Regression of glioma in rat models by intranasal application of parvovirus H-1

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Translational Relevance

The experimental infectivity but excellent tolerance of some rodent autonomous paroviruses in humans together with their oncosuppressive effects in preclinical models, speak for the inclusion of these agents in the arsenal of oncolytic viruses presently under consideration for cancer therapy. This is exemplified by our recent assessment of the antineoplastic activity of wild-type parovirus H-1PV in glioma, pancreatic ductal adenocarcinoma and non-Hodgkin lymphoma models, laying the basis for the present launching of a first phase I/II clinical trial with glioma patients. Preclinical studies show the ability of H-1PV to achieve a complete cure of some tumors in animal models, and kill tumor cells that resist conventional anticancer treatments.
Abstract

Purpose. In previous studies we have demonstrated that the apathogenic rat parvovirus H-1 (H-1PV) is capable to induce regression of advanced symptomatic rat and human gliomas in a rat model, when the virus was injected in the tumor (intracranially) or intravenously. Infection with H-1PV did not provoke any pathology in non-tumor tissue. The present study addresses the question whether also intranasal application of this oncolytic virus is suitable and sufficient for treating gliomas in this animal model.

Experimental Design. Rat (RG-2) or human (U87) glioma cells were grafted stereotactically in the brain of rats (Wistar or RNU, respectively), and after development of tumors visible by MRI, H-1PV was instilled intranasally. Tumor regression was monitored by MRI, and survival was analyzed by Kaplan-Meier analysis. Brains from sacrificed animals were analyzed for histological alterations, presence of viral DNA and proteins and infectious virions. In addition, distribution of virus to other organs was determined.

Results. A single intranasal instillation of H-1PV was sufficient to induce efficient regression of rat glioma, leading to significant prolongation of survival without any toxicity for other tissues. It is demonstrated that the virus reaches brain and other tissues, and that the viral replication-associated regulatory (and oncolysis-associated) proteins are exclusively expressed in the tumor tissue. In rats with xenografts of human glioma, oncolytic activity of H-1PV was less pronounced, however leading to significant prolongation of survival.

Conclusion. In view of an ongoing clinical trial on the use of H-1PV for oncolytic virotherapy of glioma, the option of applying the virus intranasally may be a valuable alternative to invasive routes of infection.
Introduction

For patients with malignant gliomas (glioblastoma multiforme, GBM) prognosis is still extremely poor despite significant advances in tumor imaging, neurosurgery and radiotherapy, with a five-year survival rate of less than 5% after initial diagnosis (1). Factors that contribute to the dismal prognosis associated with GBM include its infiltrative nature throughout the brain, which limits the effectiveness of surgical resection and targeting of radiotherapy, and the blood-brain barrier (BBB), which limits access of systemically administered therapeutics to the tumor. In the past decade a number of drug delivery strategies have been developed to overcome obstacles presented by the BBB. In particular, direct drug administration into the brain parenchyma, such as convection-enhanced delivery (CED) has shown promising results in both animal models and clinical trials (2-11). However, CED requires the use of potentially risky surgical procedures to position the catheter into the patient’s brain parenchyma (6, 7). The convective flow to distribute the drug through the implanted catheter can lead to significant inflammation and local edema. Another technique that holds promise for bypassing the BBB to deliver drugs to the brain while eliminating the surgical risk and spillover to normal tissue, is intranasal delivery. Intranasal delivery provides a practical, non-invasive method for delivering therapeutic agents to the brain because of the unique anatomic connections. Intranasally administered drugs reach the brain parenchyma, spinal cord, and cerebrospinal fluid (CSF) within minutes by using an extracellular route through perineural and/or perivascular channels along the olfactory and trigeminal nerves without binding to any receptor or using axonal transport (5, 6, 8, 9). In addition to bypassing the BBB, advantages of intranasal delivery include rapid delivery to the CNS, and elimination of the need for systemic delivery, thereby reducing unwanted systemic side effects. Intranasal delivery also provides painless and convenient self-administration for patients, features that encourage its use for delivering therapeutic agents into the CNS. Many therapeutic agents, including growth
factors, proteins, peptides, viral vectors, liposomes, and vaccines, have been delivered to the CNS through the nasal route and applied for the treatment of CNS disorders in both animals and humans (8, 10, 11). In brain tumors, anticancer agents such as methotrexate (12), 5-fluorouracil (13) and raltitrexed (14) have been delivered successfully to the brain using intranasal delivery. Intranasal targeting of the chemotherapeutic drug raltitrexed to the brain is significantly higher compared with intravenous administration (12, 14). However, these chemotherapeutic agents do not discriminate between tumor and normal tissue. Thus, the concentrations of drug required to kill tumor cells can also lead to toxicity in normal neural tissue. To achieve therapeutic efficacy without toxicity to normal tissue, the drugs need to preferentially target brain tumor while sparing normal tissue from damage. In this context, intranasal application of oncolytic viruses selectively targeting human glioblastoma has been successful in animal studies using oncolytic Vesicular Stomatitis virus (15). The oncolytic parvovirus H-1PV seems to be another suitable candidate by virtue of its selective toxicity for malignant (glioma) cells (16) and its impressive selective oncolytic activity in rat and human brain tumor models when applied intratumorally or intravenously (17). Parvovirus H-1PV belongs to the genus Parvovirus (PV) within the Parvoviridae family. Parvoviruses are small (diameter $\approx 25$ nm), non-enveloped icosahedral particles with a single-stranded DNA of about 5000 nucleotides (18). In permissive cells, these viruses undergo a lytic cycle with main replication and assembly steps taking place in the nucleus and typically leading to cell death by an oncotic pathway, classical apoptosis or a lysosomal mechanism (19, 20). To be permissive for lytic parvovirus replication, the target cell must originate from the right animal species, proliferate, and be in an appropriate (usually not very differentiated) state (21). As a consequence, the pathogenicity of the virus (if any) is restricted to proliferating tissues and decreases in severity from the fetus to the adult, in which infection is usually asymptomatic. The rat parvovirus H-1PV can also replicate in experimentally infected cancer patients within
the tumor tissue without causing significant clinical signs (22). On the contrary, H-1PV and related parvoviruses actually protect various animal models against oncogenesis and display oncolytic activities (16, 19, 22). Therefore, some parvoviruses such as H-1PV are considered for anticancer applications (16).

As mentioned above, we previously demonstrated the potential of H-1PV for brain tumor therapy in rat models after intratumoral or intravenous infection (17). Here we analyzed whether intranasal inoculation of H-1PV could also be used for efficient treatment of brain tumors (glioma) in these animal models. Although the mode of transmission of the virus in nature (among rats) is unclear, nasal mucosa might be a target for infection and therefore deserves to be explored in settings of virotherapy.

We report here on significant tumor regression and prolonged survival in rat glioma-bearing rats after intranasal application of H-1PV, with selective toxicity for tumor tissue and resistance of normal brain to the lytic effect of the virus. Similarly, prolonged survival was observed in human glioma-bearing immunodeficient rats after intranasal infection with the virus. These results demonstrate that intranasal application is suitable to treat gliomas in animal models. Intranasal application appears to allow efficient transfer of oncolytic parvoviruses to the brain without the need to open the skull.
**Materials and Methods**

**Cells**

The rat glioblastoma cell line RG-2 (American Type Culture Collection [ATCC], Manassas, VA, USA), cells of the human glioblastoma line U87 (ATCC), and NB-K were grown in DMEM (Sigma-Aldrich, Hamburg, Germany) supplemented with 10% FCS (Biochrom KG, Berlin, Germany) and 1% antibiotics (penicillin, streptomycin; Gibco, Invitrogen Corporation, Karlsruhe, Germany) in a 5% CO2 humidified atmosphere at 37°C. Exponentially growing cells to be injected in rat brains were trypsinized and centrifuged (146 g/10 min), and the pellet was resuspended in DMEM without supplements.

**H-1PV production and infection**

H-1PV was amplified in human NB-K cells and purified on iodixanol (5-{N-[3-(N-{3,5-bis[(2,3-dihydroxypropyl)carbamoyl]-2,4,6-triiodophenyl}acetamido)-2-hydroxypropyl]acetamido}-1-N,3-N-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide, GE Healthcare Canada Inc. Mississauga, ON) gradients as described previously (23). H-1PV was titrated on NB-K indicator cells by plaque assay and further used at multiplicities of infections expressed in plaque-forming units (pfu) per cell (20). Empty H-1PV particles were isolated by CsCl gradient centrifugation, and residual infectious particles were inactivated by UV-irradiation as described (24, 25).

**Animal Experiments**

All animal experiments were carried out in accordance with institutional and state guidelines. Female Wistar inbred rats (10 weeks old; 26 in total), and immunodeficient RNU-rats (10 weeks old; 24 in total) were obtained from Charles River (Sulzfeld, Germany).

(i) Intracerebral implantation of tumor cells. Rats were anesthetized with isoflurane (5% and 2.5% as initial and maintenance doses, respectively) and mounted to a stereotactic frame. After linear scalp incision, a 0.5-mm burr hole was made 2 mm right of the midline and 1 mm...
anterior to the coronal suture. The needle of a 10-µL Hamilton syringe was stereotactically introduced through the burr hole into the posterior part of the frontal lobe at a depth of 5 mm below the level of the dura mater, and RG-2 glioma cells or U87 human glioma cells, respectively (in each case 50,000 cells in a volume of 7 µL), were injected over 7 minutes. The needle was withdrawn slowly to minimize spreading of tumor cells along the needle tract.

(ii) Intranasal H-1PV infection of glioma-bearing immunocompetent Wistar rats. At 7 days post RG-2 cell implantation, tumor-bearing rats were infected with H-1PV by intranasal application. Animals were shortly anesthetized as above and were instilled once with $10^8$ pfu of H-1PV in 100 µl PBS, 50 µL in each nostril. Control animals received either PBS or an equivalent number ($1 \times 10^{10}$) of non-infectious particles (UV-irradiated empty H-1PV capsids) in the same volume. Infected animals were kept separately from uninfected control rats under isolator conditions. Tumor growth was monitored every 4-5 days by MRI (see below). Clinical signs and weight of animals were monitored daily. Animals developing tumor-related symptoms or a body weight drop (>20%) were euthanized according to institutional guidelines.

(iii) Magnetic resonance imaging (MRI). The animals were examined in a 2.45-T MRI scanner (Bruker BioSpin MRI GmbH, Ettlingen, Germany) using T1-weighted imaging before and after injection of 0.4 mL contrast medium (Gadodiamide, Omniscan, Amersham, Freiburg) into the tail vein. Contrast-enhanced T1 imaging was performed 5 minutes after injection. During MR examination, rats were anesthetized by isoflurane insufflation (initial dose 5%, maintenance 2%, as above).

(iv) Intranasal H-1PV infection of glioma-bearing immunodeficient RNU rats. RNU rats with U87 xenografts were treated with H-1PV through multiple intranasal instillations, at days 8, 11, 14, 17, and 20 after tumor cell implantation. The total dose of H1-PV per animal was $5 \times 10^8$ pfu.
Analyses of tissue samples

Animals from each group (glioma-bearing animals intranasally treated with PBS or with noninfectious [controls] or infectious H-1PV) were sacrificed 1, 3 and 5 days after mock or virus infection. Blood, brain and other organs (kidney, lung, heart, spleen, liver and gut) were analyzed for viral DNA.

(i) DNA extraction from tissue samples.

DNA was extracted from cryosections (10µm thick) from shock-frozen specimens from different parts of both brain hemispheres (one bearing a tumor and the other not) and from samples of peripheral organs (see above; 25 mg of tissue) using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Darmstadt, Germany). From complete blood, DNA was extracted with the QIA amp DSP blood kit (Quiagen, Hilden, Germany).

(ii) PCR detection of H-1PV DNA. PCR reactions were performed using 50 ng total DNA in 40 µl Supermix (Invitrogen, Darmstadt, Germany) and the following primers (0.2 µl of a 100 nM stock of each primer): sense primer, 5’-TCAATGCGCTCACCATCTCTG-3’ (position nt 1996–2016 within the NS gene region of the H-1PV genome) and antisense primer, 5’-TCGTAGGCTTCGTCGTGTTCT-3’ (position nt 2490–2510), yielding an amplified fragment of 516 bp. Amplification (34 cycles) was as follows: 3 min, 95°C starting reaction; cycle: 30 sec, 94°C; 30 sec, 58°C; 1 min, 72°C; final elongation 7 min, 72°C. Amplified fragments were separated by agarose (1.7%) gel electrophoresis at 120V. DNA from infected RG2 cells served as a positive control. Negative controls included H2O and the corresponding tissue from a mock-infected rat.

(iii) Dispersed cell assay. Filtered homogenates of brain and blood samples from H-1PV-infected animals were inoculated in triplicate on semiconfluent NB-K cells, seeded in 48-well plates. At the occurrence of the virus-mediated cytopathic effect (CPE), cells were harvested by trypsinization and pooled with the culture supernatant and trapped by suction onto
GeneScreen (Perkin Elmer, Rodgau, Germany) transfer membrane or analysis of amplification of viral DNA using the dispersed cell assay as described previously (26). After cross-linking with UV (using the UV Stratalinker 1800 at 254 nm, Stratagene/Agilent, Waldbronn, Germany; auto crosslink program), filters were hybridized with a radio-labelled viral DNA probe kindly provided by J.F.P Nüesch and exposed to X-ray film (Amersham HyperFilm ECL, GE Healthcare, München, Deutschland).

**Analysis of virus replication**

Brains were removed from animals sacrificed 1 and 5 days after infection with H-1PV, and the two hemispheres were analyzed separately. After weighing, tissues were homogenized in 6 ml PBS in the presence of Matrix-D beads (Q-Biogene [Heidelberg, Germany]). One ml of this mixture was centrifuged, and supernatant was filtered through 0.45 nm filters and used in serial dilutions (1:10 steps) for inoculation on NB-K and RG-2 indicator cells. When the controls reached confluence (4 d p.i.), cells were fixed with an acetate/ethanol mixture (1:3) and stained with crystal violet. Virus in the tissue was determined as the highest dilution that caused a CPE. (Titers were normalized to 1 g of brain tissue.)

**Immunohistochemistry**

For the immunodetection of parvoviral proteins, frozen 20 micrometer sections mounted on poly-L-lysine coated slides (Roth, Karlsruhe, Germany) were permeabilized with 0.1% Triton X-100 in PBS, and incubated with 5% goat serum in PBS for 1 h at room temperature to block non-specific staining. Sections were then incubated for 36 hours at 4°C with the mouse monoclonal NS-1 specific antibody 3d9 (courtesy of Dr. N. Salome) and/or the rabbit H-1PV-VP-specific antiserum (courtesy of Dr. C. Dinsart (27)). After rinsing, secondary antibodies (anti-mouse IgG conjugated with cyanin3 [red; Jackson ImmunoResearch, West Grove, USA], anti-mouse IgG conjugated with cyanin2 [green; GE Healthcare, Freiburg, Germany], or anti-rabbit IgG-conjugated with cyanin2 [Jackson ImmunoResearch, West Grove, USA])
were applied to the sections (3 h at room temperature) for revealing viral NS-1 and VP proteins, respectively. Finally, the coverslips were intensively washed and mounted with DAPI-containing medium (VESTASHIELD, Vector Laboratories, Burlingame, USA) to visualize the nuclei. Images were digitized using a Leica DMRBE fluorescent microscope (Leica, Bensheim, Germany) equipped with a digital camera and an image analysis software (analySIS, Olympus, Germany).

**Histological analyses (H.-E. staining)**

Frozen brain sections on slides were incubated for 3 min in haematoxylin (Sigma-Aldrich, Hamburg, Germany), washed with tape water, exposed for 5 min in eosin (Sigma-Aldrich) and incubated in increasing concentrations of ethanol (70%, 80%, 90% and 100%; 10 min for each concentration) followed by immersion in xylol (Merck, Darmstadt, Germany), covered using Eukitt (Kindler, Freiburg, Germany), and air-dried.

**Virus-neutralizing antibodies**

Blood samples were obtained from animals at different time points after H-1PV infection. Serial dilutions of the sera were made in MEM, and mixed with an equal volume of H-1PV suspension (corresponding to $8 \times 10^4$ pfu/well). After incubation for 30 min at room temperature, the serum-virus mixture was inoculated on NB-K cells plated in 96-well plates ($8 \times 10^3$ cells/ well). After a 20 min incubation, cultures were supplemented with MEM (containing 5% heat-inactivated FCS and 1% penicillin-streptomycin), kept for 72 hours at 37°C and processed for the determination of the number of living cells (relative to mock-infected cultures) using the MTT assay. Briefly, MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, provided by Sigma-Aldrich, USA) stock solution (5 mg/ml) was added to each culture (10 µL per 100 µL). After incubation for 3.5 h at 37°C, medium was removed, incorporated dye was solubilized with acidic isopropanol (100 µL/well), and extinction values were measured with a Multiskan EX apparatus (Thermo Electron...
Corporation, Dreieich, Germany) at 570 nm. The virus-neutralizing antibody titer was defined as the dilution of serum, which reduced the viral cytotoxic effect (i.e., fraction of living cells relative to mock-infected cultures) by 50%.

**Statistics**

Statistical significance of differences in survival rates between tumor-bearing animals that were infected with H-1PV versus mock-treated, was calculated using the logrank test, type Wilcoxon Rank sum test (28), modified for censored (sacrificed animals) survival data, and expressed as p-values. In the Kaplan-Meier curves, the sacrifice (i.e., censoring) time points are indicated by vertical bars.
Results

Intratumoral or intravenous infection with parvovirus H-1PV was recently shown to induce full regression of experimental gliomas in a rat model (17). Since there are hints that nasal epithelium might be a possible virus entry site in natural infection with parvoviruses (29), we tested whether similar glioma suppression could be achieved by instilling H-1PV in the nasal cavity of animals. We investigated this in Wistar rats bearing intracranial RG-2 cell-derived (rat) gliomas and in (immunodeficient) RNU rats with human gliomas established from U87 cells. Fifty thousand cells were implanted stereotactically in the posterior part of the frontal lobe of the brains of 26 Wistar rats and 24 RNU rats, respectively, resulting in the formation of tumors. In the absence of further treatment, the animals had to be sacrificed because of tumor-related symptoms: survival of animals after tumor cell implantation was 22 days (RG-2 glioma) or 27 days (U87 glioma) at a maximum.

For treatment experiments, animals were infected with H-1PV by the intranasal route (10^8 pfu in 100 µL, 50 µL in each nostril; one inoculation in 15 Wistar rats, multiple inoculations in 11 RNU rats) 7 days (Wistar rats) or 8, 11, 14, 17, and 20 days (RNU rats) after tumor cell implantation. Control groups were treated by instilling empty viral capsids (10^10 particles in 100 µL; Wistar: n=5, RNU: n=7) or PBS (100 µL; Wistar, n=6; RNU, n=5) in the nostrils of the animals.

As apparent from MR images in Figure 1, a single intranasal instillation of H-1PV was sufficient to achieve complete remission of intracranial gliomas in 13 of 15 RG-2-derived glioma-bearing animals (Wistar rats). This striking effect required infectious virions since empty capsids failed to protect rats from glioma growth, with animals dying of their tumor after the same interval as PBS-treated controls, i.e. 12 to 22 days after glioma cell implantation (Fig. 2A). In the case of RNU rats bearing U87 cell-derived tumors, the tumor suppressive effect of H-1PV was less pronounced but infected animals survived one week
longer than uninfected ones (none of the animals survived more than 32 days after tumor cell implantation, Fig 2B). Hence, in animals treated intranasally with infectious H-1PV, survival was significantly prolonged (RG-2 gliomas in Wistar rats, p=0.0021; U87 gliomas in RNU rats, p=0.0038) compared with animals that received PBS or inactive viral particles (Fig. 2 A,B). Survival probability was calculated by Kaplan-Meier analyses for the PBS-treated control group (Wistar rats, n=6; RNU rats, n=5) and for rats inoculated with empty viral particles (Wistar rats, n=5; RNU rats, n=7) or infectious H-1PV virions (Wistar rats, n=15; RNU rats, n=11). In the control groups, 2 animals were sacrificed for further analyses at day 3 p.i.. In the group treated with infectious H-1PV virions, animals were sacrificed for analyses at day 1 (2 Wistar rats, 1 RNU rat), 3 (4 Wistar rats, 1 RNU rat), 5 (2 Wistar rats, 2 RNU rats), and 45 (2 Wistar rats) following infection. Three surviving Wistar rats were sacrificed at day 78 p.i. to end experimentation (Fig. 2 A; 2 animals of the infected group died from tumor unrelated reasons, 18 days after tumor cell implantation). Sacrificed animals were included in the Kaplan-Meier calculation as censored animals. The highly efficient regression of RG-2-derived glioma could be confirmed in 3 additional independent experiments (data not shown). As in the previous study (17), tumor regression after H-1PV infection was not accompanied by any adverse side effects in the animals (see also below).

Paralleling previous results with intratumoral or intravenous virus inoculation (17), intranasal infection with H-1PV induced destruction of tumor tissue (oncolysis) without leading to any morphological alteration in adjacent brain tissue (Fig. 3) or any other organ (data not shown). However, it is to be noted that destruction was more efficient in RG-2 cell-derived tumors in immunocompetent animals compared to human xenografts (U87 cells) in immunodeficient animals (Fig. 3). This was also observed in previous studies using other than intranasal routes of infection (17).
Twenty-four hours after infection with full H-1PV virions, viral DNA could be detected by PCR or dispersed cell assay in the brain (Fig. 4 A,B) demonstrating that intranasally inoculated virus can reach brain tissue, with a predominant accumulation in the tumor area. A positive signal was also detected in the peripheral organs listed in Table 1 with relatively high signals in liver and spleen. This shows that also after intranasal application, the virus got widely distributed as reported for intravenous and intracranial treatment (17) irrespective of the presence of tumor tissue in the animals. In blood, viral DNA could be detected in tumor bearing animals 1 day after infection but not at days 3 and 5, indicating transient viremia. In cryosections of brain tissue, the viral cytotoxic nonstructural protein NS-1 that is required for viral DNA replication became detectable by immunohistochemistry starting from day 5 p.i., but exclusively in the tumor cell area (Fig. 5), and never in sections from peripheral organs (data not shown). Viral capsid (VP) proteins could be demonstrated at earlier times p.i. (3 days) in various brain sections, in particular in the tumor area (exemplified for RG-2 gliomas in Fig. 6) confirming the transfer to the brain of input virions as they were also detected in brain sections from animals having received empty viral capsids (data not shown). Infectious virions could be detected by inoculating brain tissue samples to indicator cells. Compared to the input of virus in the nostrils, virus titers in brain decreased with time due to rapid distribution to the whole body of the animal, irrespective of the presence of tumor tissue (data not shown).

Intranasal treatment of animals with infectious virions induced an antiviral immune response as evident from the appearance of H-1PV-neutralizing serum antibodies in immunocompetent (Wistar) rats, detectable around 10 days after infection (Fig. 7).

The data demonstrate that the intranasal route of administration of H-1PV is as successful in curing rat glioma in the RG-2 glioma model as the intracranial application (17), and superior to intravenous treatment which requires multiple injections of the virus (17). A favorable
effect of intranasal H-IPV infection could also be demonstrated in immunodeficient animals with human U87 cell-derived gliomas.
Discussion

The aim of the present study was to assess whether intranasal infection with the apathogenic oncolytic rat parvovirus H-1 (H-1PV) is sufficient for inducing glioma regression in a rat model, as we have reported previously for other routes of infection (intracranial or intravenous administration of the virus) (17). Intranasal applications have been reported for a variety of therapeutic compounds, because this route provides a practical, non-invasive delivery to the brain. Furthermore, intranasal administration overcomes the blood-brain barrier by using an extracellular route through perineural and/or perivascular channels along the olfactory and trigeminal nerves, independently of receptor binding or axonal transport (5, 6, 8, 9).

The present study shows that intranasally administered H-1PV induced full regression of (RG-2 cell-derived) rat gliomas in about two weeks p.i.. Although this time interval was about one week longer compared with intracranial or intravenous infection (17), intranasally treated immunocompetent animals showed complete tumor suppression resulting in their long-term survival as evident from Kaplan-Meier analysis (significantly \( p=0.0021 \) extended life span compared to mock-treated tumor-bearing rats). In immunodeficient RNU rats bearing a human (U87 cell-derived) glioma, a significantly prolonged survival was observed after infection with H-1PV compared to uninfected animals (\( p=0.0038 \)). However, as in our previous studies (17), oncolysis mediated by the virus was less efficient in immunodeficient animals. This is probably due to a major role of immunological factors in efficient tumor regression necessitating the presence of functional T cells (Kiprianova et al., manuscript in preparation, and (17)).

We demonstrate that the virus reached the brain and was distributed to other organs after intranasal instillation of H-1PV, as reported previously for intracranial and intravenous administration. This was evidenced by immunohistochemistry using an H-1PV capsid protein
(VP)-specific antiserum, as well as by PCR analysis of viral DNA. We observed an enrichment of input H-1PV capsids in the tumor area, which has also been found for infectious virions in animals infected intravenously (N. Thomas, unpublished results). Presence of viral DNA was transient with the notable exception of the tumor tissue. Expression of the regulatory and toxic viral protein NS-1 that is also necessary for virus replication could only be demonstrated within the tumor tissue and was not detected in other brain areas or organs from infected animals. Due to the early and efficient distribution of the virus throughout the whole body of the animals, titers of infectious virus dropped rapidly in specific areas compared to the amount of virus applied initially. Therefore, it is difficult to assess to which account the virus replicates yielding progeny virions remaining at the production site. However, since viral proteins necessary for replication are exclusively found in the tumor cells, it is most likely that replication takes place in these cells, spreading the virus within the tumor area and being distributed to other sites. Viremia seems to take place rather early after infection (within the first 24 hours), and may reappear in tumor-bearing animals at later stages, indicating virus replication in tumor cells, which is supported by the detection of the viral NS-1 protein in these cells. Altogether, these findings confirm the previously reported oncotropism of parvovirus replication. Detection of NS-1 protein in gliomas was delayed by 2 days compared to intravenous or intracranial application (17), in keeping with the delay in the onset of tumor regression observed by MR imaging.

Intranasal infection of immunocompetent rats led to induction of H-1PV-neutralizing antibodies after 10 days, which represents a delay of 3-4 days compared to the appearance of serum antibodies after intravenous or intracerebral administration (17). This might be an advantage in clinical applications of the virus when envisaging multiple infections.

In this study, we show for the first time that H-1PV is able to reach the brain (and the tumor therein) as well as other organs when deposited on the nasal mucosa. Interestingly, we
observed that virus from an infected glioma-bearing rat could be transmitted to an animal kept in another cage in the same isolator, correlating with the infection and full regression of the glioma carried by the latter (data not shown). This observation suggests that virus (excreted via saliva or urine [N. Thomas, unpublished observation, cf., (30)]) can be transmitted by air to infect neighboring rats. It is tempting to speculate that nasal mucosa may be a site of virus entry.

The herein demonstrated efficacy of intranasal application of H-1PV in oncolytic virotherapy may present an advantage in clinical settings because it is an easy and safe-to-perform treatment not requiring invasive measures. As mentioned above, the relatively late appearance of serum antibodies against the virus may allow a wider time frame for multiple applications to reach a higher total virus dose.
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References


Figure 1

Glioma regression by intranasally administered H-1PV.

MR images of three animals, each from one experimental group, are given as representative examples. Rats bearing an intracranial RG-2 glioma were intranasally treated with (A) PBS, (B) noninfectious H-1PV capsids (UV-irradiated empty capsids equivalent to 1x10^{10} particles), (C) infectious H-1PV (1 x 10^{8} pfu) 7 days after tumor cell implantation. MR imaging was performed 7 (a), 12 (b), 17 (c) or 22 (d) days after tumor cell implantation. There are no images at day 22 (d) for the control groups since no PBS- or empty virus-treated rats survived for so long. MR image for group B (empty capsids) had to be performed at day 16 due to the lack of survivors at later times. The group C animals survived symptom-free for more than 80 days after tumor cell implantation. NB: Probably due to regression of the tumor, MRI shape of brains changed slightly (C, d).

Figure 2

Kaplan-Meier analysis of the survival rate of glioma-bearing rats after intranasal instillation of H-1PV.

A: RG-2 glioma-bearing Wistar rats; B: U87 glioma-bearing RNU rats. The x-axis shows days after tumor cell implantation. Vertical bars indicate censoring of animals for analysis purposes. Red line: PBS (A: n=6; B: n=5), black line: empty H-1PV capsids (A: n=5; B: n=7), green line: infectious H-1PV virions (A: n=15; B: n=11). The difference between the H-1PV-treated group and the controls was statistically significant in both models (log rank test: A: p=0.0021; B: p=0.0038). (In the Wistar rat group, 17 days after tumor cell implantation, 2 animals of the H-1PV-infected group died from tumor unrelated reasons.)
**Figure 3**

**Histology of brains of H-1PV-infected animals.**

Haematoxylin-Eosin staining of sections from brain areas as indicated, 5 days after intranasal infection with H-1PV. Morphology of infected brains was not altered after infection (conformed by a neuropathologist) except for the glioma area in tumor bearing animals (RG-2 cell-derived tumor in Wistar rats or U87 cell-derived tumor in RNU rats, as indicated). Five days after infection, destruction of tumor cells was more pronounced in RG-2 glioma compared with U87 glioma.

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**Figure 4**

**Presence of viral DNA in brains of glioma-bearing animals intranasally infected with H-1PV.**

(A) Detection of viral DNA in different sections of brains of RG-2 cell glioma bearing rats. Section positions within the brain are indicated: 1: olfactory bulbs, 2: frontal cortex/caudate putamen area, 3: tumor area/hippocampus, 4: perirhinal cortex/subiculum, 5: cerebellum. DNA was extracted from these sections at 24 h or 72 h after infection and viral DNA was detected by PCR. The amplified product (516 bp) was visualized after agarose gel electrophoresis. The glioma was localized in section 3, which was divided in tumor-containing (3t) and –free (3) parts. N=negative control (tissue from mock-infected animals); P=positive control (DNA from H-1PV-infected RG-2 cells).

(B) Dispersed cell assay of brains from infected immunodeficient RNU rats. NBK cells were inoculated with sterile filtered brain tissue of infected animals and transferred to a membrane followed by hybridization with a NS-1-specific radioactive labeled probe. Results from the tumor bearing hemisphere of the brain and the corresponding hemisphere of a non-tumor bearing animal are shown for comparison, 1 and 5 days p.i.. Controls included H-1PV infected cells and mock-infected cells.
Figure 5

Restriction of expression H-1PV non-structural protein 1 (NS-1) to the glioma area of rat brains after intranasal infection.

Five days p.i., expression of NS-1 was detected in brain sections by immunohistochemistry, using anti-NS-1 antibodies and DAPI counterstaining as indicated. A: tumor area of a RG-2 glioma-bearing brain (NS-1 signal, red fluorescence); B: tumor area of a U87 glioma-bearing brain (NS-1 signal, green fluorescence); C: non-tumor area of the brain of an infected RG-2 glioma-bearing rat (also in U87 glioma-bearing infected RNU rats, there was no NS-1 signal in the non-tumor areas of the brain).

Figure 6

Detection of H-1PV capsid proteins in rat brain after intranasal instillation of H-1PV.

A representative Wistar rat was sacrificed 10 days after RG-2 cell implantation (i.e. 3 days after intranasal virus instillation). H-1PV capsid proteins (VP) were detected by immunostaining of brain sections using VP-specific antiserum and DAPI counterstaining (VP, green signals). A: area of olfactory bulbs, B: tumor area, C: cerebellum. Similar results were obtained in sections from brains bearing a U87 cell-derived tumor and in non-tumor-bearing rats. VP could also be detected after instillation of empty viral capsids.

Figure 7

Appearance of H-1PV-neutralizing serum antibodies after intranasal virus infection.

Blood samples were obtained from a representative animal (Wistar rat) at different days after H-1PV infection (as indicated), and virus-neutralizing antibody titers were determined by MTT test. Titers are given as reciprocals of serum dilution, defined as the dilution, which reduced the viral cytotoxic effect of H-1PV by 50%.
Figure 2

A

logrank p=0.0021

B

logrank p=0.0038
Figure 3

- Olfactory bulbs
- Cerebellum
- Frontal cortex
- Perirhinal cortex
- Hippocampus
- Tumor area
- RG2 tumor + H1
- U87 tumor + H1
Figure 7

Virus-neutralizing antibodies

Titer

days post infection

0 5 10 15 20 25 30

0 100 200 300 400 500 600 700
Kiprianova et al.

Table 1

Detection of viral DNA in blood and various organs of rats infected intranasally with H-1PV

<table>
<thead>
<tr>
<th>Rat strain</th>
<th>Days p.i.*</th>
<th>Blood</th>
<th>Kidney</th>
<th>Lung</th>
<th>Heart</th>
<th>Spleen</th>
<th>Liver</th>
<th>Gut</th>
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<tr>
<td>Wistar, with RG-2 glioma</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>3 (5)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Wistar, without tumor</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>3 (5)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RNU, with U87 glioma</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td></td>
<td>3 (5)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>RNU, without tumor</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
</tbody>
</table>

DNA was extracted from organs and blood, and viral DNA was detected by PCR analysis or dispersed cell assay.

* In the case of blood, the presence of viral DNA was analyzed at days 1 and 5, for organs at days 1 and 3 after infection.
Regression of glioma in rat models by intranasal application of parvovirus H-1

Irina Kiprianova, Nadja Thomas, Ali Ayache, et al.

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