Oncolytic Vesicular Stomatitis Virus for Treatment of Orthotopic Hepatocellular Carcinoma in Immune-competent Rats

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INTRODUCTION

HCC is the third leading cause of cancer deaths in the world, accounting for over 1 million cases annually. In the United States, its incidence has increased from 1.4 (1976–1980) to 2.4 (1991–1995) per 100,000, which may be related to an increase in chronic hepatitis C virus infection, with an estimated 16,600 new cases and 14,100 deaths in 2002 (1–3). The prognosis of untreated HCC is poor. For patients who are not candidates for surgical resection or local-regional therapies, 1- and 3-year survival rates are 44% and 0%, respectively (4). The current treatment modalities for HCC with demonstrated survival prolongation are hepatic resection and loco-regional intratumoral ablation procedures for solitary tumors. For patients with multifocal tumors localized to the liver, regional approaches such as orthotopic liver transplantation offer long-term remission with 3-year survival rates of 39–57% (5, 6). However, the supply of livers available for transplantation is very limited, and patients often face waiting periods of over 1 year. Therefore, HCC is a disease where only a small proportion of patients are candidates for hepatic resection, local ablation, or orthotopic liver transplantation, and despite available treatments, prognosis of the patients remains poor. With an estimated 3.9 million Americans infected by hepatitis C virus to date and the expected increase in HCC patients over the next 20 years, development of more effective treatment modalities is urgently needed.

Tumor-targeted replication-competent viruses represent a class of novel agents for cancer therapy. In addition to adenovirus, HSV, and influenza virus that are molecularly engineered to replicate specifically in tumor cells, viruses with inherent tumor specificities are actively being developed as oncolytic agents for cancer treatment. This group of viruses includes reovirus, autonomous parvoviruses, Newcastle disease virus, measles virus, and VSV (7). VSV is an enveloped, negative-strand RNA virus (Rhabdoviridae family) that infects a wide variety of mammalian and insect cells. Infections in humans are asymptomatic in most cases or result in a mild febrile illness (8). Recently, Stojdl et al. (9) demonstrated that VSV replicated much more efficiently in tumor cells than in normal cells in vitro, and the difference was even more striking in the presence of IFN-α. Furthermore, treatment of human melanoma xenografts with VSV in nude mice resulted in regression or growth inhibition of the established tumor (9). This finding has been postulated to be due to the fact that IFN-responsive antiviral pathways are defective in many tumors, and thus VSV can replicate within these cells even after exogenous IFN treatment while leaving normal cells relatively unaffected (10). Compared with other replication-competent oncolytic vectors, VSV is particularly appealing as its rapid replication rate (8–10 h in tumor cells), such that antitumor effects would be expected within hours of injection, and significant tumor destruction would have occurred before the initiation of any potentially neutralizing antiviral immune responses. In this study, we have evaluated a rVSV-GFP vector as a potential novel therapeutic agent for therapy of orthotopic HCC in an immune-competent animal model.

MATERIALS AND METHODS

Cell Culture. The human HCC cell lines Hep 3B and Hep G2 and the rat HCC cell line McA-RH7777 were obtained from American Type Culture Collection (Manassas, VA). Human HCC cell lines were maintained in MEM Eagle (Mediatech, Herndon, VA) and supplemented with 10% FBS (Sigma-Aldrich, St. Louis, MO). The rat HCC cell line McA-RH7777 was maintained in 10% FBS-DMEM (Mediatech). Primary human and rat hepatocytes were purchased from Biowhittaker (Walkersville, MD) and maintained on Matrigel (Becton Dickinson, Bedford, MA) and in medium as suggested by the manufacturer. Primary cells were infected with the virus 24 h after thawing. The BHK-21 cell line (American Type Culture Collection; CCL-10) used for production of VSV was maintained in 10% FBS-DMEM (Mediatech).

Viruses and Infection Assays. rVSV encoding GFP was generated using the established method of reverse genetics (11, 12). With this system, rVSV expressing additional transcriptional units can be rescued, and expression of heterologous proteins can be achieved (13, 14). Briefly, individual plasmids encoding T7 promoter-driven VSV nucleocapsid (N), phosphoprotein (P), and polymerase (L) [kind gifts from Dr. Michael Schnell (Thomas Jefferson University)] were transfected into BHK-21 cells previously infected with vTF-7.3 [kindly provided by Dr. Bernard Moss (NIH, Bethesda, MD)], a
recombinant vaccinia virus that expresses T7 RNA polymerase. In addition, a plasmid encoding the full-length antigenomic VSV RNA with insertion of a GFP transcription unit into the 3’ noncoding region of the VSV-G gene (kind gift from Dr. John Rose (Yale University)) was also cotransfected into the cells. After successful recovery of rVSV-GFP, vaccinia virus was completely eliminated by plaque purification. The titers of viral stocks were determined by plaque assay on BHK-21 cells.

For virus infection assays, 1 x 10^5 cells/well were plated in 6-well plates and then incubated with rVSV-GFP at a MOI of 0, 0.0001, 0.001, 0.1, or 1 in 1 ml of PBS supplemented with 1 mM CaCl_2 and 1 mM MgCl_2 for 30 min at room temperature. At the end of the incubation period, the virus was removed, and the cells were maintained in normal growth medium. At the indicated time points after infection, cells were monitored for cytopathic effects by bright-field microscopy, GFP expression was analyzed by fluorescence microscopy, and cell lysis was confirmed by staining with ethidium homodimer-2 (1:2000; Molecular Probes, Eugene, OR). In addition, a sample of cell culture supernatant was assayed for viral RNA genome by real-time RT-PCR using specific primers. Forty-eight h after infection, cells were fixed and stained with 0.1% crystal violet in 20% ethanol for 15 min, followed by washing with tap water to remove excess color. The plates were dried, and images were captured with a Sony DSC-F707 digital camera (Sony, Tokyo, Japan). All experiments were performed in triplicates.

**Real-Time RT-PCR.** Synthetic genomic VSV RNA was transcribed from the full-length pVSV-GFP plasmid (15) by using the T7 RNA polymerase (New England Biolabs, Beverly, MA) under the conditions recommended by the supplier. The DNA template was eliminated by extensive treatment with RNase-free DNase (Invitrogen, Carlsbad, CA). After purification and concentration, the RNA amount was estimated by spectrophotometer, and known amounts were used to determine the standard curve for real-time RNA quantification. Cells were infected with rVSV-GFP, and virus present in the supernatant was collected at the indicated time points. Cell debris was eliminated by low-speed centrifugation, and viral RNA was extracted according to the manufacturer’s instructions from 140 µl of supernatant by using the QIAamp Viral RNA kit (Qiagen, Valencia, CA). The forward (5’-TTC-GCAAGTGATGCTAAGTC-Ag-3’) and reverse primers (5’-AGGACTT-GGATATGCAGTACC-3’) hybridize at positions 1298–1318 (VSV-N gene) and 1414–1435 (VSV-P gene), respectively, in genomic sense RNA, generating a 138-nucleotide DNA fragment spanning one intergenic region. The TaqMan probe (5’-ACAAATGACCCCTATAATTCTCAGATCACC-3’) containing a fluorescent reporter dye (6-fluorescein-phosphoramidite) at the 5’ end and a fluorescent quencher dye (tetramethylrhodamine) at the 3’ end was synthesized by Gene Link (Hawthorne, NY). The real-time RT-PCR was carried out with the QuantiTect Probe RT-PCR kit (Qiagen) using the LightCycler system (Roche, Mannheim, Germany).

**Animal Studies.** All procedures involving animals were approved by and performed according to guidelines of the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine. Male Buffalo rats (6–7 weeks of age; Harlan, Indianapolis, IN) received orthotopic implant of 4 x 10^6 syngeneic McA-RH7777 rat HCC cells/20 µl Hanks’ solution. Fourteen days after tumor cell implantation, rVSV-GFP (1 x 10^6 pfu/100 µl) or an equivalent volume of buffer was injected intratumorally. To assess tumor response and intratumoral virus spread and replication, animals were euthanized at various time points after vector injection, and livers were removed for examination. In addition, groups of animals were followed for survival.

**Recovery of Virus from Tumor and Tissue Extracts.** Tumors and normal liver tissues were harvested and disaggregated under sterile conditions. The amounts of tissue were measured, and a known amount of tissue was homogenized and then subjected to a series of low-speed centrifugation steps. The supernatant was collected and used for real-time RT-PCR analysis.

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**Fig. 1.** VSV genomic RNA replication in infected HCC cell lines and primary hepatocytes in vitro. Increase in viral genomic RNA in cultures of rVSV-GFP-infected rat (McA-RH7777) and human (Hep 3B and Hep G2) HCC cell lines and primary rat and human hepatocytes at the indicated time points. The cell line BHK-21 is shown as positive control. MOIs used for all target cells were as follows: ■, MOI = 0; ●, MOI = 0.0001; ○, MOI = 0.001; □, MOI = 0.1; ○, MOI = 0.1; and ○, MOI = 1.
The suspension was centrifuged at low speed to remove cellular debris, and the supernatants were used to perform plaque assays on BHK-21 cells (sensitivity, 25 pfu/mg).

**Histology and IHC for VSV-G.** Whole livers were harvested and fixed in 4% paraformaldehyde overnight, and paraffin-embedded sections were subjected to either H&E staining for histological analysis or IHC using a monoclonal antibody against the VSV-G protein (Alpha Diagnostic, San Antonio, TX). IHC sections were counterstained with hematoxylin.

**Intratumoral GFP Expression Analysis.** Tumors were harvested 16 h after injection of rVSV-GFP or buffer control and fixed in PBS solution.
RESULTS

Oncolytic Activities of rVSV-GFP in HCC Cells in Vitro. To assess whether rVSV-GFP has the ability to preferentially replicate in and kill HCC cells, the rat (Mca-RH777) and human (Hep 3B and Hep G2) HCC cell lines, as well as nontransformed primary rat and human hepatocytes, were examined in virus infection assays. BHK-21 cells were included as positive controls. Cells were infected with the virus at various MOIs, and the supernatants were harvested at various time points after infection. Total RNAs from the culture supernatants were prepared, and the RNA samples were analyzed for the presence of genomic VSV RNA by real-time RT-PCR. The results showed that rVSV-GFP is fully capable of replicating its RNA genome in rat and human HCC cells and reached plateau within 24–48 h of inoculation, but its ability to do so is severely attenuated in normal rat and human hepatocytes (Fig. 1).

Next, we examined the ability of rVSV-GFP to induce lytic infections in rat and human HCC cells versus normal hepatocytes in vitro, again using BHK-21 cells as positive controls. The cells were infected with rVSV-GFP at various MOIs and examined for cytopathic effects by bright-field microscopy and for GFP expression by fluorescence microscopy 24 h later. In addition, cell lysis was confirmed by staining with ethidium homodimer 2 (Fig. 2A). Rat and human HCC cells transduced with rVSV-GFP at MOIs as low as 0.0001 showed typical VSV cytopathic effects, whereas the morphology of normal liver cells transduced with the virus at MOIs as high as 1 was unchanged. Additionally, infected HCC cells at all MOIs uniformly expressed GFP, whereas no expression was detected in transduced normal hepatocytes. Infection with rVSV-GFP at all MOIs also led to complete lysis of rat and human HCC cells as indicated by positive staining with ethidium homodimer 2, whereas normal hepatocytes were refractory. To confirm that these observed effects culminated in cell death, crystal violet staining was performed on HCC cell lines 48 h after infection (Fig. 2B). All HCC cells became nonviable and completely floated off from the tissue culture plates at infectious VSV doses as low as MOI = 0.0001.

Antitumor Activities of rVSV-GFP in Orthotopically Implanted HCC in Immune-competent Rats. To investigate the potential of rVSV-GFP for therapy of HCC, we tested the virus in an orthotopic solitary tumor model of HCC in immune-competent Buffalo rats. Syngeneic Mca-RH777 HCC cells (4 × 10⁶ cells/20 µl Hanks’ solution) were implanted directly into the left lateral lobe of the liver. Fourteen days later, when tumors reached 13–18 mm in diameter, 1 × 10⁶ pfu of rVSV-GFP in 100 µl or an equivalent volume of buffer were injected intratumorally. The survival curves of rats in both groups are shown in Fig. 3A, and the difference in survival between the two groups was statistically significant by log-rank test analysis (P < 0.016). Moreover, a single vector-treated tumor-bearing rat achieved long-term tumor-free survival. Histologically, no residual tumor was seen at autopsy 120 days after tumor cell implantation. Instead, abundant accumulation of ceroid pigment in foamy macrophages at the tumor implantation site was noted (data not shown).

In a separate experiment, sets of animals were sacrificed at different time points after VSV injection, and to measure tumor volumes, the livers were explanted and carefully studied to determine the actual dimensions of the tumor. The results indicated that tumor growth was significantly inhibited compared with control animals injected with buffer (Fig. 3B).

Selective Intratumoral Virus Replication and Spread. Groups of tumor-bearing animals were sacrificed at 30 min, 16 h, 3 days, and
7 days after vector injection to evaluate viral replication and spread within the tumors. To quantitatively determine the viral titers in tumor and normal surrounding liver tissues, the tissues were harvested at each time point, mechanically lysed, and centrifuged to remove cellular debris, and the supernatants were used to perform plaque assays on BHK-21 cells (Fig. 4). There were increases of infectious virus yields by 2–3 orders of magnitude in the injected tumors at 16 h versus 30 min after vector injection, indicating the virus has successfully replicated in the solid tumor mass in vivo. Importantly, infectious virus yields in the normal liver were reduced by more than 1-log over the same time period, indicating that VSV replicates selectively in the tumor. The intratumoral virus yield diminished by 2 orders of magnitude after 3 days and to insignificant levels after 7 days, suggesting that intratumoral virus replication was suppressed after an initial burst in the immune-competent host.

In addition, sets of animals were sacrificed at the same time points to evaluate intratumoral virus spread and the corresponding tumor responses. The whole liver was explanted, and paraffin-embedded sections as well as frozen sections were obtained. Buffer-injected tumors showed only minimal necrotic areas and were negative for VSV-G protein by IHC (data not shown). Large necrotic areas were apparent in the hepatic tumors injected with rVSV-GFP, and on consecutive sections, multiple foci, which are close to necrotic areas, were positive for VSV-G protein by IHC (Fig. 5A). In contrast, the surrounding normal liver tissues were negative for the virus and any signs of pathology. There was also no significant elevation of transaminases in the sera of vector-injected animals at all time points after virus injection, indicating a lack of hepatotoxicity (data not shown). Additionally, tumors were harvested, and frozen sections were subjected to analysis for GFP expression by immunofluorescence (Fig. 5B). GFP expression was observed in tumors injected with rVSV-GFP, whereas only background expression was evident within the tumor of buffer-injected animals.

**DISCUSSION**

In the present study, we demonstrate efficient rVSV-GFP vector gene expression and replication in human and rat HCC cell lines. In contrast, normal human and rat hepatocytes are refractory not only for virus replication but also for viral gene expression. Defects in the IFN-α/β signaling pathway in several tumor cell lines have been shown to be responsible for tumor-specific VSV replication and cell
lysis (9). In particular, several human HCC cell lines were found to have an intrinsically poor ability to produce and respond to type I IFNs (16, 17). The present study focuses on the use of the vector as a controlled viral agent, which will permit comprehensive mechanistic studies in vivo in the future. PKR, a double-stranded RNA-dependent protein kinase, is a critical antiviral protein whose expression is activated by IFN-α/β signaling through the IFN receptor (18). Interestingly, RAS-transformed cells appear to have a defect in PKR activity. Viruses with mutations in the viral-encoded PKR inhibitory protein (such as ICP34.5, which is encoded by the HSV-1 or NS1 in influenza A virus) have been shown to replicate in tumor cells with overactive RAS, whereas in normal cells, PKR activity would limit the replication of these viruses (19, 20). The replication of reovirus and Newcastle disease virus has also been linked to RAS overactivity through unknown mechanisms (21, 22). Therefore, genetically engineered or even certain wild-type viruses can take advantage of alterations in the IFN/PKR or RAS/PKR pathway for tumor-specific replication.

A single intratumoral injection of rVSV-GFP into large HCC tumors showed potent antitumor activity. The decision to explore the effects of VSV in an immune-competent animal model of orthotopic HCC is based on several criteria. First, because VSV can efficiently replicate in rodent cells, in vivo studies with this type of vector can be tested in a syngeneic animal model, thereby allowing a realistic appraisal of potential efficacy in immune-competent patients. Second, the orthotopic locale more realistically recapitulates the liver environment and results in a locally aggressive, spontaneously metastatic tumor. The ability of rVSV-GFP to replicate intratumorally in vivo was demonstrated by IHC staining for VSV-G protein and by an increase in viral titer over time. These data indicate that intratumoral VSV replication led to tumor destruction, slower tumor growth, and survival prolongation of treated animals. These results are consistent with those reported previously by others using intratumoral injections of VSV against sarcoma, melanoma, and breast cancer-derived s.c. tumors in syngeneic mice (23, 24).

Intratumoral VSV replication was significantly reduced by day 3 after vector injection. The emergence of T-cell-independent neutralizing IgM antibodies occurs very early after infection, by day 3 or 4 (25, 26). Due to the rapid replication rate of VSV in tumor cells, however, significant oncolytic effects have manifested before the onset of neutralizing antiviral immune responses. The role of the immune system in oncolytic virus treatment is not completely clear. Some studies using HSV as an oncolytic vector suggest that immunosuppression improves viral oncology (27), whereas in others, the immune response contributes to the clearance of virally infected tumor cells and even produces antitumor immunity (28). Whereas a genetically engineered VSV vector has been reported that expresses an additional transcriptional unit encoding the interleukin-4 cytokine gene (24), the optimal strategy on how to modulate the immune system to achieve maximal VSV oncology has yet to be elucidated.

To determine whether VSV has tumor selectivity in vivo, we injected VSV intratumorally in HCC-bearing rats and evaluated viral distribution and toxicity of normal surrounding liver tissues versus the liver tumor. Importantly, no collateral damage to the normal liver was found, and viral replication and necrosis were restricted to the tumor. RNA viruses including VSV are known to have high spontaneous mutation rates (29). Therefore, expression of unselected foreign proteins in VSV vectors might be lost rapidly. It was demonstrated previously, however, that transgenes inserted into the noncoding region of the VSV-G gene were maintained stably over multiple passages in vitro (14). In our hands, there was neither loss of viral titer over multiple passages in vitro nor antitumor efficacy in vivo. In addition, there was no uncontrollable viral replication in the treated animals because viral replication was limited to the tumor bed and subsided to negligible levels after 7 days. We also did not observe any untoward events in the animals treated at the indicated vector doses.

In conclusion, VSV replication has been shown to be tumor selective in vitro as well as in vivo and produces significant destruction of large tumors before the onset of potentially neutralizing antiviral immune responses in the host, and a single intratumoral application of the virus can significantly prolong the survival of tumor-bearing animals. Hence, this oncolytic virus system warrants further development as a novel therapeutic option for the treatment of HCC in patients.

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