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Treatment of multi-focal colorectal carcinoma metastatic to the liver of immune-competent and syngeneic rats by hepatic artery infusion of oncolytic vesicular stomatitis virus

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Viruses that replicate selectively in cancer cells hold considerable promise as novel therapeutic agents for the treatment of malignancy. We report an orthotopic model of multi-focal colorectal cancer (CRC) metastases in the livers of syngeneic and immune-competent rats, which permitted rigorous testing of oncolytic virus vectors as novel therapeutic agents through hepatic arterial infusion for efficacy and safety. Vesicular stomatitis virus (VSV) is a negative-strand RNA virus with intrinsic oncolytic specificity due to attenuated anti-viral responses in many tumors. After administration at the maximum tolerated dose, the recombinant VSV vector gained access to multi-focal hepatic CRC lesions that led to tumor-selective viral replication and oncolysis. No relevant vector-associated toxicities were noted and in particular, no damage to the hepatic parenchyma was seen. Moreover, the survival rate of vector-treated rats was significantly improved over that of animals in the control treatment group ($p = 0.015$). Our results demonstrate that hepatic arterial administration of oncolytic VSV is both effective and safe in an immune-competent and syngeneic rat model of multi-focal CRC liver metastasis, suggesting that it can be developed into an effective therapeutic modality in patients in the future.

Key words: colorectal cancer; liver metastasis; oncolytic virus; vesicular stomatitis virus; hepatic artery infusion

Colorectal cancer (CRC) is the second largest contributor to cancer deaths in the United States. A total of 105,500 new cases and 57,100 deaths from CRC are estimated for 2003.[1] Due to hematogenous spread of the tumor *via* the portal circulation, the liver is the main site of metastasis in advanced CRC, and about 20% of patients present with disease macroscopically confined to this organ at first recurrence.[2] Prognosis of patients with CRC liver metastasis is poor with an overall median survival of <12 months and virtually no survivors at 5 years.[2] There is an urgent need for novel therapeutic agents that can provide significant clinical benefit for patients with CRC metastatic to the liver.

A broad spectrum of novel molecular therapies to treat hepatic metastasis from CRC has shown promising results in pre-clinical animal models and early clinical trials.[3,4] In particular, oncolytic viruses provide an attractive new tool for treatment of solid cancers because of their ability to replicate selectively within the tumor and kill neighboring cancer cells upon tumor lysis and secondary infection.[5,6] In addition to DNA viruses that are molecularly engineered to replicate preferentially in tumor cells, oncolytic RNA viruses, including reovirus,[7] Newcastle disease virus,[8] measles virus,[9] vesicular stomatitis virus (VSV)[10] and others, are currently being developed as a novel class of anti-tumor agents because of their inherent preference for replication in tumor cells. VSV is a negative-strand RNA virus of the family *Rhabdoviridae* with inherent specificity for replication in tumor cells due to their attenuated anti-viral responses mediated by Type 1 interferons (IFN).[10,11] Natural hosts for VSV infection are cattle, horses and swine. Infections in humans are asymptomatic in most cases or result in a mild febrile illness.[12] VSV is not endemic to the North American population, implying that there will not be pre-existing neutralizing antibodies or memory cellular immune responses in most patients to interfere with its infection process after intravascular administration.[13]

For oncolytic viruses to have significant clinical benefit in advanced cancer patients with multifocal hepatic metastasis from CRC, regional or systemic delivery of the therapeutic agent through the vasculature is needed. The liver has a dual blood supply, with the portal vein supplying 75% and the hepatic artery 25% of hepatic blood flow. It is also known that in humans and animal models, malignant liver tumors have predominantly an arterial blood supply.[14] Based on these considerations, regional delivery of chemotherapeutic agents via the hepatic artery has been widely carried out in the clinic, thereby increasing response rates and reducing systemic toxicity.[15,16] In a correlate to hepatic arterial chemotherapy, several groups have investigated the potential of hepatic arterial infusion of adenoviral vectors for increased transduction of liver tumors.[17–20] Studies were initially carried out in hepatocellular carcinoma animal models and have demonstrated relative improvements in tumor cell transduction of predominantly small lesions, but the overall efficiency of gene transfer was low and significant transduction of normal hepatic parenchyma could not be avoided.[17–19] Subsequent studies showed that intra-arterial delivery of recombinant adenovirus failed to increase gene transfer to human tumor cells in a nude rat model of metastatic CRC.[20] Phase II clinical trials of hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520, also known as Onyx-015) with intravenous 5-fluorouracil and leucovorin have recently been carried out in patients with gastrointestinal carcinoma metastatic to the liver.[21,22] The combination treatment with viral doses up to 2×10^{12} particles was well tolerated by most patients, however, the clinical benefit has so far been marginal.[21,22]

VSV is particularly appealing for its rapid replication rate of 8–10 hr in tumor cells,[13] such that oncolytic effects may be manifested before the onset of potentially neutralizing anti-viral immune responses in the host. We have reported previously the effective use of recombinant VSV vector as an oncolytic agent to treat solitary nodules of hepatocellular carcinoma and CRC in the livers of immune-competent and syngeneic rats and mice by intratumoral injections, respectively.[23,24] Recently, we have observed that hepatic arterial infusion of oncolytic VSV was also capable of accessing multi-focal lesions of hepatocellular carcinoma in the livers of syngeneic rats, which resulted in substantial tumor necrosis and survival advantage of the treated animals.[25] We generated an orthotopic model of CRC in the livers of syngeneic and immune-competent rats and tested the hypothesis that multi-focal CRC lesions in the rat liver can be readily transduced by hepatic artery infusion of a recombinant VSV vector, which would result in tumor-selective viral replication, oncolysis and survival advantage of the treated animals.

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Material and methods

Cell culture

The chemically induced and poorly differentiated rat CRC cell line LMCR$^-$ was kindly provided by Dr. Steven Itzkowitz, Mount Sinai School of Medicine,[26] and maintained in DMEM (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO). The human CRC cell lines SW480 and HT-29 (American Type Culture Collection, ATCC) were maintained in McCoy's 5A (Mediatech) and supplemented with 10% FBS (Sigma-Aldrich). BHK-21 cells (ATCC) used for amplification and titration of VSV were maintained in DMEM (Mediatech) with 10% FBS (Sigma-Aldrich). All media used in this study contained 100 U/ml penicillin-streptomycin (Mediatech).

Virus generation and replication assays

Recombinant VSV encoding the lacZ gene (rVSV-lacZ)[25] or the GFP gene (rVSV-GFP)[23] was generated using the established method of reverse genetics.[27,28] Viral titers were determined by plaque assays on BHK-21 cells as described previously.[23] To evaluate viral replication in the rat CRC cell line LMCR$^-$ *in vitro*, 1×10^5 cells/well were seeded in 6-well plates overnight and then infected with rVSV-GFP at various MOI (0, 0.0001, 0.001, 0.01, 0.1 or 1). At indicated time points post infection, cells were monitored for cytopathic effects (CPE) by phase-contrast microscopy and GFP expression was analyzed by fluorescence microscopy. In addition, a sample of cell culture supernatant was collected at designated time points after infection and assayed for viral RNA genome by real time RT-PCR using specific primers as described previously.[23]

In vitro *cytotoxicity assays*

Human and rat CRC cells were plated in 24-well plates at 1×10^4 cells/well and infected with rVSV-GFP at various MOI (0, 0.0001, 0.001, 0.01, 0.1 or 1). Cell viability was measured at the indicated time points after infection using the Cell Proliferation Kit I (Roche, Indianapolis, IN). All cell viability results are expressed as percentage of viable cells compared to mock-infected control at each time point.

Animal studies

Inbred male BDIX rats (6 weeks old) were purchased from Charles River Laboratories (Indianapolis, IN) and housed at a specific pathogen-free environment under standard conditions. All procedures involving animals were approved by and carried out according to guidelines of the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine. Rats were anesthetized with 80 mg/kg ketamine (i.p.), 1 mg/kg xylazine (i.p.), and isoflurane using an inhalation anesthesia system (Vet-Equip, Pleasanton, CA). To establish multi-focal CRC liver metastases, rats were infused with 1×10^7 syngeneic LMCR$^-$ cells in 1 ml of DMEM *via* the ileocecal vein. Twelve days after tumor cell implantation, animals were anesthetized and underwent laparotomy to assess the presence of multiple tumor lesions macroscopically visible on the liver surface. Then, rVSV-lacZ vector at 1.3×10^8 plaque-forming units (pfu) in 1 ml of PBS or an equivalent volume of buffer was administered *via* the hepatic artery (see Fig. 3 for detailed description of surgical manipulations).

To evaluate liver histology and VSV-G expression staining of tumor-bearing liver sections, sets of animals were sacrificed at various time points after hepatic arterial infusion of the vector. Tissue samples were obtained using an operating microscope. In a separate experiment, groups of animals infused with vector or buffer control were followed for survival, which was checked daily in all animals.

Histology and immunohistochemical staining

At indicated time points after vector infusion into the hepatic artery, animals were sacrificed, explanted livers fixed in 4% paraformaldehyde overnight, and then paraffin-embedded. Thin sections (5 μm) were subjected to either hematoxylin and eosin (H&E) staining for histological analysis or immunohistochemistry using a monoclonal antibody against the VSV-G protein (VSV11-M, Alpha Diagnostic, San Antonio, TX). Immunohistochemistry sections were counterstained with hematoxylin.

Assessment of serum transaminases

Blood samples were collected from the inferior vena cava at the time of euthanization or before virus injection, and the levels of alanine transferase (ALT) and aspartate transferase (AST) were determined at the Chemistry Laboratory at Mount Sinai School of Medicine.

Statistical analyses

Survival curves of animals treated with VSV or buffer were plotted according to the Kaplan-Meier method. Statistical significance in different treatment groups was compared using the log-rank test. Results and graphs were obtained using the GraphPad Prism 3.0 program (GraphPad Software, San Diego, CA).

Results

Oncolytic activities of VSV in human and rat CRC cells in vitro

To assess the ability of VSV to replicate in the rat CRC cell line LMCR$^-$, cells were tested in infection assays *in vitro*. LMCR$^-$ cells were infected with rVSV-GFP at various MOI and were evaluated for CPE by bright field and for GFP expression by fluorescence microscopy 24 hr later (Fig. 1*a*). LMCR$^-$ cells transduced with rVSV-GFP at an MOI 0.01 showed typical VSV-mediated CPE and detached from the plate. At the same time, GFP expression was detected uniformly in infected cells. In addition, the supernatants of rVSV-GFP infected cells at various MOI were harvested at indicated time points after infection. Total RNAs from the supernatants were prepared and the RNA samples were analyzed for the concentrations of genomic VSV RNA by real-time RT-PCR (Fig. 1*b*). Our results showed that rVSV-GFP was fully capable of replicating its RNA genome in the LMCR$^-$ cell line and reached plateau of about 10^8 genomic RNA copies/10 μl at 24–48 hr after infection.

Next, we examined if VSV infection would result in efficient cell killing of human and rat CRC cells. We infected 2 human (SW480, HT-29) and the rat (LMCR$^-$) CRC cell lines with rVSV-GFP at various MOI. The cytotoxic effects on the cells were quantified by an MTT assay and expressed as a fraction of mock-infected cells at each time point (Fig. 2). The 2 human CRC cell lines, SW480 and HT-29 were killed efficiently by the virus and in fact, most cells were dead within 48–72 hr (Fig. 2*a*,*b*). The degree of viral killing of rat LMCR$^-$ cells was not as extensive as seen in the human CRC cell lines (Fig. 2*c*), suggesting that human CRC tumors might be more susceptible to VSV oncolysis than the rat LMCR$^-$ model.

Rat model of multi-focal CRC metastatic to the liver

The rat CRC cell line LMCR$^-$ and its syngeneic host BDIX rat closely resemble human CRC in several respects, and may therefore be a useful model to test new therapeutic agents.[26] Twelve days after infusion of 1×10^7 LMCR$^-$ cells into the ileocecal vein of syngeneic BDIX rats, 100% of animals developed numerous multifocal CRC lesions of up to 1 mm in diameter in their livers (Fig. 3).

Access to multifocal hepatic CRC lesions and tumor-selective replication of VSV after hepatic artery infusion

The maximum tolerated dose (MTD) of rVSV-lacZ administered into the hepatic artery of BDIX rats was determined. rVSV-lacZ vector in half-log dose increments were infused into non-tumor bearing animals ($n = 10$/dose level cohort) and the highest dose that resulted in no vector-associated toxicities was determined to be 1.3×10^8 pfu, which was used in all subsequent

studies. At viral doses beyond the MTD (4.0×10^8 pfu and higher) the animals showed signs of neurotoxicity including limb paralysis and altered consciousness, which was typically manifested 5 days after virus infusion.

To demonstrate access to multi-focal CRC lesions in the liver and the tumor-specificity of VSV replication, histopathological sections showing the tumor border regions with normal liver tissues were examined. Control sections obtained from PBS-treated tumor-bearing animals showed minimal spontaneous necrotic areas within the tumor lesions (Fig. 4*a*,*c*), and all samples were negative for VSV-G protein by immunohistochemical staining (Fig. 4*e*). In contrast, multiple large necrotic areas were apparent within the tumor lesions at Day 3 after virus infusion (Fig. 4*b*,*d*), which were positive for VSV-G protein in the consecutive section (Fig. 4*f*). The results indicated that the virus readily gained access to, and productively replicated within the multifocal CRC metastasis after hepatic artery administration.

Absence of significant hepatotoxicity after hepatic artery infusion of VSV

To assess potential hepatotoxicity after hepatic arterial administration of VSV at the MTD in BDIX rats, the kinetic profiles of serum transaminase levels (mean ± SD, $n = 2$ for each time point) in the same experimental animal groups above were determined (Fig. 5*a*). Mild elevations of serum transaminases (AST and ALT) were seen at Day 1 after hepatic arterial administration of rVSV-lacZ but the levels rapidly returned to baseline at Day 3. Additionally, the histology of neighboring hepatic parenchyma was completely normal (Fig. 5*b*). The results confirmed the notion that there was no remarkable hepatotoxicity associated with hepatic artery infusion of VSV at the MTD.

In vivo *anti-tumor efficacy*

To assess the potential of VSV as a therapeutic agent for multi-focal CRC metastatic to the liver, rats bearing numerous CRC tumors in their livers with sizes up to 1 mm in diameter were randomly assigned to infusion with rVSV-lacZ at 1.3×10^8 pfu ($n = 11$) or PBS control ($n = 10$) *via* the hepatic artery and survival was followed (Fig. 6). Buffer-treated rats started to die of tumor progression in 9 more days and all of them expired at

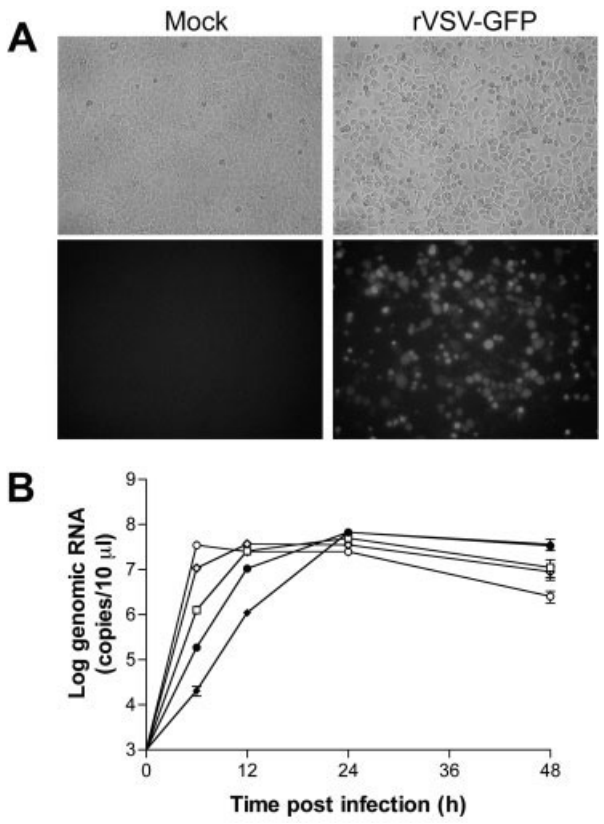

Figure 1 – VSV replication in the rat CRC cell lines LMCR$^-$ *in vitro*. (*a*) Cell morphology (*top panel*) and expression of GFP (*bottom panel*) in rVSV-GFP infected LMCR$^-$ cells (MOI = 0.01). Cells are shown in the same field under bright field and immunofluorescence microscopy at 24 hr after infection. Mock-infected cells are shown for comparison. (*b*) Supernatants of rVSV-GFP infected LMCR$^-$ cells at different MOI were collected at the designated time points and viral RNA was purified from the supernatant. Quantitative RT-PCR was carried out to determine the copy number of viral genomic RNA. MOI used for all target cells were as follows: ◆, MOI 0.0001; ●, MOI 0.001; □, MOI 0.01; ◇, MOI 0.1; ○, MOI 1. Experiments were carried out in triplicates.

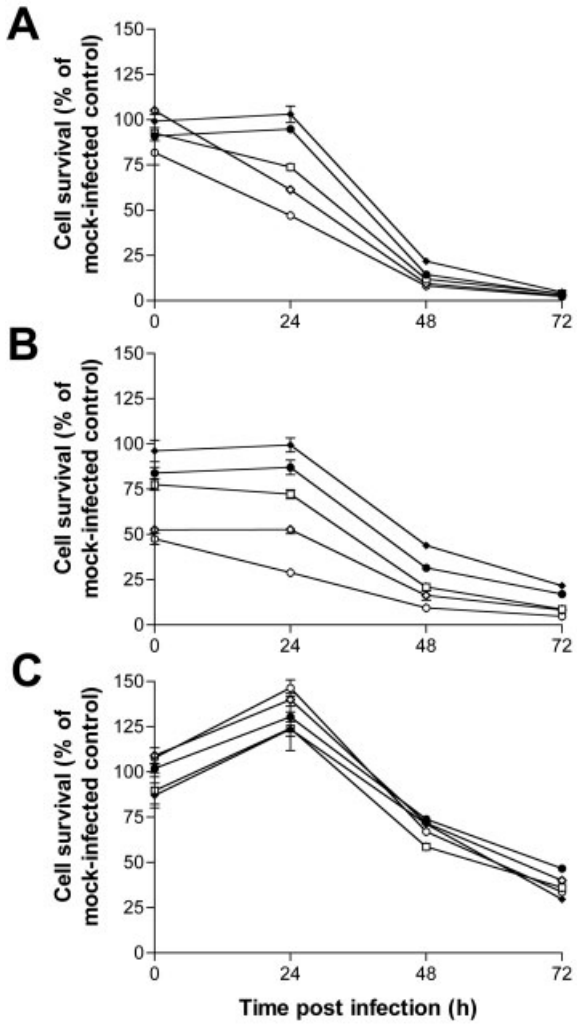

Figure 2 – Oncolytic effect of recombinant VSV vector on CRC tumor cells *in vitro*. (*a*) SW480, (*b*) HT-29 or (*c*) LMCR$^-$ cells were infected with rVSV-GFP at different MOI and, at the indicated time points, cell viability was measured using an MTT assay. The OD_{570} values of mock-infected cells were set as 100% at each time point. Data are expressed as means ± SE of triplicate experiments. MOI used for all target cells were as follows: ◆, MOI 0.0001; ●, MOI 0.001; □, MOI 0.01; ◇, MOI 0.1; ○, MOI 1. Experiments were carried out in triplicates.

19 days (15.5 ± 3.1 days). The VSV-treated rats survived for up to 24 days post vector injection (19.0 ± 4.0 days), and the differential survival rates were statistically significant by log-rank test analysis ($p = 0.015$). Necropsy was carried out in all animals to evaluate the cause of death and dissemination pattern of the tumor. Control and virus-treated animal showed several lung metastases with sizes up to 3 mm in diameter without pleural effusion. In addition, massive hepatomegaly with multiple liver metastases as well as peritoneal dissemination and bloody ascites was observed. Progressive disease seemed to be the likely cause of death in both treated and control animals. This experiment demonstrated the ability of VSV, administered via the hepatic artery, to produce significant anti-tumor activity in this aggressive, immune-competent rat model of multi-focal CRC metastatic to the liver.

Discussion

Hepatic artery infusion of the therapeutic agent to treat malignant liver tumors offers potential advantages over systemic therapy depending on the agent to be administered. Agents with high total body clearance will achieve increased concentrations in the liver tumor if given by hepatic artery infusion, whereas agents that are efficiently extracted by the hepatic parenchyma will achieve reduced systemic exposure.[29,30] We report the generation of an orthotopic multi-focal CRC model in the livers of immune-competent and syngeneic rats, which can be utilized to critically evaluate the efficacy and toxicity of novel therapeutic agents through hepatic arterial infusion.

Based on these considerations, we hypothesized that the oncolytic potential of VSV in the treatment of hepatic CRC metastasis can be elevated by hepatic artery administration of the virus in tumor-bearing rats. Our results provide conclusive evidence that hepatic arterial infusion of VSV at the MTD in tumor-bearing rats resulted in efficient viral transduction of multi-focal CRC lesions in the liver, tumor-selective viral replication and substantial oncolysis. Our *in vitro* studies show that VSV replicates in LMCR$^-$ cells to lower levels and with slightly slower kinetics compared to human CRC cells. Although the molecular mechanism has not yet been established, it is possible that LMCR$^-$ cells retain partial sensitivity to the anti-viral actions of Type 1 IFN that can lead to higher resistance to VSV infection and oncolysis. This would suggest that human CRC tumors might be more sensitive to VSV treatment than in the rat LMCR$^-$ model.

The orthotopic locale of our tumor model more realistically recapitulates the liver environment and results in a locally aggressive, spontaneously metastatic CRC tumor. Therefore, the observed prolongation of survival conferred by VSV over PBS treated control animals ($p = 0.015$) was remarkable. Importantly, no significant vector-associated toxicities were noted and in particular, no damage to the hepatic parenchyma was seen. The oncolytic activity of VSV was achieved after a single administration of the virus, and the tumor burden at the time of treatment (12 days after tumor cell implantation) was quite extensive. Because effective anti-viral immune responses are mounted typically in the host after 1 week of virus infection, multiple intra-hepatic artery infusions during this window of opportunity might lead to more oncolysis than a single injection. Although multiple dosing is technically difficult to examine in the current model, percutaneous catheterization of the hepatic artery for local-regional therapy of liver tumors in patients is a standard procedure in the clinic, which is readily applicable in re-administration of oncolytic viruses.

Although we detected large necrotic regions within multiple tumors, which led to significant prolongation of animal survival, there were no long-term survivors. To achieve much improved therapeutic outcome, the oncolytic potential of the virus will need to be significantly enhanced. The use of reverse genetics techniques to manipulate the genome of VSV can be used to improve

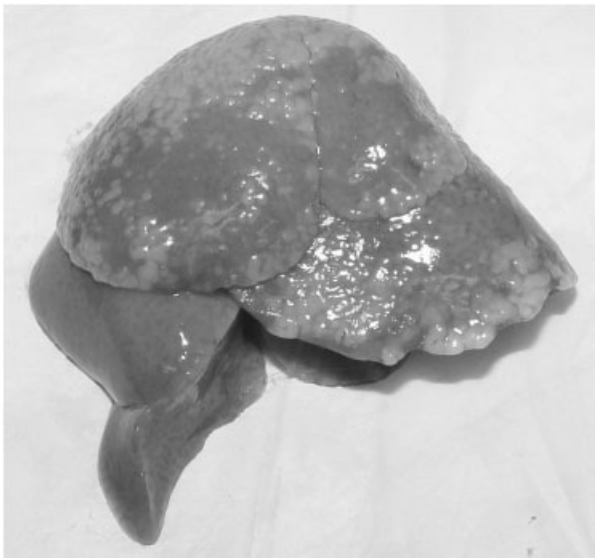

Figure 3 – Representative macroscopic picture of a rat liver with multi-focal metastatic CRC lesions. Twelve days after infusion of LMCR$^-$ rat CRC cells into the portal vein of syngeneic BDIX rats, 90–100% of animals developed numerous macroscopically visible CRC lesions (up to 1 mm in diameter) in their livers. The hepatic vessels (common hepatic artery, proper hepatic artery and gastroduodenal artery) were dissected with the aid of an operating microscope. After ligation of the gastroduodenal artery and temporal block of the common hepatic artery, 1 ml of PBS or VSV vector was administered over 15 sec into the gastroduodenal artery. The proximal site of the gastroduodenal artery was ligated to prevent bleeding, the block of the common hepatic artery was released and the presence of appropriate hepatic blood flow was confirmed.

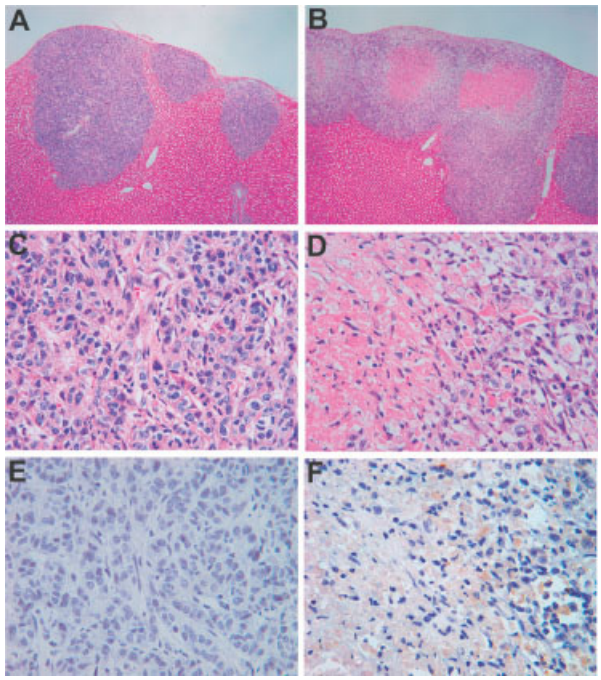

Figure 4 – Histopathology of liver sections of tumor-bearing animals after hepatic arterial infusion of VSV. H&E staining and immunohistochemical analysis of VSV-G protein expression (positive staining appears in brown) of representative sections of the liver 3 days after infusion of PBS control (*a*,*c*,*e*) or recombinant VSV vector (*b*,*d*,*f*), original magnification, 5× (*a*,*b*) or 40× (*c*–*f*).

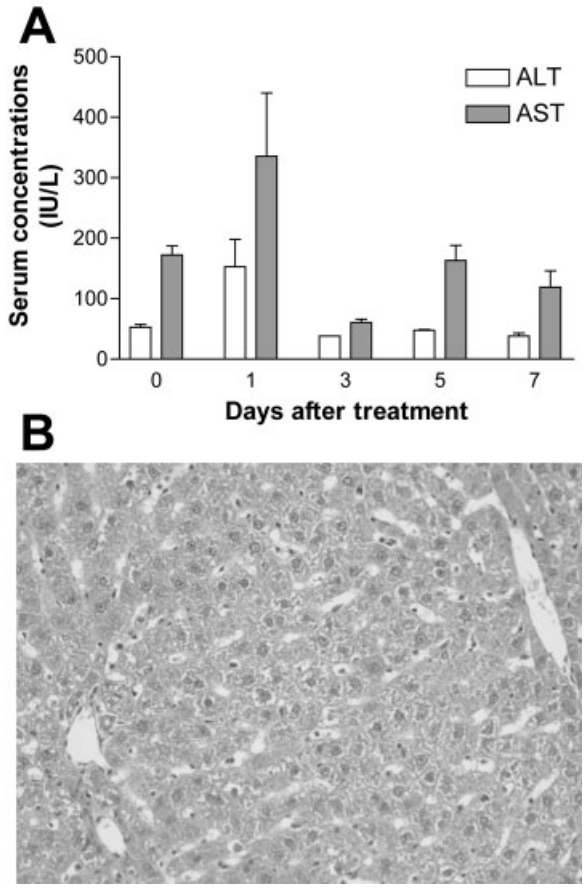

FIGURE 5 – Absence of liver damage after hepatic arterial infusion of VSV. (*a*) Serum transaminases in tumor-bearing animals after hepatic arterial infusion of VSV. Blood samples were collected at indicated time points (time point 0; before virus injection) and serum chemistry was carried out. (*b*) H&E histopathology of a representative liver section showing neighboring hepatic parenchyma obtained from recombinant VSV vector infused animals 1 day after treatment (original magnification = 40×).

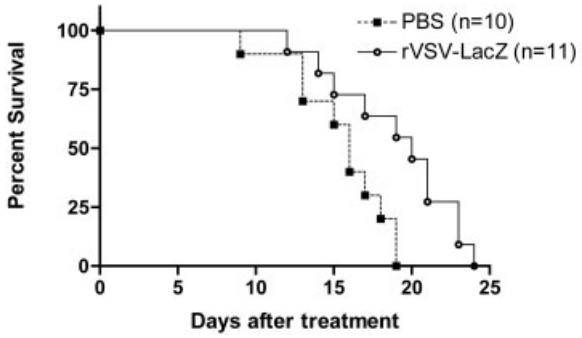

FIGURE 6 – Kaplan-Meier survival curve of rats with multi-focal CRC lesions in the liver after hepatic arterial infusion of VSV versus PBS. Tumor-bearing animals were infused with 1.3×10^8 pfu rVSV-LacZ (*solid line*) or PBS control (*dashed line*) via the hepatic artery on Day 0, and followed for survival. The survival advantage for VSV administered animals was statistically significant compared to PBS control animals ($p = 0.015$, log-rank test). The results were combined from 2 consecutive sets of animals with stratification.

its anti-tumor properties,[27,28] and it has been demonstrated recently that this might be possible.[31–33] Recombinant VSV expressing the herpes simplex virus thymidine kinase or the cytosine deaminase suicide genes have been reported by Barber *et al.*[31,32] Their results suggest that the use of the prodrug-suicide gene strategy may add synergy to the effectiveness of oncolytic VSV in cancer treatment. In an alternative approach, Bateman *et al.*[34] demonstrated that plasmid-mediated expression of viral fusogenic membrane glycoprotein efficiently killed tumor cells through induction of fusion to form large multinucleated syncytia. *In vitro*, this "bystander effect" was reported to be at least 10-fold greater than that observed with various suicide gene/prodrug systems.[34] In this regard, we reported the construction of a novel recombinant VSV capable of inducing syncytia formation between tumor cells through membrane fusion at neutral pH, which led to enhanced viral transmission and oncolysis of hepatocellular carcinoma in the livers of rats.[33] These and other transgenes can be integrated into recombinant VSV vectors to increase their potential for the treatment of multifocal hepatic CRC metastasis further.

The safety of replication-competent viruses used in humans for cancer therapy remains of paramount importance and these viruses should meet specific safety criteria. First, viral replication should be limited to neoplastic cells with minimal, if any, replication in normal cells. An essential, dose-limiting event of conventional cancer therapeutics is bone marrow toxicity. Importantly, bone marrow cultures from healthy donors produced no infectious VSV particles, even when infected at a high MOI of 10.[10] In addition, previous studies in our laboratory indicate that replication of VSV is severely attenuated in normal primary rodent and human hepatocytes *in vitro*, whereas efficient VSV replication was observed in established rodent and human CRC cell lines.[24] In our present study, there was no demonstratable replication of the virus in normal tissues in the animals after hepatic arterial infusion of VSV, and viral replication was limited to the tumors. This striking tumor-selectivity of VSV replication might explain the apparent lack of organ toxicity when administered at the MTD, and is suggestive of a relatively large therapeutic window. Second, because VSV is a cytoplasmic virus with a RNA genome, it does not enter the nucleus and does not have a DNA phase in its life cycle, thus avoiding potential complications associated with chromosomal integration.[13] A limitation of the RNA viruses lies in their high spontaneous mutation rates, which might limit their ability to deliver and express other transgenes that can augment their therapeutic potential.[35] It was demonstrated previously, however, that transgenes inserted as an additional transcriptional unit into the non-coding region of the VSV-G gene were maintained stably over multiple passages *in vitro*.[36] We found no loss of viral titer over multiple passages *in vitro* (data not shown), further indicating that VSV can be used as a stable vector. Finally, VSV is extremely sensitive to the antiviral actions of type I IFN in normal cells, providing an effective tool to suppress unwanted replication in normal tissues, if necessary.[37]

In summary, hepatic metastasis from CRC remains a significant clinical problem with limited therapeutic options. We show that hepatic arterial administration of VSV is both effective and safe in the treatment of multi-focal CRC metastases in the livers of immune-competent and syngeneic rats, suggesting that further development of this approach may have significant potential for clinical application in patients.

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