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（出國類別：研究）

外科腫瘤學之分子生物基礎研究及腹腔鏡手術

服務機關：國立成功大學醫學院附設醫院
出 國 人 職　　稱：主治醫師
姓　　名：戴運生
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主辦機關:

國立成功大學醫學院附設醫院

聯絡人／電話:

洪璨貞／06-2353535 轉 2049

出國人員:

戴運生　國立成功大學醫學院附設醫院　外科部　主治醫師

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分類號/目: J2／西醫　J2／西醫

關鍵詞:　腹腔鏡手術

內容摘要: 近來外科手術的許多創新發展，使得病人從傳統手術數後倍極艱辛的恢復過程，轉而成爲積極治療且可輕鬆快速恢復。尤其於癌症醫學方面，外科更是佔有極重要的地位，然而許多傳統手術沿襲傳統固定方式，執行上有諸多限制。所幸拜 21 世紀電腦網路科技進步所賜，目前腹腔鏡手術結合數位化儀器將外科手術推向前衛發展，讓外科醫師能夠更精準的處理病人的『痛』，而每位手術後的病人都宛如醫師創造下的藝術品，和傳統手術術後病人有顯著差別。

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正文：近來外科手術的許多創新發展，使得病人從傳統手術數後倍極艱辛的恢復過程，轉而成為積極治療且可輕鬆快速恢復。尤其於癌症醫學方面，外科更是佔有極重要的地位，然而許多傳統手術沿襲傳統固定方式，執行上有諸多限制。所幸拜 21 世紀電腦網路科技進步所賜，目前腹腔鏡手術結合數位化儀器將外科手術推向前衛發展，讓外科醫師能夠更精準的處理病人的『痛』，而每位手術後的病人都宛如醫師創造下的藝術品，和傳統手術術後病人有顯著差別。

或許一般民眾對腹腔鏡手術還很陌生，其實腹腔鏡手術也是內視鏡手術的一種，就如同胃鏡或大腸鏡一樣，利用長鏡頭深入腹腔及各個需探查及處理的器官後，即可清楚看到病源所在，再經由外科醫師熟稔的技術進行病灶切除、縫補極重建…，幾乎皆可使用腹腔鏡手術施行。

去年一整年我有機會到美國紐約市位於曼哈頓上城之 Mount Sinai 醫院進修學習，該院為紐約市 1000 床以上之大型醫院，Dr. Ganger 是國際知名腹腔鏡手術專家，其所收治極開刀病患幾乎 100%皆由腹腔鏡手術完成，而該院一般外科手術也有高達 80%病患是接受腹腔鏡手術治療。在美國發展超過 20 年且如此普及的手術在台灣亦是目前手術選擇的趨勢，但在健保局保守的給付、醫院設備及醫師訓訓練背景的條件下，提醒您在手術前仍需找有經驗的醫師討論。

其實大部分病人在初次接觸腹腔鏡手術時都相當好奇，為何腹腔鏡手術有此迷人之處，在執行經驗上無論美國

或國內病人再接受手術時，直需由外科醫師於肚子上開 4 個小於 1 公分的小洞，利用此入口可容許各種器械及數位化儀器執行手術，所以術後病人恢復相當快，和傳統手術病人腹部留下長長一條『蜈蚣疤痕』，相較有天壤之別。特別是可減少病患因手術後傷口疼痛引發之心肺合併症及術後傷口感染之危險性，且經由數位影像高倍高畫質的手術影像呈現，更讓病灶無所遁形，讓外科醫師的手術更加精細順暢。

但腹腔鏡手術也並非如神話般無所不能，其中最重要的因素和限制是外科醫師技術的熟練度和周邊設備及人員的配合，腹腔鏡手術因其手術過程精細費時，所以外科醫師需耗費較一般傳統手術更長的時間，但健保給付上並未比傳統手術高，甚至有所限制。成大醫院為南部最優秀之醫學中心，在歷任院長，尤其是前外科系楊院長及現在陳志鴻院長的支持下，外科腹腔鏡已嘉惠眾多南部病患，正如我在成大及美國的手術經驗，每每看到病患和家屬術後既驚奇又感激的表情，讓我們手術工作群的同仁感到莫大的鼓舞，早已將手術過程中需耗費加倍精神和體力的疲憊一掃而空。

身為外科醫師，我總深深期待能藉由我的手和將至美國所學先進精準的最新手術方式將患者身上的病痛減至最低，讓每位能符合腹腔鏡手術的病患，都能接受此一輕鬆的先進手術。

Topical treatment with Oxaliplatin for the prevention of port site metastases in laparoscopic surgery for colorectal cancer: An experimental study in rats

Yun-Sheng Tai, MD, Federico Cuenca Abente, MD, Ahmad Assalia, MD, Kazuki Ueda, MD & Michel Gagner, MD, FRCSC, FACS

Department of Surgery, New York Presbyterian Hospital, Weill-Cornell College of Medicine, New York, NY

Address for correspondence:

Michel Gagner, M.D., FRCSC, FACS
Chief, Bariatric Surgery
Weill-Cornell College of Medicine
New York-Presbyterian Hospital

525 E. 68th Street, Box 129, New York, NY 10021

tel:1-212-746-5294

fax:1-212-746-5236

mig2016@med.cornell.edu

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Running Head: Oxalipaltin for prevention of port-site metastases.

ABSTRACT

BACKGROUND

The development of port site metastases following laparoscopic resection of various malignancies continues to be a disturbing issue for laparoscopic surgeons. Previous studies revealed promising results with Oxaliplatin, a third-generation platinum compound, as a first-line treatment in advanced colorectal cancer. This study evaluates the effect of topical application of oxaliplatin on the development of port-site metastases in an experimental murine model.

METHODS

Nineteen female BDIX rats (immunocompetent, 6 weeks old) were subjected to a sham laparoscopic operation after $1X10^7$ viable rat colon carcinoma viable cells (LMCR) had been injected into their peritoneal cavities. Three trocars (one central camera port and 2 additional lateral ports) were introduced into the abdomen and a pneumoperitoneum was created with carbon dioxide. Ten minutes after LMCR cells were injected into the peritoneal cavity, the two lateral trocars were removed and

carbon dioxide insufflation was maintained for an additional 5 minutes to allow for tumor cells seeding. Oxaliplatin (0.198 mg/kg) was then topically applied to one trocar site intramuscularly, while the other site was left untreated. One week later, the animals were euthanized and the port sites were histologically examined for evidence of metastases.

RESULTS

The rate of tumor implantation at the muscle layer in control sites was 68% (13/19) compared to 37% (7/19) at oxaliplatin-treated sites (P =0.1). No significant differences were detected in port site metastasis rate in other non-treated layers of the abdominal wall.

CONCLUSIONS

Intramuscular topical application of Oxaliplatin may decrease the incidence of port-site metastasis in a syngeneic animal model of colon cancer. Further studies are needed to better determine its possible therapeutic role in high-risk humans undergoing laparoscopic resection of colorectal malignancies.

Laparoscopic surgery for malignancy continues to be a matter of contention. While in the beginning of the laparoscopic era the major concern was focused on technical feasibility, it wasn't until several reports describing port site recurrences appeared [1-4], that the applicability of laparoscopy in these cases was questioned. Multiple publications have demonstrated the advantage of laparoscopy over open surgery regarding postoperative recovery and better cosmetics [5-7], however, questions as to adequacy of resection, long term oncological outcome and port site recurrences, limited it's application in cancer surgery. Recently, several studies showed that the oncological results are, at least, the same as in open surgery and surprisingly, the rates of port site meatstasis were comparable [7-10]. The real incidence of this particular way of tumor spreading is not known, neither is its prognostic implication. It seems that in experienced hands the incidence does not significantly differ from that in open surgery [7, 9, 10]. However, since the greater bulk of procedures is not performed by highly skilled laparoscopic surgeons, this continues to pose a problem that should be addressed during surgery. Nonetheless, even though its occurrence is considered low (1.1%-3.9%) [11, 12], overall it's still higher than what is expected for open surgery (0.6-0.8%) [11].

The mechanisms proposed for this unwarranted phenomenon include excessive manipulation of the tumor, CO2 insufflation, air leakage through port sites

(desufflation), direct implantation with contaminated instruments and contamination while extracting the specimen [13] However, the precise mechanism is not known yet. Nonetheless, while most of these causes can, theoretically, be prevented through better surgical technique and experience, it has been noted that tumor cells are being spilled in almost half of the patients undergoing open cancer surgery [14]. This fact has drawn the attention of several investigators, who published a number of articles addressing this issue [11, 13, 15-18]. Some therapeutic agents have been tested including Povodine Iodine and 5-FU, which showed significant potency in preventing tumor growth at port sites [11, 16, 17, 19], but none displayed a total protection. In those studies, intraperitoneal irrigation or systemic administration of cytotoxic agent might prevent tumor implantation after laparoscopic surgery and port-site metastases. The use of intraperitoneal heparin also can prevent tumor implantation by reducing the presence of intraperitoneal blood [18]

On August 2002, the F.D.A. approved Oxaliplatin for the treatment of colorectal cancer in those patients refractory to 5-FU and Irinotecan. This new drug has been demonstrated to be effective in the treatment of patients with colorectal cancer [20].

The aim of our study was to evaluate the efficacy of this new cytotoxic drug as a topical treatment to prevent port sites metastasis following laparoscopic surgery for

colorectal cancer.

METHODS

Cell cultures and animals

A metastatic rat colon carcinoma cell line (LMCR) with Sialyl-Tn (STn) negative clones was used in this study. The cell line was originally derived in the Gastrointestinal Research Laboratory, Department of Medicine, Mount Sinai School of Medicine [21]. The cell line was grown in DMEM supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 microg/ml streptomycin and incubated at 37 °C in 7.5% CO2. Nineteen female BDIX rats (4-6 weeks old) obtained from Charles River Laboratories through the National Cancer Institute were used. This number of animals will allow detection of a difference of 50% in port-site metastasis incidence (80% for control group and 30% for each group) [11] with power 0.80 for a two-sided test at the 0.05 level of significance. Our Institutional Animal Care and Use Committee approved the study.

Cytotoxicity assays

In order to determine the optimal concentration of Oxaliplatin for use in the animal experiment, the oxaliplatin concentration that inhibits 50% of cell growth (IC_{50}) was determined using an MTT (3, 4, 5-dimethylthiazool-2, 5-diphenyltetrazolium bromide; Sigma) assay. 4x10E3, 8x10E3 and 1.2x10E4 cells/well were seeded into a 96-well

plate which was incubated for 24 hours. The cells were then treated with various concentrations of oxaliplatin, and incubated for an additional hour at 37 °C. Subsequently, 10 µl of MTT at a concentration of 5 mg/ml was added to each well and cells were incubated for an additional 4-6 hours. The supernatant was aspirated and 100 µl of dimethylsufoxide was added to the wells to dissolve any precipitate present. The optical density was then measured at a wavelength of 570 nm using an ELX800 plate reader (Bio-Tek Instruments, Inc.; Winooski, Vermont).

Surgical protocol

The rats were anesthetized throughout the procedures using 1.5% isoflurane (Abbott Laboratories, North Chicago, IL) and oxygen and surgery was performed under sterile conditions. One 5-mm trocar (camera port) was introduced in the middle lower abdomen using an open technique and pneumoperitoneum was created with carbon dioxide insufflation up to a pressure of 4mm Hg. Two additional "port" (2-mm trocars) were inserted in the left and right upper abdominal quadrant under direct vision (Fig.1). Following gas insufflation and trocar placement, $1X10^7$ LMCR cells in 5ml PBS were injected in the peritoneal cavity under laparoscopic vision through the 16-gauge needle attached to a 10ml syringe. After 10 minutes, the two 2-mm trocars were removed and carbon dioxide insufflation was continued at a rate of 0.4 liters/minute and a pressure of 4 mmHg for an additional 5 minutes to allow the tumor

cells to implant at the port sites. Oxaliplatin (0.198 mg/kg), 200-µl at a 500 µM concentration, was then applied topically via an intramuscular injection to one 2-mm trocar site (right side) using a 27 gauge insulin syringe. The other port site was left untreated as the control. The ports were then closed with sutures. After 1 week, the rats were euthanized, and the abdomen was examined for the presence of tumor. The port sites were histologically examined for evidence of metastases.

Statistical Analysis

The frequency of port site tumor development was compared between groups using the Fisher's exact probability test. Probabilities less than 0.05 were considered significant.

RESULTS

Cytotoxicity following 1 hour exposure to oxalipalatin

The cytotoxic effect of different concentrations of oxalipatin is illustrated in Fig. 2. All three cell numbers gave us the similar IC50 (one hour exposure) values at 100 micromole oxaliplatin.

To determine the concentration of oxaliplatin with a 100% cytotoxic effect on LMCL (-) cells, we performed another in-vitro study using oxaliplatin at 50 µm, 100 µm and 500 µm concentrations. In the wells exposed to 500 µm, about 95% of the cells died.

We therefore chose the 500 μM concentration of oxaliplatin (0.198 mg/kg) as our treatment dose.

Necropsy and microscopic findings

All rats survived for the duration of the study. During the autopsy, tumor was visible or palpable at the control port sites. All rats developed severe carcinomatosis in the abdominal cavity, including ascites in some cases. Macroscopically, the treated port site appeared to have a lower incidence of tumor nodule (Fig.3). Microscopically, neoplastic cells were present individually or arranged in small aggregates or short cords. Neoplastic cells showed moderate to marked pleomorphism and had eosinophilic cytoplasm and normal to slightly hyperchromatic nuclei. Inflammation associated with tumor cells was generally mononuclear (plasma cells, lymphocytes and few macrophages). In some areas, chronic active inflammation or granulomatous inflammation was seen. This was most likely due to the introduction of hairs into the tissue during the surgical procedure (Fig. 4).

Frequency of port-site tumor implantation

We observed that the number of animals with port-wound tumors was significantly lower at the oxaliplatin treated port sites compared with those untreated port the untreated (P=0.1) (Fig 5). In the subcutaneous tissue, cancer cells were seen in 6 of 19 (32%) treated sites compared with 7 of 19 (37%) in the untreated ports (p=1). In the

deep border skeletal muscle, cancer cells were seen in 13 of 19 (68%) treated ports and 14 of 19 (74%) untreated ports (p=1). Tumor growth was not significantly reduced in the subcutaneous tissue or the deep border skeletal muscle as a result of oxaliplatin treatment.

DISCUSSON

The cause and real incidence of port-site tumor recurrences remain unknown, and therefore, efficient ways of prevention are still evolving. On anatomical grounds it is unlikely that port tumors are the result of hematogenenous or lymphatic spread. Direct implantation of tumor cells in the port-site or wound is the most likely mechanism [16]. Even though many surgeons still question the clinical significance of metastases arising in trocar wounds following laparoscopic intervention for malignancies, there is increasing evidence, both clinical and experimental, to suggest a need for greater caution regarding its application to malignant growth [13]. It has been postulated that wound metastasis occur as a result of contamination following laparoscopic manipulation of malignant tumors, with resultant spread to abdominal wall wounds by direct transfer when trocars and instruments are withdrawn from the peritoneal cavity [22]. In this instance, the development of barrier strategies to protect the wounds during

laparoscopic surgery is supposed to be sufficient to overcome the problem. However, it is also possible that the insufflation gas used during laparoscopy mobilizes free cancer cells inside the abdominal cavity and thereby, transfer them to the wounds without any direct physical contact with contaminated instruments. In our animal model, we were able to observe how it is possible for cancer cells to become implanted while the pneumoperitoneum is maintained after trocar removal. A variety of methods have been proposed to minimize the likelihood of port site tumor implantation; i.e. wound protectors, impermeable specimen retrieval bags, and cytotoxic or cytolytic irrigations [11, 17, 23] but only one study has been performed to test the efficacy of direct injection of a cytotoxic agent. This study used intraperitoneal povidone-iodine and an appropriate dose of intraperitoneal or intramuscular methotrexate to demonstrate that a reduction in the incidence of port-site metastases might be achieved by the injection of appropriate tumorocidal agents [15]. In this study, IM methotrexate injection over the port-site can reduce the number of port sites with tumor presentation from 74% to 39%, comparable with our result from 68% to 37%.

Oxalipatin is a third-generation platinum derivative whose mechanism of action is similar to cisplatin. Oxaliplatin (l-OHP) differs from cisplatin by the presence of a diaminocyclohexane ligand in its chemical structure. This important difference in

the molecule, and hence in the DNA adducts formed, confers a different spectrum of activity when compared with cisplatin [24]. Oxaliplatin is an active drug in the treatment of advanced colorectal carcinoma that is either chemotherapy naïve or refractory to 5-FU. In advanced colorectal cancer, two randomized studies from Europe reported promising results with a combination of oxaliplatin, fluorouracil (FU), and leucovorin (LV) as first-line treatment compared with 5-FU and LV alone [25, 26]. Oxaliplatin has been licensed in Europe since 1999, but it only gained FDA approval in the United States in August of 2002. Accordingly, we chose to study oxaliplatin as a possible cytotoxic agent to prevent port-site metastases. It is critical to determine the correct dose of oxaliplatin to be used to enable the success of this modality of treatment. Therefore, we performed an in-vitro study to test the cytotoxicity effect of this drug on the cancer cell line we used in our animal model and determine the concentration to be used in-vivo. No necrosis was observed at the injection site based on the necropsy and our pathology findings. This proved that the dose of oxalipaltin chosen was not toxic to the tissues. The results of the current study demonstrate a significant reduction in the incidence of tumor metastases in the treated muscle layer. There was a borderline statistical significance between the treatment and control port-sites (P=0.1). This suggests that an effective dose of oxaliplatin applied via an intra-muscular injection may reduce the number of viable tumor cells in the

muscle layer and prevent metastasis to develop from implanted viable cells into port-site wounds. Nevertheless, the results suggest that it is difficult to control cancer cell growth in all layers of the abdominal wall using a port site injection of an anticancer drug. To solve this problem of drug delivery to other layers, the use of a biodegradable polymer [27] may be a more suitable alternative to injection. We concluded that topical Oxaliplatin may be useful in reducing the incidence of port site metastasis in our small animal model. Nonetheless, further studies are needed to improve its method of application and verify whether this cytotoxic agent can be successfully applied to clinical practice.

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FIGURE LEGENDS

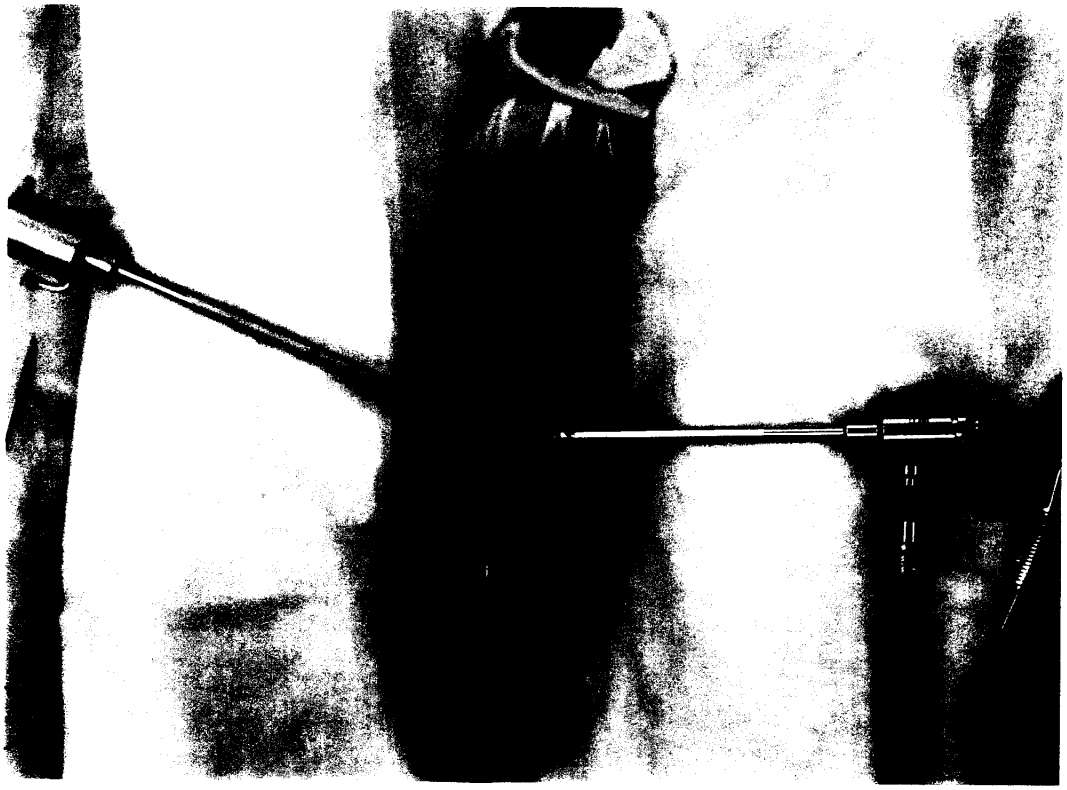
Figure 1. **Pictures of the position of the trocars placement.**

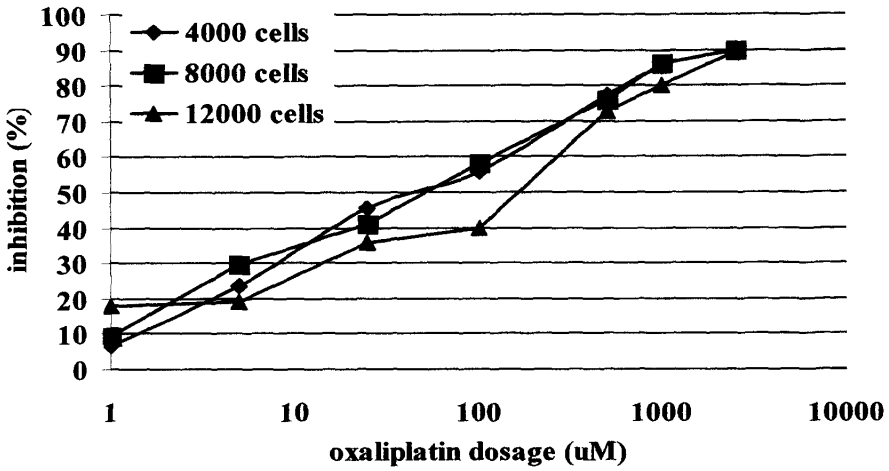
Figure 2. **Inhibition of LMCR-(dimethylhydrazine induced BD IX rat colonic adenocarcinoma on different oxaliplatin level.**

Figure 3. **Gross findings during Autopsy. Arrow indicated the ort-site metastases.**

Figure 4. **Upper panel (arrow) revealed the tumor cells in the peritoneum (100x H&E staining), lower panel (arrow) showed the tumor growth within the muscle (50x H&E staining).**

Figure 5. **Port-site tumor incidence. *P=0.1.**

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inhibition (%)
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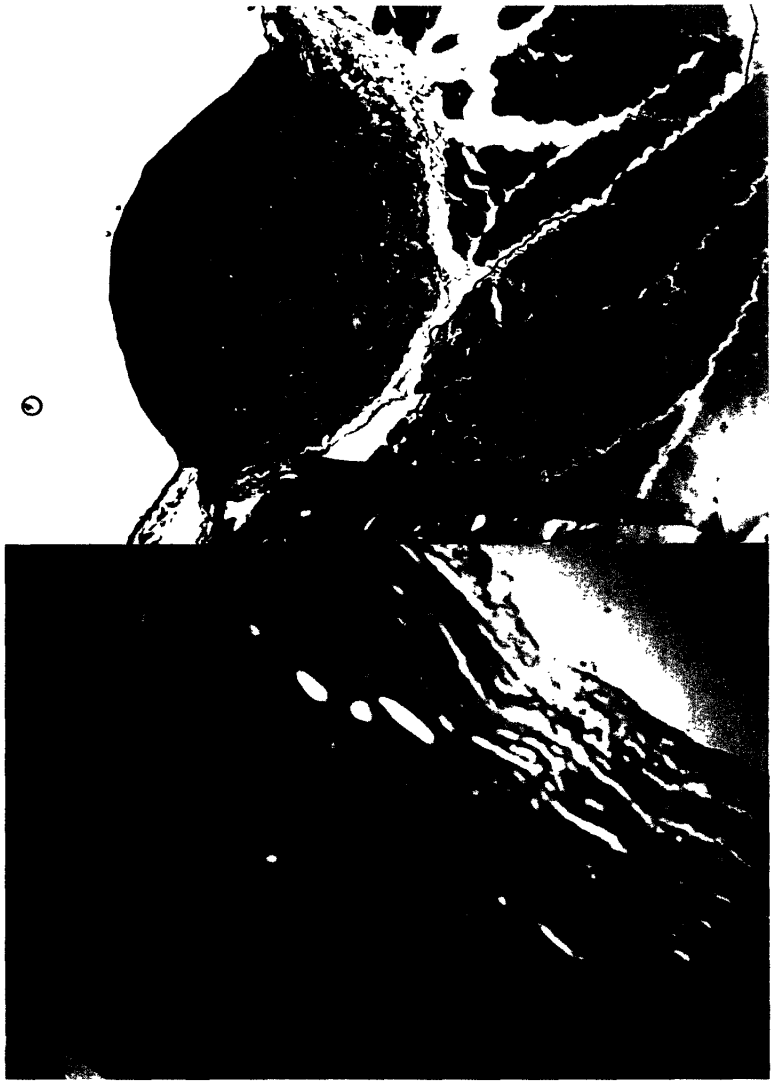

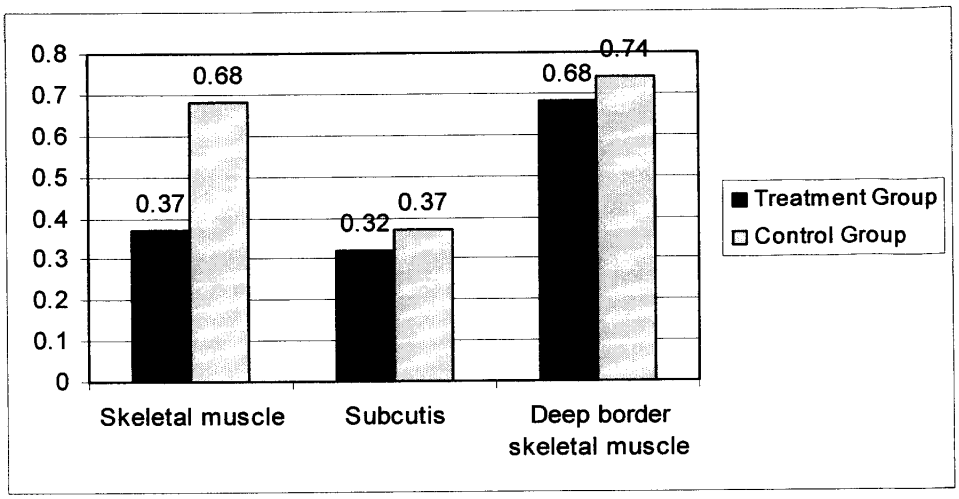
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0.68
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Skeletal muscle
Subcutis
Deep border skeletal muscle
Treatment Group
Control Group

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Oncolysis of Multifocal Hepatocellular Carcinoma in the Rat Liver by Hepatic Artery Infusion of Vesicular Stomatitis Virus

Katsunori Shinozaki, Oliver Ebert, Chryssanthi Kournioti, Yun-Sheng Tai, and Savio L. C. Woo*

Carl C. Icahn Center for Gene Therapy and Molecular Medicine, Mount Sinai School of Medicine, New York, NY 10029-6574, USA

**To whom correspondence and reprints should be addressed at the Carl C. Icahn Center for Gene Therapy and Molecular Medicine, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1496, New York, NY 10029-6574, USA. Fax: (212) 803-6740. E-mail: savio.woo@mssm.edu.*

Hepatocellular carcinoma (HCC) is a lethal malignancy with poor prognosis and few effective treatments, as well as ever-increasing frequencies in the Western world. Viruses that replicate selectively in cancer cells hold considerable promise as novel therapeutic agents for the treatment of malignancy. Vesicular stomatitis virus (VSV) is a negative-strand RNA virus with intrinsic oncolytic specificity due to significantly attenuated antiviral responses in many tumor cells. The aim of this study was to evaluate the potential of VSV, administered via the hepatic artery, as an effective and safe therapeutic agent for treating "multifocal" HCC in the rat liver. Recombinant VSV vector expressing β-galactosidase (rVSV-β-gal) was generated by reverse genetics and infused into the hepatic artery of Buffalo rats bearing orthotopically implanted multifocal HCC. Access by the virus to multifocal HCC lesions in the liver, as well as the kinetic profiles of intratumoral viral replication and spread, was established by X-gal staining of liver and tumor sections. Plaque assays were also performed to determine the infectious viral yields in tumor and normal liver tissues. Pharmacotoxicology studies, including serum chemistries and proinflammatory cytokine production, as well as organ histopathology, were performed. Buffer- or vector-treated tumor-bearing rats were followed for survival and the results were analyzed by the Kaplan–Meier method and the log-rank test. Hepatic arterial infusion of rVSV-β-gal at the maximum tolerated dose in tumor-bearing rats resulted in efficient viral transduction of multifocal HCC lesions in their livers, tumor-selective viral replication, and extensive oncolysis. Importantly, no significant vector-associated toxicities were noted and, in particular, no damage to the hepatic parenchyma was seen. Finally, survival of vector-treated rats was substantially prolonged over that of animals in the control treatment group ($p < 0.028$). Thus, hepatic arterial administration of VSV is both effective and safe in an orthotopic animal model of multifocal HCC. The results suggest that oncolytic VSV can be developed into an effective and safe therapeutic modality for patients with multifocal HCC in the future.

Keywords: multifocal hepatocellular carcinoma, oncolytic virus, vesicular stomatitis virus, cancer gene therapy

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths and the fifth most common cancer in the world, accounting for over 1 million cases annually [1–3]. In the United States, its incidence has increased from 1.4 to 2.4 per 100,000 for the periods 1976–1980 and 1991–1995, respectively [4, 5], which may be related to an increase in HCC related to chronic hepatitis C virus (HCV) infection [6]. Currently, 3.9 million people in the United States are infected with HCV, with a projection of 27,200 deaths from HCV-related HCC in the years 2010–2019 and $10.7 billion direct medical expenditures [7]. Survival of patients with HCC is dependent on the extent of both the malignancy and the underlying liver disease. Studies have reported a median survival of 7.8 months and a 3-year survival rate of 10% [8]. The treatment modalities for HCC with demonstrated survival prolongation are hepatic resection and local–regional intratumoral ablation procedures for solitary tumor nodules. For patients with multifocal lesions localized to the liver,

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regional approaches such as orthotopic liver transplantation offer long-term remission with 3-year survival rates of 39–57% [9, 10]. However, the supply of transplantable livers is very limited, and patients often face waiting periods of over 1 year, which exceeds the medium survival time by more than 4 months. HCC is therefore a disease with poor prognosis and limited treatment options, and development of new and more effective treatment modalities is urgently needed.

Oncolytic viruses provide an attractive new tool for cancer treatment because of their ability to replicate selectively within the tumor and kill neighboring cancer cells upon tumor lysis and secondary infection [11]. In addition to viruses that are molecularly engineered to replicate preferentially in tumor cells, oncolytic RNA viruses, including reovirus, Newcastle disease virus, measles virus, mumps virus, and vesicular stomatitis virus (VSV), are currently being developed as a novel class of anti-tumor agents because of their inherent preference for replication in tumor cells [12–15]. VSV is a negative-strand RNA virus of the family *Rhabdoviridae* with inherent specificity for replication in tumor cells due to their attenuated antiviral responses mediated by type 1 interferons [16]. The New Jersey and Indiana serotypes of VSV infect insects and mammals and cause the economically important disease called *vesicular stomatitis* in cattle, horses, and swine [17]. Infections in humans are asymptomatic in most cases or result in a mild febrile illness. VSV is not endemic to the North American population, implying that there will not be preexisting neutralizing antibodies in most patients to interfere with its oncolytic potential [18]. We have recently demonstrated the oncolytic potential of VSV against human and rat HCC *in vitro*

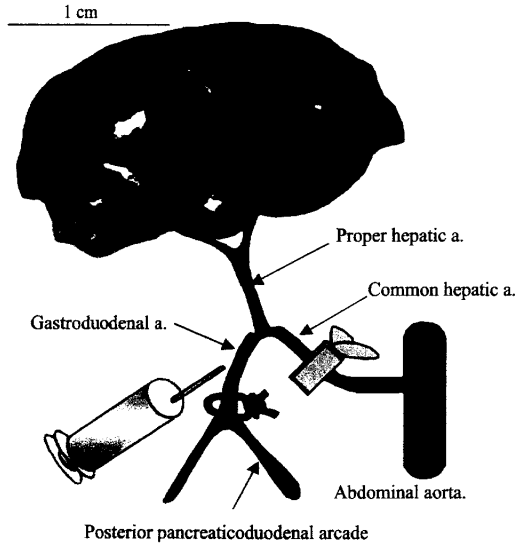

FIG. 1. Representative picture of a rat liver with multifocal HCC lesions superimposed with a schematic representation of the hepatic arterial infusion procedure. 15 days after tumor cell infusion into the portal vein, 90–100% of rats developed visible multifocal HCC lesions macroscopically (1–6 mm in diameter, 5 to 10 nodules). 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 s into the gastroduodenal artery. Then, 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.

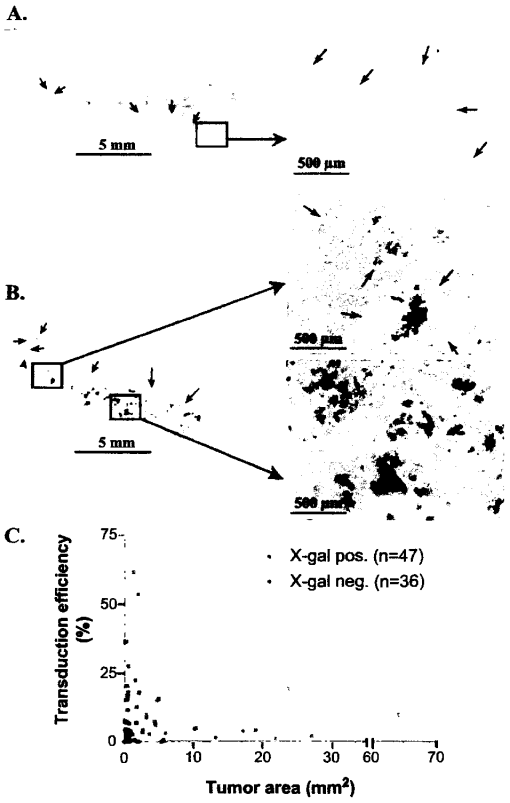

FIG. 2. Transduction and tumor-selective replication of VSV after hepatic artery administration in rats bearing multifocal HCC. Sets of animals (n = 3/time point) were sacrificed (A) at 30 min and (B) at day 1 post-virus infusion, and frozen liver sections were stained for β-gal expression. Representative sections are shown at low (left) and higher magnifications (right). Arrows indicate the borders between tumor lesions and hepatic parenchyma. (C) Relationship between tumor size and transduction efficiency. HCC-containing liver sections were analyzed by morphometric analysis to quantify total tumor and X-gal-positive areas.

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and *in vivo* [19]. We have found that recombinant VSV expressing green fluorescent protein (GFP) (rVSV-GFP) replicated efficiently in cultured human and rat HCC cells, whereas normal primary human and rat hepatocytes were refractory. When a single dose of the vector was injected intratumorally into large solitary HCC nodules orthotopically implanted in the livers of syngeneic rats, rVSV-GFP selectively replicated in the solid tumor mass and caused massive tumor destruction, which led to significant prolongation of animal survival. While the results are encouraging, patients with HCC often present with multifocal hepatic lesions and are not amenable to intratumoral injections to all lesions. In the present study, we tested the hypothesis that multifocal HCC lesions in the livers of immune-competent rats can be readily accessed by VSV via hepatic artery administration, which will lead to tumor-selective viral replication and spread, oncolysis, and survival advantage of the treated animals.

RESULTS

Multifocal HCC Model in the Livers of Immune-Competent and Syngeneic Rats

Fifteen days after portal vein infusion of 10^7 rat HCC (McA-RH7777) cells in syngeneic Buffalo rats, 90–100% of animals developed visible multifocal HCC lesions of 1–6 mm in diameter in their livers (Fig. 1). The tumor model also appeared to be liver-specific, as no metastatic lesions were detected in the other major organs.

Access to Multifocal HCC Lesions and Tumor-Selective Replication of VSV after Hepatic Artery Infusion

First, we determined the maximum tolerated dose (MTD) of recombinant VSV administered into the hepatic artery of Buffalo rats. We infused recombinant VSV in half-log dose increments into tumor-bearing animals (n = 10/dose level cohort) and determined the highest dose that resulted in no vector-associated toxicities to be 1.3×10^7 plaque-forming units (pfu), which was used in all subsequent studies.

To evaluate whether VSV administered into the hepatic artery could gain access to, and selectively replicate in, multifocal HCC lesions in the liver, we conducted a kinetics study with sacrifice of three tumor-bearing animals each at 30 min and 1, 3, 5, 7, and 10 days after infusion with recombinant VSV expressing β-galactosidase (rVSV-β-gal). We explanted the whole liver, obtained frozen sections containing liver and tumor tissues, and subjected them to staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)-containing solution. No β-galactosidase (β-gal)-expression was evident at 30 min post-vector infusion, while 1 day later widely dispersed β-gal expression in HCC lesions of various sizes was visible macroscopically (Fig. 2A). Importantly, at higher magnifications, β-gal expression was noted to be exclusively restricted to the tumors, while normal hepatic parenchyma was completely negative (Fig. 2B). X-gal staining in tumors gradually decreased after 1 day and could no longer be detected after 7 days (data not shown). Other major organs (brain, kidney, lung, and heart) stained for β-gal expression at days 1 and 3 post-virus infusion were all negative (data not shown). The results indicated that the virus readily gained access to, and productively transduced, the multifocal HCC lesions after hepatic artery administration.

To analyze the relationship between tumor size and transduction efficiency of intravascular VSV therapy in multifocal HCC disease, we examined, by morphometric analysis, X-gal-stained tumor-bearing liver sections that we obtained from three rats sacrificed at 1 day after virus infusion (Fig. 2C). We evaluated a total of 83 randomly selected tumor nodules with an average area of 3.52 ± 0.55 mm^2. Of those, we found 47 nodules (57%) to be positive for X-gal. The average fraction of X-gal-positive cells in the lesions was 10.8 ± 12.7%, not including the necrotic regions, which were significantly larger than the nontransduced tumor nodules ($P = 0.0053$). However, we found no significant correlation between tumor area and transduction efficiency.

To determine quantitatively the extents of viral replication in the tumors versus the liver, we harvested respective tissues at each time point, mechanically lysed them, and centrifuged them to remove cellular debris, and we used the supernatants to perform plaque assays on BHK-21 cells. The results indicated that there was more than a 1000-fold increase in infectious virus yield in the HCC lesions from 30 min to day 1 post-vector infusion. In contrast, infectious virus yield in the liver

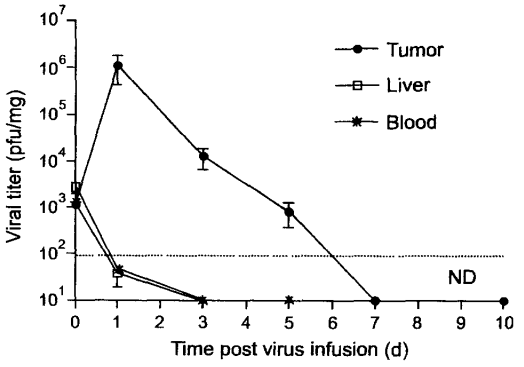

FIG. 3. Quantification of intratumoral virus replication and kinetic profiles of infectious virus yields in tumor, liver, and blood. Viral titers (pfu/mg) in samples obtained from tumor-bearing animals at 30 min and days 1, 3, 5, 7, and 10 post-virus infusion into the hepatic artery are shown (mean ± standard deviation; n = 3/time point). Tumor lesions, normal liver tissues, and whole blood were obtained for infectious virus extraction, and the samples were analyzed by plaque assay. ND, not detected (<100 pfu/mg).

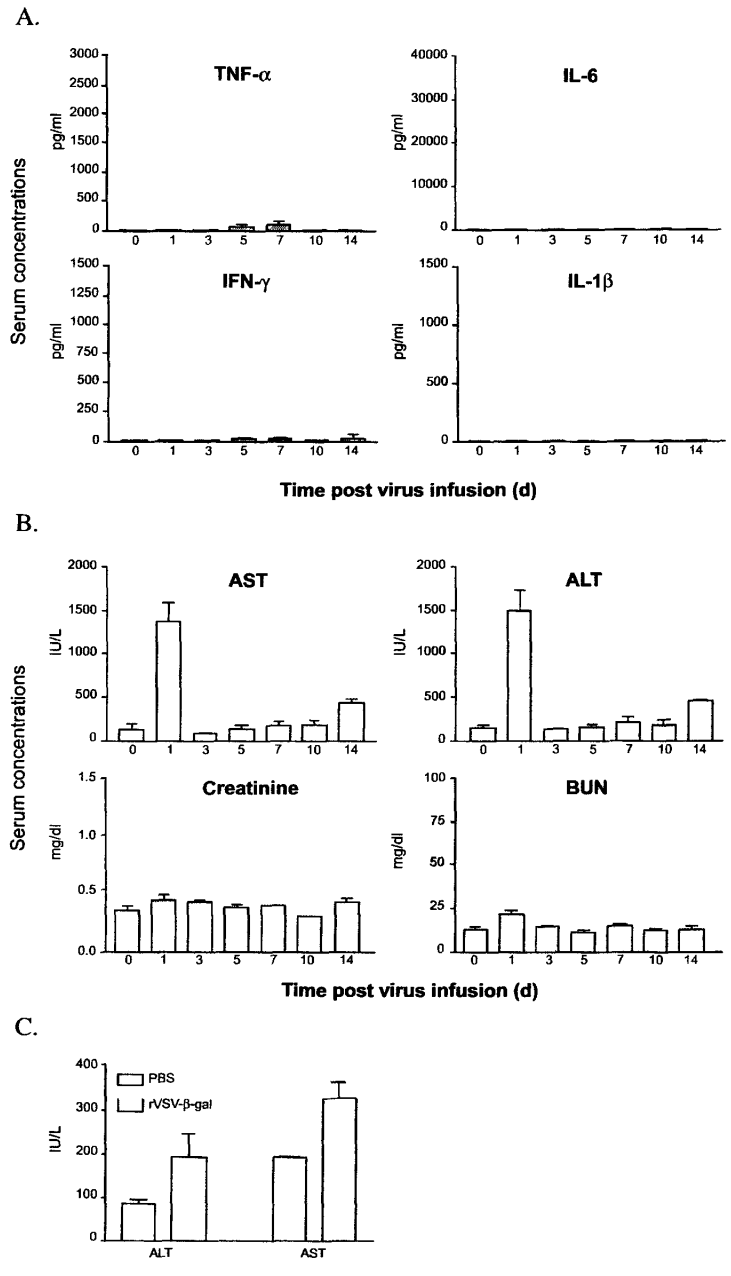

FIG. 4. (A) Serum proinflammatory cytokines and (B) blood chemistries in tumor-bearing animals after hepatic arterial infusion of VSV. Blood samples were collected from the same sets of animals as in Fig. 3 at the indicated time points (time point 0, before virus injection) and serum chemistry was performed. Serum cytokine levels were determined using an ELISA. Maximal values on each *y* axis indicate toxic thresholds for these cytokines in clinical trials reported by others [20]. (C) Serum transaminase levels obtained at day 1 from non-tumor-bearing animals infused with rVSV-β-gal or PBS control.

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was reduced by 100-fold over the same period (Fig. 3). The 5-log differential in viral titers provided unambiguous evidence that rVSV-β-gal selectively replicated within the tumor lesions. We also determined the viral titer in blood and observed a rapid decline similar to that in the liver (Fig. 3), indicating that there was no viremia after intravascular administration of VSV. Intratumoral virus yield decreased gradually after day 1 and became undetectable after day 7, indicating that intratumoral virus replication was not sustained over the long term in the immune-competent host.

Serum Proinflammatory Cytokines and Blood Chemistry

To assess the systemic proinflammatory response and toxicity after hepatic arterial administration of VSV at the MTD, we determined the kinetic profiles of blood chemistries and serum cytokine levels (mean ± standard deviation, $n = 3$ for each time point) in the same experimental animal groups as above. The blood levels of proinflammatory cytokines (TNF-α, IFN-γ, IL-6, and IL-1β) in the VSV-injected animals were comparable to those before injection of the virus at all time points and were well below concentrations associated with systemic toxicity in animals and in human clinical trials [20] (Fig. 4A). The results indicated that hepatic arterial administration of recombinant VSV at the MTD did not induce a systemic proinflammatory cytokine response in the immune-competent rat model. There were also no elevations of blood urea nitrogen (BUN) or creatinine at all time points, indicating a lack of nephrotoxicity (Fig. 4B). Significant elevations of serum transaminases (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) were seen at day 1 after hepatic arterial administration of rVSV-β-gal, although the levels rapidly returned to baseline at day 3 (Fig. 4B). To determine if the significant but transient rise in serum transaminases was caused by toxicity to the liver or oncolysis of the HCC lesions, we infused non-tumor-bearing animals with the same dose of rVSV-β-gal ($n = 3$) or PBS control ($n = 3$) in a follow-up experiment and measured serum transaminases 1 day later. The results indicated that rVSV-β-

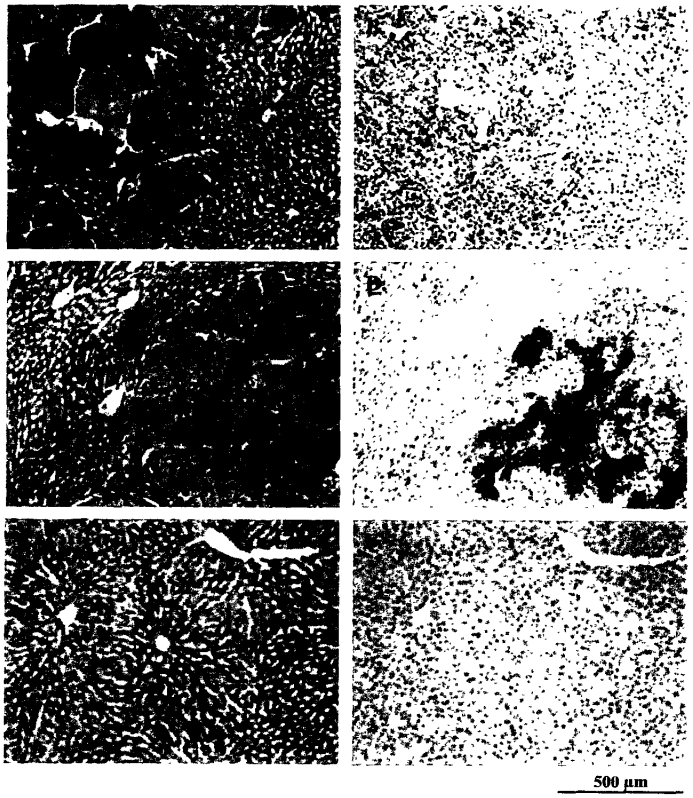

FIG. 5. Histopathology of liver sections of tumor-bearing animals after hepatic arterial infusion of VSV. H&E and immunohistochemistry against VSV-G protein of representative sections of the liver (A and B) before and (C, D, E, and F) 1 day after virus infusion. Arrows indicate necrotic areas.

gal infusion into the hepatic artery of non-tumor-bearing animals caused only mild elevations in serum transaminases compared to buffer-injected control animals (Fig. 4C). Therefore, the significant but transient elevation in serum transaminases on day 1 in tumor-bearing animals infused with rVSV-β-gal (Fig. 4B) can be attributed to massive VSV-induced oncolysis of HCC cells and subsequent release of transaminases into the circulation. Serum transaminase levels in tumor-bearing animals also increased slightly at 14 days posttreatment, which might be indicative of tumor progression.

Histopathological Examination of Tumors and Livers

To demonstrate further the tumor specificity of VSV replication and absence of liver damage, we obtained histopathological sections showing the regions of tumor border with normal liver tissues. Control sections obtained from PBS-treated tumor-bearing animals revealed minimal spontaneous necrotic areas within the tumor lesions (Fig. 5A), and all samples were negative for VSV-G protein by immunohistochemical staining (Fig. 5B). In contrast, large necrotic areas were apparent within multiple tumor lesions at day 1 post-virus infusion (Fig. 5C), which were strongly positive for VSV-G protein in the consecutive section (Fig. 5D). Histology of neighboring hepatic parenchyma was completely normal (Fig. 5E), and in the consecutive section there was no evidence for VSV-G expression by immunohistochemistry (Fig. 5F). The results confirmed the notion that there was no significant hepatotoxicity associated with hepatic artery infusion of VSV at the MTD.

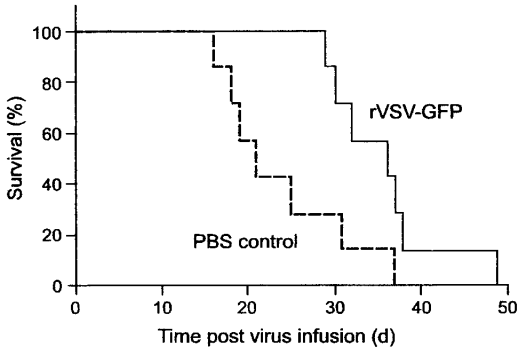

FIG. 6. Kaplan–Meier survival curve of rats with multifocal HCC after hepatic arterial infusion of VSV versus PBS. Animals with multifocal HCC were infused with 1.3×10^7 pfu rVSV-GFP (solid line) or PBS control (dashed line) via the hepatic artery on day 0 and followed for survival. The survival advantage for VSV-administered animals was statistically significant compared with PBS control animals ($P = 0.028$, log-rank test). The results were combined from two consecutive sets of animals with stratification.

***In Vivo* Anti-tumor Efficacy**

To assess the potential of VSV as a therapeutic agent for multifocal HCC, we randomly assigned rats bearing 5–10 visible HCC tumors in their livers with sizes ranging from 1 to 6 mm in diameter to infusion with VSV at 1.3×10^7 pfu ($n = 7$) or PBS control ($n = 7$) via the hepatic artery and followed their survival (Fig. 6). Buffer-treated rats started to die of tumor progression in 16 more days and all of them expired by 37 days. The VSV-treated rats survived for up to 49 days post-vector injection, and the differential survival rates were statistically significant by log-rank test analysis ($p = 0.028$). The median survival in the virus-injected animal group increased to 36 days from 21 days in the PBS-injected group.

Discussion

As HCC patients often present with multifocal lesions in their livers, it is critically important for any therapeutic strategy developed for HCC to be effective against multifocal disease. Theoretically, for gene therapy of multifocal HCC tumors, vector delivery via the vascular route would be advantageous. 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, and hepatic artery infusion is the most commonly employed method for local–regional therapy of HCC in current clinical practice [21]. Intrahepatic artery delivery of replication-deficient adenoviruses in transplanted or chemical-induced animal models of multifocal HCC has been shown to transduce predominantly small lesions of less than 5 mm in diameter with relatively low efficiencies, while significant transduction of normal hepatic parynchyma could not be avoided [22–24]. More recently, replication-conditional viruses have been examined for their oncolytic potential against HCC, including adenovirus [25–27], herpes simplex virus (HSV) [28,29], and autonomous parvovirus H1 [30]. Portal vein infusion of the replication-conditional HSV-1 mutant rRp450 has been reported to destroy multifocal HCC selectively in the Buffalo rat model without apparent liver toxicity [29].

We reported previously on the preferential replication of oncolytic VSV within large solitary HCC nodules in the liver after intratumoral administration [19]. While encouraging, this mode of administration would not be expected to be very effective against multifocal disease. In this study, we provide conclusive evidence that VSV can gain access to multifocal HCC lesions of various sizes in the liver after hepatic arterial administration, replicate to high titers, and spread efficiently within the tumors. VSV buds from the plasma membrane of infected cells at the basolateral surface [31]. This viral morphogenesis predisposes VSV to invasiveness, which may explain

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why viral replication appears not to be limited to the initial infection site and spreads efficiently in the solid tumor mass.

Intratumoral VSV replication was significantly reduced by day 3 post-vector injection before the onset of adaptive immunity. Therefore, the transient nature of viral replication may be due to antiviral responses mediated by the innate immune system. We have indeed found strong NK cell infiltrates within VSV-treated tumors at day 5 post-vector injection (data not shown), which may be responsible for eliminating VSV directly by killing of virally infected tumor cells or indirectly by secretion of cytokines. In addition, it is possible that humoral immune mechanisms may have contributed to the time-limited VSV replication. It is known that control of VSV in naïve animals strongly depends on natural or early induced antibodies [32,33]. The emergence of T-cell-independent neutralizing IgM antibodies occurs very early after infection, by day 3 or 4 [34,35]. Due to the exceptionally rapid replication rate of VSV in tumor cells, however, we observed significant oncolytic effects that manifested before the onset of neutralizing antiviral immune responses.

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. In this regard, previous studies in our laboratory indicate that replication of VSV is severely attenuated in normal primary rat and human hepatocytes *in vitro*, while efficient VSV replication was observed in established rat and human HCC cell lines [19]. In the present study, there was no demonstrable replication of the virus in normal tissues in the animals following 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 [18]. 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 [36]. It was previously demonstrated, however, that transgenes inserted as an additional transcriptional unit into the noncoding region of the VSV-G gene were maintained stably over multiple passages *in vitro* [37]. In our hands, there was loss of neither viral titer over multiple passages *in vitro* nor anti-tumor efficacy *in vivo*. Finally, VSV is extremely sensitive to the antiviral actions of type I interferons (IFN) in normal cells, providing an effective tool to suppress unwanted replication in normal tissues, if necessary [38].

Although we detected large necrotic regions within multiple tumors, which led to significant prolongation of animal survival, there were no long-term survivors. It should, however, be noted that the anti-tumor activity was achieved after a single administration of VSV, which achieved its maximal replication in the tumors after only 1 day. Multiple intrahepatic artery infusions before the onset of effective antiviral immune responses in the host over periods of 1–2 weeks will probably result in more significant tumoricidal effects than a single injection. While multiple dosing, however, is technically difficult to examine in the current model, percutaneous catheterization of the hepatic artery for local–regional therapy of liver tumors is a standard procedure in the clinic, thereby potentially allowing readministration of the oncolytic virus. To improve the antitumor efficacy of oncolytic viruses further, vectors expressing various transgenes may be used to increase their oncolytic activity [39–42]. In this regard, recombinant VSV vectors with insertion of an additional transcription unit encoding the IL-4 gene or the HSV-1 thymidine kinase suicide gene were generated and reported to exhibit considerably more oncolytic activity than a control virus expressing GFP [43]. Recently published data [44,45] presented the generation of IFN-inducing VSV variants or recombinant VSV vectors expressing IFN-β with greatly improved therapeutic indices over wild-type VSV after systemic delivery. These and other transgenes can be integrated into recombinant VSV vectors to increase their oncolytic potential further.

In conclusion, hepatic arterial administration of VSV is both effective and safe in a preclinical orthotopic animal model of multifocal HCC, suggesting that it can be developed into an effective therapeutic modality for multifocal HCC in patients in the future.

Materials and Methods

Cell culture. The rat HCC cell line McA-RH7777 was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium (DMEM; Mediatech, Herndon, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO, USA) in a humidified atmosphere containing 10% CO_2 at 37°C. BHK-21 cells (ATCC) used for amplification and titration of VSV were maintained in DMEM (Mediatech) with 10% FBS (Sigma–Aldrich) in a humidified atmosphere at 5% CO2 and 37°C. All media used in this study contained 100 U/ml penicillin–streptomycin (Mediatech).

Construction of recombinant vesicular stomatitis virus. To generate recombinant VSV expressing β-gal, the β-gal sequence was amplified by PCR from plasmid pβgal-Basic (BD Biosciences, Palo Alto, CA, USA) using the forward (5′ -ACTGTA**CTCGAG**ATGTCGTTTACTTTGACCA-3′) and reverse (5′ -GTAATC**GCTAGC**TTATTTTTGACACCAGACCA-3′) primers containing unique restriction sites (in bold) for *Xho*I and *Nhe*I, respectively. The PCR product was digested with *Xho*I and *Nhe*I and ligated into the full-length pVSV-XN2 plasmid (kind gift from Dr. J. Rose, Yale University) previously digested with the same enzymes. Recombinant VSV expressing β-gal or GFP [19] was generated using the established

method of reverse genetics [46,47]. After successful recovery, vaccinia virus was completely eliminated by plaque purification. The titers (pfu/ml) of viral stocks were determined by standard plaque assay on BHK-21 cells. To exclude potential contaminations with gram-negative bacterial endotoxin in viral preparations, the QCL-1000 Chromogenic LAL Test Kit assay (Cambrex, Walkersville, MD, USA) was performed.

Animal studies. Inbred male Buffalo rats (6 weeks of age; 122 ± 7 g) were purchased from Harlan (Indianapolis, IN, USA) and housed in a specific-pathogen-free environment under standard conditions. 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. To establish multifocal HCC lesions within the liver, rats were anesthetized with 100 mg/kg ketamine (ip), 1 mg/kg xylazine (ip), and isoflurane using an inhalation anesthesia system (VetEquip, Pleasanton, CA, USA). Subsequently, rats were infused with 10^7 syngeneic McA-RH7777 rat HCC cells in 1 ml of DMEM via the portal vein. Fifteen 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, recombinant VSV vector (1.3×10^7 pfu) in 1 ml of PBS or an equivalent volume of buffer was administered via the hepatic artery (see Fig. 1 and legend for detailed description of surgical manipulations).

To evaluate the kinetics of viral replication within the tumor lesions versus the normal liver, sets of animals were sacrificed at various time points after hepatic arterial infusion of rVSV-β-gal. Tissue samples were obtained using an operating microscope and subjected to plaque assays to determine the viral yield. In addition, histological analysis and X-gal staining were performed. In a separate experiment, groups of animals infused with vector or buffer control were followed for survival, which was checked daily in all animals.

Recovery of virus from tumor and tissue extracts. Tumor and normal liver tissues were harvested and disaggregated under sterile conditions. In addition, heparinized whole blood samples were collected and lysed by freeze–thaw. The suspensions were centrifuged at low speed to remove cellular debris and the supernatants were used to perform plaque assays on BHK-21 cells (sensitivity 100 pfu/mg).

X-gal staining. The organs were fixed in 1% paraformaldehyde for 4 h at 4°C, and then in 18% sucrose solution overnight. After fixation, the blocks were embedded in Tissue-Tek (Sakura Finetek USA, Torrance, CA, USA), immediately frozen by floating on 2-methylbutane (Sigma–Aldrich, Milwaukee, WI, USA) precooled with dry ice, and stored at −80°C until sectioning. Cryostat sections of 10-μm thickness were stained by immersion in X-gal staining solution for 16 h at 37°C. Sections were counterstained with Nuclear Fast red (Vector Laboratories, Burlingame, CA, USA).

Histology and immunohistochemical staining. At the indicated time points after vector infusion into the hepatic artery, animals were sacrificed, and explanted livers were fixed in 4% paraformaldehyde overnight and then paraffin embedded. Five-micrometer-thin sections 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, USA). Immunohistochemistry sections were counterstained with hematoxylin.

Assessment of cytokine production and serum chemistry. Blood samples were collected from the inferior vena cava at the time of euthanization or before virus injection, and the levels of rat serum cytokines were determined by an ELISA (Biosource, Camarillo, CA, USA). Serum chemistry including ALT, AST, BUN, and creatinine was performed at the Chemistry Laboratory at Mount Sinai School of Medicine.

Statistical analyses. For morphometric analysis of tumor sizes and transduction efficiencies, the software Image Pro Plus 4.5.1 (Media Cybernetics, Silver Spring, MD, USA) was used, and results were analyzed for statistical significance using an unpaired *t* test. 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, USA).

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