Engineering Oncolytic Vaccinia Viruses for Non-Invasive Optical Imaging of Tumors

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Abstract: Attenuated vaccinia viruses (VV) selectively replicate in malignant cells and confer oncolytic effect *in vivo*. Here we demonstrate that oncolytic VV may also be used as a diagnostic agent for tumor-bearing mice. A series of recombinant vaccinia viruses has been constructed expressing optical reporters to mediate emission of bioluminescent and fluorescent light which can be visualized. Data show that following systemic virus delivery the developing tumors can be non-invasively visualized in mice *in vivo*. *Renilla* luciferase and *Aquoria* GFP have been effective in imaging xenografted PC-3 prostate and orthotopic MB-49 bladder tumors. Brighter reporters, *Gaussia* luciferase and *Renilla* GFP have been used for imaging TRAMP prostate cancer and C6 subcutaneous model of glioma. The C6 imaging data have been corroborated by traditional MRI. We are also developing a VV-mediated system for tumor detection in far red or near infra red fluorescent light. Results suggest that VV-mediated imaging is a promising alternative for early diagnosis of various human cancers.

INTRODUCTION

Current methods of external imaging of internally growing tumors, e.g. X-ray, MRI, PET, ultrasonography, etc., are convenient for noninvasive imaging of the body, but they are less sensitive for monitoring the growth of small tumors, metastatic dissemination, and recurrence. To date, few methods exist that allow noninvasive and repetitive imaging of reporter gene expression in living cells and animals. Reporter genes with optical signatures may become a low-cost alternative for real-time analysis of early stages of tumor development and metastases [1-7]. Advances in technology have made it possible to noninvasively image optical reporter-labeled tumors in living animal [8, 9] and define critical pathways involved in tumorigenesis, metastasis, and evaluate the efficiency of gene therapy strategies [3, 10-12]. In particular, green fluorescence protein (GFP) and luciferase-labeled tumor/metastasis growth have been visualized and analyzed using fluorescence [7, 13-16] or bioluminescence [17-19] imaging instrumentation.

Optical imaging of GFP gene expression offers specificity, sensitivity, simplicity, and good resolution at a cellular level. No contrast agent or other compounds or treatments are needed, only UV illumination is necessary. The GFP delivered to various organs of mice is stable over long time periods allowing visualization of dynamic studies in whole body noninvasively and in real-time, and at necropsy as well. Visualization of gene expression at high resolution at the single-cell level is possible using fluorescence reporters, confocal laser, two-photon excitation, or stereo fluorescence microscopy [20, 21]. Micro-vessel development can also be followed in real time, allowing precise evaluation of tumor progression and neovascularization. The sensitivity of external imaging *in vivo* is currently limited by light scattering in intervening tissue, especially in skin, and is sufficient for relatively shallow organs, e.g., in subcutaneous labeled xenograft-bearing nude mice [22]. Nevertheless, due to rapid progress in technology whole-body, real-time visualization of GFP in the major internal organs of intact mice, including brain, liver, pancreas, prostate, and bone, has already become feasible [10]. Metastases of GFP-labeled cancer cells inoculated into mice can also be visualized in various organs throughout the body [9]. The resolution of visualized internal organs and tumors significantly depends on the intensity and spectrum of emitted fluorescence signal [7, 23, 24]. If tumor cells labeled with GFP are sufficiently bright they often could be viewed through simple video equipment located externally to the animal [23]. The use of brighter GFP fluorescence may enable detection of internal tumors and metastases labeled with these reporters in critical organs, such as the pancreas, spleen, and liver [7].

In contrast to fluorescence, bioluminescence imaging is not limited by the autofluorescence properties of living cells and does not require any external source of light for activation [25]. It rather depends on the delivery of specific substrates, such as the luciferin in case with firefly luciferase (Fluc). The emitted luciferases-mediated signals are weak, and the imaging resolution at the cellular level is low. However, it can be amplified by prolonged counting of photon emission by charge-coupled device (CCD) cameras combined with an intensifier. The major advantage of bioluminescence imaging is that the low light video camera enables detection of very low levels of bioluminescence emitted from internal parts of the body [26-28] and thus, determining

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the location of the light source. To date, Fluc and *Renilla* (Rluc) luciferases are the main bioluminescence reporters, which have been used as markers of gene expression in bacteria, yeast, plant and mammalian cells [28, 29], and in living mice [6]. The Rluc substrate (CTZ) is diffusable to many tissues upon tail-vein injection and is non-toxic for mammalian cells [30]. Previously, we reported optical imaging of virus-expressed luciferases and GFP for analyses of gene expression and viral replication in cultured mammalian cells, live insect larvae, and experimental animals [31-39]. Noninvasive bioluminescent imaging of luc-labeled murine tumor models has also been shown to correlate with the tumor volume [18] and number of labeled cancer cells injected to animal [40]. Modeling of photon diffusion through tissue indicates that bioluminescent cell counts as low as a few hundred can be detected subcutaneously, while approximately 10^6 labeled cells are required to detect signals at approx. 2 cm depth in tissue [41]. New technical solutions for detection of bioluminescence in the future will lead to significant improvement in localizing and quantifying the emitted light, and in resolution [42]. However, for noninvasive visualization and diagnosis of spontaneously emerging (non-labeled) cancer cells in a living organism development of new strategies are required.

VIRUS-BASED STRATEGY OF TUMOR IMAGING *IN VIVO*

Previously, grafted GFP- and luciferase-labeled tumor/metastasis growth *in vivo* has been visualized non-invasively [7, 13-19]. However, labeling of cultured cancer cells with optical reporters prior to inoculation was an indispensable condition of these studies. Inoculated labeled cancer cells in inoculated animals develop tumors/metastasis which can be visualized *in vivo* by optical instruments. Therefore, currently known optical imaging of tumors is effective only in grafted animal cancer models. Naturally developing tumors, caused by spontaneous or introduced (in transgenic models) mutations, can not be imaged and detected by this approach. However, it can be achieved using tumor-targeting recombinant viruses. Over last several years, attention of many investigators in the area of cancer therapy, including our laboratory, was turned to oncolytic viruses, i.e. viruses selectively lysing cancer cells [43-48]. It has become evident that oncolytic recombinant VV carrying sensitive reporters could be a safe and promising tool for specific tumor targeting and imaging. Indeed, each viral particle is able to propagate in tumor cells up to 1,000-10,000 virus copies per cell producing abundant amount of reporter proteins, e.g. fluorescence protein and luciferase, which is an important condition for optical imaging of deep tissues. Recently, we have learned that certain attenuated VV strains possess these useful attributes. Thus, oncolytic activity of systemically delivered attenuated VV in established tumors has been demonstrated in murine and rabbit tumor models [49-53]. Here, we describe our experience in using oncolytic VV as a powerful tool for optical imaging of solid tumors in several experimental animal cancer models.

RECOMBINANT VIRUSES FOR TUMOR IMAGING *IN VIVO*

To monitor the spread of vaccinia virus in live animals first we constructed the recombinant vaccinia virus VV-RG [39] expressing the fusion protein of Rluc and *Aquoria* jellyfish GFP (Fig. **1**). Replication of the VV-RG can be monitored on live animals by both fluorescent microscopy or imager and low-light video imager (bioluminescence)**.** Furthermore, we have constructed more advanced VV expressing brighter optical reporters (provided by Prolume Ltd, Pinetop, AZ). Investigators of the indicated company isolated and characterized three new *gfp* genes from the sea pansies and pens and demonstrated that these reporters emit fluorescence light of higher intensity increasing the sensitivity and enhancing GFP-mediated imaging of internal tissues of living animals [54]. The *Renilla mullerei* GFP (R-GFP) has spectral properties, such as high quantum efficiency, high molar absorbency and efficient use with universally available fluorescein filters, which seem likely to make it very useful for optical imaging. According to Prolume, Ltd., the R-GFP is 6-fold brighter than the A-GFP on a molar basis, and 3-fold brighter than the available brightest modified GFP. In addition, R-GFP is less cytotoxic than A-GFP.

New bioluminescent light emitting luciferase reporter genes have been isolated from the *Gaussia princeps*, in both natural and "humanized" (optimized in codon usage) versions [54]. The *Gaussia* luciferase (Gluc) is extremely stable to elevated temperature. In our study we have constructed a recombinant VV strain expressing both Gluc and R-GFP optical reporters. First, the Gluc and R-gfp genes were inserted into the transfer plasmid p2B8R under control of two early/late synthetic vaccinia-specific promoters and then, the resulting plasmids pBGR12 and pBGR13 were used for the construction of VV-BGR12 and VV-BGR13 virus, respectively, through homologous recombination of the B8R sequences of the VV genome as described earlier [39]. Then, mammalian cells were infected/transfected with the vaccine strain Lister (LIVP) of VV and with the transfer plasmid (pBGR12 or pBGr13). The newly constructed hyper-attenuated viruses VV-BGR12 and VV-BGR13 (Fig. **1**) emitted green fluorescent and bioluminescent lights of approximately equally high intensity. We found that bioluminescent light emitted by Gluc is significantly brighter than the Rluc (Fig. **2**). Comparing VV-BGR12 with VV-RG in identical *in vitro* conditions, cells infected with VV-BGR12 emitted significantly brighter fluorescent light than VV-RG-infected cells expressing the A-GFP (Fig. **3**).

For tumor imaging we are currently developing recombinant viruses which can induce light of far red or near infrared (NIR) wavelengths. These fluorescence proteins are of special interest in optical tumor imaging due to the low absorption in tissue at these wavelengths. While current noninvasive macroscopic imaging with GFP is limited to superficial tumors, it has been predicted that NIR fluorescent light can penetrate the tissue for several centimeters [24]. Potential of red fluorescent proteins (RFP) has already been demonstrated earlier by imaging of RFP-labeled orthotopic prostate tumors in mice [55]. We are constructing a recombinant VV- GRF1 expressing both the RFP and Gluc (Fig. **1**). Testing the constructed transfer plasmid pGRF1 carrying the optical reporters we have confirmed that the RFP is suitable for visualization of VV replication in far red light spectrum of fluorescence (Fig. **4**). We expect that the VV-GFR1 virus will have an advantage in fluorescent optical imaging due to more efficient penetration of RFP-generated far red light. In addition, we explore the possibility of using chemically syn-

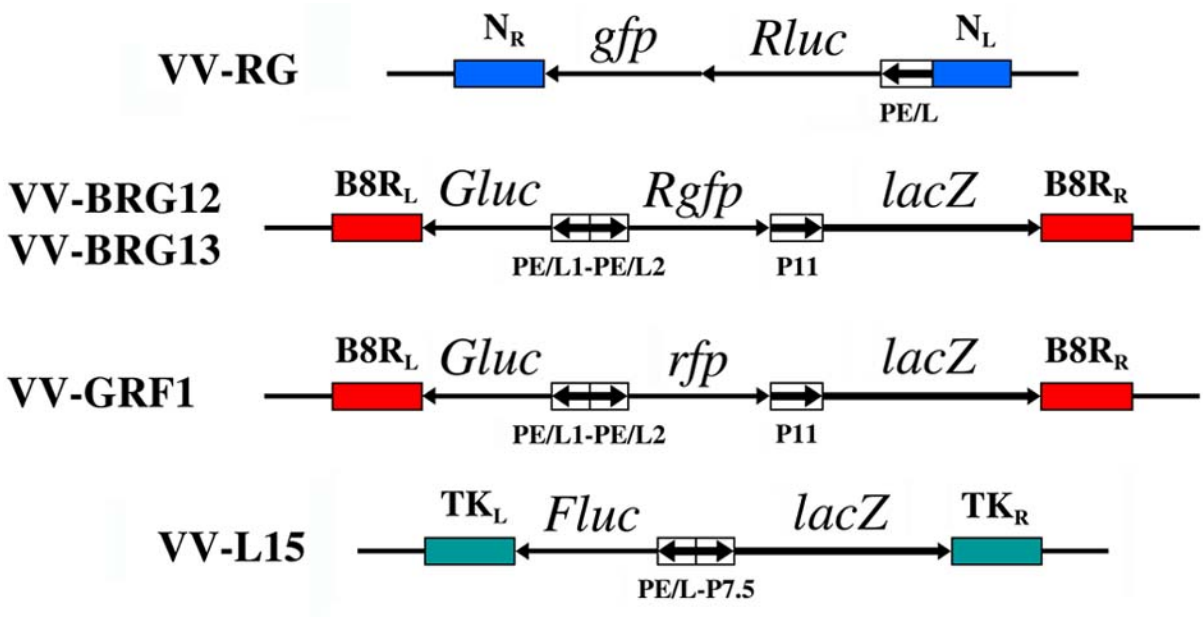

Fig. (1). Expression cassettes of the recombinant vaccinia viruses. The construction of the VV-RG virus expressing fused Rluc and GFP was described earlier [39]. The Rluc/gfp expression cassette was inserted into HindIII-N fragment of the virus genome disrupting the 150 bp F14.5L gene located between F14L and F15L of the Lister strain. Other recombinant viruses were constructed using the plasmid p2B8R (provided by Dr. T. Yilma, UC Davis, CA) and traditional infection/transfection method [39]. In the viruses VV-BGR12 and VV-BGR13 the cassette is inserted into VV B8R gene and the expression of the optical reporters, humanized Gluc [57] and *Renilla* gfp (Rgfp, humanized), is driven by two identical promoters PE/L1 and PE/L2, respectively. The VV-BGR12 virus expresses the entire Gluc gene while in the VV-RGR13 the Gluc lacks the 15 aa signal peptide and expresses the non-secreting reporter protein. In the VV-GRF1, the expression of Gluc and red fluorescence protein (rfp) was driven by PE/L1 and PE/L2. The VV-L15 expressing lacZ and Fluc was described earlier [37].

thesized fluorogenic compounds for virus-mediated tumor imaging that may offer an alternative approach for tumor labeling and imaging in far red or NIR spectra [56]. Preliminary data with the fluorogenic lacZ substrate DDAOC are promising (Fig. **5**) and future experiments on optimizing of imaging of deep malignant tissues may lead to harnessing this less explored imaging method.

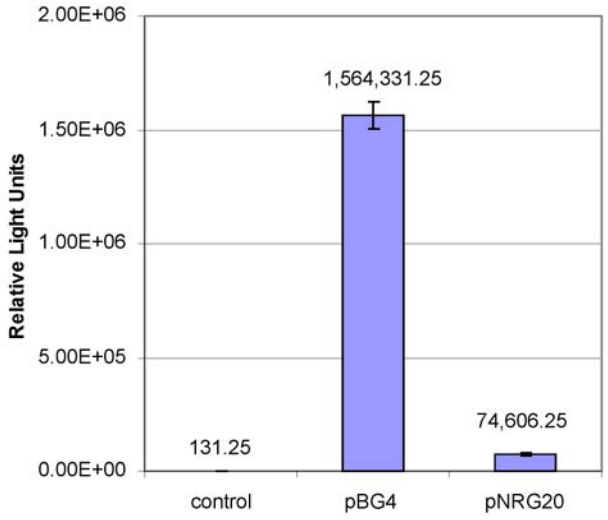

Fig. (2). Bioluminescence of *Renilla* and *Gaussia* luciferases in CV-1 cells. Cells were infected with VV (MOI=1) and then transfected with a plasmid encoding Rluc (pNRG20) or Gluc (pBG4). Light emission of the extracts was measured by luminometer (Dynatech Laboratories, Inc., Chantilly, VA). *In vivo*, the Gluc is 200-fold brighter than the Rluc [54].

VV-MEDIATED IMAGING OF UROLOGICAL TUMORS

The VV-RG virus has been tested in non-invasive imaging in several tumor models following the rules and regulation of the Institutional Animal Care and Use Committee. Experiments have confirmed that the virus selectively replicates in tumor tissues of the animal emitting bioluminescence and fluorescence lights. Visualization of an orthotopic MB-49 bladder tumor in a live mouse following inoculation with VV-RG is demonstrated in Fig. (**6**). A clearly glowing spot of luciferase-associated bioluminescence light has been observed during the whole-body optical imaging of the mouse. At autopsy, the malignant tissue of the bladder was

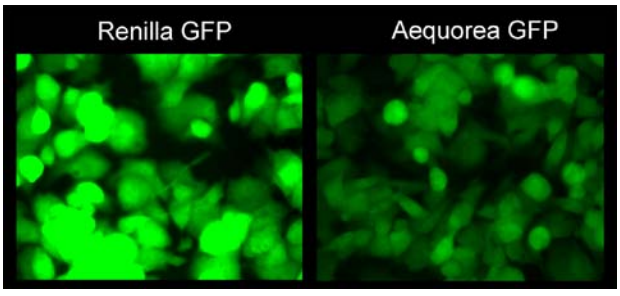

Fig. (3). Comparison of fluorescence brightness emitted by virus-encoded *Aequorea* and *Renilla* GFP. CV-1 monkey kidney cells were infected with VV (MOI=1) expressing either A-GFP (VV-RG) or R-GFP (VV-BGR12), and after overnight incubation the light emissions were compared by fluorescence microscope equipped with CCD Princeton camera.

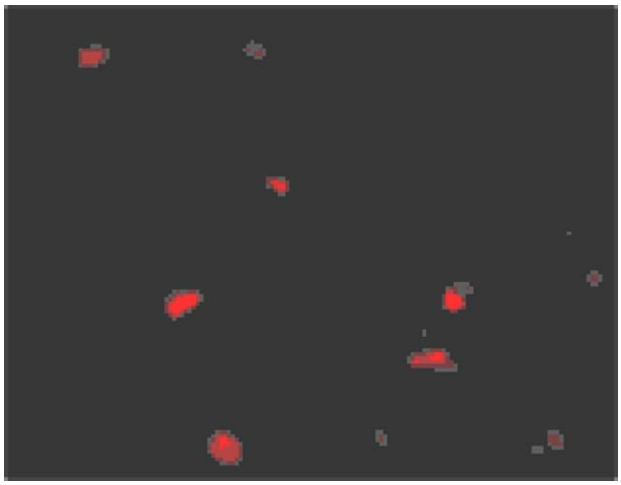

Fig. (4). Construction of recombinant VV expressing red fluorescent protein. The transfer plasmid pGRF1 carries genes for the optical reporters RFP and Gluc (see Fig. **1**). The rfp gene was isolated from the pDsRed2 plasmid provided by B.A. Tannous (Boston, MA). The image demonstrates that cultured CV-1 cells infected with VV and transfected with the pGFR1 express RFP as detected by fluorescent microscopy (Axiovert 100TV, Zeiss equipped with XBO 150 W/10FR lamp and filter N14).

identified as a source of bioluminescence (Rluc) and fluorescence (A-GFP, data not shown). Furthermore, our data indicated that recombinant VV can induce antitumor effect in orthotopic MB-49 tumor model not only through the viral lytic effect and immunologic response, but also through expression of the tumor suppressor transgene p53 [48].

Feasibility of the VV-RG mediated bioluminescent visualization of implanted tumors was confirmed in a prostate SC cancer model PC3. Whole-body optical imaging of tumor-bearing mice following systemic inoculation of the virus detected clearly glowing spots in the tumor area (Fig. **7A**). At autopsy, the bioluminescent (Rluc) and fluorescent (A-GFP) light sources were found to be associated with the tumor tissues (not shown). In other models, the feasibility of using the VV-RG virus (designated by authors as rVV-RUC-GFP or GLV-1d27) for tumor imaging and therapy has been reported elsewhere [58, 59].

The TRAMP mouse model serves as a general prototype for mimicking the pathways, parameters, and mechanisms of multistage prostate tumorigenesis in humans. Published data have proved that this mouse model is convenient and useful for cancer prevention and therapy studies [4, 60-62]. TRAMP mice characteristically express the T antigen oncoprotein by 8 weeks of age and develop pathology in the epithelium of the dorsolateral prostate. Distant site metastases can be detected as early as 12 weeks of age. The common sites of metastases are the periaortic lymph nodes and lungs, with occasional metastases to the kidney, adrenal gland, and bone. By 28 weeks of age, mice harbor metastatic prostate cancer in the lymph nodes or lungs [62]. In contrast with grafted bladder cancer model TRAMP mice develop genetically predetermined tumors/metastases.

Thus, the TRAMP model provides a consistent source of primary and metastatic tumors for molecular analysis to further define the earliest molecular events involved in the genesis, progression, and metastasis of prostate cancer.

We have also attempted to monitor the early development of the tumor in TRAMP mice using the recombinant VV-BGR13 virus. Early stage of cancer in this transgenic model can be observed in prostate gland and other genitourinary organs by 8 weeks of age and is confined to a small area (>2%) of the organ that can not be detected visually [55]. Only histological analyses of tissue sections following staining with hematoxylin and eosin can detect the cancer at this stage of cancer development. Using the VV-BGR13-expressed optical reporters, we have observed clear signals of bioluminescence light in two TRAMP mice at 8 weeks of age (see Fig. **7B-C**). Analysis of isolated glowing tissue on GFP expression showed patchy distribution of fluorescence of the tissue (Fig. **7D-E**). The unusual GFP expression pattern possibly reflects the spread of the virus through microvessels of the cancer tissue. This observation will be further explored in future studies.

OPTICAL IMAGING OF GLIOMA IN MICE

The potential of the VV-encoded Gluc- and R-GFP-mediated tumor imaging has been recently demonstrated in our lab using SC model of C6 glioma in mice. Two nude mice carrying tumors in the right flank were systemically injected with the rVV-BGR12 and after several days were analyzed by stereo fluorescence microscopy (Fig. **8**), low-light video imager, and compared with MRI data (Fig. **9**).

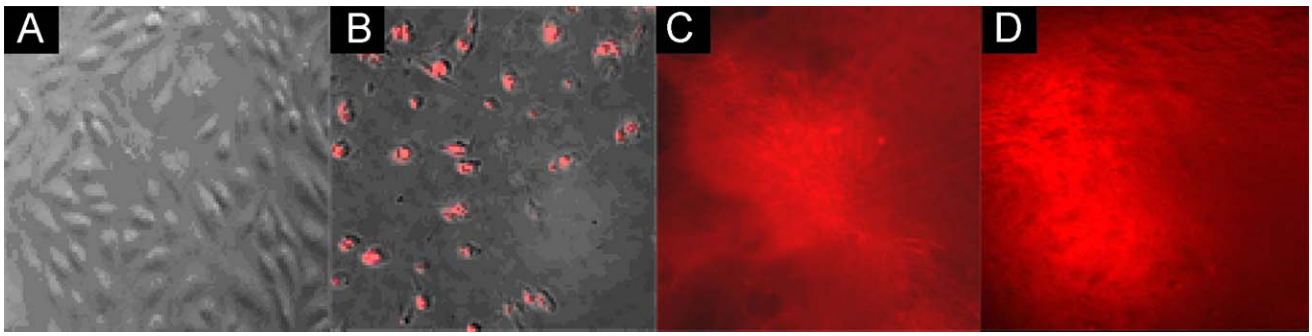

Fig. (5). VV-mediated far red fluorescence. A-B. CV-1 cells were infected with rVV-L15 (Fig. **1**) expressing lacZ, and on the following day the DDAOG substrate was added to the cells. The virus-encoded lacZ cleaved the substrate releasing a fluorogenic compound with far red fluorescence properties (**B**), while uninfected cells (**A**) had no fluorescence. **C-D**. A nude mouse carrying SC-inoculated C6 tumor (see below) was injected with the VV-L15. A week later, the the DDAOG substrate was injected to tail vein, and 10 min later the mouse was sacrificed. The tumor was removed and analyzed by fluorescence microscopy. Multiple patches of distinct red fluorescence were found in the tumor, two representative images (**C-D**) of which are shown. Microscopy was performed as described in Fig. (**4**).

Both optical reporters were effective in visualizing the tumors, and optical data matched the MRI data.

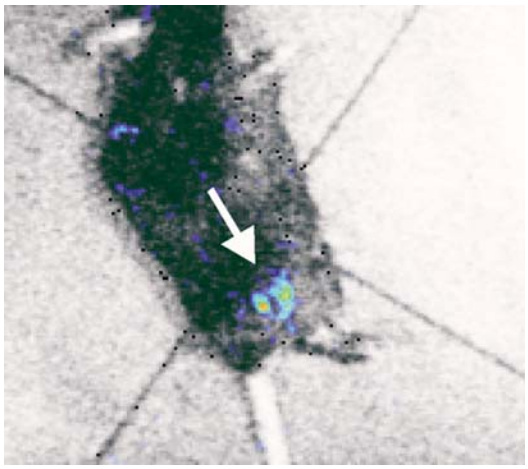

Fig. (6). Visualization of bladder MB-49 cancer in mice using VV-RG. Orthotopic bladder tumor in C57/Bl6 mouse was induced by intravesical instillation of MB-49 cancer cells. The palpable tumor was visualized after on day 3 after tail vein injection of 10^8 PFU of VV-RG virus using Hamamatsu CCD camera. Intensive bioluminescence induced by Rluc expression was detected only in the bladder area following IV injection of CTZ only in the bladder tumor area.

DISCUSSION

Preexisting Immunity

Oncolytic viruses are highly immunogenic in mammals and there was some concern that virus delivery to a pre-immunized organism could be ineffective due to a primed virus-specific immune response. Indeed, there are data in mice [63] and some data in humans [64] suggesting that pre-existing vaccinia immunity, such as that occurring in a large proportion of the adult population because of smallpox vaccination, limits the effectiveness of recombinant vaccinia vectors delivery. However, others demonstrated effective VV immunization in pre-immunized rabbits [65] and humans [66, 67]. Interestingly, under certain conditions pre-existing immunity may even enhance intratumoral virotherapy. Thus, in mice pre-immunized with HSV subsequent intratumoral administration of oncolytic HSV showed enhanced efficacy compared to HSV-naïve mice [68]. Similarly, in mice pre-immunized with adenovirus or vaccinia virus the anti-tumor effect of both intra-tumorally injected oncolytic viruses was dramatically greater when compared with other control groups [69]. The authors suggested that the enhanced anti-tumor effect was due to a redirected adaptive immune response against foreign antigens released from virus-lysed tumor cells. Mucosal routes of immunization can be also used to induce systemic CTL and antibody responses with vaccinia vectors in the face of preexisting systemic im-

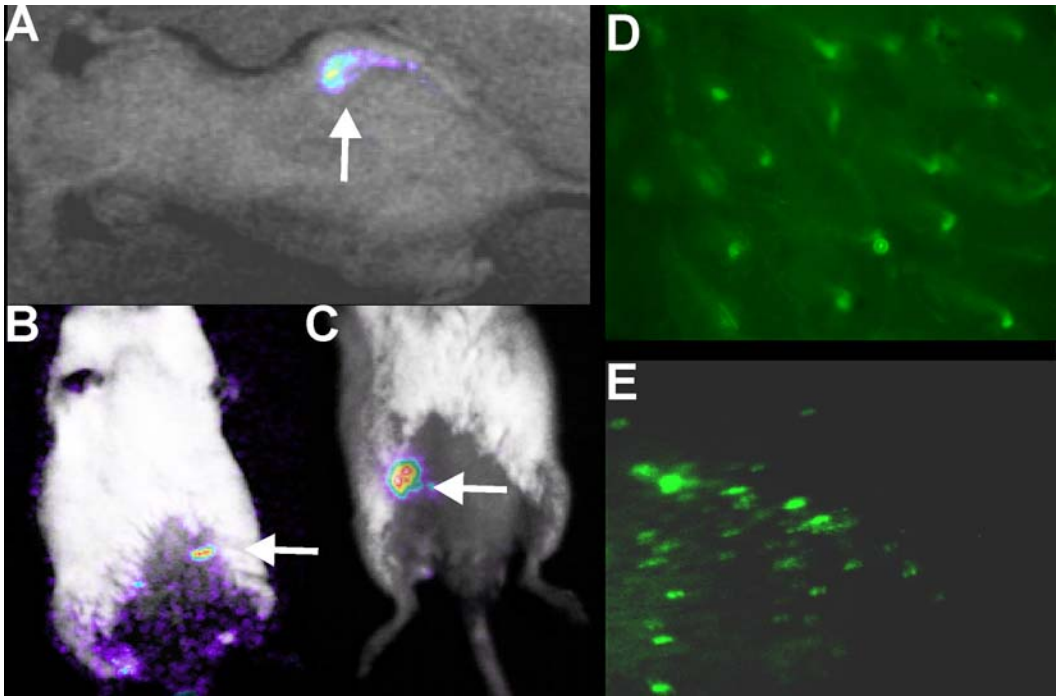

Fig. (7). Imaging of prostate tumors in mice. A. The VV-RG virus expressing fused Rluc/GFP (10^7 PFU) was intravenously injected to nude mice bearing SC human PC-3 prostate tumor in the right flank. On day 3 after virus injection, the whole bodies were monitors by low-light video imager as described in Fig. (**6**). **B-C**. VV-mediated early detection of cancer in TRAMP mice. Two TRAMP mice (8 weeks of age) were intraveinously (IV) inoculated with VV-BGR13 ($1x10^8$ PFU). Six days later, the abdominal part of the body was shaved, and 5 μg of Gluc substrate CTZ was injected IV. Accumulated bioluminescent light (5 min) was captured by Hamamatsu camera. Both mice emitted light from a single confined site (shown by arrows). Then mice were sacrificed and urogenital organs were removed for fluorescence microscopy (Axiovert 100TV, Zeiss) of R-GFP. Both mice had a small site of tissue containing multiple VV infection foci suggesting an early stage of malignancy. **D-E**. A 9-month of age TRAMP mouse was intravenously inoculated with $1x10^8$ PFU of VV-RG. A week later, when the animal succumbed from cancer all major organs were removed and analyzed for GFP fluorescence. Only sections of prostate tumor contained patches of GFP expression. A characteristic pattern of virus foci at microvessels can be seen.

munity to vaccinia [70]. It is conceivable that virus delivery to pre-immunized individuals for beneficial purposes can be achieved using alternative routes of administration. In naïve individuals, repeated systemic delivery of oncolytic viruses may be effective during 2-3 weeks after first injection. However, ultimately for extended virotherapy treatments or repeated tumor detection procedures several unrelated oncolytic viruses should be available for medicine, and studies to achieve that goal are currently in progress [71].

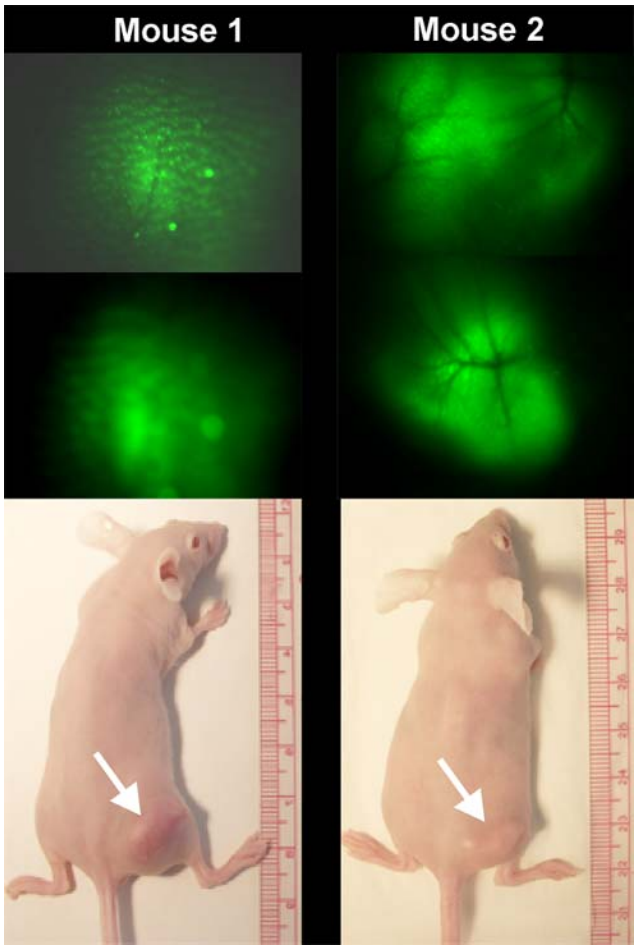

Fig. (8). Imaging of R-GFP fluorescence of tumors in a glioma model. Two subcutaneous C6 glioma-bearing nude mice (#1 and #2) were IV-injected with rVV-BGR12 ($5x10^7$ PFU), and on day 5 p.i., the body was analyzed by fluorescence microscopy. Most of tumor surfaces (white arrows) emitted a characteristic pattern of R-GFP fluorescence shown on top. Two R-GFP images of each mouse are presented.

Routes of Administration

In most preclinical and clinical tumor therapy studies oncolytic viruses have been delivered intra-tumorally (IT). Although animals and patients may clearly benefit, most cancers are multifocal and, in addition, spread of viruses after IT delivery is confined to small area of tumor tissue surrounding the path of the needle. Consequently, in the majority of studies the anti-tumor effect has been moderate or low. For effective oncolytic virus-mediated tumor diagnosis or cancer therapy apparently systemic or regional delivery of viruses to tumors through the vasculature is necessary. Traditional IV administration of viruses encounters potential hurdles including rapid clearance of the virus from the bloodstream by reticuloendothelial organs and limited viral influx into tumor tissue. Therefore, the IV route requires higher doses of viruses than, for example, systemic or regional intra-arterial (IA) virus administration.

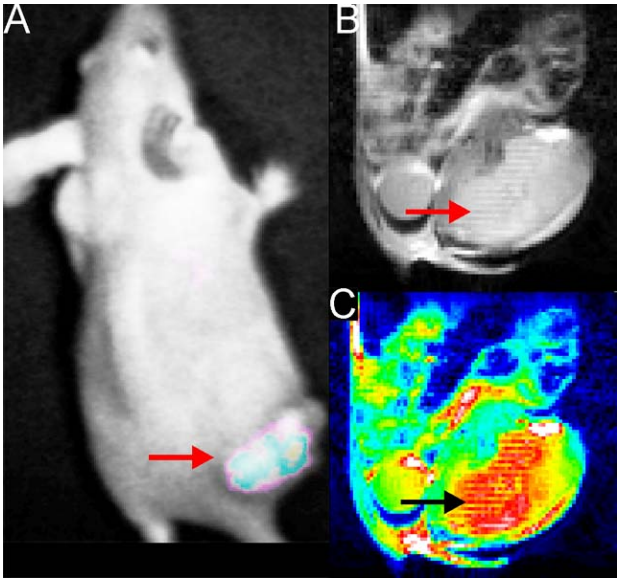

Fig. (9). Tumor detection by bioluminescence and MRI (on right). Optical imaging (on left). The C6 glioma-bearing mouse #2 of Fig. 12 was IP-inoculated with rVV-BGR12, and on day 6 p.i. the CTZ substrate was injected. Bioluminescence of the tumor was visualized as described in Fig. (**6**) (red arrow). MRI (on right): T2 weighted image of the tumor in the hind leg of the mouse #2 is seen three days later. High resolution imaging details not only the tumor but visible morphologic changes within the tumor itself. Analysis will consist of 3D volumetric analysis and reconstruction for total tumor volumes. Window-level colorization highlights the increased T2 intensity within the tumor, suggestive of increased water content (white to red).

For certain types of cancer, such as liver cancer, preferential perfusion of tumor masses can be achieved *via* the hepatic artery as a single 10-min infusion of the virus [72, 73]. To maximize the efficacy of virus-mediated of various cancer types the arterial anatomy should be defined first for subsequent arterial administration of the virus using an infusion pump or catheter. It is clear that further pre-clinical and clinical studies to evaluate the safety and efficacy of various routes of virus administration are warranted.

Mechanism of Oncolysis

Although little is known about mechanisms of virus-mediated oncolysis there is sufficient evidence to suggest that it involves host defense and anti-viral mechanisms, like Toll-like Receptors (TLR), double-stranded RNA (dsRNA)-activated protein kinase (PKR), RNase, and interferon (IFN) signaling pathways [71, 74, 75]. A critical role for type I IFN and activation of NK cells in the innate immune control of VV infection has recently been demonstrated in a murine model of infection [68, 76]. The importance of these signal-

ing pathways for virus survival are supported by unique strategies acquired by poxviruses to prevent activation of the host anti-viral response targeting TLRs [77, 78], expression of type I and II IFN viroceptors, TNF-α, IL-1β or CC chemokines [79-82]. The cellular status of c-Jun NH2-terminal kinase (JNK) function also dramatically affected oncolytic VV replication and vaccinia virus-mediated host cell killing [83]. Other oncolytic viruses such as VSV showed preferential replication in cells with an activated Ras-ERK pathway and defective IFN pathway [84]. Tumor selectivity of myxoma virus depended on overexpression of Akt in human cancer cells which facilitated virus replication and oncolysis [85].

Alternatively, there is overwhelming evidence in support of immune system protection of the host against tumor development, and IFNs play a pivotal role in this process [86-90]. Thus, IFN-γ insensitive mice lacking important components of the IFN signaling, e.g. the IFNGR1, transcription factor STAT1, or the IFN-γ gene, developed cancer more rapidly than control mice [91, 92]. In human patients, systemic administration of IFN-β or IFN-γ produced regression of vascular tumors, including Kaposi sarcoma, pulmonary hemangiomatosis, and hemangiomas [93-95]. Thus, it seems that defects of the IFN pathway which is linked with the TLR signaling pathway and anti-viral protection may be indeed crucial in rendering cancer cells sensitive to virus infection. Currently ongoing studies conducted in many laboratories may shed light on genetic mutations and/or deregulation of gene expression that are involved both in antiviral state and in the process of tumorigenesis.

Tumor tissues represent sites of immune privilege modulated mainly by transforming growth factor-β (TGF-β) in which immune response and host defense functions are significantly dampened [96]. Consequently, immune surveillance mechanisms in tumors are compromised resulting in failure to recognize or properly respond to danger signal of malignant cells, or infections. Colonization of malignant masses by microorganisms is frequently observed in medical practice and considered to be a secondary effect caused by cancer development. However, we do not exclude an alternative cause/effect relationship when at penetration sites of infectious agents, virus, bacteria, etc. may locally deliver antagonists of anti-viral (or anti-bacterial) defense and immune systems. These antagonists may compromise immune surveillance of randomly developing transformed cells resulting in escape from destruction and tumorigenesis. Thus, according to this hypothesis, besides oncogenic viruses, such as HBV or HPV, lytic infectious viruses, such as pox- or herpes-viruses, may also be causative agents of cancer development. Future studies may shed light on this assumption.

CONCLUSIONS

The ultimate goal of this work is to explore the feasibility of a virus-based method for noninvasive visualization of malignant tumors and metastases in living organisms, especially at early stages of malignancy. Here we report that oncolytic tumor-targeting properties of attenuated recombinant VV carrying genes of highly sensitive optical reporters may provide a powerful tool for optical imaging of solid tumors and metastases in experimental animal models. In this system the bioluminescent optical reporter (luciferase) induces emission of low intensity photons which are collected and amplified by a sensitive CCD/Intensifier camera. The instrument allows visualization of low light-emitting sources radiating from the tumors located in deep organs throughout the mouse body. The refined visualization of detected tumors/metastases at the cellular level is achieved by using fluorescent reporters, e.g., bright GFP or RFP. Further technological improvements, like combination with laser-induced fluorescence [97], the ultra-fast laser [98], or dual photon imaging [99], in near future may increase the sensitivity, depth of detection, and spatial resolution of proposed virus-mediated tumor diagnosis.

The recombinant viruses used for tumor imaging are non-toxic and can be applied for human use as well. Underlying molecular mechanisms of viral oncolyses remain to be investigated. Cancer cells have undergone drastic genetic alterations which provide them certain advantages for rapid growth over normal cells. However, these growth advantages in cancer cells often associated with loss of critical components of the intracellular pathways of defensive mechanisms against danger signals and thus, cells become highly sensitive to infection of many viruses. Therefore, engineered attenuated oncolytic VVs are likely to be effectively harnessed as diagnostic tool for different types of cancer. The imperative requirement of that is hyper-attenuation of the replication-competent virus strain.

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ABBREVIATIONS

CTZ	=	Coelenterazine
DDAOG	=	A conjugate of beta-galactoside and 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)
Fluc	=	Firefly luciferase
GFP	=	Green fluorescence protein
Gluc	=	*Gaussia* luciferase
gfp	=	Green fluorescence protein gene
IA	=	Intra-arterial administration
IP	=	Intraperitoneal administration
IT	=	Intra-tumoral administration
IV	=	Intravenous administration
lacZ	=	*E. coli* β-galactosidase gene
MOI	=	Multiplicity of infection
MRI	=	Magnetic Resonance Imaging
NIR	=	Near infrared
ORF	=	Open reading frame
PFU	=	Plaque forming unit
p.i.	=	Post inoculation
RFP	=	Red fluorescence protein

Rluc = *Renilla* luciferase

SC = Subcutaneous administration

TK = Thymidine kinase

VV = Vaccinia virus

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