Toxicity and tropism of oncolytic VSV vectors in glioblastoma gene therapy

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List of abbreviations

AV	Attenuated virus
BSA	Bovin serum albumin
Caspase	Cysteine-aspartic proteases
CD3	Cluster of differentiation 3
CD11b	Cluster of differentiation molecule 11b
CNS	Central nervous system
DAB	Diaminobenzidine
DAF	Decay-accelerating factor
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
FITC	Fluorescein Isothiocyanate
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GP	Glycoprotein
H&E	Hematoxylin and eosin
HIF-1α	Hypoxia-inducible factor 1α
HSV-1	Herpes simplex virus type1
IFN	Interferon
Kb	kilobase
LCMV	Lymphocytic choriomeningitis virus
LCMV-GP	Lymphocytic choriomeningitis virus-glycoprotein
mRNA	Messenger RNA
MV	Myxoma virus
NDV	Newcastle disease virus
NKs	Natural killer cells
P53	Protein 53
PBS	Phosphate Buffered Saline

PCLs	Packaging cell lines
PFA	Paraformaldehyde
P.I.	Post infection
PI(3)K	Phosphatidylinositol 3-kinase
PS	Phosphatidyl serine
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
TdT	Terminal deoxynucleotidyl transferase
TP53	Tumor protein 53
TUNEL	TdT-mediated dUTP nick end labeling
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus
VSV-GP	Vesicular stomatitis virus with the glycoprotein of LCMV
VSV-WT	Vesicular stomatitis virus wild-type
WHO	World Health Organization

Summary

Glioblastoma multiforme is the most common and most fatal primary brain tumor in adults. Despite using advanced conventional therapeutic approaches like surgical resection, radiotherapy and chemotherapy only marginal improvement with a median survival of about 14 months is observed. Thus, finding urgent novel therapeutic strategies to target and kill GBM cells are desperately needed. In this respect, gene therapy provides new perspectives for treating cancer, although the safety and efficient delivery of genes into target cells still need critical evaluation in further experiments. In particular, oncolytic viruses are of interest due to their ability to infect and replicate in tumor cells.

In this thesis, we analyzed the toxicity and efficiency of Vesicular stomatitis virus (VSV)-based oncolytic virotherapy for the treatment of GBM. Vesicular stomatitis virus (VSV) has the potential to significantly improve the prognosis of aggressive malignancies such as brain cancer but its intrinsic neurotoxicity limited clinical development so far. The cytopathogenicity of vesicular stomatitis virus (VSV) has been attributed mainly to the glycoprotein of VSV (VSV-G). By exchanging the VSV-G with a glycoprotein from a different virus, the tropism of the virus could be changed. Thus, the glycoprotein from the Lymphocytic choriomeningitis virus (LCMV-GP) was used for pseudotyping VSV as this virus is not neurotoxic.

By analyzing mouse brains infected with either VSV wild-type or VSV pseudotyped with LCMV-GP, it was obvious that VSV-WT induced a strong inflammatory response mainly consisting of CD3 positive lymphocytes and CD11b positive microglia cells. In contrast, VSV-GP infected brains showed no significant increase in inflammation compared to PBS injected control mice. Further, our results from immunostaining with Caspase-3 and TUNEL assays demonstrated significantly more apoptotic brain cells in the VSV-WT compared to the VSV-GP group. By using confocal microscopy analysis we verified that VSV-WT infected mainly neurons, while VSV-GP did not show infection of brain cells at all. Intravenous injection of VSV-GP virus into mice that harbored, intracerebral U87 gliomas lead to specific infection of the glioma cells, while normal brain cells were spared.

In conclusion, replication-competent VSV-GP vectors are promising vehicles for future gene therapy of brain cancer as they specifically and efficiently infect brain tumor cells and do not induce a neurotoxic damage to the normal brain tissue.

1 Introduction

1.1 Cancer and cancer development

Cancer is defined as a group of diseases characterized by uncontrollable abnormal cell growth and division, leading to formation of malignant tumors. Tumor cells can further spread from the primary tumor to other organs/lymph nodes through the blood and lymphatic vessels to establish metastatic disease.

The knowledge of many aspects of cancer has increased substantially in the last decades, which has improved treatment options for some cancers. However, many molecular mechanisms mediating tumor initiation and progression are still unclear.

According to a review published by Hanahan and Weinberg all cancers have six common hallmarks. These hallmarks describe a series of abilities that a normal cell must acquire to transform into a cancer cell. These are unlimited replicative potential, evasion of growth inhibitory signals, autonomy of growth signals, resistance to apoptotic signals, ability to promote angiogenesis and to invade other tissues or metastasize (1). Recently, Hanahan and Weinberg published an updated version of the hallmarks of cancer adding two new ones, the ability to escape from immune attacks and deregulated metabolism in order to sustain continued growth (2).

1.1.1 Oncogenes and tumor suppressor genes

The phrase “Cancer is a genetic disease at the cellular level” is a good description for cancer provided by Lauren Pecorino (3). Thus, similarly to all genetic diseases, DNA alteration or mutation is the main reason for causing cancer (3). DNA mutations may arise either spontaneously or may be caused by environmental factors. The cancer-critical genes are divided into two broad categories: oncogenes and tumor suppressor genes. Oncogenes are growth-promoting genes often mutated or amplified in cancer leading to production of a higher amount of protein and therefore act in a dominant manner for tumor formation. Dominant means a mutation in one allele is enough for cancer development (3). Tumor suppressor genes work in the opposite way. They encode proteins that inhibit cell growth and tumor formation. Tumor suppressor mutations are

recessive, which means that both alleles of the gene must be mutated to induce an effect (3). The first discovered and one of the most crucial tumor suppressor proteins is protein 53 (p53) encoded by the tumor protein 53 (*TP53)* gene (4, 5). *TP53* gene is mutated almost in 50 % of cancers. P53 plays an anticancer role in the body, as it initiates programmed cell death in case of damaged DNA. Further it can provide genomic stability and inhibition of angiogenesis (6).

1.1.2 Angiogenesis

For receiving nutrients and oxygen and for disposal of metabolic waste products, tumor cells rely on blood vessels as normal cells do. When tumors enlarge beyond a certain size, the cells residing in the core of the tumor mass will not get sufficient oxygen and nutrients. As a consequence they will get hypoxic and upregulate *Hypoxia Inducible Factor-1α (HIF1α)*. HIF1α is a gene regulatory protein, which enables the cells to cope with lower oxygen and nutrient levels by activating transcription of pro-angiogenic factors such as *vascular endothelial growth factor (VEGF)* (7-10). VEGF is a signal protein that stimulates proliferation of endothelial cells to form new blood vessels and thereby initiate angiogenesis (8).

1.1.3 Metastasis

Metastasis is the most fatal aspect of cancer, which is responsible for 90% of cancer-associated deaths (8). The metastatic process is started with entering of tumor cells into the blood or/and lymphatic vessels by crossing the basal lamina and the endothelial lining of the vessels. Tumor cells that entered lymphatic vessels get access to lymph nodes and give rise to lymph-node metastases (8). Tumor cells which have entered the bloodstream are transported with the blood flow and finally get trapped in other organs and start to establish new tumors at these distant sites (8, 11). Change in expression of the cell adhesion molecules, ability to escape from the body's immune system and activation of enzymes that break down the extracellular matrix are important factors which are necessary to establish metastatic disease (11).

1.2 Brain cancer

A brain cancer is an intracranial solid neoplasm, either primary by developing in the brain and adjacent structures or a metastasis from a different organ.

The most common primary brain tumors are:

- Gliomas
- Meningiomas
- Pituitary adenomas
- Nerve sheath tumors (tumors of cranial and spinal nerves)

Primary brain cancer originates from the main cell types, which are resident in the brain and are used for classification of the central nervous system tumors. Thus, gliomas are arising from glial cells of the brain and can be subdivided into astrocytomas, oligodendrogliomas and ependymomas (12). Astrocytomas and in particular glioblastoma, the most malignant astrocytoma, are the most frequent gliomas. Gliomas are classified by the World Health Organization (WHO) based on the level of malignancy. According to the WHO, a benign astrocytoma is a grade I tumor and the most malignant astrocytoma, glioblastoma, is a grade IV tumor. At least 50 % of all gliomas and 12-15 % of all primary intracranial tumors are glioblastoma (13).

1.2.1 Glioblastoma multiforme (GBM)

The annual incidence of glioblastoma multiforme (GBM) varies between 3 – 6 per 100,000 people (14).

Some risk factors are defined for glioblastoma:

- Ionizing radiation (15)
- Sex: for unknown reasons, GBM occurs more commonly in males (3:1)
- Age: the incidence rate increases with age
- Ethnicity: caucasian origin are diagnosed more often with GBM
- A history of low-grade astrocytema

- Familial tumor syndromes: Li-Fraumeni syndrome, Turcot syndrome, tuberous sclerosis and neurofibromatosis are associated with an increased risk of developing GBM (16)

After several decades of intensive research, molecular studies confirm certain patterns of mutations, which are present in varying proportions of GBMs. Frequent genetic alterations in human GBMs include dysregulation of growth factor signaling via amplification and mutational activation of receptor tyrosine kinase (RTK) genes, in particular of EGFR; activation of the phosphatidylinositol-3-OH kinase (PI (3)K) pathway and inactivation of the p53 and retinoblastoma tumor suppressor pathways (17). Despite improvement in understanding of the molecular changes, the prognosis of GBM patients is still dismal.

1.3 Therapy

1.3.1 Conventional therapy

A couple of factors complicate GBM treatment including resistance of brain tumor cells to conventional therapy, damage and low repair capacity of brain cells as well as the blood-brain barrier which represents a penetration problem for drugs (18, 19).

The first stage of glioblastoma treatment involves neurosurgical resection of the tumor followed by radiotherapy, chemotherapy and recently also anti-angiogenic therapy. However, long-term tumor control is impossible, because all malignant gliomas are highly infiltrative with single tumor cells invading into the cortex and migrating along white matter tracts. These cells escape conventional therapies. Therefore, prognosis for GBM patients is still poor, with a median survival time about 14 months (20). The dismal prognosis of glioblastoma encourages researchers to find new treatment modalities for malignant gliomas. Molecular targeted therapy, immunotherapy and virotherapy/gene therapy are novel therapeutic approaches in the treatment of GBM. As gene therapy is the focus of the present thesis, this subject will be introduced in detail in the following paragraphs.

1.3.2 Gene therapy

Gene therapy can be defined as the use of nucleic acids as drugs. The combination of virus-based methods (1960s) with recombinant DNA technology (1970s) has established a new way in genetic medicine that could potentially compensate for errors in the diseases, which are associated with DNA deficiency (21). In the 1980s some pioneer research groups used gene therapy to replace various deficient proteins such as arginase in children with hyperargininemia and hemoglobin in thalasemia (22). According to the data from *Journal of gene medicine* approximately 1,700 clinical trials have been recorded with using a number of techniques for gene therapy (Figure 1.1).

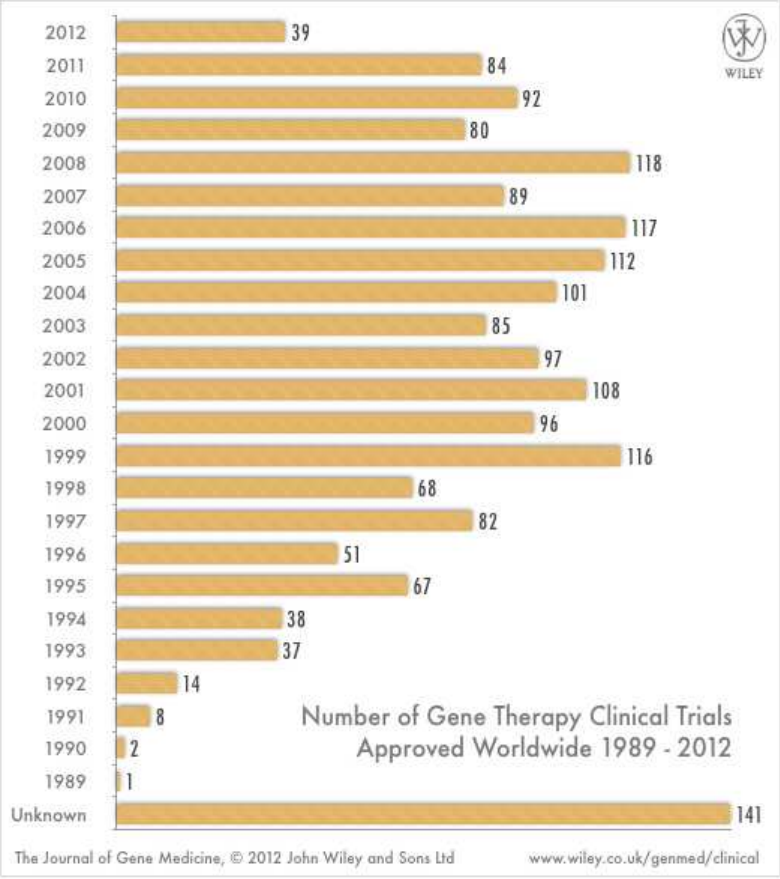


Figure 1.1. The world wide clinical gene therapy trials from 1989 to 2012 (23).

1.3.2.1 Gene therapy vectors

In order to deliver genes into target cells, two kinds of vectors can be employed as vehicles for gene transfer:

1. Viral delivery vectors
2. Non-viral delivery vectors

An optimal gene delivery system can be described as a system with several important functions. The system should efficiently transduce target tissue and ensure gene transcription in the cell and at the same time be safe without causing cytotoxic side effects. In addition the vector should be easily to manufacture (24).

From the onset of gene therapy, viral vectors have been recognized as the most efficient vehicles for transferring genes to human cells, although non-viral vectors showed some advantages as feasibility of large scale production and low host immunogenicity.

1.3.2.1.1 Viral delivery systems

Various classes of viruses have been applied as a tool for gene therapy by using their natural ability to enter the genome of the infected cell and introduce their DNA into the cell with high efficiency (Figure 1.2). Therefore it is possible to take advantage of the properties of the virus by introducing an ideal gene into the virus and deliver this gene into a target cell (25).

Using replication defective viral vectors is very important to ensure that the viruses used as vectors are safe. By replacing genes which allow virus replication with therapeutic genes is defined as replication deficiency. In this regard viral vectors can be produced only in special cell lines called “packaging cell lines” (PCLs) (26), which harbor the genes for the assembly of the virus and the therapeutic gene on a separate plasmid. Viral vectors, with regards to having different replication strategies are subdivided in two main categories:

1. Integrating vectors
2. Non-integrating vectors

The difference between integrating viruses and non integrating vectors is that the former insert themselves into the recepient’s genome and can therefore be inherited stably to the

progeny, for example lentiviral vectors, while the latter form an extrachromosomal genetic element, such as adenoviral vectors (24).

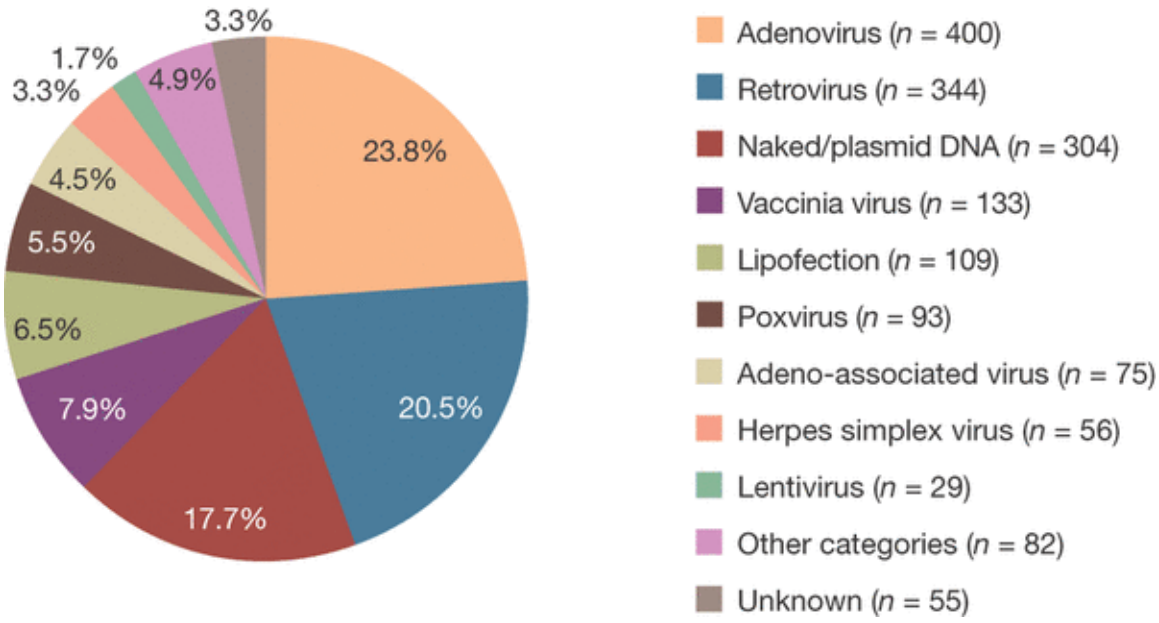


Figure 1.2. Vectors used in gene therapy clinical trials, 2012 (27).

1.3.2.1.2 Oncolytic viruses

The concept of oncolytic virotherapy is to employ virus properties enabling to infect and eradicate malignant cells while in contrast non-lytic vectors express transgenes that generate an anticancer effect. Several oncolytic viruses were used for targeting cancers during the 1950s and 1960s (28). One of the first viruses that showed promising results was a vaccine strain of rabies virus, which was used for melanomatosis patients (29). Further, the oncolytic efficacy of adenovirus serotype (type 4), the flavivirus West Nile virus (strain Egypt 101), the paramyxoviruses, mumps and Newcastle disease virus (NDV) were confirmed after using them on human tumors (30-32). Despite providing promising therapeutic potential in humans, the approach was abandoned due to toxicity as the viruses were not selective for tumor cells, but also infected normal cells. Later,

researchers took advantage of recombinant technology and made viruses, which were selective for tumor cells. The first selective oncolytic virus that was applied for cancer therapy in an experimental model was herpes simplex virus type1 (HSV-1) in 1991. In 1996 the engineered adenovirus Onyx-015 was used in a clinical trial for cancer patients (33).

Currently, oncolytic gene therapy is applied in a broad range of cancers. Based on information from a recent study, 7% of the world wide clinical gene therapy trials from 1990 to 2004 were carried out on GBM patients (34).

Vector delivery to the tumor cells can be intratumoral, intracavitary, or intravascular. Gene products of oncolytic viruses can inhibit growth of residual tumor cells after surgery in combination with other therapies (33).

1.3.2.1.2.1 VSV oncolytic viruses

Vesicular stomatitis virus (VSV) is a virus from the family of *Rhabdoiviridae*, a common laboratory virus used for research purposes and so far the only Rhabdovirus applied in cancer targeted therapies (28, 35). VSV causes oral disease in insects, cattle, pigs and horses, however is considered to be attenuated in humans. VSV has several serotypes, classified as Vesicular stomatitis Indiana virus, Vesicular stomatitis New Jersey virus and Vesicular stomatitis Alagoas virus (36). The genome of the virus is a single molecule which contains 11.2kb of negative sense RNA (Figure 1.3). Five genes are placed between 3′ leader (le) and 5′ trailer (tr) regions in the order 3′-N-P-M-G-L-5, encoding five major proteins: Glycoprotein (G), large polymerase (L), phosphoprotein (P), matrix (M) and nucleocapsid (N) (Fig 1.3) (37, 38). The glycoprotein (G) resides on the cell surface and contains sialic acid, which is necessary for cell surface attachment and budding. The nucleocapsid protein (N), the phosphoprotein (P) and polymerase or L protein are responsible for viral transcription and subsequently constitute the viral genome replication complex. The Matrix or M protein is a multi-functional protein which consists of 229 aminoacids. It has an important role in budding and apoptosis. It also acts as a critical player in the oncolytic activity of VSV (39).

VSV has many advantages that make it a promising therapeutic candidate: (1) VSV has a short and highly cytolytic replication cycle which kills a wide range of tumor cells

rapidly; (2) it is easy to produce and genetically stable which allows generation of high titers; (3) it is sensitive to the host antiviral response, which mediates its cancer selectivity (40); (4) it is not a human pathogen so there is not any preexisting immunity in humans that could limit clinical applications (39, 41-43).

As of yet, there is not any clinical trials initiated with VSV in cancer patients, because VSV is highly neurotoxic, which is one of the disadvantages limiting its application in clinical trials (44-46). However, several research groups have attempted to develop attenuated viruses (AV). The major concern is that whether AVs would be effective enough for treatment (47-49) as the reduction of cytotoxicity of the virus can only be achieved by limiting also its replication ability and oncolytic activity (50).

Since the tropism of a viral vector is determined by its envelope protein which interacts with a specific receptor on the cell surface, we can assume that neurotropism of VSV is attributed to the glycoprotein (VSV-G) (51-54). The glycoprotein of VSV binds to phosphatidylserin (important component of the cell surface membrane), which is highly concentrated in myelinated part of neurons (39).

By exchanging the VSV-G with a glycoprotein from a different virus, the tropism of the virus could be changed in order to minimize the neurotropism, but at the same time to maximize tropism for tumor cells. This approach will be discussed in the following chapter.

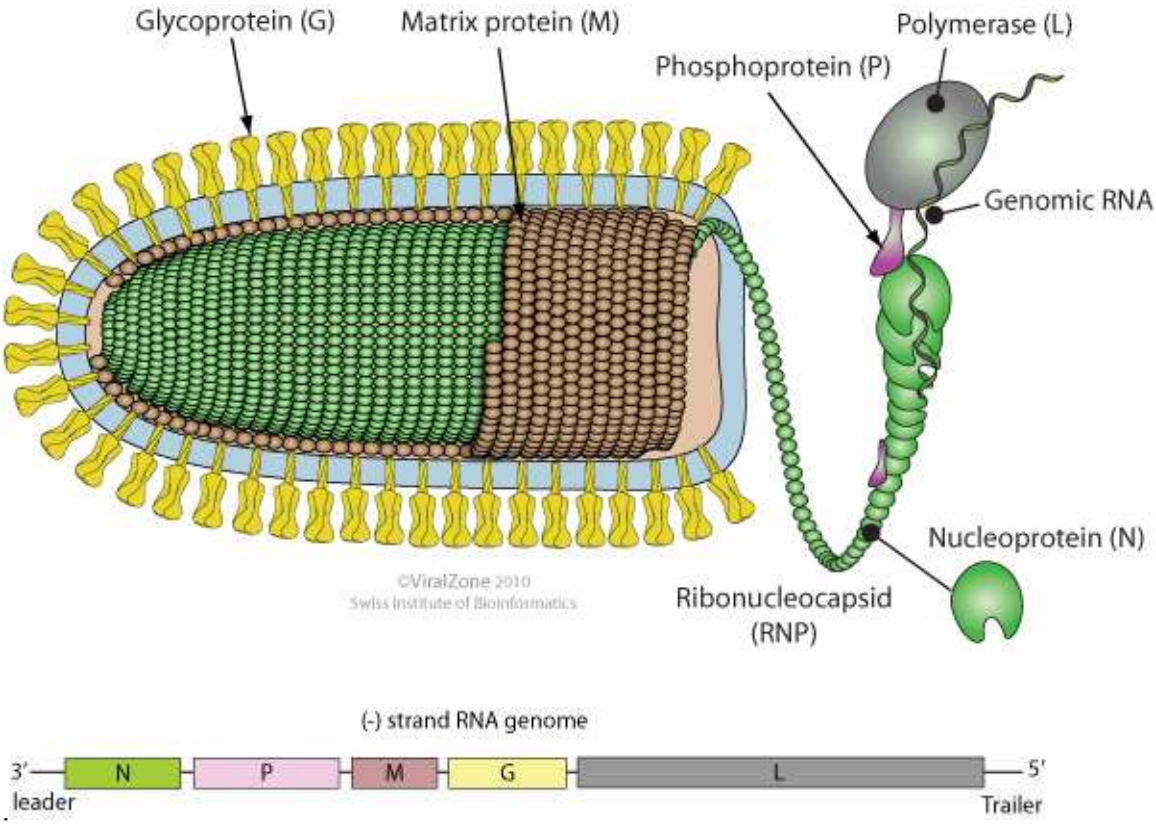


Figure 1.3. Enveloped, bullet shaped VSV and negative-stranded RNA linear genome of VSV. The genome of VSV is about 11 kb in size, which Encodes for 5 distinct proteins: Glycoprotein (G), large polymerase (L), phosphoprotein (P), matrix (M) and nucleocapsid (N) (55).

1.3.2.1.2.2 Modification of viral tropism: Pseudotyped VSV

The exchange of the virus glycoprotein by a glycoprotein from a different virus is called pseudotyping and changes the tropism of the chimeric viral particle. Using this strategy, VSV can accommodate glycoproteins from other viruses, after mixed infections of cells. Consequently, the cell tropism of the novel virus would be determined by the receptor specificity of the introduced glycoprotein (56).

Miletic and Von Laer *et al.* have in a collaborative effort established several novel chimeric viruses that carry the envelope glycoproteins (GP) of the lymphocytic choriomeningitis virus (LCMV). They showed that LCMV-GP pseudotypes can be efficiently incorporated into the gammaretroviral and lentiviral vector envelope (57, 58). These pseudotypes are stable, but unlike the vesicular stomatitis virus G protein, the LCMV glycoproteins are not cytotoxic/neurotoxic (59). Moreover, Miletic *et al.* (2004,

2007) showed that LCMV-GP pseudotyped lentiviral vectors delivering a suicide gene confirm high transduction efficacy and killing of glioblastoma in a rat glioblastoma model (58, 60).

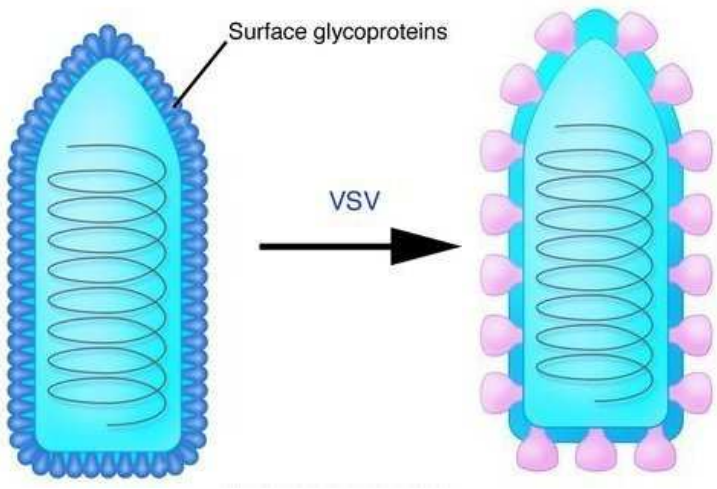


Figure 1.4. VSV and VSV / LCMV-GP. By reverse genetic techniques, the virion glycoproteins were changed. Consequently, the cell tropism of VSV / LCMV-GP would be determined by the receptor specificity of the interduced glycoprotein.

Therefore, the LCMV-GP would be an optimal candidate for pseudotyping with VSV in order to eliminate neurotropism. The VSV normally packaged with the glycoprotein VSV-G, revealed the ability to incorporate the LCMV-GP glycoprotein into the viral envelope, generating a pseudotyped virus (VSV-GP; Figure 1.4). The VSV-GP was first generated as a replication deficient virus and proved not to infect neuronal cells. In addition it was still infecting and killing cancer cells as efficiently as wild-type VSV. Hence the oncolytic VSV-GP pseudotypes are currently under development for the treatment of melanoma, brain and ovarian cancer (54).

2 Aims

Based on the information provided in the previous sections, the following hypothesis and aims are outlined in the presented thesis:

Hypothesis

Although replication deficient VSV-GP has been proven to be devoid of neurotoxicity (54), replication competent virus has to be tested as this will be used as the final therapeutic agent in order to maximize the therapeutic effect for glioblastoma treatment. By injecting replication competent VSV wild-type (WT) and VSV-GP into the brains of normal mice, our collaboration partner showed that VSV-WT even when using low virus titers killed the mice, whereas all mice injected with high titers of VSV-GP survived (Muik A. *et al.*, unpublished observations). Therefore we hypothesize that VSV-WT due to infection of normal brain cells induces a lethal encephalitis, while in contrast VSV-GP is sparing normal brain cells and thus is the ideal vector for gene therapy of glioma.

Aim

Because malignant gliomas only rarely metastasize outside the skull, novel locoregional treatment modalities such as gene therapy are potentially interesting.

The aim of this thesis is to compare neurotoxicity of replication competent wild-type VSV and VSV pseudotyped with the non-neurotropic LCMV-GP and to identify whether VSV-GP is an attractive candidate for gene therapy of glioma.

3 Materials

Table 3.1: chemicals, solutions and reagents

Chemical/solution	*Catalogue nr.*	*Supplier*
Bovin serum albumin(BSA)	A4503	Sigma-Aldrich, St. Louis, Missouri, USA
EDTA	ED2SS	Sigma-Aldrich, St. Louis, Missouri, USA
Ethanol, absolute	32221	Sigma-Aldrich, St. Louis, Missouri, USA
Extravidin-Cy3 (1:100)	125K6195	Sigma-Aldrich, St. Louis, Missouri, USA
Fat pen	H-4000	Vector laboratories, Inc. Burlingame, California, USA
Haemotoxyline	RBA-4213-00A	CellpathLTd, UK
Hydrogen peroxide	H1009	Sigma-Aldrich, St. Louis, Missouri, USA
Isopentane	M3263-1L	Sigma-Aldrich, St. Louis, Missouri, USA
Paraformaldehyde (PFA) powder	76240	Fluka, Sigma-Aldrich, St. Louis, Missouri, USA
Tissue-Tech O.C.T Compound	90501	Sakura Finetek, USA
Triton X-100	T8787	Sigma-Aldrich, St. Louis, Missouri, USA
Vectashield Mounting Solution w/DAPI	H1200	Vector Laboratories, Burlingame, California, USA
Xylenes	33817	Sigma-Aldrich, St. Louis, Missouri, USA

Table 3.2: Commercial kits

Article	*Cataloque number*	*Producer*
DAB detection kit	K3468	DAKO laboratories, CA, USA
In Situ Cell Death Detection Kit, POD (peroxidase)	1684817	Roche applied science, Penzberg, Upper Bavaria, Germany
M.O.M kit (peroxidase)	PK-2200	Vector laboratories, Inc. Burlingame, CA, USA
Vectastain ABC kit	PK-6200	Vector laboratories, Inc. Burlingame, CA, USA

Table 3.3: Buffers/solutions prepared in the lab

Citrate buffer,10 mM (pH=6)	2.94 g Sodium citrate di-hydrate mixed with 250 µl Tween 20, pH adjusted with HCl, add MilliQ H2O up to 1 liter
Dulbecco's Phosphate Buffered Saline (PBS) 1x	1 volume of Dulbecco's Phosphate Buffered Saline (10x), 9 volumes of autoclaved MilliQ H2O
Ethanol,96%	96 ml, absolut Ethanol diluted with MilliQ H2O up to 100 ml
3% H_2O_2	0.1 ml 30%hydrogen peroxide to 0.9 ml 1xPBS
4% Paraformaldehyde/PBS	40g PFA powder diluted in 1L PBS and heated in steamer until it become clear
Permeabilisation solution for Tunel assay	0.1% Triton X-100, 0.1% sodium citrate

Protein/serum block for IHC	950 µl TBST/5% BSA+50 µl serum from species secondary antibody
30% Sucrose/PBS	150 g sucrose mixed with 50 ml 10x PBS, diluted with MilliQ H2O up to 500 ml
TBS Tween/buffer (pH=7,2 - 7,6)	50 Mm Tris base,300 Mm NaCl, 0,05% Tween 20 was mixed add MilliQ H2O up to a total volume of 1 liter, pH adjusted with HCl

Table 3.4: Primary antibodies

Anti-X antibody, application	***Dilution***	***Source species and clonality***	***Catalogue nr.***	***Supplier***
CD3	1:500	Rabbit polyclonal IgG	ab5690	Abcam, Cambridge, United Kingdom
CD11b	1:300	Rabbit polyclonal IgG	ab75476	Abcam, Cambridge, United Kingdom
Cleaved Caspase-3	1:50	Rabbit Monoclonal	D175 5A1E	Cell signaling technology, MA,USA
GFAP	1:200	Mouse monoclonal IgG1	MAB3402	Millipore, Billerica, MA, USA
NeuN	1:200	Mouse monoclonal IgG1	MAB377	Millipore, Billerica, MA, USA

Table 3.5: Secondary antibodies

Antibody	*Dilution*	*Source species and clonality*	*Catalogue nr.*	*Supplier*
Biotinylated anti rabbit IgG	1:100	Goat anti-rabbit IgG	BA-1000	Vector laboratories, Inc. Burlingame, CA, USA
anti-GFP, Alexa Flour 488 conjugate	1:200	Rabbit IgG fraction, polyclonal	A21311	Invitrogen laboratories, Oregon, USA

Table 3.6: Hardware

Name	*Catalogue nr./Model*	*Supplier*
Confocal microscope	LSM 510 Meta	Carl Zeiss Microimaging GmbH, Oberkocken, Germany
Cryostate	Leica CM3050S	Leica Biosystemes, Germany
Fluorescence microscope	Axioplan II	Carl Zeiss Microimaging GmbH, Oberkocken, Germany
Optical microscope	Nikon ECLIPSE, E600	Nikon, Tokyo, Japan

Table 3.7: Software

Program	*Version*	*Supplier*
Adobe photoshop	CS5	Adobe Systems Inc., San Jose, California, USA
Microsoft Office Excel	2003	Microsoft Corporation , Redmond, Washington, USA
NIS-Elements BR Image software	4	Nikon, Tokyo, Japan
Zeiss LSM Image Examiner (LSM 510META)	4.2	Carl Zeiss Microimaging GmbH, Oberkocken, Germany

4 Methods

All procedures were performed at room temperature unless otherwise stated.

4.1 Immunostaining of paraffin sections

3 µm thick sections from paraffin-embedded formalin-fixed tissue were deparaffinized in a xylene bath (Sigma-Aldrich, Missouri, USA) for 2x5 minutes, in absolute ethanol (Sigma-Aldrich, Missouri, USA) for 2x3 minutes, in 96% ethanol (Table 3.3) for 2x3 minutes and finally in distilled water for 2 min. For antigen retrieval, depending on the primary antibody, sections were heated either Citrate buffer (pH=6, Look at table 3.3) or EDTA 1mM (pH=6, Sigma-Aldrich, Missouri, USA) for 5 min in the microwave and subsequently in the steamer for 25 min. The sections were then cooled down on the bench for 25 min and thereafter put under running water for 10 min. The tissue was encircled with a fat pen (Vector laboratories, CA, USA) and TBS w/Tween (Table 3.3) was added immediately to avoid tissue drying. The sections were blocked with a mixture of 950 µl TBST/5% BSA and 50 µl of serum derived from species of the secondary antibody. The sections were incubated with monoclonal antibodies, which were diluted in TBS/5% serum (of secondary antibody) and were kept at 4 ºC over night.

On the second day the sections were rinsed with TBS w/Tween for 2x5 min. A solution of 3% H_2O_2 (0.1 ml 30% hydrogen peroxide to 0.9 ml 1x PBS) was prepared and added to the sections for 5 min. A biotinylated antibody (Table 3.5) was used as secondary antibody dilution 1:100 in TBS Tween/buffer and incubated with avidin biotin complex (ABC) from Vectastain ABC kit (Vector Laboratories, CA, USA) for 30 min at room temperature. Peroxidase activity was determined by DAB solution. DAB solution was prepared by adding one drop of DAB chromogen substrate to 1 ml of buffered substrate (DAKO laboratories, CA, USA). The index was analysed by NIS-Elements BR Image software (Version 4, Nikon, Japan). For each slide, ten areas were randomly counted at different magnifications.

4.2 TUNEL assay

TUNEL (TdT-mediated dUTP nick end labeling) is a specific technique for labeling distinctive size of DNA fragments, which are found in the nuclei of apoptotic cells. In this procedure the enzyme terminal deoxynucleotidyl transferase (TdT) adds chains of labeled deoxynucleotide to the 3'-OH ends of DNA fragments.

The brain sections were deparaffinized as described before under the immunostaining section. The tissues were encircled with fat pen and pretreated with proteinase K (nuclease free, In Situ Cell Death Detection Kit- POD, Roche Applied Science) for 15 minutes at room temperature. After rinsing three times with PBS (Table 3.3) the endogenous peroxidase was inactivated with 3% H_2O_2 for 7 minutes. Permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) was freshly prepared and added to the tissues for 2 min, while they were kept on ice. Tunel reaction mixture was prepared according to the manufacturer's protocol (In Situ Cell Death Detection Kit- POD, Roche Applied Science). 100 µl label solution was removed for 2 negative controls and total volume of enzyme solution (50 µl) was added to the remaining 450 µl label solution to obtain 500 µl Tunel reaction mixture. The slides were incubated with the reaction mixture in a humidified chamber at 37°C for one hour, and subsequently washed three times with PBS. Sections were incubated with converter-POD (In Situ Cell Death Detection Kit- POD, Roche Applied Science) at room temperature for 30 minutes, followed by incubation with diaminobenzidine (DAB) solution. Apoptotic cells were analyzed using NIS-Elements BR Image software (Version 4, Nikon, Japan). For each slide, we randomly counted ten sections, using a light microscope at a magnification of ×20. The positive cells with condensed chromatin masses, were counted as apoptotic cells.

4.3 Snap-Freezing and cryosectioning of tissue samples

Mouse brains were first fixed in 4% Paraformaldehyde/PBS solution (Table 3.3) overnight and then incubated in 30% sucrose/PBS solution (150 g sucrose mixed with 50 ml 10x PBS, diluted with MilliQ H2O up to 500 ml) for three days at 4 ºC. Coronal slices of mouse brains were prepared using tissue scalpels and were positioned on a thick paper and coated with Tissue-Tech O.C.T Compound (Sakura Finetek, USA). The tissue was

snap-frozen in isopentane (2-methyl butane) chilled with dry ice (-70 ºC) and stored at -80 ºC until it was used.

Serial coronal sections with a thickness of 10 µm were cut on a cryostate. From the serial sections, every 10th section was examined under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Sections that showed green fluorescence, indicating virus infection (GFP) were selected for further analysis. Sections were stored in a freezer (- 80 ºC) until studied.

4.4 Double Immunofluorescence of cryosections

To investigate which cell types were infected by the viruses, the cryosections were stained with antibodies against neurons (NeuN) and astrocytes (GFAP). Anti-GFP antibody was used to enhance the GFP signal indicating virus infection. DAPI was used to stain nuclei.

Before staining, the slides were warmed up to 37 ºC for 20 minutes and thereafter fixed with 4% paraformaldehyde/PBS (Table 3.3) for 15 min. After washing with PBS for 10 min, the tissue was encircled with a fat pen and was blocked with a mixture of IgG blocking reagent (M.O.M kit, Vector laboratories, Burlingame, CA, USA) dilute in PBS for 1 hour. Thereafter, 200µl protein concentration (M.O.M kit) was mixed with 2.5 ml PBS and added to the sections for 5 min. After tipping excess solution off the sections, the primary antibodies, NeuN for neurons (1:200 dilution; Millipore, Billerica, MA, USA) and GFAP (1:200 dilution; Millipore, Billerica, MA, USA) for astrocytes were used. After adding primary antibody, the slides were put into a humidified chamber at 4 ºC overnight.

On the next day, sections were washed 3x for 10 min with PBS. Thereafter biotinylated anti-mouse IgG (1:250, M.O.M kit) was used as a secondary antibody for 1 hour. After rinsing with PBS 2x for 10 min, Extravidin-Cy3 (1:100, Sigma-Aldrich, Missouri, USA) was added to the sections for 30 min. Finally, the sections were incubated with anti-GFP antibody (Invitrogen laboratories, USA) dilute 1:200 in PBS for 60 min and subsequently washed with PBS 3x for 10 min. For microscopy the sections were mounted with a glue containing DAPI (Vector Laboratories, CA, USA).

4.5 Confocal imaging

In confocal or fluorescence microscopy, the specimen is excited by light of different wave lengths which is detected by the microscope's camera. In consequence in a regular fluorescence microscope we get a big and unfocused background of the image. In contrast, the principle of confocal imaging is the use of a pin hole to reject out-of-plane unfocused light. In consequence it creates sharp images of a specimen with less background. The confocal microscope is equipped with a laser as light source, which has the highest intensity, to be detected while all other light is deprived. When light is emitted by the specimen and focused through the pin hole it is measured by a detector (photomultiplier tube) connected to a computer. Only one point (pixel) in the specimen is observed at a time, and the computer builds up an image as a chosen number of pixels detected.

Zeiss LSM 510 Meta (Carl Zeiss, Oberkochen, Germany) located in the Molecular Imaging Center (MIC) of department of Biomedicine, Bergen was used for experiments performed in this project. Zeiss LSM 510 Meta is equipped with several lasers, of which the UV laser was used to excite at 358 nm (DAPI), the Argon laser was used to excite at 488 nm (FITC) and the HeNe laser was used to excite at 543 nm (Cy3). Pictures were taken from areas were green fluorescence (FITC) was observed, indicating virus infection. Single pictures as well as stacks, representing serial pictures with different depths (z-axis), were taken. Pictures were then analyzed with the Zeiss LSM Image Examiner (Version 4.2, Carl Zeiss, Oberkocken, Germany).

4.6 Statistics

Statistical analysis was carried out using the program Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, Washington, USA). The student's t-test was used, and a p-value of <0.05 was considered statistically significant.

5 Results

5.1 Toxicity and efficiency of oncolytic VSV vectors

5.1.1 Histological analysis reveals strong inflammation in VSV-WT injected mice

We received paraffin-embedded brain tissue from our collaborator, who performed the injection experiments (Alexander Muik, Frankfurt, Germany). To directly compare the neurotoxicity of VSV-WT and VSV-GP, histological slides from either VSV-WT or VSV-GP infected mice brains at day 3 and day 7 post infection (p.i.) were stained with Hematoxylin and eosin (Figure 5.1). PBS injected animals were used as a control. Part A of Figure 5.1 illustrates the injection tracks of VSV-WT and VSV-GP vectors administrated intracerebrally compared to PBS injected controls. VSV-WT shows a stronger inflammatory response close to the injection site compared to VSV-GP and PBS, both after 3 and 7 days p.i.

To investigate whether the viruses spread from the injection site to distant areas in the brain, we looked at the brain hemisphere contralateral to the injection site (Figure 5.1 B). VSV-WT showed perivascular accumulation of inflammatory cells as well as spreading of immune cells into brain tissue at both days p.i. Inflammatory cells were often observed in direct contact to neurons (Figure 5.2). In contrast, VSV-GP only showed a few single inflammatory cells around vessels and no significant inflammation in the brain tissue. PBS injected control animals did not show any sign of inflammation.

Interestingly, in the VSV-WT group there was sign of edema with loosely textured brain tissue around the inflammatory spots at day 7 after infection which was not observed at day 3 p.i. (Figure 5.1 B).

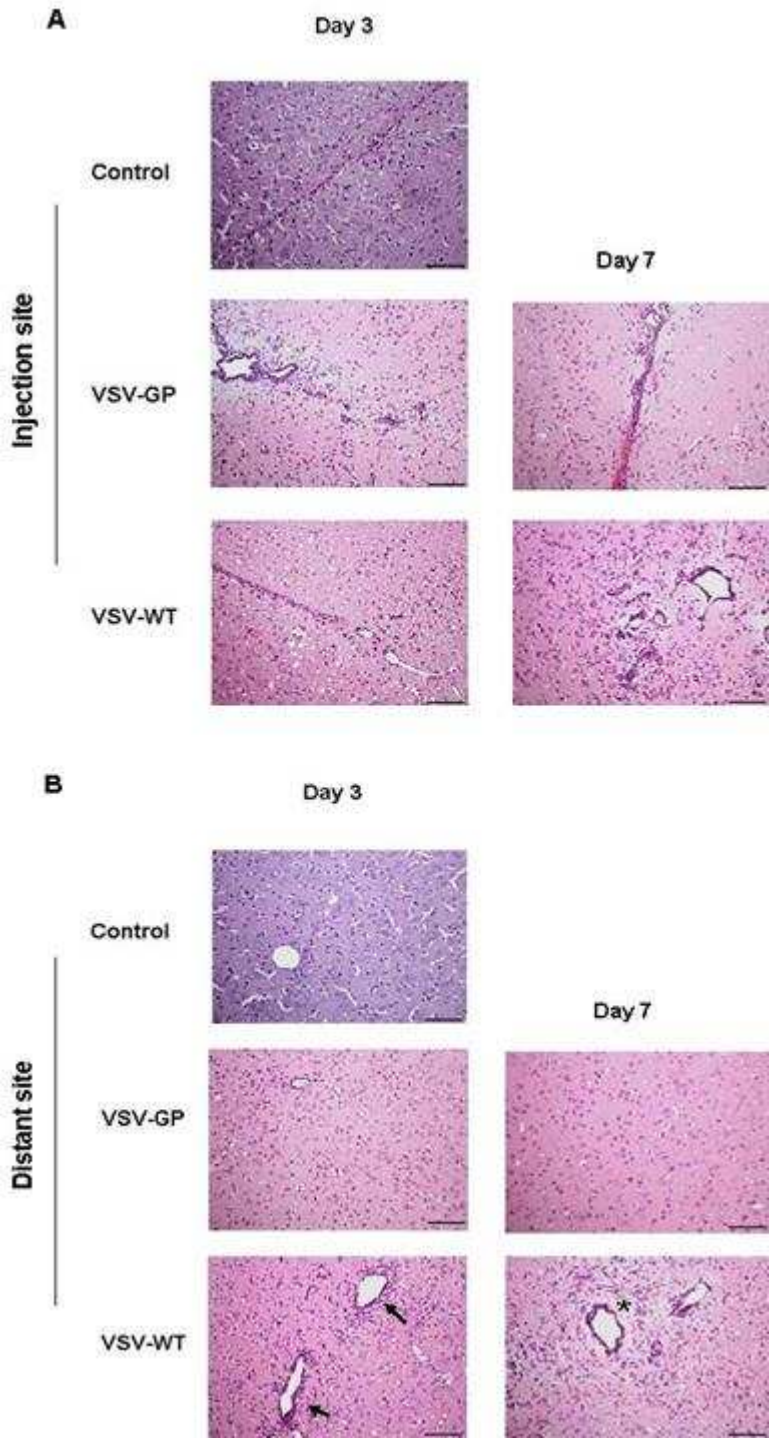


Figure 5.1. H&E stained brain sections of mice injected with VSV-WT, VSV-GP or PBS intracerebrally. The upper panel shows the injection tracks (A). The lower panel illustrates the contralateral hemisphere (B). Black arrows reveal inflammation around vessels at distant sites from injection track. Edema is visible around vessels in VSV-WT after 7 days p.i. (*). (Magnification: ×20). [Scale bars: 100 μm].

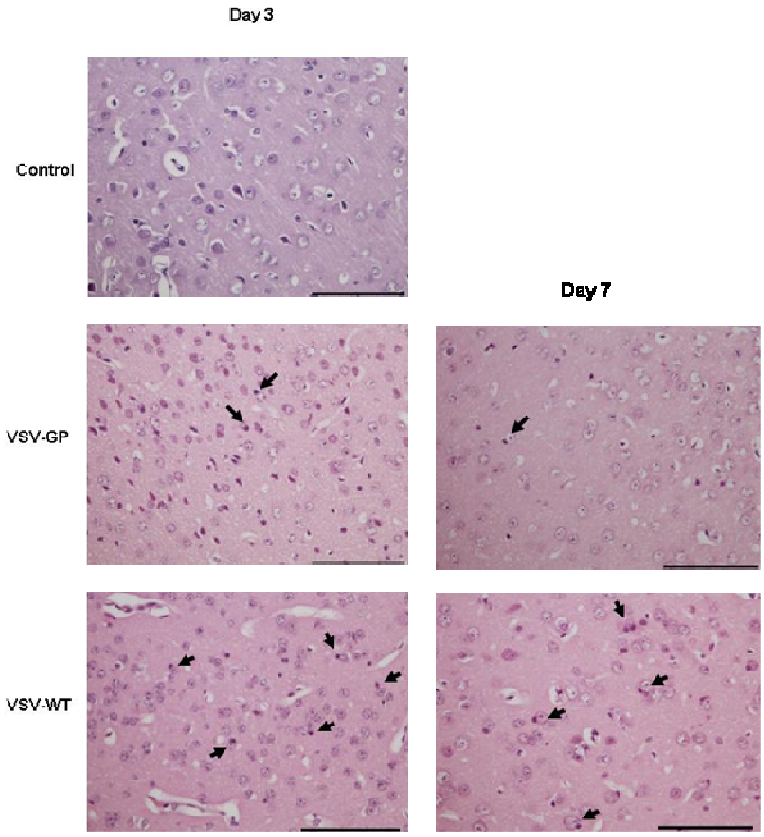


Figure 5.2. H&E stained brain sections of mouse. The injection area was avoided. Black arrows reveal Inflammatory cells, which are in direct contact to neurons. The amount of inflammatory cells, which were in direct contact with neurons was high in VSV-WT day 3 p.i.. (Magnification: ×40). [Scale bars: 100 µm].

5.1.2 Inflammation is composed of CD3 positive T cells and CD11b positive microglia

For the following experiments we analyzed brain tissue distant from the injection site in order to identify responses that are specific to spread of the virus and not related to reactive changes of the injection.

To identify the type and quantity of immune cells causing the inflammation in VSV-WT injected animals, we performed immunohistochemistry. Virus infections most often induce a T cell mediated immune response and therefore we stained the sections with the T cell marker CD3 (Figure 5.3). VSV-WT brains showed a strong infiltration of CD3 positive lymphocytes around vessels and in the brain tissue similar to what was observed on H&E sections. Thus, T lymphocytes seemed to be the major cell population causing inflammation in VSV-WT injected animals. In contrast VSV-GP injected animals

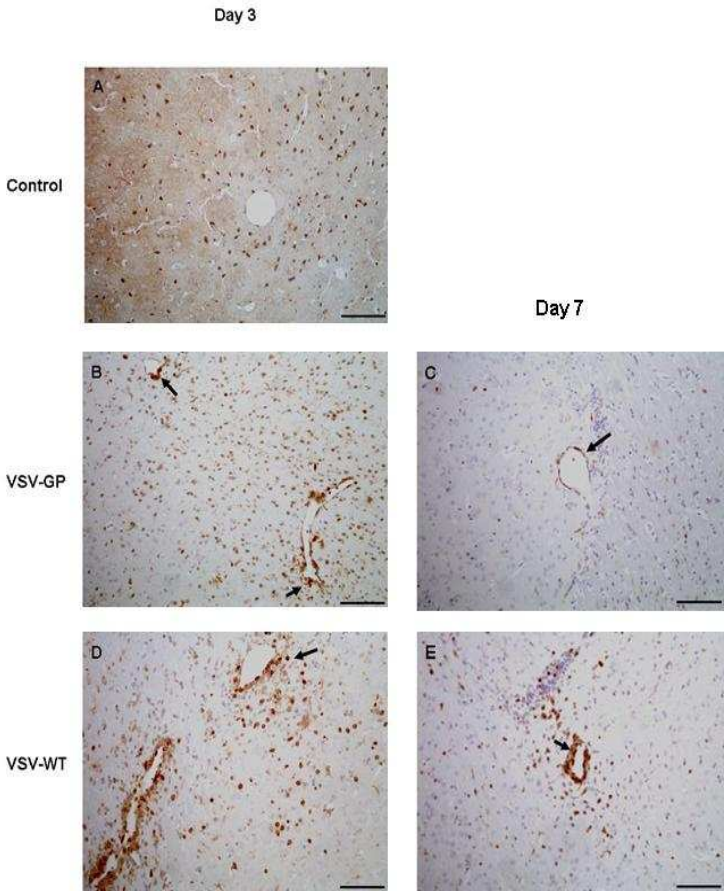


Figure 5.3. Immunostaining for CD3. Black arrows indicate lymphocytes. Left panel of the top row shows PBS treated brain tissue (A). Two panels of the center row show VSV-GP 3 days p.i. (B) and VSV-GP 7days p.i. (C). Two panels at the bottom show VSV-WT 3 days p.i. (D) and VSV-WT 7 days p.i. (E). VSV-WT brains showed a strong infiltration of CD3 positive lymphocytes around vessels and in the brain tissue. In contrast VSV-GP brains tissue showed only few CD3 positive lymphocytes around vessels and in the brain. Infiltration of lymphocytes was almost absent in PBS injected animals. (Magnification: ×20). [Scale bars: 100 μm]

showed only few CD3 positive lymphocytes around vessels and in the brain. Infiltration of lymphocytes was almost absent in PBS injected animals (Figure 5.3 A).

Quantification of the number of CD3 positive cells at day 3 p.i. revealed 48.2 ± 19.2 lymphocytes for VSV-WT infected brains, while VSV-GP infected brains showed significantly less infiltration of lymphocytes (5 ± 4.2; $p<0.0001$; Figure 5.4). At day 7 p.i. a strong reduction of the number of lymphocytes was observed in VSV-WT injected animals (10.5± 10.9) compared to day 3 p.i. Also less infiltration of lymphocytes in VSV-GP infected brains was observed from 3 to 7 days p.i. The difference of the VSV-GP group compared to the control group (PBS injected) at both days p.i. was not statistically significant ($p=0.0911$) indicating that VSV-GP did not induce a significant T-cell response (Figure 5.4).

To analyze whether the resident immune cells of the brain, microglial cells, are participating in the inflammatory response, we stained sections for CD11b, a microglial marker. VSV-WT injected animals showed a slightly stronger activation of microglia

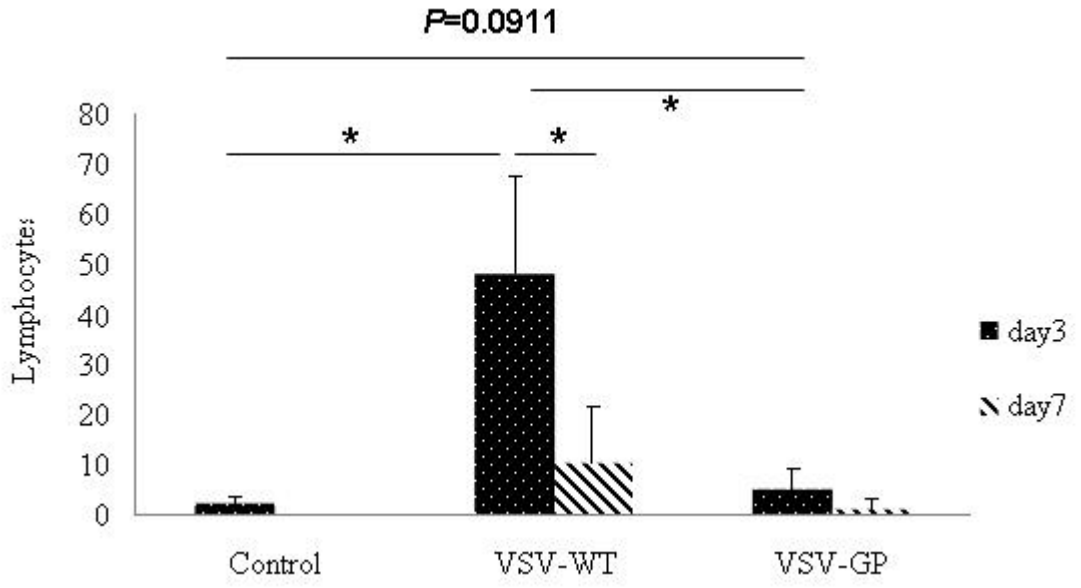


Figure 5.4. The amount of lymphocytes was quantified at 3 and 7 days p.i. in VSV-GP and VSV-WT. 10 random microscopic fields were counted in ×20 magnification. The injection area was avoided. Significantly less lymphocytes were counted in VSV-GP infected animals at day 3 p.i. compared to VSV-W (p<0.0001). A strong reduction in the number of lymphocytes was observed from 3 days p.i. to 7 days p.i. in VSV-WT (p<0.0001). T cell counts of VSV-GP compared to the control group were not statistically significant (p =0.0911). (*) p<0.0001.

around vessels and in the brain tissue compared do VSV-GP injected animals at day 3 p.i. (Figure 5.5 D and B). At day 7 p.i., the difference in quantity of microglial cells was even more evident (Figure 5.5 C and E). PBS injected animals showed only a few single microglial cells throughout the sections (Figure 5.5 A).

Quantification of microglial cells revealed similar results, which was analyzed from immunohistochemical stainings. The number of microglial cells was 13.2 ± 5 for VSV-WT at day 3 p.i., while VSV-GP showed significantly less microglial cells 3 days p.i. (3.9 ± 3; p<0.0001; Figure 5.6). Interestingly reduction of the microglials was not very significant from day 3 to day 7 p.i. in VSV-WT (9.4 ± 10.8; p=0.3889). The microglial cell count for VSV-GP compared to control was not statistically significant (p=0.6549; Figure 5.6) at both days p.i.

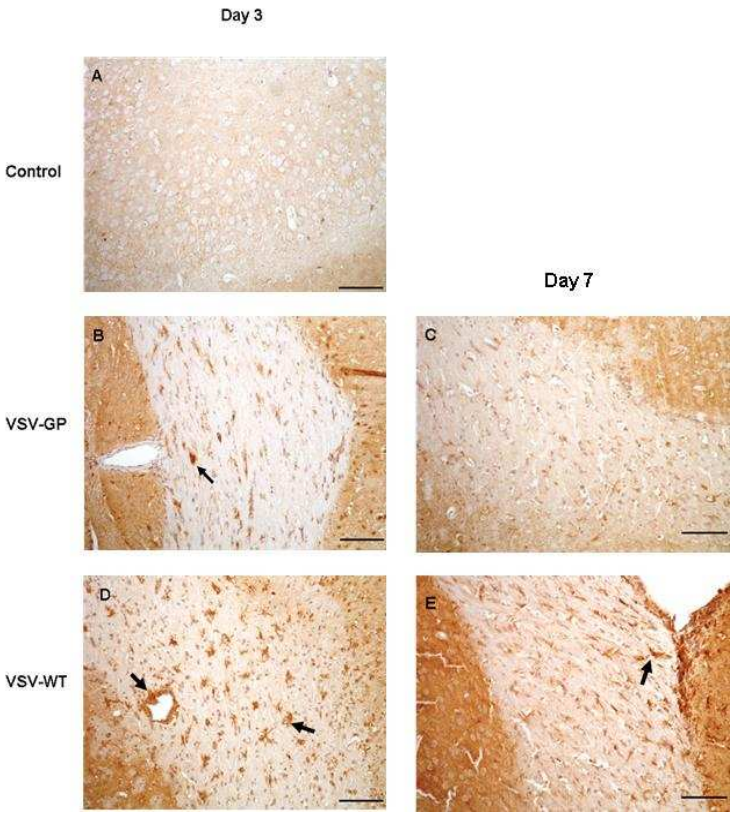


Figure 5.5. Immunostaining for CD11b (macrophage-1 antigen). Black arrows indicate microglia cells which show a strong immunostaining. Left panel of the top row shows PBS treated brain tissue (A). Two panels of the center row show VSV-GP 3 days p.i. (B) and VSV-GP 7days p.i. (C). Two panels at the bottom show VSV-WT day 3 p.i. (D) and VSV-WT day 7 p.i. (E). VSV-WT brain tissues showed a stronger activation of microglia around vessels and in the brain tissue compared do VSV-GP injected animals at day 3 p.i. At day 7 the difference in quantity of microglial cells was even more evident. (Magnification: ×20). [Scale bars: 100 μm].

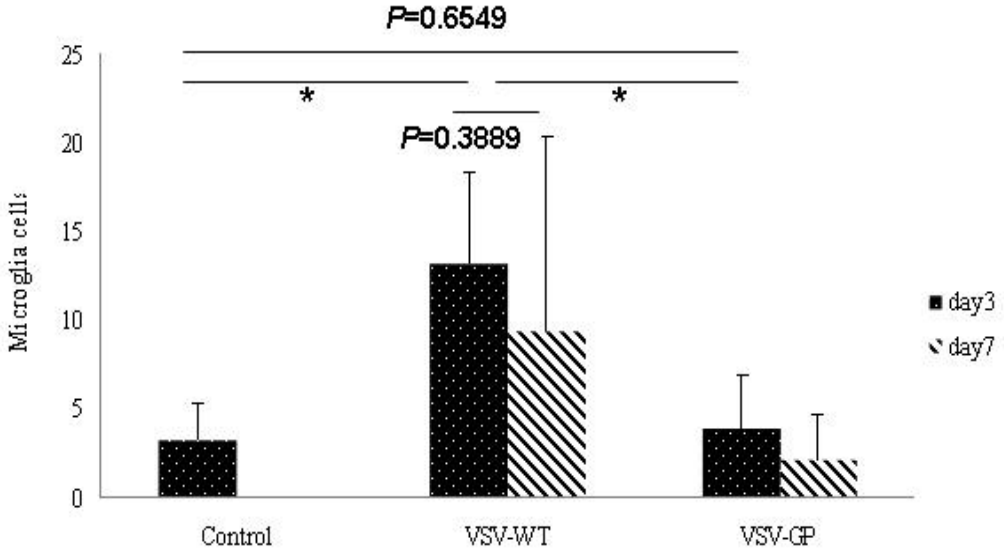


Figure 5.6. The number of microglial cells was determined by light microscopy. 10 random microscopic fields were counted, the area around the injection site was not considered. Significantly less microglial cells were observed in VSV-GP compared to VSV-WT at 3 days p.i. (p<0.0001). The numbers for VSV-WT at day 3 compared day 7 p.i. were not significantly different (p=0.3889). The numbers for VSV-WT compared to control group were significantly different (p<0.0001). The cell counts for VSV-GP compared to the control, were not statistically significant (p=0.6549). (*) p<0.0001.

5.1.3 Significant cell death of brain cells is only detected in VSV-wt injected mice

In addition, we tested toxicity of both viruses by the use of cleaved Caspase-3 antibody, to investigate apoptosis of normal brain cells. By analyzing immunostained sections, more apoptotic cells were found in VSV-WT compared to VSV-GP injected mice brains both after 3 days and 7 days p.i. (Figure 5.7). No apoptotic cells were detected in PBS injected mice brains (Figure 5.7 A).

Quantification of the number of apoptotic cells showed 7.9 ± 3.5 for VSV-WT, while less numbers of positive cells were counted for VSV-GP at 3 days p.i. (1 ± 1; $p<0.0001$; Figure 5.8). A strong reduction in the number of apoptotic cells was observed from 3 days to 7 days p.i. in VSV-WT (1.2 ± 2.1; $p<0.0001$). The differences compared to the control group (PBS injected) were statistically significant both for VSV-WT ($p<0.0001$) and VSV-GP ($p=0.0114$; Figure 5.8) at day 3 p.i.

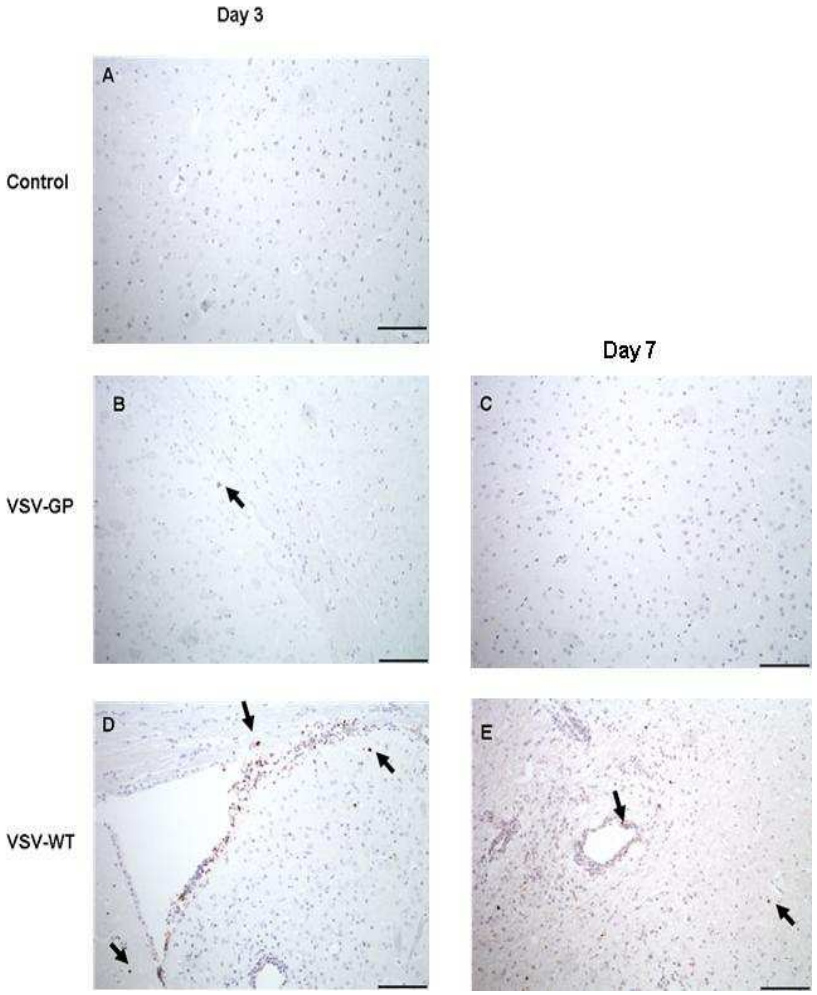


Figure 5.7. Immunostaining for cleaved Caspase-3 (A-E). The pictures indicate more apoptotic cells in VSV-WT both after 3 and 7 days post infection. Left panel of the top row shows PBS treated brain tissue (A). Two panels of the center row show VSV-GP 3 days p.i. (B) and VSV-GP 7days p.i. (C). Two panels at the bottom show VSV-WT 3 days p.i. (D) and VSV-WT 7 days p.i. (E). The amount of apoptotic cells were higher in VSV-WT compared to VSV-GP both after 3 days and 7 days p.i.. No positive cell was detected in control group. (Magnification: ×20). [Scale bars: 100 µm].

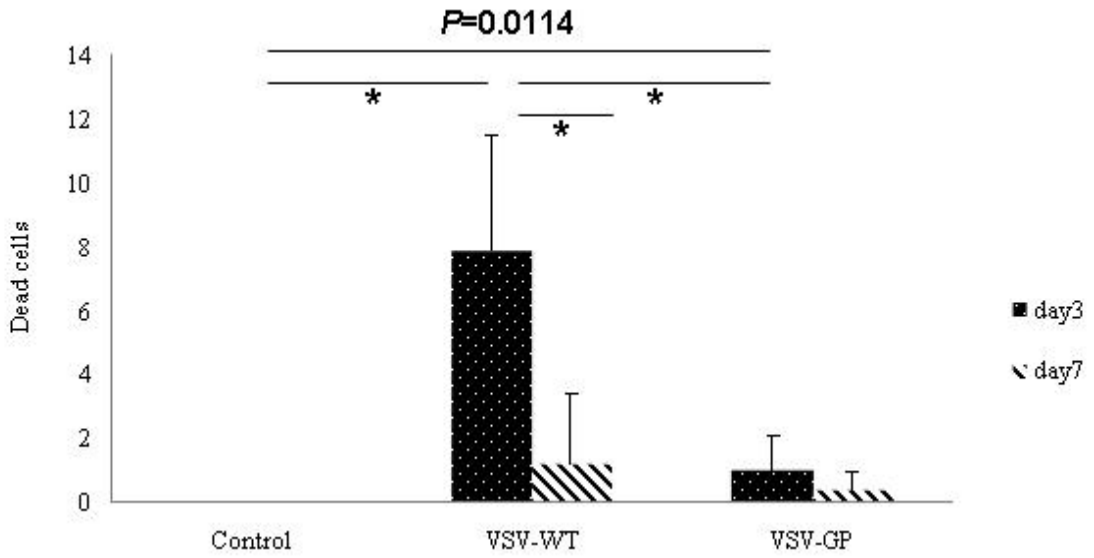
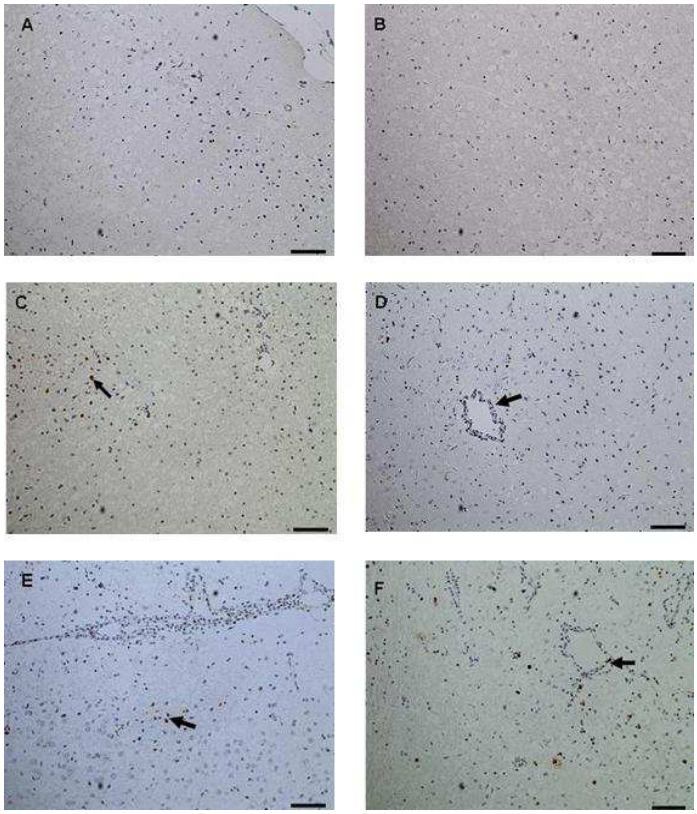


Figure 5.8. 10 different areas were counted for apoptotic cells by cleaved caspase-3 immunostained sections. The injection site was avoided. Significantly less apoptotic cells were counted in VSV-GP compared to VSV-WT at 3 days p.i. (p<0.0001). Strong reduction of apoptotic cells was observed at day 3 to day 7 p.i. in VSV-WT (p<0.0001). The difference compared to the control group (PBS injected) was statistically significant both for VSV-WT (p<0.0001) and VSV-GP (p=0.0114). (*)p<0.0001.

We extended these findings by examining cell death using the TUNEL assay. The TUNEL assay aims at detecting fragmented DNA as indicator of apoptotic events. Also with this method, we found more apoptotic cells in the VSV-WT compared to the VSV-GP samples both after 3 days and 7 days p.i (Figure 5.9). No positive cells were detected in the control group (Figure 5.9 A). Normal brain tissue, which was treated with DNase but without transferase used as negative control (Figure 5.9 B).

Figure 5.10, displays the quantification of apoptotic cells in the TUNEL assay. 4.8 ± 2.7 apoptotic cells were counted for VSV-WT at day 3 p.i., while VSV-GP infected brains showed significantly less apoptotic cells (1.6 ± 2.1; p<0.0001; Figure 5.10). At day 7 p.i. a strong reduction of the number of apoptotic cells was observed for VSV-WT (1.9 ± 1.2) compared to day 3 p.i. Less apoptotic cells were seen in VSV-GP infected brains at day 7 compared to day 3 p.i. A significant decrease in the number of apoptotic cells was observed from 3 days p.i. infection to 7 days p.i. in VSV-WT (p<0.0001). The numbers were significantly different for both VSV-WT (p<0.0001) and VSV-GP compared to the control group (p=0.0281; Figure 5.10).

Figure 5.9. TUNEL assay on paraffin sections of mouse brains (A-F). Shown are VSV-WT 3 days p.i. (E), VSV-GP 3 days p.i (C), VSV-WT 7 days p.i. (F), VSV-GP 7days p.i. (D), control (A) and negative control (B, treated with DNase but without transferase). Higher cell numbers in the VSV-WT compared to the VSV-GP samples both after 3 days and 7 days p.i. No positive cells were detected in control group. (Magnification: ×20). [Scale bars: 100 μm].



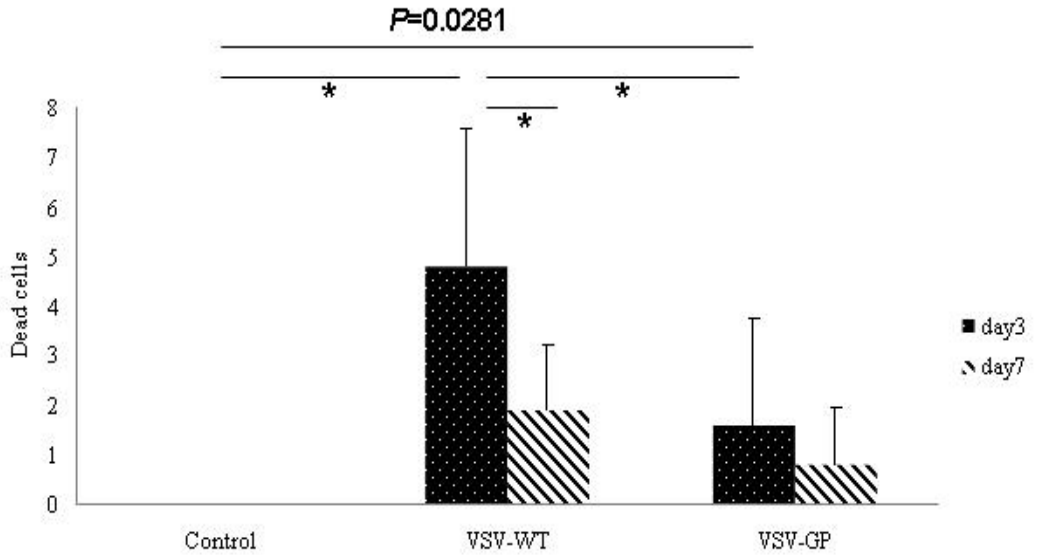


Figure 5.10. The number of dead cells was determined by TUNEL assay, counting 10 different microscopic fields, the area around injection site was avoided. Significantly less apoptotic cells cells were counted for VSV-GP compared to VSV-WT at 3 days p.i. (p<0.0001). Significant reduction of dead cells was observed from day 3 to day 7 p.i. in VSV-WT (p<0.0001). Numbers were significantly different for both VSV-WT (p<0.0001) and VSV-GP compared to control group (p=0.0281). (*) p<0.0001.

5.2 Infection of neurons is only detected in VSV-WT injected mice

To analyze the tropism of both vectors for normal brain cells, we used mouse brains infected with VSV-WT and VSV-GP delivering GFP in order to identify infected cells. We received fixed brain tissue from our collaborator, who performed the injection experiments (Alexander Muik, Frankfurt, Germany). The brain tissue was then frozen and cryosections were prepared as described in the Methods. Neurons were stained with anti-NeuN and astrocytes with anti-glial fibrillary acidic protein (anti-GFAP). The GFP signal was enhanced by staining with an anti-GFP antibody and sections were analyzed by confocal laser scanning microscopy.

By analyzing serial sections, we observed that VSV-WT transduced NeuN-positive neurons (Figure 5.11) in several areas, while we did not find any glial fibrillary acidic protein (GFAP)-positive cells infected. This indicates that neurons are the primary targets of VSV-WT. When screening sections from VSV-GP infected brains we could not detect any GFP positive cells even after staining with anti-GFP antibodies.

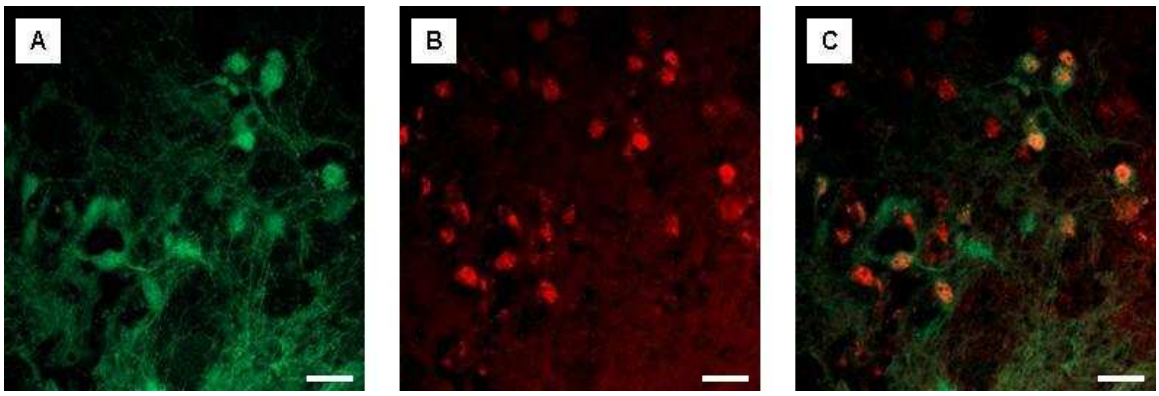


Figure 5.11. A representative microscopic field from VSV-WT infected normal brain cells *in vivo* (A-C). Mouse brains were first fixed in 4%Paraformaldehyde/PBS solution overnight and then incubated in 30% sucrose/PBS solution for three days at 4°C. Coronal slices of mouse brains were prepared. Neurons were stained with anti-NeuN. Anti-GFP antibody was used to enhance the GFP signal indicating virus infection. (A) GFP, (B) NeuN, (C) Overlay. (Magnification: ×40). [Scale bars: 100 μm].

5.3 Experimental brain tumors are efficiently transduced with VSV-GP

As VSV-GP did not infect normal brain cells and did not show neurotoxicity either, this vector is a promising candidate to be used for gene therapy of brain tumors. To assess the ability of replication-competent VSV (VSV-GP) to infect and replicate within brain cancer cells, recombinant viruses were administered intravenously to mice harbouring U87 gliomas. The intravenous approach was used in order to test the potential of VSV-GP to enter the brain and infect tumor cells upon systemic delivery. The tumor cells were transduced with DsRed, while the virus packaged GFP in order to identify infected tumor cells by fluorescence. This experiment was performed by our collaborator (Alexander Muik, Frankfuert, Germany) and we got fixed brain tissue to analyze. 3 days p.i histological slides were prepared for confocal microscopic analysis to determine infection efficacy of VSV-GP. By analyzing the tumor area (DsRed positive), we detected efficient infection of tumor cells by VSV-GP virus (GFP) (Figure 5.12). This demonstrates that the virus can even home to the brain after systemic delivery and specifically infect the tumor cells. Normal brain cells were not transduced as also observed in the previous experiment.

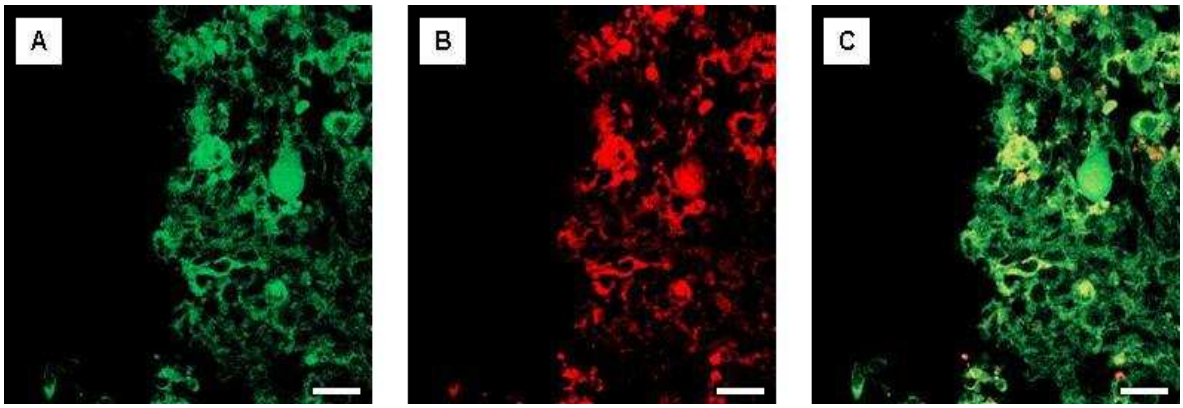


Figure 5.12. Distribution of intravenously administered recombinant vesicular stomatitis virus (VSV-GP) expressing green fluorescent protein (GFP) in U87 tumors xenografted in the brain of immunodeficient mice. (A) GFP, (B) DsRed positive tumor mass, (C) overlay. (Magnification: ×40). [Scale bars: 100 μm].

6 Discussion

In most gene therapy studies, a "normal" gene is inserted into the genome to replace an "abnormal," disease-causing gene. To treat a disease or correct a genetic deficiency, a carrier molecule called a vector must be used to deliver the therapeutic gene to a certain cell type. The goal in using gene therapy for the treatment of cancer is to develop new agents/viruses that have a high killing potency for malignant cells and less pathogenicity to normal cells.

To increase specificity of gene delivery, different strategies have been developed: (1) transcriptional targeting by using Adenovirus and Herpes simplex virus (61, 62); (2) cellular targeting by Adenovirus and Herpes simplex virus (63, 64); (3) Attenuation by deletion of viral genes or gene fragments of Adenovirus, Herpes simplex virus, Vaccinia virus, Poliovirus and Measles virus (65-70). However, the improvement of tumor specificity is often associated with a decrease in efficacy. Therefore, still better methods are warranted that can improve both, efficacy and specificity of killing/gene transfer.

Oncolytic (onco=cancer; lytic=killing) viruses provide a promising tool for gene therapy, since they are innocuous, non-disease causing, replication competent viruses, which can replicate in and kill a large amount of human tumor cells (44). They may keep their tumor selectivity in attenuated forms as well as wild-type forms. Further, tumor-selectivity can be provided by engineering of the viral genes (71).

The most attractive approach to increase tumor-selectivity is to use viruses that possess inherent tumor-selectivity. A good example is vesicular stomatitis virus (VSV), which is highly sensitive to inhibition by interferon. Thus normal cells remain almost intact from infection while tumor cells which have deficiency in the Interferon (IFN) antiviral signaling network are rapidly eradicated. However, VSV is neurotoxic which certainly limits its application for brain tumors or brain-related diseases. The neurotoxicity is related to the VSV glycoprotein, which is critical for virus entry into the cells (51). Thus the pseudotyping of VSV with the glycoprotein of LCMV, which has been shown to spare infection of neurons (54), might be a promising approach to eliminate neurotoxicity. Therefore, in this thesis wild-type VSV vectors and VSV-pseudotyped with glycoprotein of LCMV were compared.

6.1 Immune responses to intracerebral VSV-WT and VSV-GP infections

We have investigated the immune responses following infection of mouse brains with VSV and VSV pseudotyped with glycoproteins of LCMV. We identified CD3+ T cells and CD11b+ microglia as major cell populations causing inflammation in VSV-WT injected mice. Similar results have been obtained in previous studies (72, 73). In addition to T cells, the authors in the study by Christian *et al*. identified NK cells as contributors to the inflammatory response (72). In the study by Bi *et al*. the authors found that production of nitric oxide, an innate immune mechanism, also participated in the immune response (73). Interestingly, in the present study the quantity of immune cells decreased from 3 days to 7 days p.i., although mice normally succumb 7-10 days after infection (Muik et al. unpublished observation). A hypothesis could be that the cytotoxic effect of the virus comes after the peak of inflammation and induces a cytotoxic edema in the brain, which is lethal. This is supported by our observation, that the brain tissue showed edema at 7 days p.i., which was not observed at 3 days p.i.

In contrast, CNS infection with VSV-GP, which has not been studied in detail before, showed only a minor inflammatory response at the injection site and only few CD3+ T cells at distant sites. Thus, this virus did not induce a significant immune response compared to VSV-WT. This is also reflected by a survival study where intracerebral injections of VSV-WT caused death of all mice even at low viral titers (10E2), while all mice injected with VSV-GP even at high titers (10E7) survived (Muik *et al*., unpublished observations).

6.2 Cytopathic effect of VSV-WT vectors for brain cells

In our study, we observed that VSV-WT induced considerable cell death in normal brain cells by using cleaved Caspase-3 and TUNEL immunostainings. In contrast, VSV-GP infection induced apoptosis in only few single cells, which were statistically significant much less compared to VSV-WT infected brains. Caspase-3 is a key molecule in the apoptotic pathway of cells and its cleavage clearly indicates that this pathway is activated (74). The TUNEL assay represents a method, which detects DNA fragmentation as a result of the activation of apoptotic signaling cascades (75). Thus these two methods

detect different steps in the apoptotic pathway and thus taken together clearly indicate that VSV-WT, in contrast to VSV-GP, induces apoptosis in normal brain cells.

The strong cytopathic effect of VSV has been related to the viral glycoprotein and the viral matrix protein. Moreover, it was found that these two substantial proteins trigger apoptosis through distinct pathways. Apoptosis was associated with the formation of syncytia between infected cells (giant polynucleated cells) suggesting that the fusogenic properties of the VSV-G protein was involved (76). This fusogenic protein undergoes a reversible conformational change upon contact with the acidic milieu of the Golgi apparatus in the secretory pathway. Thus, it is its fusion active form when it reaches the plasma membrane and therefore is able to perform cell–cell fusion (77, 78). In all fusogenic viruses the fate of resulting syncytia is apoptosis (79-82).

Other studies observed that the amino acid substitutions M51R, V221F and S226R in the M protein, which inhibit both cellular transcription and nucleocytoplasmic mRNA transport, probably are responsible for cytotoxic properties of VSV (83-89). However, the results in this thesis clearly show that exchange of the VSV glycoprotein by a glycoprotein of a different virus can eliminate neurotoxicity, which indicates that the observed cytotoxic properties of the Matrix protein (M) are strongly dependent on a functional VSV glycoprotein and most likely on its fusion activity (76).

6.3 Neurons are a target of VSV-WT, but not VSV-GP vectors

Infection of mouse brains with VSV-WT and VSV-GP viruses delivering GFP enabled us to analyze which cell types are infected by the different virus strains. While VSV-WT showed infection of neurons, no infection of brain cells with VSV-GP could be detected.

In previous studies using the glycoprotein of VSV-G pseudotyped with lentiviral vectors, Miletic *et al*. also showed infection of neurons, while lentiviral vectors pseudotyped with LCMV-GP did not infect neuronal cells, but few glial cells (58). This was confirmed by another recent study using replication deficient VSV (54).

The tropism of viruses has been attributed to the cellular receptors. VSV shows a very broad cell tropism and replicates rapidly in various cell lines that are derived from

different species and tissues. The presence of phosphotidylserin (a universal component of cell-surface membrane) correlates with the extensive tissue tropism of VSV (82).
The glycoprotein of LCMV binds with high affinity to alpha-dystroglycan, a receptor on the cell membrane. Alpha-dystroglycan is a dystrophin-associated glycoprotein, which is widely expressed in most tissues (54, 90). Thus, it is somewhat surprising that LCMV is sparing neurons as alpha-dystroglycan is also expressed on neuronal and glial cells (54). One hypothesis could be that LCMV needs a co-receptor to infect cells which is not present on neurons. In this regards, some studies have shown that certain LCMV variants inefficiently interact with alpha-dystroglycan and also infect alpha-dystroglycan-negative cells (91). Further it has been observed that LCMV upon injection into the brain primarily infects glial cells and at later time points when the infection becomes persistent spreads to neurons (92). The infection of neurons might then be dependent on certain properties of the LCMV which are not present when only the glycoprotein is used for pseudotyping with a different virus.

Although we did not observe infection of normal brain cells by VSV-GP, we might have missed infection of a few glial cells as this has been reported before (54). Also the identification of a few apoptotic cells by cleaved Caspase-3 and TUNEL staining which were significantly more compared to PBS injected controls indicates that a few brain cells were infected and killed by the virus. However, these cells seem not to be neuronal cells and the effect is not toxic enough to induce symptoms or even lethality.

6.4 Efficiently transduction of brain tumors by VSV-GP

In the majority of clinical trials performed so far, oncolytic viruses have been administered via intratumoral injection. A smaller number of studies have examined regional or intravenous delivery. An ideal oncolytic virus for cancer should show effective spread to multiple sites within the tumor upon systemic delivery or it should be modifiable to be efficient in tumor targeting. This has been shown in the present thesis. Following intravenous administration of the recombinant virus VSV-GP, efficient and specific infection of brain cancer cells was observed.

In a study that was conducted in 2006, the effectiveness and toxicity of VSV Δ M51, which contains a single – amino acid deletion in the M protein was evaluated *in vitro* and *in vivo*. VSV Δ M51 infected and killed 14 different glioma cell lines and when administered intravenously to nude mice bearing human gliomas, significant regression of tumors was observed. Interestingly no evidence of toxicity was found, probably due to modification of the M protein (93).

In another study the efficacy of two oncolytic viruses, myxoma virus (MV) and an attenuated vesicular stomatitis virus VSV Δ M51 were compared *in vivo* with using the same dose and intratumoral administration for both viruses. Remarkable shrinkages were observed for both viruses when they injected directly into the tumor mass. Looking at details confirmed VSV Δ M51 was somewhat more effective than MV (94). However, the efficacy of these viruses might not be sufficient for the human situation, as the mutated M protein significantly reduces the replication and lytic potential of the virus. In contrast, VSV-GP viruses used in the presented thesis still have the full replication and lytic abilities.

A drawback of systemically delivered virus for glioblastoma therapy is pointed out by Xueqing Lun *et al.* First, the spread of the virus within the tumor may be limited to due the immune system's response. Secondly, although the leaky blood-brain barrier of the main tumor mass allows the virus to enter the tumor, invasive glioma cells might escape therapy as they remain in areas where the blood-brain-barrier is still intact (93). Thus, the future animal experiments with clinically more relevant glioma models and clinical studies in patients might show which route of delivery will be most efficient in killing glioblastoma cells with oncolytic vectors.

6.5 Conclusion

In summary, replication competent LCMV-GP pseudotyped VSV vectors mediate specific and efficient infection of glioblastoma *in vivo* upon systemic delivery without signs of neurotoxicity. In contrast, VSV-WT vectors are neurotoxic and induce a T-cell mediated immune response with infiltration of CD3 positive T cells and activation of CD11b positive microglia. Analysis of tropism for normal brain cells explains the difference of these two vectors regarding neurotoxicity as VSV-WT vectors infect neurons, while VSV-GP vectors do not infect normal brain cells. Thus VSV-GP vectors are promising candidates for future gene therapy applications of brain tumors due to their ability to specifically infect and replicate in brain tumor cells.

7 Future perspectives

This work shows that VSV-GP oncolytic vectors efficiently and specifically infect glioma cells in vivo without causing significant neurotoxicity. However, future studies in clinically relevant animals are warranted in order to evaluate the efficacy of this vector in angiogenic as well as invasive tumor areas. These studies should also clarify whether a systemic or local delivery will be more effective. The results of these future experimental studies will be an important basis for a potential clinical application of VSV-GP oncolytic vectors.

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