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ORIGINAL ARTICLE

Use of an oncolytic vaccinia virus for the treatment of canine breast cancer in nude mice: preclinical development of a therapeutic agent

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Mammary cancers together with cancers of the skin account for about 60% of the total cancers occurring in dogs. The veterinary options for therapeutic management of canine mammary cancer are limited and prognosis for such patients is poor. In this study, we analyzed the functionality of the oncolytic vaccinia virus strain GLV-1h68 as a possible therapeutic agent for canine mammary cancer. Cell culture data demonstrated that GLV-1h68 efficiently infected and destroyed cells of the canine mammary adenoma cell line ZMTH3. Furthermore, after systemic administration this attenuated vaccinia virus strain primarily replicated in canine tumor xenografts in nude mice. The efficient tumor colonization process resulted in inhibition of tumor growth and drastic reduction of tumor size. This is the first report demonstrating that vaccinia virus is an effective tool for the therapy of canine mammary cancers, which might next be applied to dogs with breast tumors.
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Introduction

Malignant tumors of the mammary glands occur with a higher incidence than any other form of cancer in female dogs.[1,2] The traditional treatments such as surgery or radiotherapy are very unlikely to result in drastic changes in patient status.[3] Despite surgical intervention, 40–60% of dogs with mammary cancer will experience tumor-related death within the first 2 years.[4] In addition, the use of chemotherapeutic drugs in dogs has produced complete and partial remissions of disease in only some isolated cases.[5,6] These facts emphasize the need for the development of new therapies for cancer in dogs. One of the most promising new strategies in this field could be the oncolytic virotherapy.[7,8] The concept that viruses may be useful in the eradication of cancer has existed since the early twentieth century.[9,10] However, during the last 10 years numerous reports have confirmed that intratumorally or systemically delivered viruses such as Newcastle disease virus,[11,12] reovirus,[13,14] lentivirus,[15] herpes simplex virus,[16,17] enterovirus,[18] Sindbis virus,[19] Semliki Forest virus,[20] Seneca Valley virus[21] and vaccinia virus[22,23] can display an antitumor activity *in vivo*. In comparison, vaccinia virus has significant advantages as a live viral vector: (1) large foreign gene-carrying capacity; (2) broad host cell range; (3) replication exclusively in the cytoplasm, with no risk of chromosomal DNA integration and (4) natural tropism for targeting tumors on systemic administration.[22,24]

Very recently, Zhang *et al.*[22] have described the construction and characterization of a new recombinant vaccinia virus (LIVP strain), GLV-1h68, and its functions as a simultaneous diagnostic and therapeutic agent. The data demonstrated that GLV-1h68 has an improved safety profile when compared with the wild-type LIVP strain and is successful in oncolytic virus-mediated tumor therapy of human breast tumor xenografts in nude mice.

In the present study, GLV-1h68 was tested as a potential agent for treating canine mammary adenoma. Here, we describe that GLV-1h68 virus successfully infected, replicated and lysed the canine adenoma ZMTH3 cell line in cell culture. In addition, we analyzed

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the ability of GLV-1h68 to prevent cancer growth in mice with tumors derived from ZMTH3 cells.

Materials and methods

Cell culture

African green monkey kidney fibroblasts (CV-1) were obtained from the American Type Culture Collection (ATCC). ZMTH3 is an immortalized canine mammary pleomorphic adenoma cell line.[25]

Cells were cultured in Dulbecco's modified Eagle's medium supplemented with antibiotic solution (100 U ml^{-1} penicillin G and 100 U ml^{-1} streptomycin) and 10% fetal bovine serum (Invitrogen GmbH, Karlsruhe, Germany) for CV-1 and 20% fetal bovine serum for ZMTH3 at 37 °C under 5% CO_2.

Virus strain

GLV-1h68 is a genetically stable oncolytic virus strain designed to locate, enter, colonize and destroy cancer cells without harming healthy tissues or organs.[22] GLV-1h68 is based on the vaccinia virus LIVP strain, which was used as a vaccine against smallpox. Zhang *et al.*[22] have modified this virus by inserting three marker genes encoding *Renilla* luciferase-green fluorescent protein (RUC-GFP) fusion, β-galactosidase and β-glucuronidase into the F14.5L, J2R (encoding thymidine kinase (TK)), and A56R (encoding hemagglutinin) loci of the viral genome, respectively.[22]

Cell viability assay

CV-1 and ZMTH3 cells were seeded onto 24-well plates (Nunc, Wiesbaden, Germany). After 24 h in culture, the cells were infected with GLV-1h68 using multiplicities of infection (MOI) of 0.1 and 1. The cells were incubated at 37 °C for 1 h, then the infection medium was removed and subsequently the cells were incubated in fresh growth medium. The amount of viable cells after infection with GLV-1h68 was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Taufkirchen, Germany). At 24, 48, 72 or 96 h after infection of cells, the medium was replaced by 0.5 ml MTT solution at a concentration of 2.5 mg ml^{-1} MTT dissolved in RPMI 1640 without phenol red and incubated for 2 h at 37 °C in a 5% CO_2 atmosphere. After removal of the MTT solution, the color reaction was stopped by adding 1 N HCl diluted in isopropanol. The optical density was then measured at a wavelength of 570 nm. Uninfected cells were used as a reference and were considered as 100% viable.

Fluorescence microscopy

Cells were stained with Hoechst 33342 (1 μg ml^{-1}) to visualize the nuclei of all cells. The virus-infected cells were analyzed with a fluorescence microscope (Leica DMR HC; Leica, Wetzlar, Germany) and images were captured with an electronic camera (Diagnostic Instruments, Sterling Heights, MI). Digital images were processed using META-MORPH (Universal Imaging, Downingtown, PA) and Photoshop 7.0 (Adobe Systems, Mountain View, CA).

Measuring apoptosis and necrosis

Apoptosis and necrosis levels were determined using an annexin V-PE labeling kit (Becton-Dickinson, San Diego, CA). ZMTH3 cells were grown on 24-well plates (Nunc) and infected by GLV-1h68 at an MOI of 2. At various time points, infected and non-infected ZMTH3 cells were harvested by trypsin-EDTA treatment (PAA Laboratories GmbH, Pasching, Austria), washed twice in phosphate-buffered saline (PBS) and resuspended in fluorescence-activated cell sorting buffer. For discrimination between apoptosis and necrosis, ZMTH3 cells were stained using 5 μl annexin V-PE and 5 μl 7-amino-actinomycin-D (7-AAD) per 100 μl cell suspension for 15 min at room temperature in the dark. A minimum of 10^5 cells was then measured using an Epics XL flow cytometer (Beckman Coulter GmbH, Krefeld, Germany). Annexin V-positive and 7-AAD-negative cells qualified as apoptotic cells.

Viral replication

For the viral replication assay, CV-1 and ZMTH3 cells grown in 24-well plates were infected with GLV-1h68 at an MOI of 0.1. After 1 h of incubation at 37 °C with gentle agitation every 20 min, infection medium was removed and replaced by fresh growth medium. After 1, 12, 24, 48, 72 or 96 h, the cells and supernatants were harvested. Following three freeze–thaw cycles, serial dilutions of the lysates were titered by standard plaque assays on CV-1 cells. All samples were measured in triplicate.

Bioluminescence imaging

For monitoring studies of the distribution of the GLV-1h68 virus in tumor-bearing mice, animals were analyzed for the presence of virus-dependent luciferase activity. For this purpose, mice were injected intravenously with a mixture of 5 μl of coelenterazine (Sigma; 0.5 μg μl^{-1} diluted ethanol solution) and 95 μl of luciferase assay buffer (0.5 M NaCl, 1 mM EDTA and 0.1 M potassium phosphate, pH 7.4). The animals were then anaesthetized with 4% isoflurane (Forene; Abbott, Ludwigshafen, Germany) in a knockout box and were maintained in an anesthesia module aerated with 1.5% isoflurane/oxygen. The mice were imaged using the CCD camera-based NightOWL LB 981 Imaging System (Berthold Technologies, Bad Wildbad, Germany). Photons were collected for 2 min from dorsal views of the animals, and the images were recorded using Image WinLight 32 software (Berthold Technologies).

GLV-1h68-mediated therapy of ZMTH3 xenografts

Tumors were generated by implanting ZMTH3 cells (2.5×10^6 in 100 μl PBS) subcutaneously on the right hind leg of 6- to 8-week-old female nude mice (NCI/Hsd/Athymic Nude-*Foxn1*^{nu}; Harlan Winkelmann GmbH, Borchen, Germany). Tumor growth was

recorded weekly in two dimensions using a digital caliper. Tumor volume was calculated as ((length × width^2)/2). On day 11 after tumor cell implantation (tumor volume, ~500 mm^3) or on day 17 (tumor volume, ~1000 mm^3) respectively, a single dose of GLV-1h68 virus (5×10^6 plaque forming units (p.f.u.) in 100 µl PBS) was injected either in the tail vein (i.v.) or in the retro-orbital (r.o.) sinus vein. For r.o. injection, animals were anaesthetized intraperitoneally using ketamin (75 mg kg^{-1}; Pfizer, Karlsruhe, Germany) and xylazine (20 mg kg^{-1}; Bayer, Leverkusen, Germany). The animals of the control groups were injected i.v. or r.o. with PBS only.

The significances of the results were calculated by two-way analysis of variance with Bonferroni comparison post-test using the GraphPad Prism software (San Diego, CA). The post-test was only performed when analysis of variance revealed significance. Results are displayed as means ± s.d. *P*-values of <0.05 were considered significant.

All animal experiments were approved by the government of Unterfranken and conducted according to the German animal protection guidelines.

Histology of the tumors

For histological studies, tumors were excised and snap-frozen in liquid N_2, followed by fixation in 4% paraformaldehyde/PBS pH 7.4 for 16 h at 4 °C. Tissue sectioning was performed as described by Weibel *et al*.[26] GLV-1h68 was labeled using polyclonal rabbit anti-vaccinia virus (anti-VACV) antibody (Abcam, Cambridge, UK), which was stained using Cy3-conjugated donkey anti-rabbit secondary antibodies obtained from Jackson ImmunoResearch (West Grove, PA). Phalloidin-tetramethyl rhodamine isothiocyanate (TRITC) (Sigma) was used to label actin. The fluorescent-labeled preparations were examined using the Leica MZ 16 FA Stereo-Fluorescence microscope equipped with Leica DC500 digital camera. Digital images were processed with Photoshop 7.0 (Adobe Systems) and merged to yield pseudo-colored images.

Plaque reduction neutralization assay

Neutralizing vaccinia virus antibody titers in canine sera were tested by a plaque reduction neutralization assay. Sera from cancer-bearing dogs were heat treated for 30 min at 56°C to inactivate complement. Ten-fold serial dilutions of sera in infection medium (Dulbecco's modified Eagle's medium with 2% fetal bovine serum) were mixed with 100 p.f.u. of GLV-1h68 in a total volume of 250 µl and incubated for 2 h at 37 °C and 5% CO_2. As a control for infectious virus input, 100 p.f.u. of GLV-1h68 was mixed with infection medium and treated equally. A commercially available rabbit vaccinia virus antibody (Abcam) was used as a positive control. Following incubation, confluent CV-1 monolayers (grown on 24-well plates) were infected in duplicate with each dilution. After 1 h, wells were overlaid with 1.5% carboxymethyl-cellulose medium. At 48 h post-infection, the CV-1 cell monolayers were stained using 0.13% crystal violet solution and the number of plaques in each well was determined.

Results

Analysis of the oncolytic potential of GLV-1h68 against canine cancer cells

To test the ability of GLV-1h68 virus to infect and lyse canine cancer cells, we first performed a cell viability assay as described in Materials and methods. At 96 h post-GLV-1h68 infection at an MOI of 0.1 and 1, both ZMTH3 and CV-1 (positive control) cells were eradicated, with only 12 and 15% surviving the treatment, respectively (Figure 1).

As demonstrated above, the infection of ZMTH3 with GLV-1h68 led to killing of ZMTH3 cells. To discriminate between apoptosis and/or necrosis, we used the annexin V-PE apoptosis detection kit I (BD Biosciences, Pharmingen, San Diego, CA) and stained cells were analyzed by flow cytometry. At 6, 24 and 72 h after infection, we found that less than 2% of the dying cells (staining positive for annexin V-PE and/or for 7AAD) were stained by annexin V-PE only (data not shown). Therefore, virus-infected ZMTH3 cells were killed by necrosis rather than apoptosis. Under these experimental conditions, no evidence for induced cell death through apoptosis was detected.

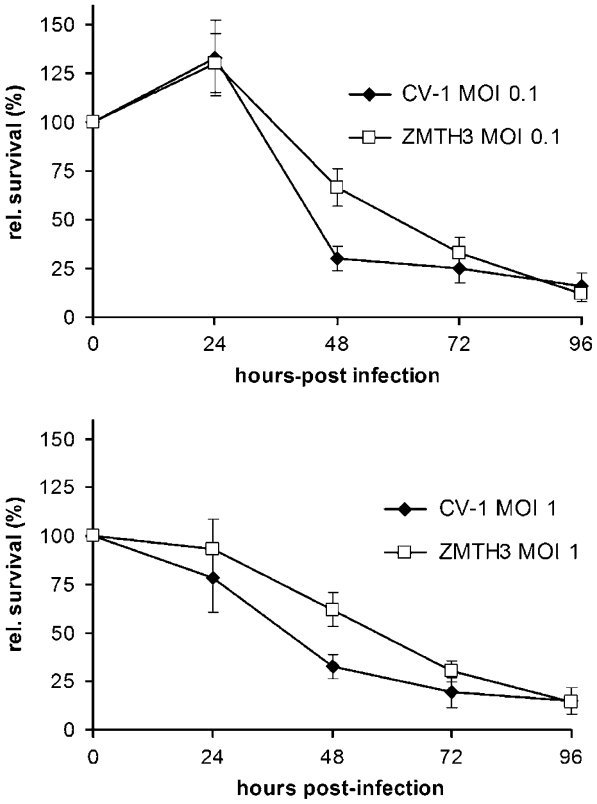

Figure 1 Viability of ZMTH3 and CV-1 cells after GLV-1h68 infection using an MOI of 0.1 and 1, respectively, monitored over 4 days. The amount of viable cells after infection with GLV-1h68 was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Taufkirchen, Germany). Values are shown as percentages of respective controls.

Replication of GLV-1h68 in ZMTH3 cells

In addition to the cell viability data, replication of GLV-1h68 in CV-1 and ZMTH3 cells was analyzed (Figure 2). The data demonstrated that GLV-1h68 can efficiently infect ZMTH3 cells and virus replication is similar to that observed in CV-1 cells under these conditions. Although the cell-associated virus titer in ZMTH3 peaked at 48 h p.i. (4.22×10^6 p.f.u. per well), the maximum yield in the supernatant was observed at 96 h p.i. (4.17×10^6 p.f.u. per well). At 24 and 48 h after infection, the majority of virus was cell associated. However, the highest virus titers were identified in supernatants at 96 h after infection, correlating with cell death and virus release (Figures 1 and 2).

GLV-1h68-mediated expression of GFP in ZMTH3 cells

We also analyzed the replication of GLV-1h68 in ZMTH3 cells by fluorescence microscopy (Figure 3). The virus-dependent GFP expression was assessed daily over a period of 4 days. In these experiments, ZMTH3 exhibited the strongest GFP expression at 72 and 96 h, whereas the CV-1 cells expressed GFP optimally at 48 and 72 h. In addition, using Hoechst 33342 staining, a nearly complete DNA degradation of infected CV-1 and ZMTH3 cells was observed at 72 and 96 h post-infection, respectively. These effects appeared to require GLV-1h68, as non-infected cells did not degrade or express GFP (data not shown).

Systemic administration of GLV-1h68 causes the regression of solid canine breast tumors in nude mice

To test the therapeutic capacity of GLV-1h68 against an induced canine breast cancer, five groups of 5–6 nude mice at the age of 8 weeks were implanted with ZMTH3 cells. At 10 days post-implantation, all nude mice developed tumors with sizes between 400 and 500 mm^3. At day 11, 5 tumor-bearing mice (group 1 and 4; $n = 5$), or at day 17, 18 mice (groups 2, 3 and 5; $n = 6$) were injected either in the tail vein (i.v.; groups 1 and 2) or in the r.o. sinus vein (r.o.; group 3) with 5×10^6 p.f.u. of GLV-1h68. The mice of the control groups 4 (i.v.) and 5 (r.o.) were injected with PBS only. All animals were monitored by weekly tumor size measurements and some animals were observed by fluorescence and/or luminescence imaging.

First, we examined the efficacy of GLV-1h68 to target tumors *in vivo*. For this purpose, at day 7 post-injection, two mice of each group were observed under the low-light Imager (NightOWL LB 981; Berthold Technologies) to detect luciferase-catalyzed light emission in the presence of intravenously injected coelenterazine (Sigma). The luciferase expression is dependent on the vaccinia virus

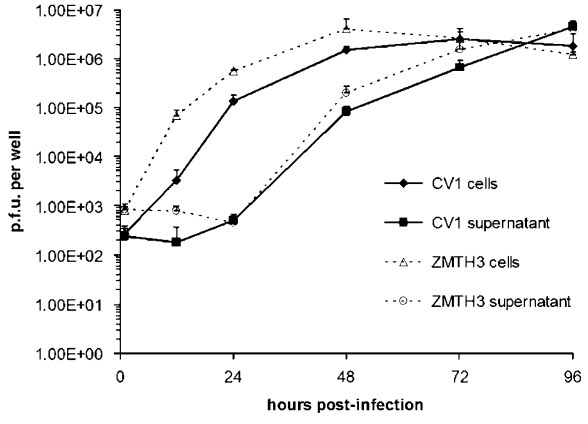

Figure 2 Comparison of the replication capacity of GLV-1h68 virus in CV-1 and ZMTH3 cells after infection with GLV-1h68 virus using an MOI of 0.1. Cells and supernatants were collected for the determination of virus titer at various time points. Viral titers were determined as p.f.u. per well in duplicate by plaque assay in CV-1 cell monolayers.

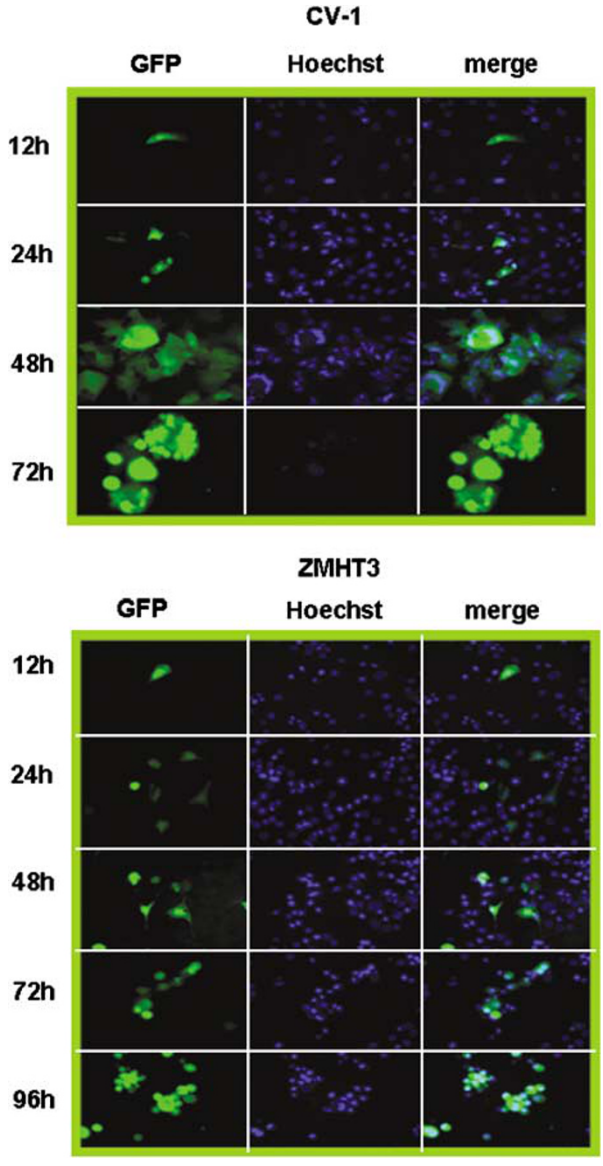

Figure 3 Expression of green fluorescent protein (GFP) in infected cells was detected by direct fluorescence. Staining of cellular DNA with Hoechst 33342, and colocalization of GFP with cellular DNA are shown in the merged imaged. The numbers of intact cells were visualized by Hoechst 33342 staining. All the pictures of the set were taken at the same magnification.

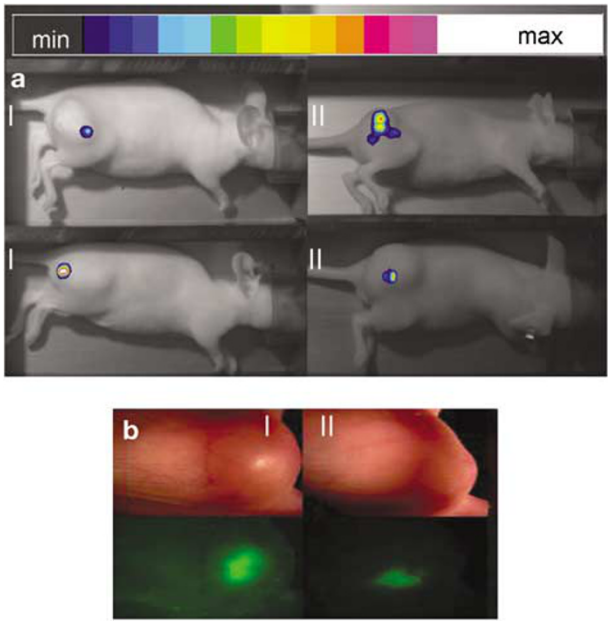

Figure 4 Luminescence and fluorescence imaging of ZMTH3 tumor-bearing mice. (**a**) Luminescence images were taken 7 days after virus injection. Two mice of group nos. 2 (I) and 3 (II) are shown. (**b**) Fluorescence images of live mice were taken 21 days after virus injection. One representative mouse of group nos. 2 (I) and 3 (II) is shown.

replication *in vivo*. As demonstrated in Figure 4a, luminescence was detected in all animals tested. The imaging data indicated a massive viral replication within the tumors.

In addition, at day 21 after injection GFP signals could be observed within regressing tumor tissue when GLV-1h68-injected mice (Figure 4b) were observed under fluorescence microscope (Leica MZ 16 FA).

The tumor measurement data showed that the GLV-1h68 infection caused a highly efficient inhibition of tumor growth and a significant decrease in the size of canine breast tumor xenografts in nude mice (Figure 5). Examination over time revealed that, after virus injection, the tumor growth was arrested within 3 weeks (groups 1–3) when compared with the uninfected control groups 4 and 5. In all cases, the virus treatment led to a significant tumor regression. Furthermore, 35 days after virus injection a complete tumor regression, reduced to the starting tumor volume of about 1000 mm^3, was observed in animals of group 2 (Figure 5). However, the tumor regression after the r.o. virus application (group 3) was not as fast when compared with that of groups 1 and 2.

To compare the viral distribution after i.v. or r.o. application, at day 35 after injection, four animals from groups 2 and 3 were analyzed either for viral distribution by standard plaque assay using CV-1 cells or by immunohistochemical staining of the tumors. The plaque assays revealed the presence of only few virus particles in lung and spleen of i.v injected mice (Table 1). In contrast, the organs of r.o. injected mice were free of virus particles. However, the highest virus titers were identified in tumor tissues of all mice (Table 1). These findings were in agreement with our luminescence imaging data demonstrating that GLV-1h68 locate and multiply almost exclusively in tumor tissue (Figure 4a).

We analyzed the effect and the presence of GLV-1h68 in regressing tumors by immunohistochemical staining (Figure 6). The data revealed that in all cases the tumors were completely infected with the vaccinia virus, which led to oncolysis and destruction of tumor tissue. Additionally, the speed of tumor regression seemed to be affected by the route of injection. Surprisingly, however, we did not find any difference in the tumor colonization of GLV-1h68 using the two different injection routes (Figure 6).

Taken together, these experiments have demonstrated an optimal efficacy of oncolytic virotherapy by GLV-1h68 in this tumor model.

The absence of vaccinia virus-neutralizing antibodies in sera *of cancer-bearing dogs*

To prepare for future virus applications directly to tumor-bearing dogs, we tested for the presence of vaccinia virus-neutralizing antibodies in canine sera, using plaque reduction neutralization assays. The experiments were carried out with 10 canine sera collected from dogs bearing tumors in different organs (data not shown). In summary, no specific neutralizing activity against GLV-1h68 was detected in any of the sera. In contrast, the rabbit anti-vaccinia virus (anti-VACV) antibody (Abcam) used as a positive control showed a significant neutralizing activity (data not shown). These data demonstrated the absence of specific GLV-1h68-neutralizing antibodies from all tested sera derived from dogs.

Discussion

The increase in the incidence of cancer in dogs is associated with longer life expectancy resulting from advances in pet nutrition and overall advances in veterinary care. The number of dogs with cancer in the USA alone is estimated to be over 1 million per year. The available cancer treatment options for dogs include surgical removal of the tumors, radiation therapy, hyperthermia, photodynamic-, immuno- and chemotherapy and are often suboptimal.[27] Therefore, the development of new therapies and diagnostics for cancer in dogs is essential. One of the most promising novel cancer therapies for humans is oncolytic virotherapy.[7,8] This method is based on the use of viruses, which accumulate in tumor tissues and cause antitumor activity by oncolysis. As there are significant similarities between human and canine cancers—such as breast and prostate cancer—it may be possible to utilize this therapy, for example, for the treatment of mammary cancers in dogs.

In this article, we assessed the suitability of a novel recombinant vaccinia virus, GLV-1h68, to infect, replicate in and lyse canine adenoma cells. GLV-1h68 was engineered by inserting expression cassettes encoding a RUC-GFP fusion protein, β-galactosidase and β-glucur-

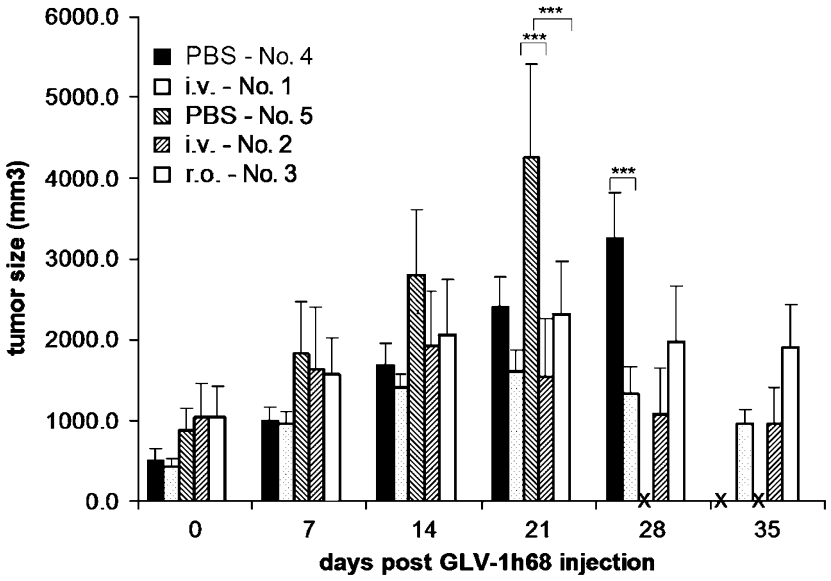

Figure 5 Effect of the intravenous virus injection on the tumor growth in nude mice. Here, 6- to 8-week-old female nude mice were subcutaneously inoculated with 2.5×10^6 ZMTH3 cells on the right hind leg. Tumor-bearing mice were injected with a single dose of GLV-1h68 virus (5×10^6 p.f.u. in 100 μl phosphate-buffered saline (PBS)) either in the tail vein (i.v. group nos. 1 and 2) or in the retro-orbital sinus vein (r.o. group no. 3) either 11 (group no. 1) or 17 days (groups nos. 2 and 3) post-injection. The animals of the control group nos. 4 (i.v.) and 5 (r.o.) were injected with 100 μl PBS at day 11 or 17, respectively. Tumor volume was monitored weekly and the animals were euthanized when tumors reached a volume of 3500–4000 mm^3 (marked by X at later time points). Two-way analysis of variance (ANOVA) with Bonferroni post-test was used to compare the two corresponding data points at day 21 or 28 of the two groups. $P<0.05$ was considered as statistically significant $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Table 1 Distribution of GLV-1h68 in tissues of ZMTH3 tumor-bearing nude mice

Tissue/organ	*Group 2 (i.v.)*	*Group 3 (r.o.)*
Lung	$1.37 \times 10^2 \pm 1.06 \times 10^2$	ND
Spleen	24 ± 12	ND
Tumor	$6.18 \times 10^6 \pm 2.16 \times 10^6$	$5.33 \times 10^5 \pm 1.8 \times 10^5$

Abbreviation: ND, not detected (detection limit <10 p.f.u. per organ).

Tumor-bearing mice were injected either in the tail vein (i.v.; group 2) or in the retro-orbital sinus vein (r.o.; group 3) with 5×10^6 p.f.u. of GLV-1h68. All mice were killed 35 days after virus injection. The data were determined by standard plaque assays on CV-1 cells using aliquots of the homogenized organs and were displayed as mean p.f.u. per organ or tissue ($n=2$). For each organ, two aliquots of 0.1 ml were measured in triplicate.

onidase into the genome of the LIVP strain and is highly attenuated when compared with the wild-type strain.[22] Very recently, it was shown that GLV-1h68 is able to specifically infect some human and mouse cancer cells.[22,28] In addition, 2 weeks after intravenous administration in mice, most of the GLV-1h68 virus was isolated from tumors but almost no virus was detected in other organs, in contrast to the wild-type LIVP and wild-type WR strains.[22] Therefore, GLV-1h68 colonization results in reduced toxicity, allowing extended survival of tumor-bearing nude mice. Moreover, Zhang *et al.*[22] demonstrated that a single GLV-1h68 injection causes regression and complete elimination of human breast tumor xenografts in nude mice.

In the current study, we tested the sensitivity of the canine cancer cell line ZMTH3 to a GLV-1h68 infection. We demonstrated for the first time that GLV-1h68 can effectively infect, replicate in and lyse the canine adenoma ZMTH3 cells in cell culture. This cell line exhibited a nearly complete lysis by GLV-1h68 vaccinia virus at 96 h p.i. Interestingly, the canine cells supported efficient GLV-1h68 replication at least as well as the best vaccinia virus producer cell line CV-1, but resulted in somewhat delayed destruction of ZMTH3 in comparison to CV-1 cells. These findings also confirmed the data of Lin *et al.*[28] demonstrating that an efficient viral replication of GLV-1h68 is essential for cytotoxicity of the virus in cell cultures.

Vaccinia virus can kill cells either by apoptosis or by necrosis.[29] The pathway choice seems to be dependent on the MOI as well as the host cell type. Our data indicate that at an MOI of 2, GLV-1h68 induces necrosis in ZMTH3 cells at 72 h p.i. There are only a few reports that demonstrate that vaccinia virus infection can cause necrosis.[30] However, the mechanism of tumor cell elimination through GLV-1h68 might differ in animals.

Our animal studies demonstrated that tumor-bearing nude mice can overcome infection by GLV-1h68 and the virus preferentially replicates in tumor tissues. In addition, in all cases using the canine tumors, the virus treatment led to a significant tumor regression at day 21 post-virus injection. Moreover, on day 35 after injection

all canine breast tumors tested showed vaccinia virus patches over the tumor surface, which were also associated by massive destruction of the tumor tissue.

In our experiments, we have used two different routes for injection of the GLV-1h68 virus into nude mice. The data demonstrated that the i.v. tail vein injection led to a faster and more efficient tumor regression when compared with the virus application through the r.o. sinus vein. On the other hand, the r.o. injected mice lost less weight and appeared healthier in comparison to i.v. injected mice (data not shown). A possible explanation for this could be that there was a more efficient virus clearing from infected organs after r.o. in contrast to i.v. injection. Alternatively, the i.v injection may have led to a better tumor targeting and faster replication of the virus, as mice are heat treated to allow dilation of the veins, whereas body temperature decreases when mice are anesthetized for r.o. injection. The difference in body temperature may have consequences on viral distribution and therefore tumor colonization as was demonstrated earlier.[31]

However, the tumor regression seems to be relatively independent of tumor size (Figure 5) and number of replication-competent virus particles reaching the tumor after injection (Table 1). This observation supports the hypothesis that tumor regression after viral colonization is at least in part mediated through host defense mechanisms, most likely by the innate immune system, as tumor regression occurred in nude mice deficient in T-cell and B-cell function. Therefore, we have analyzed the localization of macrophages in vaccinia virus-infected tumors (data not shown). Surprisingly, despite significant differences in the size of regressing tumors after r.o. or i.v. injection we could not show relevant differences in macrophage distribution (data not shown). One possible explanation could be a strong induction of innate immune responses by both the tumor cells and by the virus particles.[32] In these experimental settings, it was not possible to distinguish between components of the innate immune system directly involved in the tumor cell destruction or in the elimination of vaccinia virus particles.

In summary, our study provides the first evidence that the oncolytic virus GLV-1h68 resulted in the elimination of canine adenoma cells both in cell culture and in tumor-

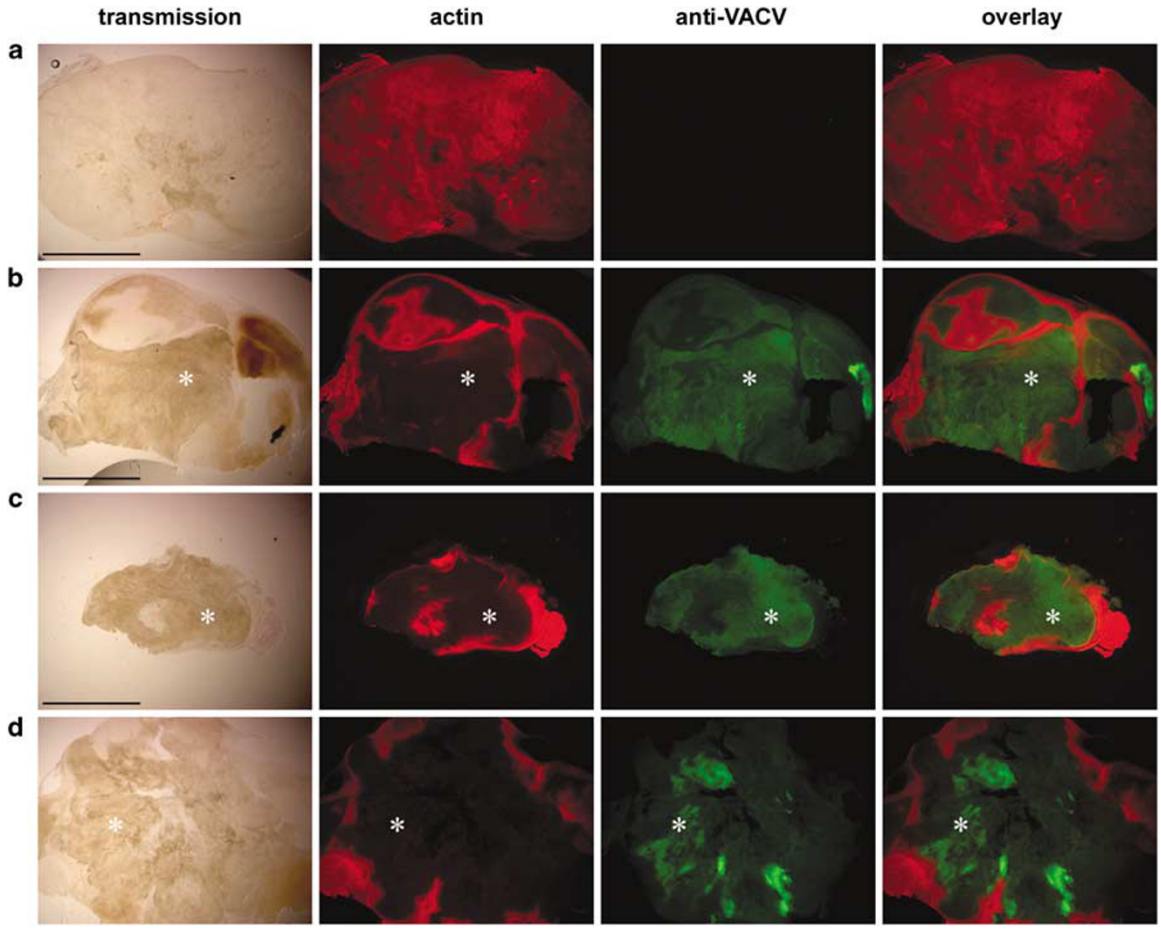

Figure 6 Immunohistochemical staining of canine ZMTH3 tumors. Mice bearing ZMTH3 tumors were injected either with phosphate-buffered saline (PBS) (**a**) or with 5×10^6 p.f.u. of GLV-1h68 (**b**–**d**). At day 25 (**a**) or 35 (**b**–**d**) after injection, whole tumor cross sections (100 μm) of control tumor with a size of $\sim 3000\,mm^3$ (**a**) as well as a tumor after i.v. injection $\sim 1600\,mm^3$ (**b**) a tumor after i.v. injection $\sim 600\,mm^3$ (**c**) and a tumor after r.o. injection $\sim 2500\,mm^3$ (**d**) were labeled with Phalloidin-TRITC (red) and anti-VACV antibody (green). Necrotic tissue destruction in infected ZMTH3 tumors was indicated by asterisks. Scale bars represent 5 mm.

bearing animals. In addition, all canine sera tested in this study did not contain neutralizing antibodies against vaccinia virus (data not shown). Taken together, these findings suggest that GLV-1h68 vaccinia virus strain has the potential to become a successful therapeutic vector for dog patients with mammary cancer.

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