pfu. The combination treatment exhibited a significantly greater antitumor efficacy than any single vector treatment on both inoculated and noninoculated sides ($P < 0.05$ versus any group for both sides, days 9, 11, and 15). IL-12, vHsv-IL-12; IL-18, vHsv-IL-18; B7.1-Ig, vHsv-B7.1-Ig. Bars, SE.

treated with vHsv-IL-12 plus vHsv-IL-18 or all three vectors combined, the latter treatment being more efficacious than the former ($P < 0.05$, days 8-13).

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

To investigate whether the enhancement of antitumor efficacy by the combination therapy requires T cells, s.c. Neuro2a tumors were generated in the left flanks of athymic mice and inoculated with 3×10^5 pfu of each vHsv vector or the combination of all three armed vHsv vectors (1×10^5 pfu of each) on days 0 and 3 (n = 6 per group). Both the

vHsv-IL-12 group and the combination group showed a significantly greater antitumor effect compared with other treatment groups ($P < 0.05$, days 7-11 for both vHsv-IL-12 and combination groups). In contrast to the results using A/J mice, vHsv-null, vHsv-B7.1-Ig, and vHsv-IL-18 showed no significant effect, suggesting that T-cell-mediated responses are largely responsible for the antitumor effect exhibited by these vectors at this dose. The superior antitumor efficacy of vHsv-IL-12 to other vectors in athymic mice is compatible with the known function of IL-12 that involves activation of natural killer cells (27). The combination therapy, however, did not increase the antitumor efficacy compared with the vHsv-IL-12 alone treatment, indicating that the enhancement effect by combining with vHsv-IL-18 and vHsv-B7.1-Ig may involve T-cell-mediated immune responses (Fig. 6A).

Furthermore, we tested whether the strong antitumor efficacy by the combination therapy observed in the contralateral noninoculated tumors in A/J mice involves T-cell immune responses. Bilateral s.c. Neuro2a tumors were generated in

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

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

Discussion

The present system (termed HsvQuik) using BAC and the two recombinases (FLP and Cre) has multiple advantages over conventional homologous recombination techniques for the construction of armed oncolytic HSV-1 vectors, such as an insertion of desired transgenes into the intended locus with high probabilities and precision and selection of recombinant BAC plasmid clones in *E. coli*. The HsvQuik system allowed us to create four different oncolytic HSV-1 vectors (three armed and one unarmed control) simultaneously in a relatively short period. The transgene expression mediated by oncolytic HSV-1 vectors is transient in a host cell due to eventual cell destruction, yet the expression level within the tumor may be high due to an amplified gene delivery (28). Moreover, the destruction of tumor cells during viral oncolysis elicits specific antitumor and antiviral immune responses. Therefore, unlike replication-defective viral and nonviral vectors, it is difficult to predict the effect of the transgene expression in oncolytic HSV-1 vectors, especially if it might affect host immune responses, viral replication, and/or viral spread. The HsvQuik system allowed screening and simultaneous comparison of the *in vivo*
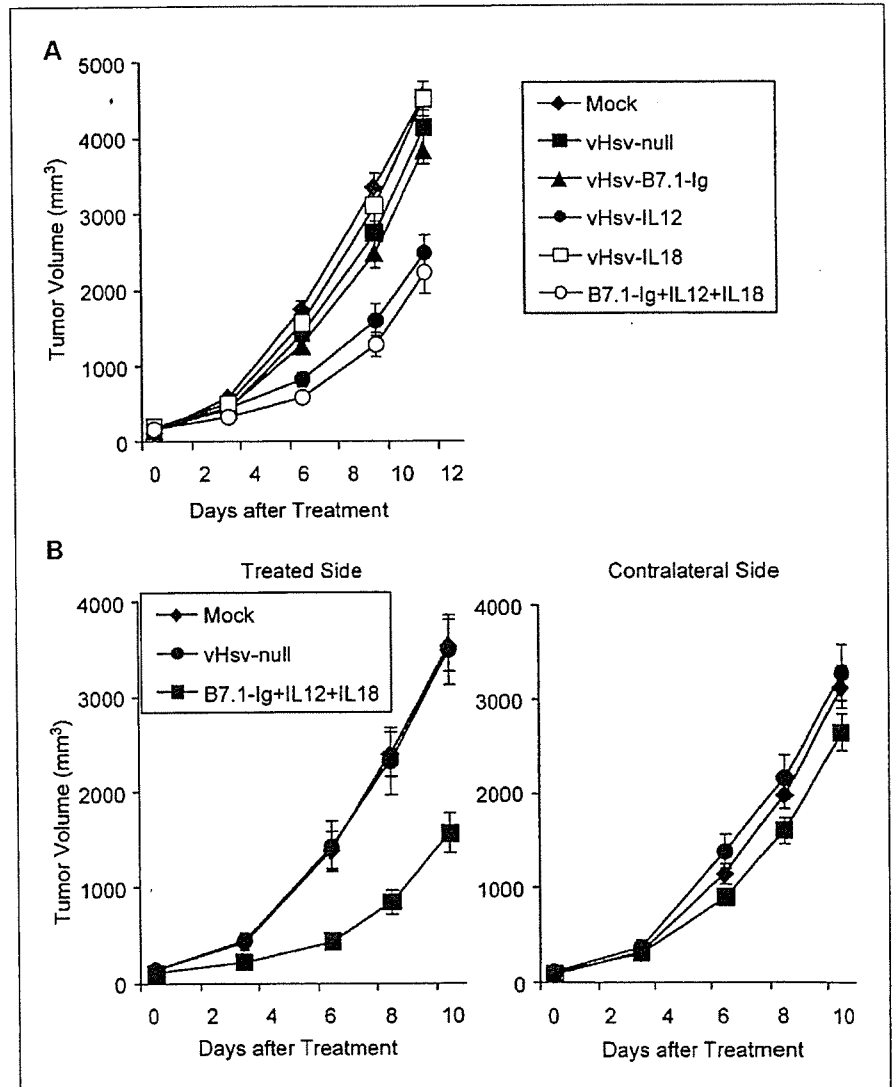
Fig. 6. Efficacy of the combination therapy in athymic mice. *A*, unilateral s.c. Neuro2a tumors in athymic mice were inoculated with 3×10^5 pfu of each vHsv vector or the combination of all three armed vHsv vectors (1×10^5 pfu of each) on days 0 and 3 ($n = 6$ per group). Both the vHsv-IL-12 group and the combination group showed a significantly greater antitumor effect compared with other treatment groups ($P < 0.05$, days 7-11 for both vHsv-IL-12 and combination groups). *B*, bilateral s.c. Neuro2a tumors were generated in athymic mice, and the left tumors alone were inoculated with mock, vHsv-null (3×10^5 pfu), or the three armed vHsv vectors combined (1×10^5 pfu each) on days 0 and 3 ($n = 8$ per group). The combination therapy resulted in a significant growth inhibition of inoculated tumors ($P < 0.05$ versus mock and vHsv-null, days 6-10), but no significant effect on the growth of contralateral tumors. Bars, SE.

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effects of various transgenes expressed in the same oncolytic HSV-1 backbone.

Antitumor immune responses play important roles in the *in vivo* antitumor activities of oncolytic HSV-1 vectors, which supports the usage of immunostimulatory genes for arming the HSV-1 vectors (4, 6, 29, 30). We compared the efficacy of oncolytic HSV-1 vectors armed with murine soluble B7-1, murine IL-12, or murine IL-18 using HSV-1-sensitive A/J mice and syngeneic, poorly immunogenic Neuro2a tumors and showed that vHsv-IL-12 was the most efficacious at a low dose of 1×10^4 pfu. The superior efficacy was unlikely due to higher expression levels of IL-12 *in vivo*, because the serum and intratumoral concentration levels of IL-18 exceeded those of IL-12. It has been reported that NV1042, an oncolytic HSV-1 vector expressing murine IL-12, was more efficacious than a murine granulocyte macrophage colony-stimulating factor–expressing oncolytic HSV-1 vector with the same backbone (NV1034) in certain animal tumor models (10). In athymic mice, only vHsv-IL-12 (3×10^5 pfu) but not other vHsv vectors showed a significant growth suppression of the inoculated tumors, indicating that the augmented efficacy by IL-12 expression may be mediated, at least in part, via non-T-cell mechanisms, including an activation of natural killer cells (27), which may be a functional advantage over soluble B7-1 and IL-18 at low dose levels. In addition, it has been suggested that antitumor actions of NV1042 were partly exhibited via antiangiogenic effects of IL-12 (31). In A/J mice at a high dose of 2×10^6 pfu, the efficacy of a single armed vHsv vector was not significantly different from that of vHsv-null in both inoculated and noninoculated tumors regardless of the transgene, indicating that an addition of a single immunostimulatory gene expression may not significantly augment the efficacy of oncolytic HSV-1 vectors when a sufficient amount is administered. HSV-1 vectors have inherent ability to down-regulate MHC class I expression of infected host cells, which may undermine the immunostimulatory potentials of oncolytic HSV-1 vectors armed with cytokines. We have shown previously that tumor cells infected with an α47-deficient HSV-1 vector had enhanced MHC class I expression and showed increased stimulation of immune cells (20).

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

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

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

The replicative capabilities of vHsv vectors were remarkably lower than MGH-1 in Vero cells for reasons yet to be elucidated. Although the structures of vHsv vectors were confirmed by Southern blot analyses, a small possibility remains as to an unintended mutation being introduced during the BAC plasmid construction. The parental MGH-1 is considerably attenuated compared with wild-type HSV-1 due to the double mutations in

the γ34.5 and *ICP6* genes. The low rate of replication may be beneficial for evaluating the effect of transgene expressions in experimental animals. In clinical practice, however, to obtain an efficient therapeutic effect from a small portion of vector-infected tumor cells, it may be preferable to retain good replicative capability, providing a large amplification of gene delivery as well as effective oncolytic activities. Furthermore, it is important for the backbone structure of armed oncolytic HSV-1 vectors to have a large therapeutic window by keeping the viral replication highly selective to tumor cells, because the transgene expression might increase the toxicity. A triple-mutated oncolytic HSV-1 vector, G47Δ, constructed from G207 by creating a further deletion within the α47 gene and the overlapping *US11* promoter, was shown to have enhanced viral replication in tumor cells while preserving the safety features.(20). A BAC-mediated oncolytic armed HSV-1 vector construction system with G47Δ as the backbone has been developed (47).

Published studies have shown that suppression of innate immunity can enhance the oncolytic virus replication and antitumor efficacy (48, 49). Whether the expression of the immunostimulatory transgenes significantly affected the *in vivo* replication of vHsv vectors is yet to be determined. A possibility remains that immune stimulation is beneficial when the oncolytic virus replicates relatively poorly. We observed that

systemic administration of immunostimulatory cytokines did not alter the intratumoral replication of G47Δ.[6] The results suggest that immune stimulation would be beneficial for oncolytic virus therapy if an effective T-cell-mediated antitumor immunity can be obtained.

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

Acknowledgments

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[6] T. Todo, Y. Ino, unpublished data.

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悪性神経膠腫の治療戦略 2006

西 川 　 亮

Treatment Strategies for Malignant Gliomas—2006

by

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In the treatment of glioblastoma (GBM), postoperative radiotherapy has been recognized as standard therapy, whereas the addition of chemotherapy has been a controversial issue. A meta-analysis based on 12 randomized trials suggested only a small benefit. The recent trial by the European Organisation for Research and Therapy of Cancer (EORTC) and National Cancer Institute of Canada Clinical Trial Group was the first study to demonstrate unequivocally that the addition of temozolomide to radiotherapy provides a statistically significant survival benefit in GBM. For anaplastic oligodendroglioma and oligoastrocytoma, two separate trials by EORTC and the Radiation Therapy Oncology Group clearly demonstrated that chemotherapy by procarbazine, lomustine, and vincristine, plus radiotherapy does not prolong survival but does increase the incidence of progression-free survival. The combined loss of 1p/19q identifies a favorable subgroup of oligodendroglial tumors, and no genetic subgroup could be identified that benefited with respect to survival from adjuvant PCV. In low-grade gliomas, older age, astrocytoma histology, presence of neurologic deficits, largest tumor diameter, and tumor crossing the midline were important prognostic factors for survival, and these factors can be used to identify low-risk and high-risk patients. Taken together, these evidences reported recently provide the most up-to-date treatment strategies for malignant gliomas.

転換期を迎えた膠芽腫・退形成性星細胞腫の化学療法

膠芽腫・退形成性星細胞腫に対する化学療法は，1970 年代に米国で行われた大規模臨床試験から始まるといって過言ではない．まず，手術後に放射線照射も化学療法も行わない群を対照としたランダム化比較試験が行われ，放射線照射あるいは化学療法単独治療による，統計学的に有意の生存期間延長効果が証明された[15]．そして さらに放射線照射に化学療法（BCNU あるいは MeCCNU）を併用する方法が検証されたが，放射線照射単独群との間には統計学的に有意の生存期間延長効果は認められなかった[15][16]．有意の生存期間延長効果は認められなかったわけであるが，実際の生存曲線をみると放射線照射に BCNU を併用した群の生存期間が最も優れていたことから，膠芽腫・退形成性星細胞腫においては，放射線照射に BCNU を併用する方法が手術後の治療として推奨されることになった．わが国においても，術後

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