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 oncology 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 nontdividing 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 nontdividing cells (7, 8). The oncolytic HSV-1 virus G207, which has been extensively tested in animal studies and is currently in clinical trials, has been constructed using a combination of these two approaches. This virus contains deletions in both copies of the 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 nontdividing 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 leukaemia 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, 22). 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-
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 cells 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 desirable 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% FBS (purchased from Harlan (Indianapolis, IN). MDA-MB-435 cells were cultured in standard conditions and were harvested in log phase with 0.05% trypsin.

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.

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 mutagenized virus stock was then screened for the ability to conferr 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-
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
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 from both viruses, as well as the syncytial formation from infection of Fu-10 (Fig. 2, panel ACV), indicating that the efficient cell membrane fusion from Fu-10 requires HSV DNA replication. These results indicate that Fu-10 retains the safety properties of the parental G207 virus, in that syncytial formation— 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. At a dose of 0.1 pfu/cell, the titer of Fu-10 was approximately five times higher than the titer of G207 at early time points before complete CPE occurred (Fig. 4A). At a lower dose (0.01 pfu/cell), Fu-10 grew to at least a 10-fold higher titer than G207 at all of the time points examined (Fig. 4B). The difference in the titer was even more obvious at later time points, when the titer from Fu-10 was almost 100 times higher than the virus titer from G207 (Fig. 4B). These results indicate that Fu-10 has an enhanced ability to replicate relative to G207.

To determine whether the syncytial formation after Fu-10 infection directly contributed to the enhanced virus replication seen in Fig. 4, A and B, we compared the replication of G207 versus Fu-10 in an experimental setting where the Vero cells were sparsely seeded in 10-cm dishes (1 × 10^4 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. 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 × 10^4 cells/dish) and infected with the indicated viruses at 0.1 pfu/cell, and harvested 48 h after infection; bars, ± SD.
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 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 administrated, 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). How-

![Fig. 6. Comparison of the cytotoxic ability of Fu-10 and G207 on tumor cells in vitro.](Image)

![Fig. 7. In vivo antitumor effect of Fu-10 and G207 in a breast cancer lung metastasis model.](Image)
Table 1 Number of lung tumor nodules after oncolytic HSV therapy

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Fu-10</th>
<th>G207</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>29</td>
<td>77</td>
</tr>
<tr>
<td>2</td>
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<td>36</td>
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<td>0</td>
<td>10</td>
<td>35</td>
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<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>82</td>
</tr>
<tr>
<td>Mean</td>
<td>2.6±b</td>
<td>22.2±</td>
<td>69.2</td>
</tr>
</tbody>
</table>

* P < 0.01 compared with control group.
* P < 0.05 compared with G207 group.

ever, attempts to increase the oncolytic potency of the virus itself, by strategies such as combining the virus with a prodrug converting enzyme, have been less successful (see, for example, Ref. 42). This is probably because the potential enhancement of antitumoral effect from prodrug activation is offset by the inhibitory action of GCV on replication.

In the current study, we have incorporated a membrane fusion function into the well-characterized oncolytic HSV G207. The new oncolytic HSV has demonstrated a dramatically enhanced antitumoral efficacy in both in vitro and in vivo animal experiments. Several advantages of such a combined strategy may have contributed to this enhanced synergistic antitumoral activity. First, unlike the HSV-thymidine kinase/GCV therapy, which can reduce oncolytic virus replication, syncytial formation seems to be a natural part of HSV infection. Therefore, the process of cell membrane fusion is predicted not to interfere with virus replication. However, our data have indicated the opposite: acquiring the syncytial phenotype may have increased virus replication and release. Second, unlike in vitro cultured tumor cells that are relatively homogeneous, many tumors contain cells of different lineages in which some may be resistant to HSV infection because of, for example, lack of viral receptors on the cell surface. The combined antitumor action from two completely different mechanisms (direct viral oncolysis and cell membrane fusion) should significantly reduce the occurrence of virus-resistant tumor cells, because those cells that become resistant to oncolytic virus infection/replication may be indirectly destroyed by syncytial formation. In support of this, our studies on six cells of different species and origins have indicated that the Fu-10-mediated syncytial formation can occur in a very wide range of cells. Additionally, although oncolytic viruses can rapidly spread in cultured tumor cells, viral spread within a solid tumor mass is often limited (43). Both antiviral immunity and the relatively large size of the viral particles may contribute to the inefficiency of viral spread in vivo. The bystander effect from the fusogenic function of Fu-10 infection, which is achieved by the fusion of Fu-10 infected cells and the surrounding noninfected tumor cells, will undoubtedly additionally increase the oncolytic efficacy. Lastly, although recent publications have shown that certain viral glycoproteins such as gibbon ape leukemia virus envelope glycoprotein can cause efficient tumor cell membrane fusion and, therefore, represent potentially attractive antitumor agents (22, 44), it is pivotal that these molecules be efficiently and specifically delivered into the tumor mass before their antitumor potential can be materialized—an issue that has not been properly addressed. The discovery of a syncytial mutant of oncolytic HSV may have solved this problem.

The safety of the parental G207 virus has been well established in animal models. For example, no serious toxicity was found when up to 1.5 × 10^7 pfu of G207 virus was injected into the brain of HSV-sensitive mice (33). In primates (Aotus), the virus was found safe when it was directly injected into the animal brain at doses as high as 1 × 10^7 pfu (34). In clinical trials, a moderate dose of G207 is also well tolerated by patients (35). Although the enhanced potency of this new oncolytic HSV will undoubtedly bring potential clinical benefit, the importance of retaining the original safety properties of G207 is paramount. There is no doubt that uncontrolled syncytial formation from an oncolytic HSV, especially delivered systemically, will cause undesirable side effects. Our demonstration that blocking viral DNA replication in Fu-10 infected cells completely abolished its syncytial-forming ability strongly suggests that Fu-10 retains the safety profile of G207. In addition, i.v. injection of Fu-10 in a relatively large dose was also well tolerated in mice, indicating that the new virus is not noticeably more toxic than the parental G207 virus.

An interesting and potentially significant observation regarding Fu-10 is its increased ability to be released into culture medium. More than 90% of Fu-10 was released from cells, which is in sharp contrast to G207 in which only a small fraction (<1%) was released into the culture medium. Although low virus release from infected cells is common for HSV type 2, during HSV-1 infection, usually ~50% of mature virions are released into the medium. Although it is unclear why G207 has such a low level of release and how the cell membrane fusion from Fu-10 has changed the viral release pattern, a relevant implication for its increased oncolytic potency is that the enhanced virus release may have partly contributed to a more widespread diffusion of the virus in the tumor masses.

Studies on oncolytic HSV in immune-competent animals have shown that destruction of tumor cells by viral replication frequently elicits significant antitumor immunity, which may contribute, at least in part, to the final outcome of the therapy (10). Because our in vivo therapeutic experiment was done in immune-deficient nude mice, the enhanced therapeutic effect on the metastatic breast cancer from Fu-10 probably arises entirely from the virus infection itself (i.e., the combined action of viral replication and cell membrane fusion in the tumor mass) and not from contributions of the host antitumor immune response. These results indicate that merely improving the design of oncolytic viruses such as Fu-10 may have the potency to destroy metastatically established tumors.

One of the major limitations facing the administration of viral vectors including oncolytic HSV is that their in vivo administration frequently elicits a strong antivector immune response that can substantially reduce the transduction efficiency of subsequent administration of the same vectors (45, 46). This is especially disadvantageous when systemic and repeated delivery is required for the therapeutic application (47). Therefore, it is likely that in immune-competent animals, the therapeutic benefit of the treatment strategy in our experiment will likely be heavily affected. We are also currently developing novel strategies such as delivering oncolytic HSV through liposome formulation of cloned HSV in the bacterial artificial chromosome (48). It is expected that repeated delivery of a potent oncolytic HSV through strategies that allow repeated administration will bring clear clinical benefits to cancer patients, especially for those with metastatic diseases.

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