Potent Systemic Antitumor Activity from an Oncolytic Herpes Simplex Virus of Syncytial Phenotype

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ABSTRACT

Conditionally replicating (oncolytic) viruses, which selectively replicate in tumor cells but not in normal cells, show great promise as antitumor agents for cancer therapy. The principal antitumor activity of these viruses derives from their replication within tumor cells, which results in cell destruction and the production of progeny virions that can spread to adjacent tumor cells. However, one potential limitation of this approach is that viral gene deletions conferring tumor selectivity also result frequently in reduced potency of the virus in tumors. Therefore, strategies designed to enhance the potency of current oncolytic viruses will likely increase their chance of clinical success. Here we report the construction of an oncolytic herpes simplex virus (HSV) of which the infection also causes strong cell membrane fusion (syncytial formation). In vitro characterization on a variety of human tumor cells of different tissue origins showed that the plaques from this virus (Fu-10) are phenotypically unique and are significantly larger than those from the parental G207 virus, a well-characterized oncolytic HSV lacking fusogenic function. Furthermore, the syncytial formation caused by this virus depended on HSV replication, indicating that cell membrane fusion will only occur in dividing cells (such as tumor cells) where the virus can undergo a full infection cycle but not in normal cells where the viral replication is restricted. Systemic administration of Fu-10 into mice with established lung metastatic breast cancer resulted in a dramatic therapeutic effect. These studies demonstrate that incorporation of fusogenic function into an oncolytic virus can significantly increase the potency of viral oncolysis; this may lead to an enhanced clinical performance, especially in late-stage cancer patients.

INTRODUCTION

Certain human viruses have a strong ability to infect and kill their target cells during their natural life cycle. These viruses can be genetically engineered for oncolytic purposes (i.e., specifically killing tumor cells) and, therefore, provide an attractive therapeutic approach for cancer treatment. Among the viruses that have been modified for oncolysis are adenoviruses, HSV-1, and some RNA viruses such as reovirus (reviewed in Refs. 1, 2). For HSV-1, two genetic approaches have been used to construct oncolytic forms of the virus. The first method consists of inactivating the function of a viral gene (ICP6) that encodes the large subunit of ribonucleotide reductase, an enzyme required for efficient viral DNA replication (3–5). This enzyme is expressed abundantly in tumor cells but not in nondividing cells; as a consequence, the virus preferentially replicates in—and kills—tumor cells. The second approach consists of deleting the viral γ34.5 gene, which functions as a virulence factor during HSV infection (6). Mutations in this gene also result in a block to viral replication in tumor cells. The second approach consists of deleting the viral γ34.5 locus and an insertional mutation in the ICP6 gene by the Escherichia coli lacZ gene (9–11). Therefore, oncolytic forms of HSV constructed from these genetic manipulations can destroy tumor cells by a direct cytopathic effect (CPE), because they replicate within the tumor cells but remain restricted in their ability to replicate in nondividing normal cells (12–14).

Although oncolytic HSVs have clinical potential as antitumor agents, evidence indicates that current versions of these viruses may have somewhat limited therapeutic benefit. For example, whereas both inhibition of tumor growth and improved survival of experimental animals have been observed in xenografted human tumors (9, 11, 15–17), only a fraction of the animals appear to be cured by the administration of current forms of oncolytic HSV. At least two factors may contribute to the suboptimal oncolytic efficacy of these viruses. First, gene deletions that confer viral replication selectivity also frequently reduce the potency of the virus in tumors. For example, the complete elimination of endogenous γ34.5 function from HSV significantly reduces viral replication potential and, therefore, may compromise the ability of the virus to spread within the targeted tumors (18). A second limitation of current oncolytic viruses is that, unlike other therapeutic strategies (such as prodrug enzyme delivery), the antitumor activity of oncolytic HSV does not induce a significant bystander effect, a process that can result in the killing of nontransduced cells after death of the transduced neighboring cells. The bystander effect is considered to be crucial for effective antitumor therapy, because it compensates for the limited efficiency of vector delivery and spread. Therefore, it is likely that additional improvements on both the potency and killing ability of these oncolytic viruses may be a requisite to obtaining a clear clinical benefit.

Several viruses have been described that kill their target cells through multinucleated syncytial formation, a process involving membrane fusion between infected and uninfected cells. The viral components that contribute to syncytial formation are mainly the fusogenic membrane glycoproteins (FMGs). It has been demonstrated recently that the fusogenic property of FMGs may be applicable to cancer therapy (19, 20). For example, transduction of a COOH-terminal truncated form of the gibbon ape leukemia virus envelope glycoprotein (19, 21) into a range of human tumor cells results in efficient cell destruction through the process of syncytial formation (20). Furthermore, the bystander killing effect from this protein is at least 10 times higher than the effect from the suicide genes HSV-thymidine kinase or cytosine deaminase (20, 21). However, it is expected that FMGs must be efficiently delivered into tumor cells in a controlled fashion before their potential therapeutic benefit can be materialized, a prerequisite that has not yet been satisfactorily addressed.

We hypothesized that the combined action of direct oncolysis and syncytial formation would result in a virus with an extremely potent and effective antitumor effect. Previous studies have shown that although infection of wild-type HSV isolated from patients does not cause significant cell fusion in vitro, cells infected with certain spontaneously occurring syncytial mutants fuse extensively either with each other or with uninfected cells (23, 24). Analyses of these syncytial mutants have uncovered nonlethal mutations that affect expres-
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...sion of several viral glycoproteins, such as gB and gK (24–27). Therefore, we reasoned that an oncolytic virus capable of conferring a syncytial phenotype could be selected from a well-characterized oncolytic HSV. Furthermore, because the viral glycoproteins (including gB and gK) are late genes of which the expression is dependent on viral DNA replication, such an oncolytic virus will maintain the safety of the original virus, because syncytial formation will only occur in tumor cells (where the virus can undergo a full infection cycle) but not in normal nondividing cells (where the virus replication is restricted and very little glycoproteins are expressed).

Here we report the isolation and characterization of an oncolytic form of HSV-1, denoted Fu-10, which is capable of conferring a syncytial phenotype. Investigations with a panel of human cancer cell lines showed that Fu-10 infection lead to widespread syncytial formation in all of these cell types in vitro and correlated with an enhanced killing of tumor cells. Syncytial formation was dependent on the initiation of viral DNA replication, because blocking viral replication completely prevented cell membrane fusion. Systemic injection of Fu-10 into mice resulted in effective treatment of established metastatic breast cancer in the lungs. These results demonstrate that Fu-10 not only has substantially enhanced oncolytic potency but has also fully maintained the desired safety of the original oncolytic virus G207.

MATERIALS AND METHODS

Cell Culture and Viruses. African green monkey kidney (Vero) cells; human embryonic fibroblasts (HF 333.Wc); and the human tumor cell lines U-87 MG (glioblastoma), A-549 (non-small cell lung carcinoma), and HepG2 (hepatocellular carcinoma) were obtained from American Tissue Culture Collection (Rockville, MD). The human breast adenocarcinoma line MDA-MB-435 was kindly provided by Dr. Dihua Yu (M. D. Anderson Cancer Center, Houston, TX), and the human prostate cancer line DU 145 was a gift from Dr. Tim Thompson (Baylor College of Medicine, Houston, TX). Vero, U-87 MG, DU 145, and MDA-MB-435 cells were cultured with DMEM containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, and 1% sodium pyruvate. The viruses were routinely grown and titrated in Vero cells. Random mutagenesis of G207 was performed according to a procedure published previously (28), with modifications. Briefly, Vero cells in a six-well plate were infected with G207 at 0.01 pfu/cell. After viral infection (1 h), DMEM containing 2% FBS and 5% CO2 was added to the cells. Virus harvested 48 h later was used to infect fresh Vero cells after a series of 10-fold dilutions. Viral plaques that showed significant cell fusion (i.e., syncytial formation) were detected under a low-power microscope and isolated. One sample showed consistent and homogeneous syncytial phenotype after five rounds of plaque purification; this virus was designated Fu-10. Viral stocks of Fu-10 and G207 were prepared by infecting Vero cells at 0.01 pfu/cell. Cells were harvested when either a complete CPE (Fu-10) or complete syncytial formation (Fu-10) was observed across the entire culture flask. Cells were harvested 48 h later for trypsinization. The number of surviving cells was counted on a hemocytometer after trypan blue staining. The percentage of cell survival was calculated by dividing the number of viable cells from the infected well by the number of cells from the well that was left uninfected. The experiments were done in triplicate, and the averaged numbers were used for the final calculation.

Animal Experiments. Six-week-old male Hsd athymic (nu/nu) mice were purchased from Harlan (Indianapolis, IN). MDA-MB-435 cells were cultured in standard conditions and were harvested in log phase with 0.05% trypsin-EDTA. The cells were washed twice with serum-free medium before they were resuspended in PBS at a concentration of 4 × 10⁶ cells/ml for establishing lung metastatic breast tumors, a procedure of orthotopic inoculation of breast tumor cells was adapted from published work (31) with modifications. To increase the chance of tumor metastasis to the lung, mice were bilaterally injected with 4 × 10⁶ MDA-MB-435 cells (in a volume of 100 μl) into the fat pad of the secondary mammary. Eleven weeks after tumor cell implantation, the breast tumors in the originally injected mammary fat pad that had grown to extremely large sizes (~1.5 cm in diameter) were surgically removed. The mice were randomly divided into three groups of five animals. One week after the surgical operation, one group of mice received i.v. injection of 5 × 10⁶ pfu of Fu-10 in 200 μl volume through the tail vein; the second group of mice received the same dose of G207; mice in the third group were injected with 200 μl of PBS. A repeat injection was given 1 week later. Four weeks after viral injection, the animals were sacrificed, and pulmonary tumor nodules were counted after infusing the lungs with India ink (32).

RESULTS

Isolation of an Oncolytic HSV Syncytial Mutant. The well-characterized oncolytic HSV G207 was subjected to random mutagenesis through incorporation of the thymidine analogue BrdUrd during its replication in Vero cells. The mutated virus stock was then screened for the ability to confer a syncytial phenotype on infection of Vero cells. Plaques that were predominately formed from syncytial formation were collected, and one isolate, designated Fu-10, consistently showed a strong syncytial phenotype after a few consecutive passages. This virus was then purified to homogeneity through mul-

Growth Characterization of Fu-10. Triply seeded Vero cells in 48-well plates were infected with either Fu-10 or G207 at 0.1 or 0.01 pfu/cell for 1 h. Cells were washed once with a saline-glycine solution (pH 3.0) to remove unabsorbed and uninternalized viruses, and then washed twice with PBS. Cells were harvested at 0, 12, 24, 36, and 48 h after infection. The cells and supernatant in each well were harvested together and were subjected to freeze/thaw and sonication. The virus titers were titrated on Vero cells by a plaque assay. For the quantification of virus release, the supernatant and cells were harvested and titered separately. The percentage of virus released was calculated by dividing the amount of virus in the supernatant by the total amount of the virus (supernatant plus cell-associated virus).

Determination of ICP6 Mutation through PCR Analysis. The presence of the inserted lacZ cassette in the ICP6 gene in Fu-10 was verified by PCR amplification. Virus DNA was extracted from purified virions according to our method published previously (29, 30). PCR was performed according to the protocol from GeneAmp XL PCR kit (Perkin-Elmer, Branchburg, NJ). The PCR reactions were carried out in a total volume of 20 μl containing 6 μl of 3.3X buffer, 1 μl of DNA sample, 1.2 mm MgCl2, 800 μM deoxynucleotide triphosphate, 0.5 μM primers, and 0.4 μl Taq DNA polymerase. The amplification was performed in two steps using a PTC-2000 thermal cycler (MJ Research, Watertown, MA): for the first 17 cycles, denaturation at 94°C for 45 s and annealing/extension at 68°C for 3 min; for the last 13 cycles, the annealing/extension was increased incrementally by 15 s/cycle. PCR primers were designed from sequences of the ICP6 gene flanking the site of the lacZ insertion: 5′-AGTATGTAGCGAGCCTGTG (forward) and 5′-CCGTTGCTGTCGTTGATC (reverse). The PCR amplification was expected to result in a product that was either 3.3 kb (indicating the presence of lacZ gene in the correct location in ICP6) or 250 bp (without the lacZ gene insertion). To Vero Oncolytic Assay. Each of the five human tumor cell lines were seeded into 48-well plates and were infected with either Fu-10 or G207 at 0.1 and 0.01 pfu/cell, or were left uninfected. Cells were harvested 48 h later for trypsinization. The number of surviving cells was counted on a hemocytometer after trypan blue staining. The percentage of cell survival was calculated by dividing the number of viable cells from the infected well by the number of cells from the well that was left uninfected. The experiments were done in triplicate, and the averaged numbers were used for the final calculation.

...on the initiation of viral DNA replication, because blocking the fusion pattern and plaques to develop. To block HSV replication, ACV was added into the culture medium at a final concentration of 100 μM. Photos of the infected cells were taken at different time points after infection.
multiple rounds of plaque purification from which 100% of plaques displayed a syncytial phenotype.

To confirm that Fu-10 maintained the lacZ insertional mutation in the ICP6 gene, we designed a pair of primers to anneal to ICP6 sequences that flanked the inserted lacZ gene cassette. PCR amplification using these primers generated identical DNA fragments of ~3.4 kb from both Fu-10 and parental G207 DNA (Fig. 1), indicating the lacZ insertion present in G207 had been maintained in the Fu-10 mutant.

**Phenotypic Characterization of the Syncytial Fu-10 Mutant.**

To characterize phenotypically the newly isolated oncolytic virus, Vero cells were infected with either G207 or Fu-10 at 0.001 pfu/cell. As shown in Fig. 2, the syncytial phenotype of plaques after Fu-10 infection was strikingly different from the ordinary plaques derived from infection of G207. By 24 h after infection, the infection foci of G207 were relatively small and were composed mainly of round cells that are characteristic of a standard HSV infection. In contrast, the plaques from Fu-10 infection were composed entirely of cells that were fused together, such that the boundaries of individual cells were almost invisible. Each plaque from Fu-10 infection covered an area equivalent to several hundred cells, in sharp contrast to those from G207 infection in which substantially fewer cells were involved. By 48 h, the infection foci from G207 infection had gotten larger, but by 72 h, the monolayer still had not reached 100% CPE. However, the cells infected by Fu-10 displayed a markedly different phenotype; by 48 h, the cells in the entire dish were fused together (and appeared like a “sheet of paper”), and by 72 h, the cells appeared contracted (resembling a “fishing net”). These data showed that Fu-10 was phenotypically different from the parental G207 virus and, because of its additional cell membrane fusion feature, had a much stronger cell-killing ability than G207 when tested in Vero cells.

The safety of the oncolytic G207 virus has been well documented (33–35); retaining this safety profile of G207 is mandatory for additional potential therapeutic applications of Fu-10. Because BrdUrd-mediated random mutagenesis generally results in multiple mutations per viral genome, it was possible that Fu-10 may have acquired additional altered viral properties, such as the reversion of its ability to replicate in normal nondividing cells. To address this particular concern, we compared the replication ability of G207 and Fu-10 in normal human cells of either quiescent or cycling state. Primary human fibroblasts were plated in 12-well plates in duplicate. One set was treated with 20 μM of lovastatin, a drug that induces cell-cycle arrest but does not interfere with HSV replication (36). Both arrested and untreated (i.e., cycling) cells were then infected with Fu-10, G207, or w.t.17 at 1 pfu/cell and harvested at 36 h after infection. As expected, replication of w.t.17 in arrested primary fibroblasts was only slightly affected when compared with that in unarrested cells (Fig. 3). In contrast, replication of G207 was reduced more than one log of magnitude when the cell cycle was arrested. This was even

![Fig. 1. Detection of the lacZ insertional mutation in ICP6. Fu-10 or G207 DNA was subjected to PCR analysis; the 3.4-kb PCR product, corresponding to the insertion of lacZ in the ICP6 gene, is indicated by the arrow. PCR products were electrophoresed on a 0.8% agarose gel with HindIII-digested A DNA (Marker); sizes of the bands are indicated on the right in kilobases (kb).](image1)

![Fig. 2. Phenotypic characterization of Vero cells infected with Fu-10. Vero cells were infected with either Fu-10 or G207 in the presence or absence of ACV in the culture medium. Photos were taken at 24, 48, or 72 h after initial virus infection, as indicated; the photos of cells in the presence of ACV were taken 72 h after infection.](image2)

![Fig. 3. Replication of Fu-10 is severely inhibited in nondividing human embryonic fibroblasts. Human embryonic fibroblasts (HF 333.We) were seeded duplicately into 12-well plates at 1 × 10⁵ cells/well. One set of cells was treated with 20 μM lovastatin in serum-free medium for 30 h. Both untreated and the lovastatin-arrested cells were infected with the indicated virus at 1 pfu/cell. Viruses were harvested 36 h after infection and quantified by plaque assay on Vero cells. The figures represent the average of two independent experiments; bars, ± SD.](image3)
more apparent for Fu-10, in which virus replication was reduced more than three logs of magnitude in the lovastatin-treated cells. These results demonstrate that Fu-10 has retained the selective replication property of the original G207 virus.

Earlier studies have shown that the phenotype of the majority of syncytial HSV-1 mutants is attributable to aberrant expression of glycoproteins (24–27), which are expressed during late-stage viral infection, and, therefore, their expression depends on the initiation of viral DNA synthesis. To test if the syncytial phenotype of Fu-10 also required the initiation of viral DNA replication, we blocked viral DNA synthesis with ACV, a strong inhibitor of HSV replication. Inclusion of ACV in the culture medium efficiently blocked the CPE and therefore killing–is restricted to tumor cells.

**Growth Characterization of Fu-10.** We next compared the replicative abilities of G207 versus Fu-10. Vero cells were infected with the viruses at either 0.1 or 0.01 pfu/cell, and harvested at 12-h intervals after infection. The viruses harvested at each time point were titrated by plaque assay. A, Vero cells were sparsely seeded in 10-cm Petri dishes (1 × 10^3 cells/dish) and infected with the indicated viruses at 0.1 pfu/cell, and harvested 48 h after infection; bars, ± SD.

![Fig. 4. Comparison of growth curves of Fu-10 and G207.](image)

A and B, Vero cells were seeded in normal density and infected with Fu-10 or G207 at 0.1 pfu/cell (A) or 0.01 pfu/cell (B). The viruses were harvested at 12-h intervals after infection and were titrated by plaque assay. C, Vero cells were sparsely seeded in 10-cm Petri dishes (1 × 10^3 cells/dish) and infected with the indicated viruses at 0.1 pfu/cell, and harvested 48 h after infection; bars, ± SD.

Inclusion of ACV in the culture medium efficiently blocked the CPE and therefore killing–is restricted to tumor cells.

**Comparison of in Vitro Tumor Cell Killing by Fu-10 versus G207.** We next assessed the syncytial phenotype of Fu-10 in a panel of human tumor cells that varied in their tissues of origin: A-549 (lung), DU 145 (prostate), HepG2 (liver), MDA-MB-435 (breast), and U-87 MG (glioblastoma). Fu-10 showed a clear syncytial phenotype in all five of the tumor cell lines tested, although the degree and pattern of the cell fusion varied from cell type to cell type (Fig. 5, top panels). Fu-10 showed the highest fusogenic efficiency in A-549 (lung cancer) and HepG2 (liver cancer) cells, as assessed by both the intensity of fusion and the number of cells that were fused together in each of the infection foci. Infection of Fu-10 resulted in a modest syncytial formation in MDA-MB-435 (breast cancer) cells, and only.

![Fig. 5. Phenotypic characterization of Fu-10 in tumor cells. Tumor cells of different origins were infected with either Fu-10 or G207 at 0.001 pfu/cell and photographed 48 h after infection. Cell lines: A-549 (non-small cell lung carcinoma); DU 145 (prostate); HepG2 (hepatocellular); MDA-MB-435 (breast); and U-87 MG (glioblastoma).](image)
a minor phenotype in DU 145 (prostate cancer) and U-87 MG (glioblastoma) cells. Infection of G207, on the other hand, did not cause noticeable syncytial formation in any of these tumor cells (Fig. 5, bottom panels).

To additionally characterize the ability of Fu-10 to destroy these tumor cells, we infected the cells with either Fu-10 or G207 at a relatively low multiplicity of infection (0.1 and 0.01 pfu/cell), which assesses both the inherent cytotoxicity of the input virus as well as the ability of the virus to replicate and spread in these cells. The cytotoxic effect of the virus infection on the tumor cells was quantified by calculating the percentage of cells that survived after the virus infection. In general, Fu-10 had a significantly stronger ability to kill these tumor cells than G207; this was particularly evident at an infection dose of 0.1 pfu/cell (Fig. 6A).

Notably, there was a strong correlation between the intensity of syncytial formation (observed in the experiment presented in Fig. 5) and the degree of cytotoxic effect on the individual tumor cells during Fu-10 infection. For example, Fu-10 displayed the strongest killing effect in A-549 and HepG2 (Fig. 6, A and B); these two cell lines also had the most extensive syncytial formation (Fig. 5). Remarkably, even at an infection dose of 0.01 pfu/cell, Fu-10 completely obliterated the number of HepG2 cells in the well, whereas >50% of cells survived the same dose infection of G207. The other three tumor cell lines appeared relatively less sensitive to Fu-10 infection. In MDA-MB-435 and DU 145 cells, where only a modest syncytial formation was seen, twice as many cells were killed in the well infected with Fu-10 as compared with the cells infected with G207 when the viruses were used at a dose of 0.1 pfu/cell. Fu-10 showed a marginally better cell killing than G207 in the U-87 MG line when the cells were infected at 0.1 pfu/cell (28% versus 44% cell survival) but did not show any detectable cytotoxic effect when a lower dose (0.01 pfu/cell) was used.

**Therapeutic Effect of Fu-10 on Metastatic Breast Cancer.** To test the systemic antitumor effect of Fu-10, a metastatic lung model was established through orthotopic implantation of tumor cells in nude mice. Six-week-old nude mice were bilaterally injected with $4 \times 10^6$ MDA-MB-435 cells in the fat pad of the secondary mammary. The mice were left for 12 weeks to allow lung metastasis to develop before they received the first injection of $5 \times 10^6$ pfu of Fu-10 or G207, or PBS only as a control. A repeat injection with the same dose of the viruses was given 1 week later. Four weeks after the initial virus administration, the lungs were removed and examined for metastatic tumors. As shown in Fig. 7, in the negative control group where only PBS was administered, the bilateral inoculation of MDA-MB-435 led to widespread metastatic tumor nodules all over the lungs. Each mouse from this control group had, on the average, 69.2 metastatic nodules on the surface of the lung (Table 1). Systemic administration of the G207 virus resulted in a significant reduction in the number of nodules compared with the PBS control (22.2 versus 69.2 nodules, respectively; $P < 0.01$). Strikingly, the antitumor effect from the administration of Fu-10 was even greater than that of G207; the average number of tumor nodules displayed by Fu-10 injected mice was only 2.6/animal. No tumor nodules were visible in the lungs from two of five mice in the group where Fu-10 was administered; the other three animals showed a greatly reduced number of metastatic tumor nodules (Table 1). Although there was one mouse from G207 injection group that appeared tumor-free in the lung, on the average, there were still >22 tumor nodules in lungs from each mouse in this group. These results demonstrate that, in correlation with the *in vitro* studies, Fu-10 has a significantly enhanced oncolytic effect on disseminated metastatic breast cancer after being systemically injected into experimental animals.

**DISCUSSION**

A variety of strategies have been explored to improve the potency of oncolytic HSV. For example, the combined administration of oncolytic HSV with radiotherapy and chemotherapy can result in a synergistic antitumor effect (4, 37, 38). In addition, insertion of immune modulator genes such as IL-4, IL-12, and B7.1, which function by boosting the antitumor immunity of the body after virus mediated oncolysis, into the viral genome has also substantially increased the antitumor efficacy of the oncolytic virus (39–41).
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Table 1 Number of lung tumor nodules after oncolytic HSV therapy

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<thead>
<tr>
<th>Mouse no.</th>
<th>Number of lung metastatic tumor nodules</th>
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<td>Fu-10</td>
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<td>1</td>
<td>3</td>
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<td>2</td>
<td>9</td>
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*P < 0.05 compared with control group.
†P < 0.05 compared with G207 group.

The safety of the parental G207 virus has been well established in animal experiments. Several oncolytic HSV agents (22, 44), it is pivotal that these molecules be efficiently materialized—an issue that has not been properly addressed. The discovery of a syncytial mutant of oncolytic HSV may bring clear clinical benefits to cancer patients, especially for those with metastatic diseases.

ACKNOWLEDGMENTS

We thank Dr. Malcolm Brenner for his continuous support and helpful discussions, and C. Ma for technical support.

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*Cancer Res* 2002;62:2306-2312.