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Original Research Article

Vesicular Stomatitis Virus (VSV) Therapy of Tumors

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Summary

Vesicular stomatitis virus (VSV) is an essentially nonpathogenic negative-stranded RNA virus, the replication of which is extremely sensitive to the antiviral effects of interferon (IFN). We demonstrate here that VSV selectively induces the cytolysis of numerous transformed human cell lines in vitro, with all the morphological characteristics of apoptotic cell death. Importantly, VSV can also potently inhibit the growth of p53-null C6 glioblastoma tumors in vivo without infecting and replicating in normal tissue. With our previous findings demonstrating that primary cells containing the double-stranded RNA-activated protein kinase PKR and a functional IFN system are not permissive to VSV replication, these results suggest that signaling by IFN may be defective in many malignancies. Thus VSV might be useful in novel therapeutic strategies for targeting neoplastic disease.

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Keywords Interferon; oncolytic virus; PKR; tumor, VSV.

INTRODUCTION

Vesicular stomatitis virus (VSV),[1] the prototypic member of the family Rhabdoviridae, is an enveloped virus with a negative-stranded RNA genome that causes a self-limiting disease in livestock and is essentially nonpathogenic in humans (*1*). Its simple genetic composition, the fact that it encodes five gene products, and its ability to grow to high titers in most tissue culture cell lines have made it one of the most extensively characterized of all RNA viruses. These studies have made clear that, although most tissue culture cell lines appear permissive to VSV, the virus is extremely sensitive to the antiviral actions of the interferons (IFNs), a family of cytokines produced in response to infection, which act by inducing the expression of >30 cellular genes (*1–6*). The importance of IFN in innate immunity to VSV infection has been demonstrated in studies with mice rendered defective in type I IFN signaling. For example, mice lacking functional *IFNAR1* or *STAT1* genes are remarkably susceptible to lethal infection by VSV, as well as by many other types of virus (*3–5*). Despite these advances in our understanding of the importance of IFN in host defense, the critical IFN-induced genes responsible for inhibiting replication of virus, including VSV, remain largely unknown.

Recent data from our laboratory, however, have demonstrated that embryonic fibroblasts and mice lacking the IFN-inducible double-stranded RNA-dependent protein kinase, PKR, are extremely susceptible to VSV infection, confirming that this kinase is an essential and nonredundant component of antiviral host defense (*2*). That VSV is capable of replicating in a majority of mammalian cell lines, but not in primary cells unless PKR function or IFN signaling is defective, implies that critical host defense mechanisms required to prevent VSV replication are impaired in cells permissive to this virus, which includes nearly all immortalized and malignant cells.

In this study, we show that several human cancer cell lines undergo rapid cytolysis when infected by VSV. Pretreating the majority of these cells with IFN only partially protects them from VSV replication and cytolysis. We also show that VSV selectively and potently inhibits the growth of highly malignant p53-defective rat C6 gliblastoma tumors in a nude mouse model. These studies indicate that VSV could provide an attractive and effective therapy against malignant disease, especially when considering the nonpathogenic nature and genetic malleability of this well-characterized virus.

EXPERIMENTAL PROCEDURES

Cell Lines, Virus, and Reagents. The BC-1 cell line was a kind gift from Dr. William Harrington (University of Miami, Miami, FL). All other cell lines were obtained from the American

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[1] Abbreviations: FCS, fetal calf serum; IFN, interferon; pfu, plaque-forming units; PKR, double-stranded RNA-activated protein kinase; TUNEL, terminal deoxynucleotidyl transferase–mediated fluorescein isothiocyanate–dUTP nick-end labeling; VSV, vesicular stomatitis virus.

Type Culture Collection (Manassas, VA). HL 60 and K562 cell lines were maintained in RPMI supplemented with 10% fetal calf serum (FCS), and the BC-1 cell line was maintained in Iscove's modified Dulbecco's medium/10%FCS. All other cell lines were maintained in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FCS. Plaque-purified VSV (Indiana strain) was used in all experiments. Viral titers were determined by standard plaque assay of serially diluted virus samples on BHK-21 cells. Except where mentioned, all other reagents were obtained from Sigma Chemicals (St. Louis, MO).

Viral Infections and Cell Viability Analyses. Plaque-purified VSV was used to infect cells in serum-free medium for 30 min at 37 °C (*2*). After virus absorption, cells were washed twice in phosphate-buffered saline and subsequently incubated in complete medium for the indicated times. Viability was determined by the cells' ability to exclude Trypan blue dye. Apoptosis was determined by terminal deoxynucleotydyl transferase–mediated fluorescein isothiocyanate–dUTP nick-end labeling (TUNEL) with the In Situ Cell Death Detection Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions.

Mice and Tumor Studies. Four- to 6-week-old athymic female nu/nu mice were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained in a specific pathogen-free environment. To induce tumor formation, we injected 2×10^6 C6 glioblastoma cells subcutaneously. After palpable tumors ($\sim$25 mm^2) had formed, cohorts of five mice each were injected intratumorally with serial doses of VSV, 2×10^7 plaque-forming units (pfu)/dose, 4 days apart. Control mice were injected intratumorally with heat-inactivated VSV. When the tumor burdens of the control animals became excessive, all the mice were killed, and the explanted tumors were either propogated in tissue culture or cryofrozen for determination of viral titers and histopathological analyses. Organs from these mice were also examined for the presence of virus by standard plaque assay.

RESULTS AND DISCUSSION

We previously observed that embryonic fibroblasts lacking PKR, but not wild-type fibroblasts containing the kinase, were susceptible to VSV infection and viral-mediated apoptosis (*2*). VSV is also known to replicate in a wide variety of malignant cells and does not appear to cause important disease in humans (*1*). We therefore speculated that mechanisms of host defense involving PKR might be defective in cells permissive to VSV.

To further examine the ability of VSV to induce cell death in other transformed human cell lines, including those derived from breast (MCF7), prostate (PC-3), or cervical tumors (HeLa), as well as various cells derived from hematological malignancies (HL 60, K562, Jurkat, BC-1), we infected those cells with VSV as described in Experimental Procedures. We observed that VSV efficiently replicated and induced cytolysis of every established cell line tested, including BC-1, which is positive for human herpesvirus-8 (HHV-8), overexpresses Bcl-2, and is largely

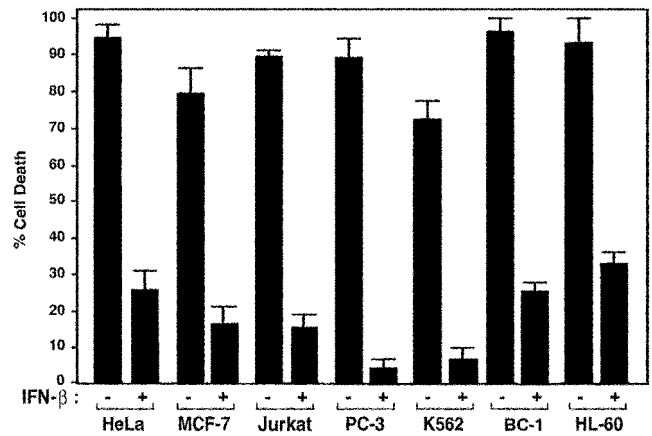

Figure 1. Several human cancer cell lines are permissive to VSV replication and lysis. MCF-7, BC-1, Jurkat, HL-60, K562, PC-3, and HeLa cells were treated with or without 1000 U/ml hIFNβ (Research Diagnostics Inc., NJ) for 18 h and subsequently infected with VSV at a multiplicity of infection of 1. At 48 h post infection, viability was assessed by Trypan Blue exclusion analysis. Data represent the mean of triplicate samples $\pm$ SD from one of two experiments with similar results.

resistant to a wide variety of apoptotic stimuli and chemotherapeutic strategies (Fig. 1) (*7*). The apoptotic nature of this cell death was confirmed by using TUNEL (data not shown). Supernatants were taken from infected cells and examined for VSV yield by the standard plaque assay. As shown in Table 1, all tested cell lines were remarkably permissive to VSV replication. In fact, pretreatment with 1000 U/ml human IFN-β was unable to completely protect many of these cell lines from viral

Table 1

Viral yields from several human cancer cell lines infected with VSV at a multiplicity of infection of 1 (supernatants from cells treated as in Fig. 1 were analyzed for viral yield by standard plaque assay 48 h postinfection)

Cell line	hIFNβ	Titer (pfu/ml)
HeLa	−	1.2×10^7
	+	8.4×10^3
MCF-7	−	1.9×10^7
	+	2.7×10^4
Jurkat	−	3.2×10^7
	+	1.3×10^3
PC-3	−	2.9×10^7
	+	1.2×10^3
K562	−	3.4×10^6
	+	4.4×10^5
BC-1	−	5.2×10^7
	+	1.2×10^5
KL-60	−	3.5×10^6
	+	2.0×10^4

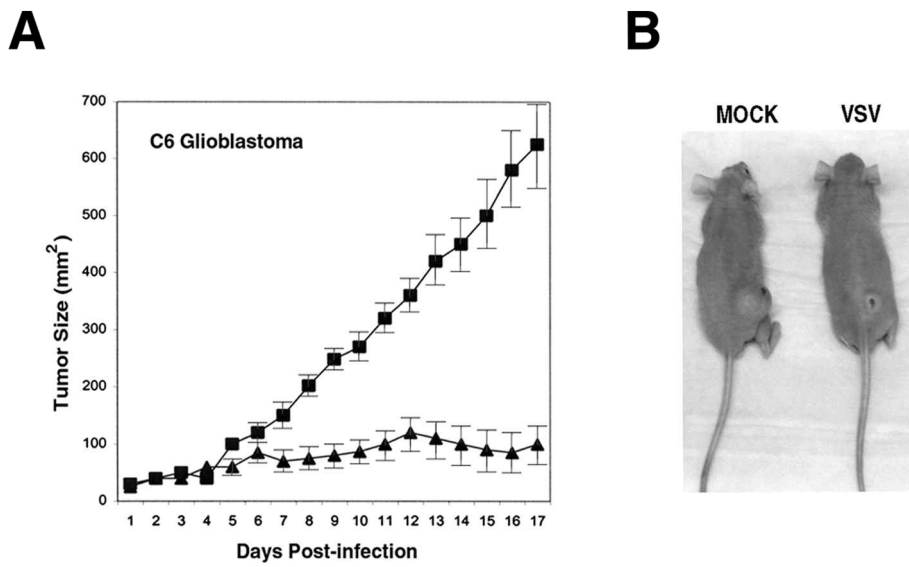

Figure 2. VSV inhibits growth of C6 glioblastoma tumors in nude mice. (A) One week after subcutaneous implantation with 2×10^6 C6 glioblastoma cells into nude mice, tumors ($n = 3$) were injected with two doses of VSV, 5×10^7 pfu/dose, 4 days apart (▲). Control tumors ($n = 3$) received equivalent amounts of heat-inactivated VSV (■). Tumor volumes were measured daily for 3 weeks. (B) Representative mice from this experiment were photographed.

replication and cytolysis, suggesting that IFN signaling may be defective in these cells.

These data indicated that VSV may be useful as an oncolytic virus in the treatment of cancer. Similar strategies have been used with viruses such as adenovirus, which has been genetically modified to preferentially replicate in p53-deficient human tumor cells (*8–10*). To start evaluating the use of VSV in antitumor therapy, we subcutaneously implanted athymic nude mice with 2×10^6 C6 glioblastoma cells. When palpable tumors had formed (~7–14 days postinoculation, when the size of the tumors had reached ~0.25 mm^2), the mice were infected intratumorally with VSV (2.5×10^7 pfu/ml) and monitored daily. Injection with the same amount of virus was repeated after 4 days. Administration of VSV resulted in marked repression of tumor growth in all animals tested within 17 days, when tumors in the control animals exceeded the acceptable tumor burden (Fig. 2A, B). Similar results were obtained with a single intratumoral injection of VSV (data not shown). These data highlight the potent efficacy of VSV against tumors both in vitro and in vivo.

Hematoxylin/eosin (HE)–staining of paraffin-embedded sections prepared from samples of VSV-infected C6 glioblastoma tumor tissue indicated widespread cell death; the retrieved tumors had a considerable lack of vascular infiltration and gave little evidence of tumor cell infiltration into the surrounding tissue (data not shown). Indeed, we were routinely successful in generating robust ex vivo growth of uninfected tumors but were unable to establish any cell lines from implanted, VSV-infected tumors (data not shown). To examine whether VSV spread beyond the virus-inoculated tumor, various tissues from the VSV-treated animals, as well as the tumors themselves, were analyzed for the presence of residual, replicating VSV. Interestingly, examination of VSV-infected tumors for VSV 21 days after infection revealed the presence of residual virus (2×10^4 to 3.5×10^5 pfu/g) in tumor tissue derived from the C6 glioblastoma cells. However, virtually no virus (< 10 pfu/g) was detectable in the lung, brain, kidney, spleen, or liver of mice receiving VSV therapy after this period. These data show that VSV replication is restricted to tumor lineage and complements our observations confirming that no overt anomalies or sickness was apparent in the treated animals during the period of study.

Our results strongly suggest that VSV may be useful in treating human cancers. The mechanisms by which VSV is restricted to replication in tumor cells, although largely unknown, may involve disruption of key host defense mechanisms such as the IFN pathway and possibly PKR action. That PKR autophosphorylation and the phosphorylation of eukaryotic initiation factor 2 (eIF2α) occur in VSV-permissive cell lines, however, implies that signaling pathways downstream of PKR and independent of eIF2α are disrupted in these cells (data not shown). Alternatively, perhaps VSV can overcome the block by PKR on translation in malignant cells by a mechanism that remains to be determined. Interestingly, studies have shown that expression of activated Ras reportedly renders PKR inactive, although the mechanism by which this occurs remains unknown (*11*, *12*). Recently, Coffey et al. (*13*) exploited this observation and showed that reovirus could preferentially replicate in cells transformed by Ras and might be useful as a therapy for tumors containing the activated Ras oncogene. Importantly, our data presented here, as well as results obtained by other sources, demonstrate that VSV replicates not only in those cell lines containing activated Ras

but also in tumors harboring other promoters of oncogenesis, such as overexpressed Myc, Bcl-2, or defective p53. These genetic defects occur in >90% of all known tumors, indicating that VSV could be used to treat a wide range of malignancies (*14*). Also noteworthy is that VSV lacks any known ability to contribute towards transformation of the cell, has a very simple genetic constitution, and is essentially nonhazardous to humans (*1*). Moreover, a lack of prior exposure to VSV in humans means that this treatment may not be markedly impeded by previous contact with the agent, as is the case when using other virus vectors (*8–10*, *13*).

Indeed, the immunobiology associated with VSV infection is considerably well characterized (*1*). Although cytotoxic T cell responses are generally considered to be critical for resolving viral infections, substantial evidence indicates that it is the antibodies that play an indispensable role in the early phase of VSV infection. For example, B cell–deficient mice are highly susceptible to low doses of virus, although mortality can be prevented by transferring naive B cells to the mice before challenge as well as by administering immune serum after challenge (*15–17*). The type-specific VSV G protein is the viral antigen giving rise to the neutralizing antibodies that are critically important in eliminating VSV infection. This knowledge may be useful in designing recombinant VSV viruses to avoid host immune responses that may affect virus spread and tumor killing. The findings reported in this study have recently been complemented by Stojdl et al., who also demonstrate the potential of VSV as an oncolytic agent (*18*).

Another advantage of using VSV as an antitumor agent is its ability to be genetically manipulated. A family of recombinant viruses could feasibly be constructed, from which one could generate and isolate individual members presenting various G protein epitopes on their surface. In this way, rapid IgM responses that occur after 6–8 days and subsequent T cell–dependent and independent IgG responses could be avoided by administering a different viral psuedotype at repeated times until the tumor is eliminated. In addition, recombinant VSVs could be generated to express foreign proteins of choice, such as suicide cassettes or selected cytokines that enhance tumor rejection and stimulate antitumor T-lymphocyte responses.

Collectively, the data presented here show that VSV preferentially replicates and destroys immortalized or tumorigenic cells, which implies that this virus may be useful as a novel anticancer agent in the treatment of cancer.

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