Philipps-Universität Marburg

Analysis of a capsid-modified and conditionally replicating, oncolytic adenoviral vector as a novel treatment for human glioblastoma multiforme.

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Martin E. Wohlfahrt
geboren am 22. Dezember 1975 in Bielefeld, Deutschland

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"Es ist nicht genug, zu wissen, man muss auch anwenden.
Es ist nicht genug, zu wollen, man muss auch tun."

("It is not enough to know, one must also apply. It is not enough to will to do, one must also do.")

Johann Wolfgang von Goethe (*Wilhelm Meisters Wanderjahre)*

Gewidmet meinen Eltern -
für Geduld und Vertrauen.

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Erstgutachter: Prof. Dr. Klaus Lingelbach

Zweitgutachter: Prof. Dr. Wolfgang Garten

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ABBREVIATIONS

Ad5	Adenovirus serotype 5
Ad35	Adenovirus serotype 5
Ad5/35	Chimeric adenovirus (Ad5 capsid + Ad35 fiber)
AF	Alexa Fluor
AGT	O6-alkylguanine-DNA alkyltransferase
ATCC	American Type Culture Collection
BCNU	Carmustine
bp	Base-pairs
CAR	Coxsackie- and adenovirus receptor
CDKN2A	cyclin-dependent kinase inhibitor 2A
DISC	Death-inducing signaling complex
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
EMT	Epithelial-mesenchymal transition
FADD	Fas-Associated protein with Death Domain
FBS	Fetal bovine serum
FLIP	FLICE-inhibitory protein
Fig.	Figure
FITC	Fluorescein isothiocyanate
GBM	Glioblastoma multiforme
GPI	Gycosyl-phosphatidylinositol
GFP	Green fluorescent protein
GSC	Glioma-derived stem-like cells
IAP	Inhibitor-of-apoptosis proteins
i.t.	Intratumorally
i.v.	Intravenously
IFN	Interferon
IR	Inverted repeat
IRES	Internal ribosomal entry sequence
LoVo	Human colon cancer cell line
µg	Microgram
mg	Milligram
µl	Microliter
ml	Milliliter
µM	Micromolar
mM	Millimolar
µm	Micrometer

MMP	Matrix metalloprotease
MOI	Multiplicity of infection
MITC	3-methyl-(triazen-1yl)imidazole-4-carboxamide
NOD	Non-obese diabetic
PBS	Phosphate-buffered saline
pfu	Plaque forming units
PI3K	Phosphatidylinositol 3-kinase
RNA	Ribonucleic acid
RSV	Rous sarcoma virus
s.c.	Subcutaneously
SCID	Severe combined immunodeficiency
SF767	Human glioblastoma cell line
SV 40	simian virus 40
T98G	Human glioblastoma cell line
TMZ	Temozolomide
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
U-87 MG	Human glioblastoma cell line
WHO	World Health Organization

1. INTRODUCTION

1.1. Glioblastoma multiforme

Glioblastoma multiforme is the most aggressive form of human brain tumors due to its growth, invasiveness and neurological destructivity.

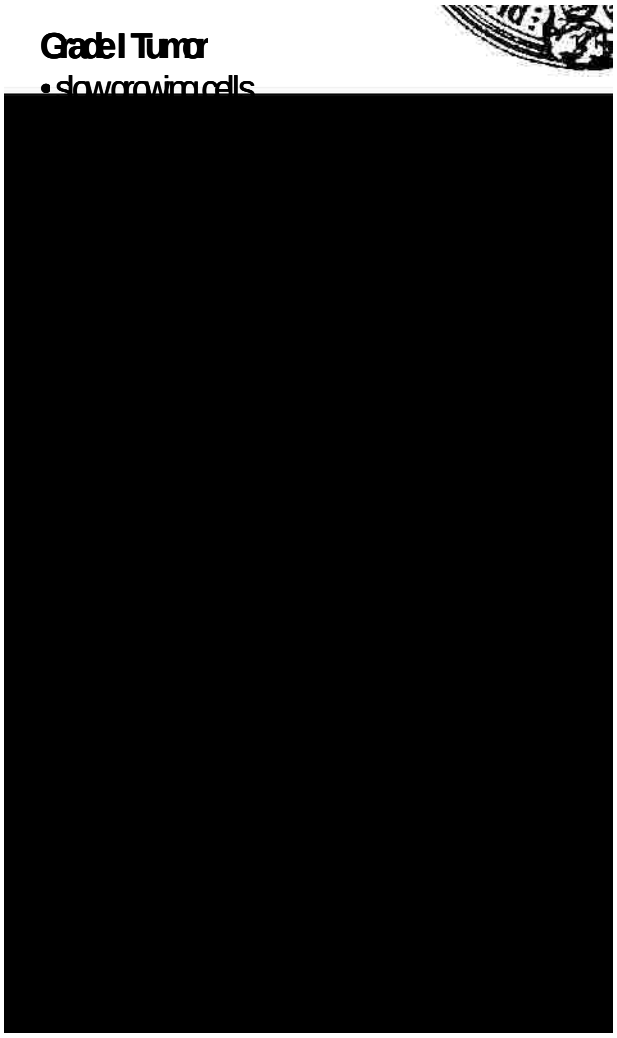


Table 1: WHO brain tumor grading system Human glioblastoma is considered a grade IV tumor because of its rapid growth, high vascularization and formation of necrotic tissue surrounded by highly invasive abnormal cells.

The standard WHO-2007 name for this brain tumor is “Glioblastoma” (GBM); in the tumor grading system GBM is considered to be a grade IV tumor [Table 1].

GBM is the most common and most aggressive type of primary brain tumor, accounting for 52% of all primary brain tumor cases and 20% of all intracranial tumors. The average life expectancy of a patient diagnosed with this form of brain tumor is on average not more than one year (1).

1.1.1 Symptoms

Symptoms of GBM resemble general common symptoms for brain tumors like headache, seizure, nausea and vomiting and hemiparesis but due to the location

of most GBM tumor masses in temporal and frontal lobe, a progressive memory-, personality-, or neurological deficit is typical. The kind of symptoms produced depends highly on the location of the tumor but not necessarily on its pathological properties. The severity of symptoms depends mostly on the location and size of the tumor. It is not uncommon that tumors remain asymptomatic until they reach a certain size.

1.1.2. Diagnosis

A tumor can be visualized using Magnetic Resonance Imaging (MRI). When viewed with MRI, glioblastomas often appear as ring-enhancing lesions [Figure1]. Definitive diagnosis of a suspected GBM on CT or MRI requires resection of tumor tissue via stereotactic biopsy or a craniotomy. Because the tumor grade is based upon the most malignant portion of the tumor, biopsy or subtotal tumor resection can result in undergrading of the lesion.

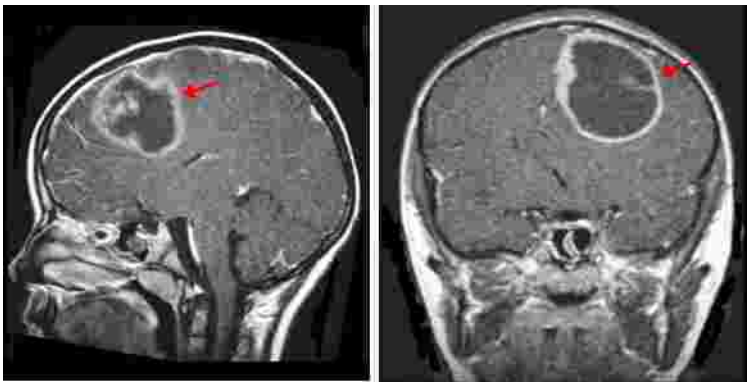


Figure 1: Sagittal and coronal contrast-enhanced MRIs of the brain showing the glioblastoma multiforme mass. Images by Neuroradiology of the Washington University in St. Louis School of Medicine.

1.1.3. Causes

Events or substances that lead to direct GBM development are not yet identified. Most glioblastoma tumors appear to be sporadic and without any genetic predisposition. No links have been found between glioblastoma and smoking or diet (2, 3). A link between exposure to electromagnetic fields or the use of cellular phones and GBM development

has been suggested but recent studies show no evidence for an existing correlation (4, 5).

Recently, first evidence for a viral association has been discovered. Simian virus 40 (SV40) and cytomegalovirus have been found in many tumors of patients leading to the conclusion that viral infected tissue causes GBM development (6, 7). There also appears to be a link between ionizing radiation and glioblastoma (8). GBM is more common in males, although the reason for this is not clear (9).

Other risk factors for GBM include having a lower-grade astrocytoma (brain tumor), which occasionally develops into a higher-grade tumor. Occurrence of GBM appears to be age related as it increases at ages over 50 years. Furthermore having one of the following genetic disorders is associated with an increased incidence of gliomas: Neurofibromatosis, Tuberous sclerosis, Von Hippel-Lindau disease, Li-Fraumeni syndrome and Turcot syndrome.

1.1.4 Pathogenesis

Glioblastoma multiforme is characterized by the presence of small areas of necrotizing tissue surrounded by anaplastic cells (pseudopalisading necrosis) [Figure 2].

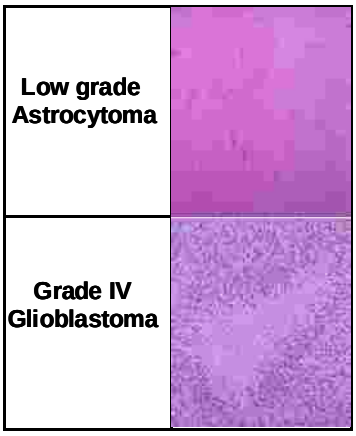


Figure 2: fixed tissue section of human brain tumor sections stained with hematoxylin & eosin (*H&E)*

Glioblastoma differs from Grade 3 astrocytomas by developing a dense network of hyperblastic blood vessels. While oligodendrogliomas form from gray matter, GBM has the ability to rise from gray or white matter of the brain. However the majority of GBM arises from the deep white matter and quickly infiltrates the brain due to a highly invasive tumor front. The tumor may extend to the meningeal or ventricular wall, leading to a detectable high protein content of cerebrospinal fluid (CSF) (> 100 mg/dL), as well as an occasional pleocytosis of 10 to 100 cells, mostly lymphocytes.

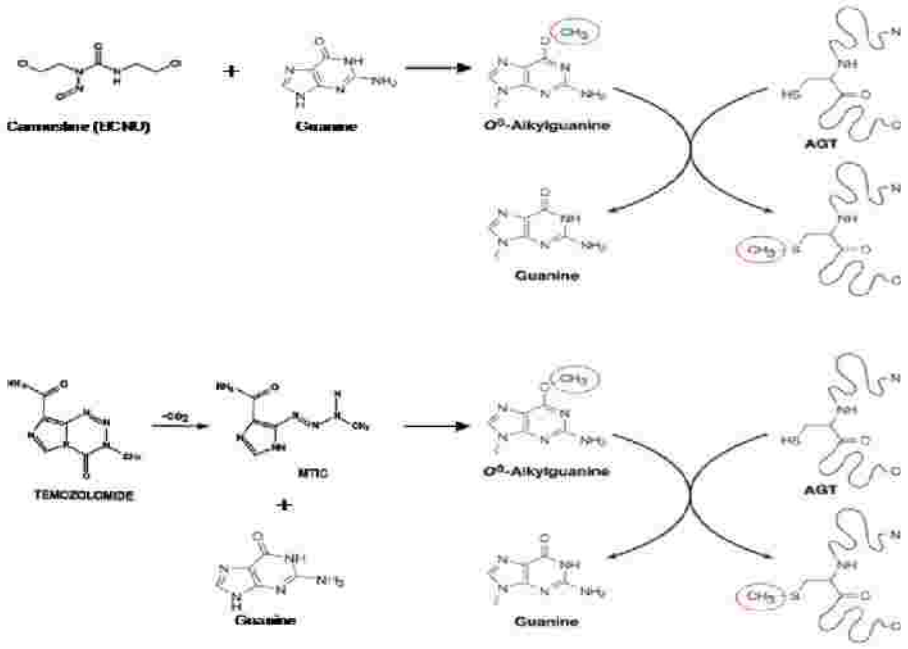
Malignant cells can spread to the spinal cord and cause menigeal gliomatosis. However, even though the tumor invasion front is very aggressive actual metastaziation is extremely rare. About 50% of GBM occupy more than one lobe of a hemisphere or are bilateral. The tumor may take on a variety of appearances, depending on the amount of hemorrhage, necrosis, or its age. An MRI or CT scan will usually show a nonhomogeneous mass with a hypodense center and a variable ring of enhancement surrounded by edema.

1.1.5 Treatment

Although clinical research has been very active to find better treatment methods for globlastoma multiforme for the last years, the prognosis of glioblastoma remains extremely poor. Several complicating factors cause GBM to be a difficult tumor to treat. Foremost, the brain is a sensitive and complex organ susceptible to damage caused by surgery and chemotherapy. GBM tumor cells on the other hand show a very high resistance to chemotherapy and other conventional therapies. The blood brain barrier limits treatment possibilities since many drugs are unable to pass it in order to act directly on the tumor (10).

The common therapy for patients with glioblastoma multiforme is surgery, radiation therapy and the treatment with chemotherapeutic agents that induce DNA damages, derogate the DNA-mismatch-repair-system and therefore lead to abortive repair and cell death. The most frequently used chemotherapeutics are Carmustin (BCNU) and Temozolomide (TMZ). Alkylating agents are most active in the resting phase of the cell therfore these drugs are cell cycle non-specific. Their cytotoxic effect has mainly been attributed to alkylation of the O^6-position of guanine. Chloroethylation at the O^6-position of guanine produces an N1-deoxyguanosinyl-N3-deoxycytidyl crosslink. Multiple interstrand crosslinks between guanine and cytosine eventually lead to single- and double-stranded DNA breaks which in turn are processed by the DNA mismatch repair system (MMR). The MMR causes induction of p53 and p21 followed by apoptotic and necrotic cell death (11-13) [Figure 3].

A.



B.

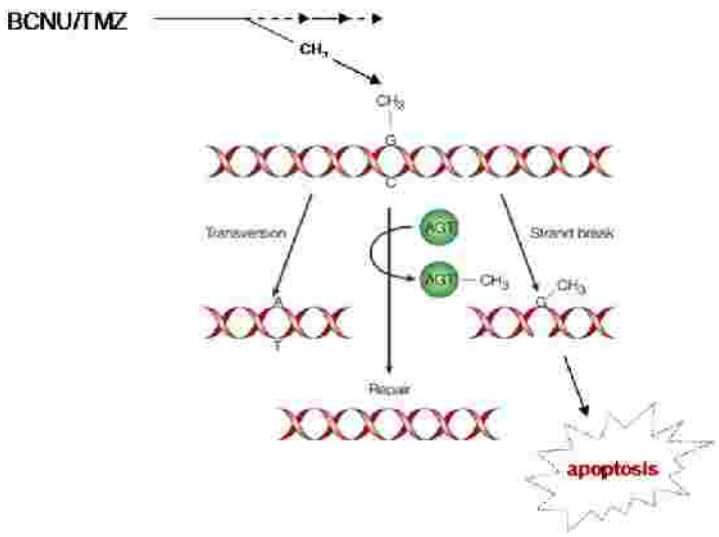


Figure 3: (A) Alkylating agents like Carmustine (BCNU) or 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MITC), the product of the prodrug temozolomide (TMZ), have the ability to attach an alkyl group to the O^6 -position of guanine resulting in O^6-alkylgluanine. The induced alkylation can be reversed by the MGMT encoded repair enzyme O^6-*alkylguanine*-DNA alkyltransferase (*AGT*) as shown.

(B) Chemotherapeutic treatment with BCNU and TMZ. The chemotherapeutic alkylating agents attack the cellular DNA of the cancer cell by guanine alkylation. The resulting formation of O6-alkylguanine (if not repaired by the cell) leads eventually to intrastrand cross-links, *1(N3-cytosine)-2(N1-guanine),* and strand breaks which triggers the initiation of cellular apoptosis.

Meta-Studies have shown that the average life expectance of up to 13% of patients diagnosed with GBM can be extended to an average of 2 years if the patients are treated with chemotherapeutic agents (14). However the appearance of recurrent GBM tumors in patients occurs often and recurrent GBM tumors tend to be less sensitive to chemotherapeutics like Wong et al. showed in another study were they point out that only a total of 6% recurrent GBM tumors responded to chemotherapy (15). Multiple attributes of human glioblastoma multiforme lead to this observed aggressive and invasive growth its high resistance against the common methods of treatment and the occurrence of recurrent, highly resistant forms after first treatment (16,17).

Drug resistance may arise through several distinct DNA repair mechanisms that can restore the integrity of BCNU and TMZ-induced alkylated DNA bases. Treatment of glioblastoma multiforme via surgery is critical but mostly only provides limited benefit due to the erratic, aggressive tumor migration with a diffuse progression pattern of the tumor invasion front that allows only a fractional removal of the tumor. In more than 90% of cases occurs a rise to a recurrent tumor by a residual pool of invasive cells that develops immediately adjacent to the resection margin again (18). In addition there seems to be a multitude of resistance mechanisms that can be developed during gliomagenesis and the GBM tissue itself seems to become highly heterologous.

Wilson et al. demonstrated that high levels of a DNA repair enzyme O-6-methylguanine-DNA methyltransferase (AGT/MGMT) in GBM cells prevent them from critical DNA damage induced by chemotherapy (19-21). It was shown in a study by Jaeckle et al. that GBM-patients with high MGMT- levels and –activity had an average life expectance of 8 months while the average life expectance of GBM-patients with low MGMT-activity was almost 4 times higher (22). Kokkinakis et al. demonstrated the loss of the p53 inducible cell cycle control point as another common mechanism of resistance can be found in glioblastoma (23). Further deletions of genes like cyclin-dependent kinase inhibitor 2A (CDKN2A) or the loss of the allele 10q leads to a weakened impact of chemotherapeutic agents on those cells (24-28).

1.1.6 Stem-like cells in human Glioblastoma as factor of tumor-resistance and – recurrence

Recent findings identified the existence of a stem cell-derived origin for gliomas (29). In particular, glioma-derived stem-like cells (GSCs) have been isolated from both human brain tumors (30) and several glioma cell lines (31). GSCs are crucial for the malignancy of gliomas and may represent the consequence of transformation of the normal neural stem cell compartment (31, 32).

Treatment of GBM with standard methods appears to lead to selection for GSCs as those stem-like cells show strong radiation- and chemotherapy resistance. It has been shown that GSCs can be detected and isolated by using the marker CD133. Liu et al. demonstrated that CD133 postive cells show higher mRNA levels of genes that inhibit apoptosis and are therefore significantly resistant to chemotherapeutic agents like TMZ or BCNU (*33)*.

Furthermore only small fractions of GSCs are necessary to regenerate the tumor. Recurrence of GBM tumors is therefore most likely triggered by resistant GSCs after surgery and subsequent therapy as it has been shown that CD133 expression is significantly higher in recurrent GBM tissue compared to newly diagnosed tumors (*34)*.
These alterations in the genome during gliomagenesis together with cellular chemotherapy resistance mechanisms lead to a dismal prognosis and significantly shortened average life expectancy in GBM patients. Therefore research for novel treatment strategies as a supportive procedure in addition to surgery, radiation therapy and chemotherapy that extends the average life expectance of patients diagnosed with GBM is necessary.

1.2. Oncolytic viral Therapy

1.2.1 History of oncolytic viral gene therapy

Until the early twentieth century, cancer therapy referred to excision of the tumor by surgery. Radiotherapy, chemotherapy, and immunotherapy were shortly to be introduced as new treatment strategies. Viruses began to be employed for cancer therapy at the end of the nineteenth century. However during this time there was no real concept of the nature of a virus yet. Although Beijerinck et al. reported in 1898 that after Chamberland candle filtration (through which bacteria could not pass), the agent causing tobacco mosaic disease could amplify itself in living, growing plant tissue (35).

In the same year the foot and mouth disease virus, was reported to be the first "filterable agent" to be implicated in an animal (36) followed by human yellow fever as the first human filterable agent disease in 1901 (37). However the precise viral identity was still unclear until electron microscope imaging of viral particles was possible (38) and the advent of cell and tissue culture systems allowed *ex vivo* virus propagation in the late 1940s, which lead to a better understanding of viral principles (39,40). *Ex vivo* culture of human cells had become possible in 1948, and attempts to implant these cells into laboratory rodents followed, providing the first opportunity to test the *in vivo* antitumor activity of an oncolytic virus under controlled laboratory conditions.

Moore et al. were the first to investigate oncolytic viruses with a newly developed rodent sarcoma cancer model in 1949. Using an *in vivo* tumor model with the "Russian Far East encephalitis virus" they where able to selectively seek out and destroy cancer cells in a living animal. In this virotherapy key experiment Moore found that, in certain instances, the mouse sarcoma 180 could be completely destroyed, giving first proof of principle for the oncolytic potency of an oncolytic virus in a mouse model. However, the virus did also show not to be safe for patient admission eventually causing fatal encephalitis in all animals (41).

In the years that followed, many other human pathogens were investigated for oncolytic activity employing rodent models, including Bunyamwera, dengue, yellow fever, West Nile virus, Semliki Forest virus, mumps, vaccinia- and adenovirus (42- 45). Many of these were also evaluated in clinical trials showing that complete tumor regression was much more likely to occur in the mouse than in the patient. However proof of activity in rodent models quickly became a standard experiment to establish proof of principle for oncolytic activity of viruses before clinical testing. In early clinical trials Hepatitis viruses were among the first to be used for therapy. As early as 1897 it had been noticed that viral hepatitis had ameliorating effects on a variety of human diseases. Then in 1949, when two patients with Hodgkin's disease were observed to go into brief remission after contracting viral hepatitis, clinical trials were undertaken (46).

Table 2: Overview of first significant trials in clinical virotherapy
Modified from Kelly et al "History of Oncolytic viruses:Genesis to Genetic Engineering"

In the early years the Egypt 101 isolate of West Nile virus was used in more than 150 virus therapy trials against a wide range of cancers [Table 2].

In most trials intra-tumoral virus replication was confirmed in most patients, but tumor responses were rare. Immunosuppressed patients with leukemia or lymphoma were more likely to respond to therapy, but were also at higher risk of fatal neurotoxicity. Due to the lack of efficacy and safety most of these early trials were abandoned while herpes viruses, paramyxoviruses, picornaviruses, and adenoviruses emerged as potential candidates for gene therapy. Identified as an oncolytic agent in preclinical models in the 1950s, adenovirus was found to have relatively modest side effects if administered,

leading to inflammation but no encephalitis or lethal side effects thus becoming a candidate for clinical trials for the treatment of cervical cancer quickly (44). If administered into the patient areas of necrosis were present in tumors within 10 days and in those who responded to administration, cancerous tissue was shed in large amounts. Unfortunately infections were quickly eradicated by the host immune system and survival was not significantly prolonged.

It became apparent that even though viruses appeared to have tremendous potential adaptation and, ultimately, genetic engineering of viruses was necessary to have a significant impact on cancer patients. This became a possibility in the early 1990s when recombinant DNA technology became a widely used method. At present there is a focus on engineering paramyxo-,herpes-, picorna-, pox- and adenoviruses, and most of the oncolytic viruses currently in clinical testing are attenuated derivatives of prevalent human pathogens genetically engineered to further attenuate their pathogenicity, increase their oncolytic potency, or enhance their specificity for cancer tissue (47-53). The aim is to create a specifically tailored virus to infect cancer cells while leaving normal cells unharmed. The engineering of such viruses involves ensuring that the viruses can only replicate inside cancer cells, lyse s when they exit and ensuring a higher dosage at the site of the tumors. This goal can be reached by transductional targeting, which means altering the viral surface to ensure that virus binds predominantly to cancer cells but not healthy tissue. Another approach is using specific virus activating promoters that are known to be highly active in cancer cells like Cox-2 or hTERT to ensure transcriptional targeting. However, even with those newfound abilities and methods to engineer viral genomes to produce a new generation of safer, specific oncolytics, a true therapeutic frontrunner has yet to emerge.

1.2.2 Adenoviral vectors in oncolytic virotherapy

Adenoviral vectors (Ad) are currently the most commonly used viral vector system in the field of gene therapy and have successfully been used to transduce a wide variety of cell types (54). This is due to several advantages adenoviral vectors have over other viral systems. The viral genome is easy to manipulate, they have a large insertion capacity of up to 7.5 kb in a non-replicating Ad-System and can be concentrated to titers up to 10^{10} - 10^{13} plaque forming units (pfu)/ml. In addition adenoviruses are attractive vectors

because they do not integrate into the host chromosome and have a broad tropism, infect both dividing and non-dividing cells and have a high stability in vivo. Another beneficial attribute contributing to their employment in antitumor therapy is that adenoviruses possess a lytic life cycle that can be exploited for oncolysis. They are also based on pathogenic human viruses and are immunogenic when used *in vivo*.

1.2.3 Adenovirus structure and life cycle

Adenoviruses were first discovered in 1953 as agents which spontaneously caused degeneration of primary cell cultures from human adenoid tissue (55). Since then more than 47 human serotypes of the adenoviridae family have been identified and adenoviruses have been shown to be responsible for a variety of illnesses including upper respiratory disease, epidemic conjunctivitis and infantile gastroenteritis (56).

Most studies into the structure of adenoviruses have been done with human serotypes 2 and 5, and have revealed that adenoviruses are icosahedral particles (20 triangular surfaces and 12 vertices) 70-100nm in diameter [Figure 4].

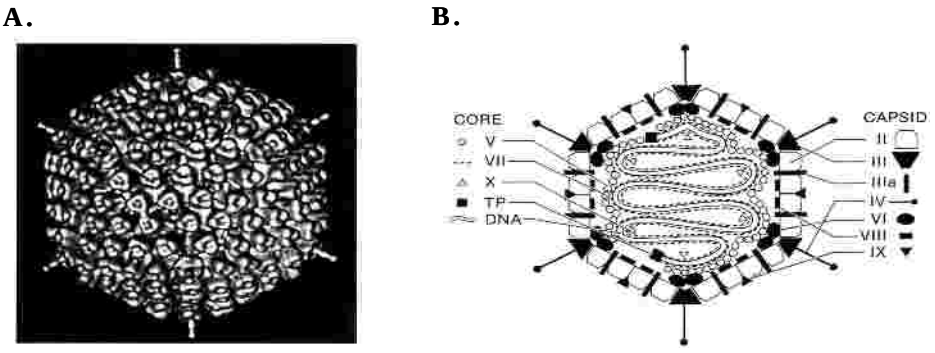


Figure 4: (**A**) Three dimensional model of an adenovirus virion viewed along an icosahedral three-fold axis. (**B**) Representative section of an adenovirus virion illustrating the current understanding of polypeptide component and DNA interactions. This figure was modified from Shenk.T (1996) Adenoviridae: The viruses and their replication. *Fundamental Virology*, 979-1016. Eds: Fields.B.N, Knipe.D.M & Howley.P.M. Lippincott-Raven Publishers, Philadelphia

The virion has a protein shell (capsid) made up of 252 capsomere subunits composed of 240 hexons and 12 pentons. Each hexon is surrounded by 6 neighboring subunits whilst each penton is surrounded by 5 neighboring subunits and has a fiber projecting from its vertex. Within the capsid are 4 polypeptides alongside a single copy of the double

stranded DNA genome covalently attached at its 5' end to the terminal protein polypeptide [Figure 5].

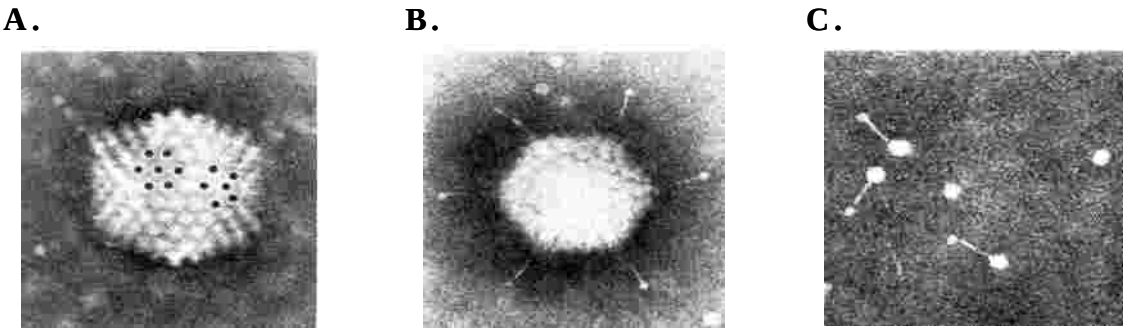


Figure 5: Electron microscopy images of adenovirus type 5. **(A)** A hexon surrounded by six hexons and a penton surrounded by five hexons are marked by dots. **(B)** Six fibers of twelve are visible projecting from penton capsomeres. **(C)** Free penton capsomeres containing penton base and fiber are visible. Magnification is X 285 000. This figure was modified from Shenk.T (1996) Adenoviridae: The viruses and their replication. Fundamental Virology, 979-1016. Eds: Fields.B.N, Knipe.D.M & Howley.P.M. Lippincott-Raven Publishers, Philadelphia

The adenoviral genome [Figure 6] is typically around 36 000 bp in length and has inverted terminal repeat (ITR) sequences of around 100-140 bp at each end which play a role in DNA replication as they contain viral origins of replication. A cis-acting packaging sequence is present within several hundred base pairs of the left hand ITR and directs interaction of the genome with encapsulating proteins. The genome contains 5 early transcription units (E1A, E1B, E2, E3 and E4), two delayed early units (IX and IVa2) and one major late unit that is processed to generate 5 families of late RNAs (L1-L5). It has been demonstrated, with the exception of E4 (57), that each early and late transcription unit encodes a series of polypeptides with related functions. Two E1A proteins are known to activate transcription and induce the cell to enter the S phase of the cell cycle (50). Two E1B proteins are known to interact with E1A gene products to induce cell growth (50). Three E2 proteins are known to function in DNA replication (56).

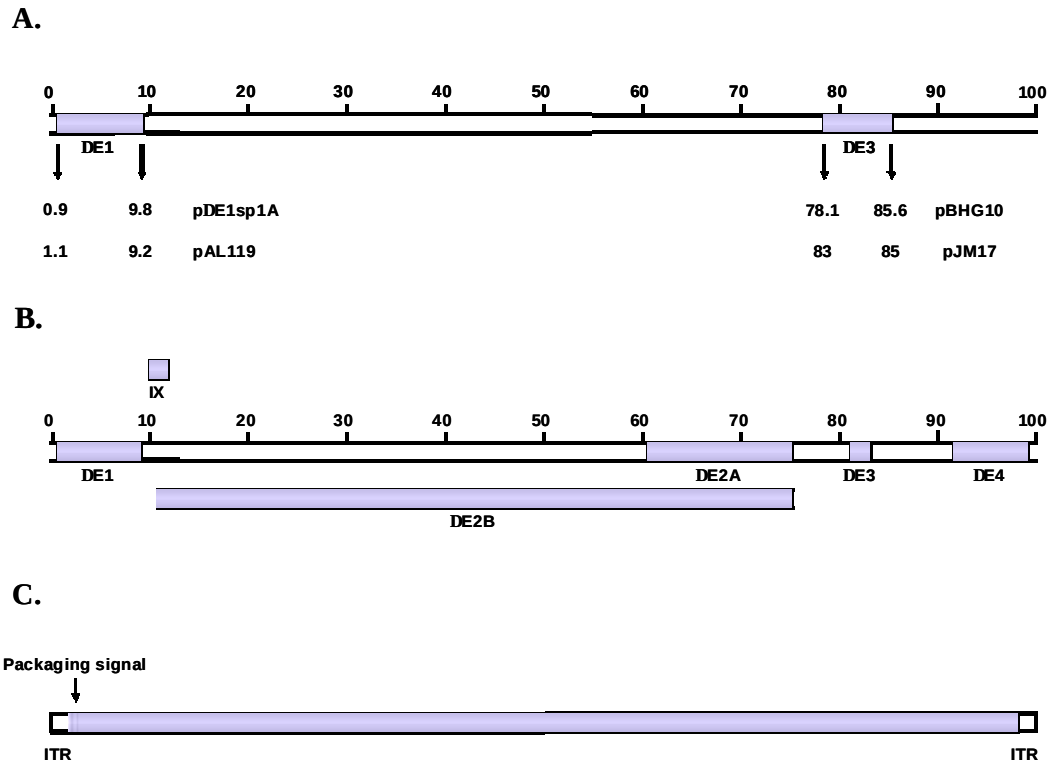


Figure 6: Adenoviral Genome. Schematic representation of the genomes of first generation, second generation and gutless adenoviruses (respective map units are indicated). (**A**) First generation RAd genomes are deleted in E1 and E3 although the extent of each deletion may vary. Expression cassettes are generally inserted into the E1 region but may alternatively be inserted in E3. The extent of E1 and E3 deletions (relative map units) present within classically used plasmids is indicated. (**B**) Second generation RAd genomes have additional viral coding regions deleted and these may include E2A (DNA polymerase), E2B (precursor terminal protein or DNA binding protein), E3, E4 or IX in various combinations. Expression cassettes are generally inserted into the E1 region and the E3 region may be re-introduced. (**C**) Gutless adenoviral genomes retain only the ITR and packaging sequences from wild type adenovirus. The remainder of the genome is comprised of expression cassette(s) and stuffer DNA of varied origin.

E3 proteins play a role in modulation of the anti-viral host response to adenoviruses (58). Late proteins are either capsid components, or proteins involved in capsid assembly (50). The adenovirus life cycle [Figure 7] begins when the adenovirus fiber knob binds to a high affinity cell surface receptor (59, 60) called the coxsackie and adenovirus receptor (CAR). For nearly all serotypes either CAR or CD46 (61) serve as the primary HAdV receptors on most cell types. The adenovirus then undergoes receptor-mediated endocytosis and this is mediated by interactions between an RGD motif within the penton base and cell surface $a_v ß_3$ and $a_v ß_5$ integrins (62,63). Once internalised, a drop in

pH within the endosome results in a conformational change in capsid structure followed by interaction of internal Ad proteins with the endosomal membrane, endosome disruption and release into the cytoplasm (64-65) whereupon it becomes localized to the nucleus through a process that involves microtubules and dynein (66). To enable this a stepwise disassembly of adenovirus particles is necessary which involves fiber release, penton base dissociation, DNA capsid scaffold protein degradation or shed, and elimination of the capsid stabilizing minor protein (67). When the capsid reaches the nuclear membrane the genome enters the nucleus, associates with the nuclear matrix through interaction with the terminal protein (68) and the process of early gene transcription begins.

The process of early gene transcription begins with the production of the viral E1A transactivator from a constitutive E1 promoter and has 3 main consequences. The first consequence is entry of the cell into the S phase of the cell cycle, which is when DNA replicates, and this is achieved through a number of ways including inhibition of the retinoblastoma tumor suppressor (pRb) by E1A, inhibition of the p53 tumor suppressor by E1B-55K and direct inhibition of apoptosis by the Bcl-2 homologue E1B-19K. The second consequence is the inhibition of host anti-viral responses and this is done by inhibition of a and ß interferon responses by E1A proteins and VA RNAs, retention of MHC I molecules in the endoplasmic reticulum by E3-gp19K, inhibition of tumor necrosis factor alpha (TNF-a

down regulation of Fas cell surface expression by the E3 14.5K/10.4K complex, which inhibits Fas mediated apoptosis of virus infected cells, and inhibition of FLICE (caspase 8) which plays a role in TNF and Fas mediated apoptosis (69). The third consequence is the synthesis of gene products needed for viral DNA replication.

Once the early gene products are synthesized the processes required for virus production are able to begin. DNA replication occurs within the nucleus and after transcription of the delayed early IX and IVa2 transcripts the major late promoter becomes activated by the IVa2 gene product and promotes production of late RNA species. The late RNA species are translated to produce capsid proteins within the cytoplasm but capsid assembly does not occur until these proteins are translocated to the nucleus. Virus assembly and genome packaging then occurs in the nucleus and adenovirus cannot be released from the cell until it is lyzed. This cell lysis requires

disruption of intermediate filaments (which are components of the cytoskeleton) such as vimentin and cytokeratin K18, and results in the collapse of the cell and lysis (70).

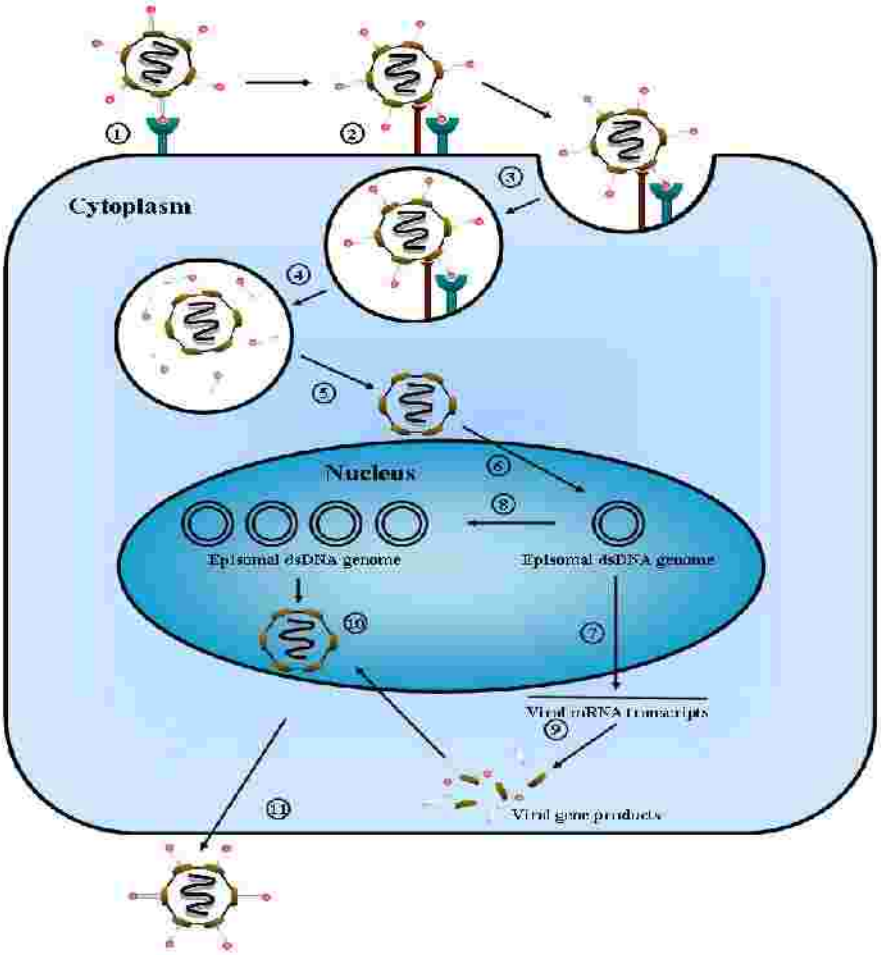


Figure 7: Adenovirus life cycle. The adenovirus knob binds to its primary receptor (CAR) (1) after which the penton base interacts with the secondary receptors (avb3/avb5 integrins) (2) that in turn trigger the process of endocytosis (3) . Once endocytosed acidification of the endosome triggers a conformational change in the viral capsid (4) that is then released into the cytoplasm and translocates to the (5) nucleus. The viral genome then enters the nucleus (6) and from its episomal location undergoes transcription (7) and then replication (8). Viral gene products are then produced in the cytoplasm following translation (9) and capsid proteins localize to the nucleus where virus assembly occurs (10). Virus can then be released from the cell following lysis. Modified from Stone *et al.* 2000.

1.3 The oncolytic adenoviral vector Ad5/35.IR.E1A/TRAIL

Although adenoviruses have become a popular choice in current intracranial gene therapy they show only inefficient gene delivery into the brain in experimental studies, which implies a lower possible clinical efficacy than expected. This leads to the necessity to develop improved viral gene therapy vectors for a better transduction, intratumoral viral spread and transgene expression. Ad5/35.IR.E1A/TRAIL is a recently developed adenoviral vector system featuring several modifications that are supposed to overcome the observed limitations of viral vectors in intracranial oncolytic therapy. In addition the vector promises to show a better biosafety due to its viral replication limitation to cancer cells (71, 72) [Figure 8].

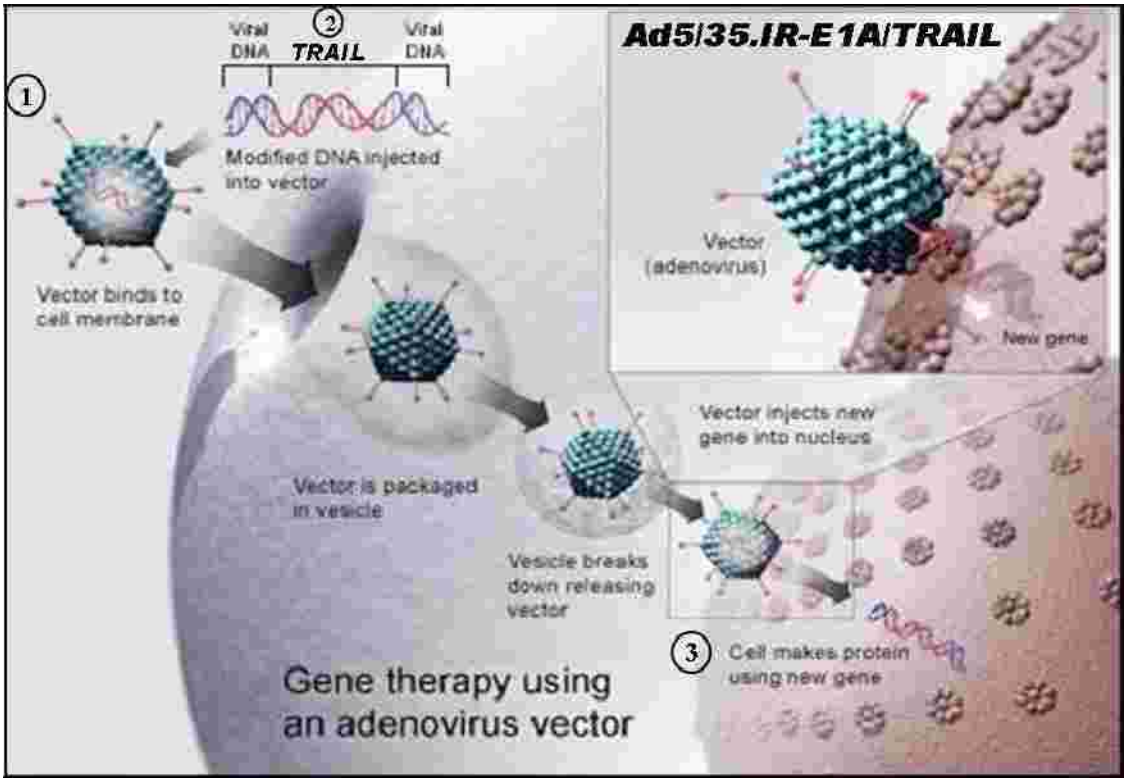


Figure 8: Ad5/35.IR-E1A/TRAIL. A modified adenoviral vector for treatment of human glioblastoma featuring **(1)** improved transduction via capsid modification, **(2)** the oncolytic transgene TRAIL and **(3)** a tumor specific transgene expression. Picture modified from *U.S. National Library of Medicine*

1.3.1 The chimeric Ad5/35 capsid

Ad5/35.IR.E1A/TRAIL combined the adenoviral capsid of serotype 5 (Ad5) with fiber proteins of the wild-type serotype 35 (Ad35) (73-75) [Figure 9]. This modification leads to a change of the viral receptor from the coxsackievirus and adenovirus receptor (CAR) to the human CD46, a membrane-bound regulatory protein that protects tissues from complement mediated damage (76-78). This leads to an important alteration in the tropism of the vector (79). CAR expression has been shown to tend to correlate inversely with the malignant potential of tumor cells including glioblastoma (61, 80-82) which leads to a reduced transduction efficiency of the adenoviral serotype 5 which is commonly used in virotherapy. The membrane protein CD46 on the other hand has been shown to be expressed in a variety of different cancer cell lines and primary tumor cells and its utilization results in a potentially increased transduction efficiency of a broad spectrum of different malignant tumor cells (83+84).

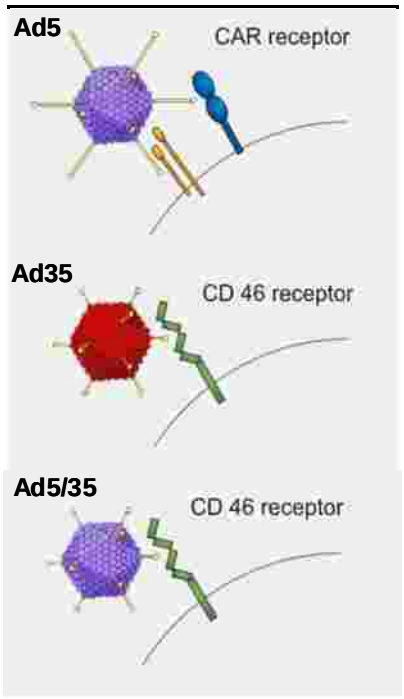


Figure 9: Adenoviral serotype 5 and 35 with their corresponding cellular receptors. The chimeric Ad5/35 virus features a serotype 5 capsid with the shorter fiber knob of serotype 35.

1.3.2 The transgene: Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)

In 1995, TNF related apoptosis-inducing ligand (TRAIL), also known as Apo-2L (85), was first identified based on its sequence homology to other members of the TNF superfamily (86). TRAIL is a type II transmembrane protein and shows highest homology to CD95L, exhibiting 28% amino acid identity with amino acid identity in the extracellular receptor-binding motif. TRAIL triggers apoptosis upon engagement of one of its two agonistic receptors, DR4 (death receptor 4) (87) and DR5 (death receptor 5), both type-I transmembrane proteins homologous to other members of the TNFR family (88+89).

On the other hand two main antagonistic receptors exist, namely TRID or TRAIL-R3 and TRUNDD or TRAIL-R4 (84, 85, also described as DcR1 (decoy receptor 1) and DcR2 (decoy receptor 2), respectively. DcR1 contains an incomplete homophilic death domain (DD) and is unable to transduce a death signal. Similarly, DcR2 lacks a cytoplasmic domain and is bound to the cell surface via a glycosyl-phosphatidylinositol (GPI) anchor and does not mediate apoptosis upon ligation. Transient overexpression of DcR1 or DcR2 in TRAIL-sensitive tumor cells prevents cell death triggering by TRAIL (90+91), and recent evidence indicates that tumor and normal cells can acquire resistance to TRAIL-induced killing by up-regulating TRAIL antagonistic receptors (92-95).

In case of TRAIL binding to DR4 and DR5 ligand-induced crosslinking initiates recruitment of various adaptor molecules through DD interactions. This includes Fas-associated death domain (FADD), which directly binds procaspase-8 to the ligated receptor to form the death-inducing signaling complex (DISC). This, in turn, promotes trans-catalytic activation of procaspase-8 and -10 which are activated by autoproteolytic cleavage and initiate the caspase cascade leading to apoptosis (96) [Figure 10].

TRAIL expression can selectively induce apoptosis in tumorigenic or transformed cells, but not in normal cells, highlighting its potential as therapeutic application in cancer treatment. Therefore TRAIL has gained considerable interest in oncology since it displays specific antitumoral activity against a wide range of tumor cells including glioblastoma (97-100). Furthermore first experiments in rodents and primates have not shown any significant side effects (101+102).

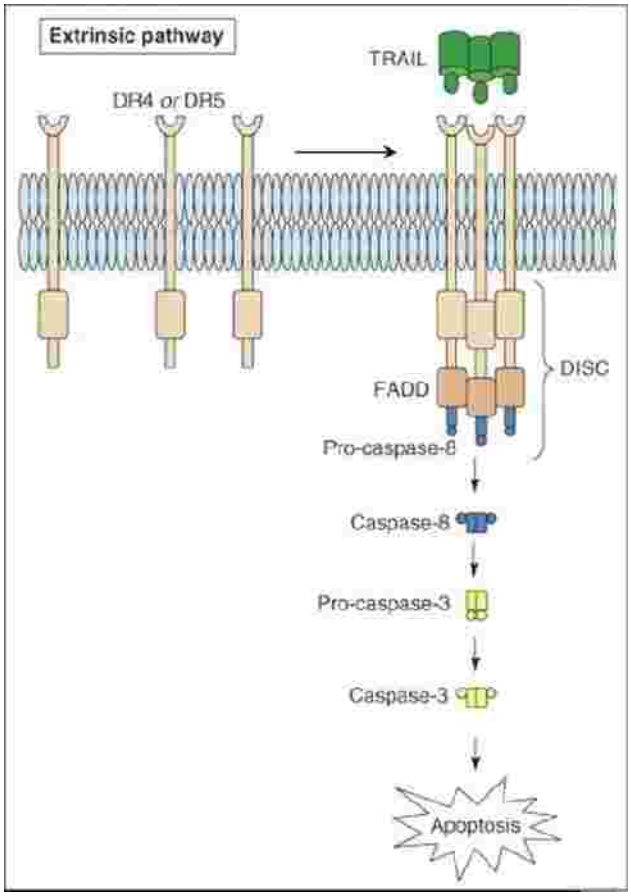


Figure 10: TNF related apoptosis-inducing ligand (TRAIL) mediated induction of apoptosis. TRAIL binds to DR4 or DR5 receptors. The resulting cross-linking recruits Fas-associated death domain (FADD) which in turn binds pro-caspase-8. The resulting complex is described as the "death-inducing signaling complex" (DISC). Activation of Pro-caspase-8 by the DISC initiates the caspase-cascade which induces apoptosis.

1.3.3 Tumor specific viral replication and transgene expression

To minimize cytotoxicity and avoid viral spread into non cancerous tissue, nonreplicating adenoviral vectors have been commonly used in the past (103+104). In these vectors the E1A region of the viral genome has been deleted (AdE1-) which renders the virus unable to synthesize the gene products necessary for viral DNA replication.

Recently a new concept for tumor-specific gene expression that is based on homologous recombination between inverted repeats (IR) in adenovirus genomes has been developed (Ad.IR) (105). The IRs inserted into the E1 region of AdE1– vectors mediate predictable genomic rearrangements depending on viral DNA replication. This system

uses adenoviral vectors deleted for all E1A and E1B genes which are capable of low level replication of their viral DNA despite the deletions in tumor cells but not in non-dividing cells.

In case of Ad5/35.IR-E1A/TRAIL inverse homologous elements flank an RSV promoter with a bicistronic expression cassette located downstream of the adenovirus packaging signal (?) and the viral inverted terminal repeats (AdITR). Since the promoter is located upstream of the bicistronic expression cassette in inactive 3'-5' orientation, transgene expression is not possible. However in case of viral replication, homologous recombination can occur and the elements mediate the formation of genomic derivatives containing the promoter in a transcriptionally active position [Figure 11]. This leads to transcription of the transgenes TRAIL and E1A. Expression of E1A allows for efficient, tumor-specific viral replication and production of progeny virus, whereas expression of TRAIL upon viral replication and TRAIL-mediated apoptosis confers efficient release of progeny virus and viral spread.

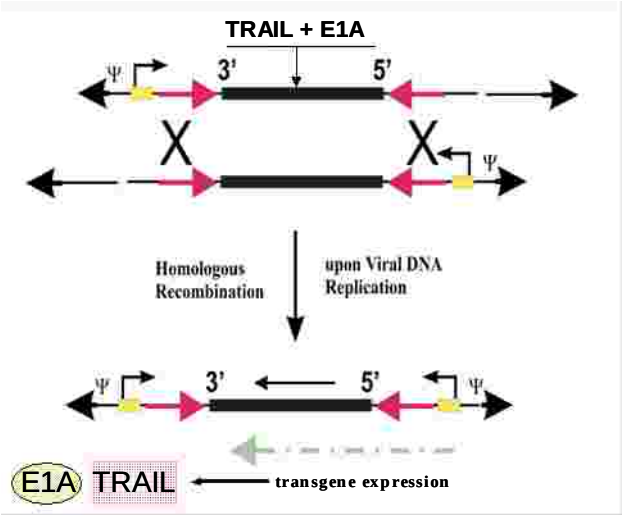


Figure 11: Tumor specific viral replication. Homologous recombination through elements that flank a bicistronic expression cassette allows to transcriptional activation which leads to E1A expression which is the essential product for tumor-specific viral replication.

2. CONCEPTUAL FORMULATION

In this study we wished to determine whether a novel virotherapeutic approach with a modified adenoviral vector utilizing a chimeric capsid consisting of a serotype 5 capsid and serotype 35 fiber is generally applicable in case of human glioblastoma. We propose an enhanced transduction ability of the chimeric capsid as compared to commonly used Ad serotype 5. Furthermore we want to evaluate if the tumor specific expression of the transgene TRAIL allows for improved infection and cell killing of human GBM cells compared to wild-type serotypes 5, 35 and an adenoviral Ad5/35 construct that does not express TRAIL. In addition, we want to verify the oncolytic potential of Ad5/35.IR.E1A/TRAIL in GBM tumor tissue *in vivo* after intratumoral injections into subcutaneously xenografted glioblastoma tumors in NOD/SCID mice. We suggest that an oncolytic adenoviral vector that allows efficient infection of glioblastoma cells, replicates specifically in infected tumor tissue and expresses ligands that induce apoptosis in the tumor shortly after infection or block the tumor invasion activity of glioblastoma could provide a promising therapeutic option.

3. MATERIAL & METHODS

3.1 Material

3.1.1 Consumables

1.5 ml Tubes	Fisher Scientific, Hampton, NH, USA
96-well plates	Fisher Scientific, Hampton, NH, USA
Cell Strainer (70 µm)	BD Biosciences, San Jose, CA, USA
Centrifuge Tubes	Corning, Corning, NY, USA
Conical Tubes	BD Biosciences, San Jose, CA, USA
Dishes, TC-treated	Corning, Corning, NY, USA
Electroporation Cuvettes, 1mm	BTX, Holliston, MA, USA
FACS tubes	BD Biosciences, San Jose, CA, USA
Filter units (0.22 µm)	Millipore, Billerica, MA, USA
Glass-pipettes	Bellco, Vineland, NJ, USA
Gloves	Kimberly Clarke, Roswell, GA, USA
Pasteur Pipettes	Fisher Scientific, Hampton, NH, USA
PCR tubes	CLP, San Diego, CA, USA
Petri Dishes (non TC-treated)	Fisher Scientific, Hampton, NH, USA
Pipette Tips	Island Scientific, Bainbridge, WA, USA
Plastic-pipettes	Fisher Scientific, Hampton, NH, USA
Polypropylene tubes	BD Biosciences, San Jose, CA, USA
TC flasks	BD Biosciences, San Jose, CA, USA

Table 3: General consumables

3.1.2. Lab equipment

ABI 3730 DNA Analyzer	ABI, Foster City, CA, USA
Binocular (Optix)	Olympus, Japan
Centrifuges	
(5415D Tabletop)	Eppendorf, Hamburg, Germany
(5415C Tabletop)	Eppendorf, Hamburg, Germany
(RC3B Plus, Sorvall)	Fisher Scientific, Hampton, NH, USA
(GS-6R)	Beckman, Fullerton, CA, USA
Electroporation (Gene Pulser Xcell)	BioRad, Hercules, CA, USA
FACSCalibur	BD Biosciences, Rockville, MD, USA
FACSVantageSE	BD Biosciences, Rockville, MD, USA
Fluorometer (DyNA Quant 200)	Hoefer, San Francisco, CA, USA
Gel Electrophoresis Chamber (SEA 2000)	Ellard Instrumentation, Monroe, WA, USA Elchrom Scientific, Cham, Switzerland

Gel Illuminator (Universal Hood II)	BioRad, Hercules, CA, USA
Heatblock (Dry bath incubator)	Fisher Scientific, Hampton, NH, USA
Hemocytometer	Hausser Scientific, Horsham, PA, USA
Hotplate (PC-351)	Corning, Corning, NY, USA
Incubators	
(Forma Scientific)	Fisher Scientific, Hampton, NH, USA
(Isotemp)	Fisher Scientific, Hampton, NH, USA
(ShelLab)	ShelLab, Cornelius, OR, USA
Liquid Nitrogen Tank (Locator 4plus)	Barnstead/Thermolyne, Dubuque. IO, USA
Microscope (CK40)	Olympus, Japan
Microwave (Carousel 1200 Watts)	Sharp, Mahwah, NJ, USA
PCR Thermocycler	
(GeneAmp PCRSystem 9700)	ABI, Foster City, CA, USA
(2720 ThermalCycler)	ABI, Foster City, CA, USA
(DNA Engine DYAD)	MJ Research (BioRad)
PH-Meter	Beckman, Fullerton, CA, USA
Pipet-Aid	Drummond Scientific, Broomall, PA, USA
Pipettors	
(Pipetman P)	Gilson, Middleton, WI, USA
(Eppendorf Research)	Eppendorf, Hamburg, Germany
Power Supply	
(EC-135)	ECApparatus Corp., Holbrook, NY, USA
(EC-105)	ECApparatus Corp., Holbrook, NY, USA
(Model 200/2.0)	BioRad, Hercules, CA, USA
Scale (Accu-413)	Fisher Scientific, Hampton, NH, USA
Shaker	
(Orbit Shaker)	Barnstead/Lab-line, Dubuque, IO, USA
(MaxQ 3000)	Barnstead/Lab-line, Dubuque, IO, USA
TaqMan Machine	
(7500 Real Time PCR System)	ABI, Foster City, CA, USA
Thermomixer	Eppendorf, Hamburg, Germany
Vortex	
(Mini)	Fisher Scientific, Hampton, NH, USA
(Type 16700)	Barnstead/Thermolyne, Dubuque, IO, USA
Waterbath	
(Sheldon)	ShelLab, Cornelius, OR, USA
(Aquabath)	Barnstead/Lab-line, Dubuque, IO, USA

Table 4: General lab equipment

3.1.3 Antibodies

The following antibodies were used for immunofluorescence experiments and flow cytometry (Table). Listed are the antibody target, the host where the antibody was raised in, the dilution factor used in the experiments, and the vendor where the antibody was purchased from using the appropriate catalog number. If not stated otherwise, antibodies were directed against human antigens.

Antibody target	Host	Dilution	Vendor	Catalog number
CAR	mouse	1:50	Abcam	Ab 9891-1
CD 46	mouse	1:50	BD Biosciences	clone J4.48
Hexon (adenovirus)	goat	1:100	Chemicon	MAB8052
MHC class I	mouse	1:50	BD Biosciences	clone G46-2.6
IgG-	mouse	1:50	BD Biosciences	555749
α goat IgG/Texas Red	rabbit	1:200	Abcam	Ab 6739-1
α-mouse IgG/FTIC	rat	1:200	BD Biosciences	550003
a- mouse polymer (chromagen)	goat		Dako	K4004

Table 5: primary- & secondary antibodies used for immunofluorescence experiments in this study. FITC=fluorescein isothiocyanate

3.1.4 Adenoviruses

The following adenoviruses were used in this study. Listed are viruses, the transgenes they carry, the promoters used for expression of transgenes, and their origin.

Adenovirus	Transgene/s	Promoter for transgene expression	Origin (reference)
Ad5/35.IR-E1A/TRAIL	E1A, TRAIL	RSV	Sova et al., 2004
Ad5.IR-E1A/TRAIL	E1A, TRAIL	RSV	Sova et al., 2004
Ad5/35-GFP	GFP	RSV	Shayakhmetov et al., 2000

Ad5/11-GFP	GFP	RSV	Shayakhmetov et al., 2000
Ad5/11-GFP	GFP	RSV	Shayakhmetov et al., 2000
Ad35	-	-	Holden strain (ATCC)
Ad5	-	-	Reference strain (ATCC)

Table 6: Adenoviruses used in this study. TRAIL=tumor necrosis factor-related apoptosis-inducing ligand, GFP=green fluorescent protein, RSV=rous sarcoma virus,

3.1.5 Oligonucleotides

All oligonucleotides were purchased as lyophilized, salt-free stocks from Operon. The following tables list oligonucleotides used for the detection of adenoviral l genomes by qRT-PCR. The official gene symbol (by Human Genome Organization (HUGO) Gene Nomenclature Committee) was used for oligonucleotide (primer) names.

Primer	Direction	Sequence
Ad5 hexon	Forward	5' TACTGCGTACTCGTACAAGG 3'
Ad5 hexon	Reverse	5' AGAGCAGTAGCAGCTTCATC 3'
HPRT1	Forward	5' AGTTCTGTGGCCATCTGCTT 3'
HPRT1	Reverse	5' GCCCAAAGGGAACTGATAGTC 3'

Table 7: Oligonucleotides used in this study

3.1.6. Mouse strain

All animal experiments in this study have been performed with the strain “NOD.CB17-Prkdcscid/NCrCrl” (NOD/SCID). In this strain the SCID mutation has been transferred onto a non-obese diabetic background. Animals homozygous for the SCID mutation have impaired T and B cell lymphocyte development. The breeding pairs were obtained through Charles River Laboratories Wilmington, MA, USA).

3.1.7 Cultured cells and culture media

The listed cells and culture media were used throughout this thesis. For cell passaging, cells were detached from tissue culture plates (BD Falcon) with trypsin solution (Gibco) and then washed with PBS (Gibco). To determine cell numbers, cultures were counted

using a hemocytometer. 1% Penicillin/Streptomycin (Gibco) was added to all media. Cells were propagated in a 1:3 ratio and cultured in DMEM containing 10% FBS, Non-Essential Amino Acids Solution, and L-glutamine (Gibco). All cells were cultured at 37°C, 5% CO_2, and 95% humidity in cell culture incubators. Cells were frozen in cryo tubes (Greiner) in 50% FBS, 40% of indicated medium and 10% dimethyl sulfoxide using a cell freezer with isopropanol

Cell type	Source	Description	Medium
HEK-293	Microbix, Toronto, Canada (Graham et al., 1977)	human embryo kidney cells, transformed by adenovirus serotype 5 E1A	DMEM (Gibco), 10% FBS (Gibco), 2 mmol/L L-glutamine (Gibco)
AE25	Kovesdi, I. (Bruder et al., 2000)	human lung adenocarcinoma epithelial cells, adenoviral E1-complementing cell line derived from A549 cells	DMEM (Gibco), 10% FBS (Gibco), 2 mmol/L L-glutamine (Gibco)
T98G	Stein, GH (Stein et a. 1979)	human glioblastoma cell line with with hyperpentaploid chromosome count.	DMEM (Gibco), 10% FBS (Gibco) L-glutamine (Gibco) Non-Essential Amino Acids Solution (Gibco)
SF767	Brain Tumor Research Center (University of California San Francisco, CA) (Berens et al., 1990)	human glioblastoma cell line	DMEM (Gibco), 10% FBS (Gibco) L-glutamine (Gibco) Non-Essential Amino Acids Solution (Gibco)
U-87 MG	Ponten, J. (Beckman et al. 1971)	human glioblastoma cells, classified as grade IV as of 2007	DMEM (Gibco), 10% FBS (Gibco) L-glutamine (Gibco) Non-Essential Amino Acids Solution (Gibco)

Table 8: Cells and their cell culture media. FBS=fetal bovine serum

3.2 Methods

3.2.1 Surface protein immunofluorescence analysis using flow cytometry

Immunofluorescence analysis of CAR and CD46 expression was performed by flow cytometric analysis. U-87 MG, T98G, and SF767 cells were trypsinized and pelleted via centrifugation. Cell pellets were resuspended in ice-cold PBS with 1% FBS in order to block unspecific antibody binding. $2x10^5$ cells were incubated with with a mouse α-human CD46 IgG primary antibody (BD Biosciences; San Jose CA USA) or mouse α-

human Coxsackie Adenovirus Receptor (hCAR) primary antibody (Abcam Inc., Cambridge, MA , USA) in 5ml round bottom tubes (BD Falcon) in a total of 100µl for 45 min on ice. All subsequent incubation steps were carried out in the dark. Cells were washed with 3 ml PBS+1%FBS and centrifuged at 400xg for 5 min at 4°C in between. This was followed by a 45 minute incubation with a secondary FITC labeled α-mouse IgG antibody (BD Biosciences; San Jose CA USA). One well containing cells of the same cell line was harvested but only incubated with the secondary antibody as a negative control. After incubation the cells were washed, fixed with 4% paraformaldehyde and analyzed via flow cytometry. The BD FACSCanto™ flow cytometer (Becton Dickenson) running the FACSDiVa software was used to analyze samples. Unspecific background of individual channels was determined using fluorophor-labeled isotype controls and color compensation was achieved with single color-stained samples. Figures were generated using CellQuest for Macintosh (Becton Dickinson).

3.2.2. Adenovirus propagation and preparation

Adenoviruses were propagated on HEK-293 cells in 150 mm dishes in a total volume of 20ml. For propagation of Ad5/35.IR-E1A/TRAIL and Ad5.IR-E1A/TRAIL the more apoptosis-resistant AE25 cell line was used. Cells were 90-100% confluent when infected. For initial infection, replication competent adenovirus (RCA)-free aliquots of virus-stocks were used in an approximate MOI of 10-25 pfu/cell. 5ml fresh medium was added the next day. When cells were rounded and started to de-attach (approximately 48 hours after infection), they were harvested in the culture medium by repeated pipetting. Cell-containing medium was transferred to a 50ml blue cap tubes (BD Falcon) and these then subjected to 4 cycles of freezing in liquid nitrogen and thawing at 37°C in a water bath. Tubes were centrifuged at 400xg (Beckman Coulter) and the supernatant was collected. Virus-containing supernatant was propagated on fresh HEK-293 or AE25 cells in a ratio of 1:3-1:4 until 30 150mm dishes were infected. Here, cells were collected when rounded, but before they started to detach (approximately 36 hours after infection). Cells were collected, pelleted (400xg) and then taken up in 1ml phosphate-buffered

saline (PBS [Gibco BRL]) per plate. After 4 cycles of freezing and thawing, virus was isolated by ultracentrifugation. The first ultracentrifugation (2 hours, 14°C, 35,000 RPM, SW41 rotor [Beckman Coulter]) was performed in a Caesium chloride (CsCl) step gradient. The following CsCl concentrations were layered above each other in 12ml ultra-clear tubes (Beckman Coulter):

i) 0.5 ml 1.50 g/cm3 CsCl (45.41g CsCl + 54.49 ml H2O)
ii) 3.0 ml 1.35 g/cm3 CsCl (35.18g CsCl + 64.82 ml H2O)
iii) 3.5 ml 1.25 g/cm3 CsCl (26.99g CsCl + 73.01 ml H2O).

5ml of viral supernatant were layered on top of the gradient and then tubes were centrifuged in a SW41 rotor for 2 hours at 35,000 RPM at 14°C (Beckman Coulter). Three clearly separated bands were obtained. Adenovirus appeared as a narrow, opaque white band in the lower 1/3 of the CsCl step gradient (Fig.12).

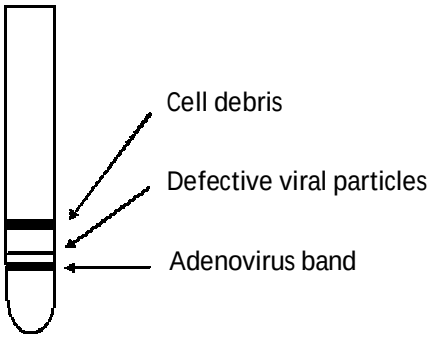


Fig 12: Cesium chloride separation of adenovirus from defective particles and cell debris.

Adenovirus fractions of individual tubes were isolated and combined. 4ml were mixed with 8 ml 1.35 g/cm3 CsCl in ultra-clear tubes and then centrifuged at 35,000 rpm overnight. The virus band was isolated from the bottom 1/4 of the tube and then dialyzed in a 50kDa cut-off dialyzing tube (Spectrum Laboratories) against 1,000 ml of 10 mM Tris pH 7.5, 10mM MgCl2, 250mM NaCl and 10% glycerol overnight at 4°C with one change of dialyzing buffer. The virus was then collected and stored in 25µl or 50µl aliquots at -80°C.

3.2.3 Adenovirus titering by spectrophotometry

The adenovirus particle titer was determined on viral DNA. A 25µl aliquot from a fresh adenovirus stock was added to 475 µl TE buffer (10mM Tris pH 8.0, 1mM EDTA pH 8.0 [Sigma]) with 0.1% SDS (Sigma). The sample was thoroughly mixed using a vortexer (Baxter) for 5 min and then centrifuged at 14,000 RPM using an Eppendorf table centrifuge for 5 min. The optical density (OD) of supernatant was assessed at 260 nm in a spectrophotometer (Becton Dickenson). Viral particle titer/ml was calculated by multiplying the OD with $2x10^{13}$ as described by Mittereder at al. 1996.

3.2.4 Adenovirus titering by plaque assay

HEK-293 cells were used to determine the plaque-forming unit titer of adenovirus stocks. Cells were plated in 6-well plates and infected when 90-100% confluent. An aliquot of the adenovirus stock was thawed on ice and then serial diluted in regular HEK-293 medium using the following dilutions: 10-2, 10-4, 10-6, 10-8, 10-9, 10-10. Cells were then infected in duplicates with 1ml virus dilution/well and incubated for 24 hours at 37°C. Medium was removed and cells then overlayed with 3ml of a warm (45°C) mix of 2xDMEM (Gibco) and 1.2% agarose (Sigma) (1:1 vol/vol) supplemented with 10% FBS. Cells were overlaid with 1 additional ml on days 4 and 9 after the first overlay. Plaques were counted on days 10 and 14 post infection and final titer was determined by multiplication of individual plaques with appropriate dilutions. The mean titer of duplicates was used.

3.2.5 Crystal violet cytotoxicity assay

Cells were plated in 24-well dishes and kept in culture until they were nearly confluent. After estimation of the total number of cells per well via “Helber” counting chamber, the cells were infected with an MOI of 1 or 10. Over a period of 6 days one well per viral serotype 5 and 35, Ad5/35.IR.E1A/GFP and Ad5/35.IR.E1A/TRAIL was stained every 24 h.p.i. Prior to crystal violet staining, the medium was removed and the cells were fixed

for 10 min in 4% paraformaldehyde at room temperature. Fixed cells were washed with PBS and incubated for 5 min in 1% crystal violet solved in 70% ethanol (Sigma), followed by three rinses with water. Air-dried cells were photographed.

3.2.6 MTT assay

An MTT stock solution of 5mg/ml (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was constituted in PBS (Gibco) and stored at -20°C. MTT assays were carried out in 96 well plates (Fisher). At 4 or 8 days post infection 20µl of MTT stock solution (in PBS) was added to each well and cells were incubated for 2h at 37°C. Medium was removed, cells washed twice with PBS and air-dried. Then 100µl of DMSO/well was added per well and incubated for 30min at RT in order to dissolve crystals. Absorbance was measured in plate reader (EL 340, Bio-Tek Instruments) at 546nm.

3.2.7 In situ apoptosis detection using the TUNEL assay

The TUNEL (terminal dUTP nick-end labeling) assay can be used to detect apoptotic cells. Cells of the two human GBM lines U-87 MG and SF767 were grown in 24-well cell culture dishes. After the mono-cell layer was nearly confluent, the average number of cells per well was estimated using a “Helber” counting chamber (improved Neubauer, Fisher Scientific). Cells were infected with adenovirus serotype 35 wild-type, Ad5/35.IR-E1A/TRAIL or the vector Ad5.IR-E1A/TRAIL with an MOI of 1 pfu/cell.
Wells of each cell line were harvested 48 and 96 hours post- infection. Induced apoptosis was detected via visualization of occurred DNA fragmentation by labeling of free 3’- OH termini with FITC with an in situ cell death detection kit (Roche, Palo Alto, CA, USA). The cells were collected after trypsin treatment, fixed with 4% paraformaldehyde and processed following the protocol of the kit manufacturer. The labeled cells were analyzed using flow cytometry. The BD FACSCanto™ flow cytometer (Becton Dickenson) running the FACSDiVa software was used to analyze samples. Unspecific background of individual channels was determined using fluorophor-labeled

isotype controls and color compensation was achieved with single color-stained samples. Figures were generated using CellQuest for Macintosh (Becton Dickinson).

3.2.8. qPCR for viral genomes

Confluent cells of the U-87 MG and T98G cell lines were transduced with an MOI of 100 using Ad5/35.IR-E1A/TRAIL or Ad5/35.IR-E1A/GFP. The experiment was stopped 3 hours or 3 days post infection. Cells were washed with PBS, trypsinized, washed with RPMI+10% FBS and washed again with PBS twice. Nucleic acids from cell pellets were isolated using the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's protocol. 1ng DNA per reaction was used for further analysis. A standard curve for genomic DNA (isolated from SKOV3-ip1 cells) was prepared based on the equation that one copy number equals 3pg of genomic human DNA (6pg/diploid cell). Primers against Ad5 hexon were used to assess the viral copy number in viral genome preparations from fixed numbers of viral particles (assessed spectrophotometrically), serially diluted and spiked with 1 ng genomic DNA of SKOV3-ip1 cells to generate a standard curve. All reactions were performed in triplicate in a total reaction volume of 15µl using ImmoMix (Bioline), SYBR green (Bioline) and 3 µmol/l of each primer, and carried out in the GeneAmp 7900 instrument (Applied Biosystems). The following parameters were used for amplification and melting curve analysis: 95°C (10 minutes), followed by 40 cycles of 60°C (2 minutes), 95°C (15 seconds), 60°C (15 seconds), 95°C (15 seconds). Ct values were calculated using the Sequence Detection System software (Applied Biosystems). Under these conditions at least 10 copies could be detected for each replicate. Levels of hexon were standardized per DNA genomic copy number using primers against housekeeping gene HPRT1. All primer sequences are supplied in Table 7.

3.2.9. Detection of apoptotic/necrotic tissue and viral capsid protein in xenografted tumor tissue after viral treatment

U-87 cells were harvested and 2 x 106 cells were resuspended in 200 µl PBS.
The cells were mixed with 200 µl Matrigel™ (BD Biosciences; San Jose CA USA) and injected subcutaneously into both flanks of NOD/SCID mice. Pre-established s.c. tumors were treated with injections of 1×10^8 pfus of the onclolytic adenovirus Ad5/35.IR-E1A/TRAIL or the adenoviral serotype 35 directly into the xenografted tumor. Mice were sacrificed 2 weeks after viral application and tumor tissue samples were embedded in OCT (Tissue-Tek) and frozen. Tumor tissue was sliced (0.8 microns) using the Leica CM 1850 cryostat (Leica Microsystems) and then transferred onto superfrost slides (Fisher Scientific). Slides were fixed in acetone (Fisher Scientific) for 10 min at -20oC. After two rinses with PBS (Sigma) slides were blocked with 2% milk powder (BioRad) in PBS for 20 min at room temperature. Microdissections were processed and analyzed for apoptotic/necrotic tissue using the “in situ cell death detection assay” (Roche) following the protocol of the kit manufacturer. Adenoviral hexon capsid proteins were detected using a goat α Ad-hexon capsid protein antibody (Chemicon AB 1056, Temecula, CA USA) and a secondary Rabbit α goat IgG H&L (Texas Red) antibody (Abcam Inc. Cambridge, MA USA).

3.2.10. Immunohistochemistry on tumor sections

Tumor sections of animal xenografts were snap frozen embedded in OCT compound (Tissue-Tek) on dry ice. OCT embedded tissues were then stored at -80oC and equilibrated to -20oC for at least 1 hour prior to sectioning. Tumor tissue was sliced (0.8 microns) using the Leica CM 1850 cryostat (Leica Microsystems) and then transferred onto superfrost slides (Fisher Scientific). Slides were fixed in acetone (Fisher Scientific) for 10 min at -20oC. After two rinses with PBS (Sigma) slides were blocked with 2% milk powder (BioRad) in PBS for 20 min at room temperature. Microdissections were processed and analyzed for surface proteins using the “K1390 Envision system” (Dako) following the manufacturer’s protocol. CD46 was detected with the a- CD46 (mouse a-human) antibody (abcam) followed by a secondary peroxidase labeled polymer

conjugated goat a- mouse antibody. Parallel to this sections were incubated with either a mouse a- human MHCI antibody as a positive control or an Igg negative control antibody. After application of the substrate-chromogen cells were counterstained using Gill's hematoxylin counterstain.

3.2.11 Animals and treatment protocol

U-87 cells were harvested and 1x106 cells were resuspended in 100 µl PBS. The cells were mixed with 100 µl Matrigel™ (BD Biosciences; San Jose CA USA) and injected subcutaneously into the flanks of Four- to 6-week-old male athymic NOD/SCID mice using a 271/2 G syringe (Becton Dickinson, NJ, U.S.A.).

For each wild-type virus serotype, viral vector and one negative control (NeCo) 5 mice received injections into both flanks. Tumor growth was measured every other day using a caliper. Once the tumors reached an average size of 55 mm^3 ±15%, viral treatment was initiated: 1×10^8 pfu of Ad5, Ad35, Ad5/35.IR-E1A/GFP or Ad5/35.IR-E1A/TRAIL were injected intratumorally. The same dose was given 48 hours after the first injection leading to a total application of 2×10^8 pfu. Further increase or reduction of the tumor volume was measured using a caliper. In a second approach 1×10^4 U-87 MG cells were transduced with the wild-type serotype Ad5, Ad35 or the vector Ad5/35.IR-E1A/TRAIL using an MOI of 100. 12 h.p.i. the cells were harvested and resuspended together with 9.9×10^5 uninfected U-87 MG in 100 µl PBS. The cells were mixed with 100 µl Matrigel™ and injected subcutaneously into the flanks leading to an injection of 1×10^6 U-87 MG cells with 1% transduced cells. For each wild-type virus serotype, viral vector and one negative control containing no previously infected cells 5 mice received injections into both flanks tumor growth was monitored every 48 hours using a caliper. Differences amongst groups of tumors were compared statistically using the Mann Whitney U Test. All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center.

3.3. Suppliers

Company	Location
Abcam	Cambridge, UK
American Type Culture Collection (ATCC)	Manassas, VA, USA
Amersham	Little Chalfont, UK
Applied Biosystems	Foster City, CA, USA
BD Falcon	San Jose, CA, USA
BD Pharmingen	Mississauga, ON, Canada
Beckman Coulter	Fullerton, CA, USA
Becton Dickinson	Franklin Lakes, NJ, USA
Bio-Tek Instruments	Winooski, VT, USA
Bioline	Taunton, MA, USA
Biomeda	Foster City, CA, USA
BioRad	Hercules, CA, USA
Calbiochem	Nottingham, UK
Cell Biolabs	San Diego, CA, USA
Charles River Laboratories	Wilmington, MA, USA
Chemicon	Temecula, CA, USA
Dako	Glostrup, Denmark
Fisher Scientific	Pittsburgh, PA, USA
Gibco	Carlsbad, CA, USA
GraphPad Software	San Diego, CA, USA
Greiner	Monroe, NC, USA
Invitrogen	Carlsbad, CA, USA
Leica Microsystems	Wetzlar, Germany
Lonza	Basel, Switzerland
Microsoft	Redmond, WA, USA
Nalgene	Rochester, NY, USA
Operon	Huntsville, AL, USA
Panasonic	Secaucus, NJ, USA
Qiagen	Valencia, CA, USA
R&D Systems	Minneapolis, MN, USA
Roche	Palo Alto, CA, USA
Sigma	St. Louis, MO, USA
Southern Biotech	Birmingham, AL, USA
Spectrum Laboratories	Rancho Dominguez, CA, USA
Stem Cell Technologies	Vancouver, Canada
Thermo Scientific	Rockford, IL, USA
Tissue-Tek	Torrance, CA, USA
Tree Star, Inc.	Ashland, OR, USA
Vector Labs	Burlingame, CA, USA

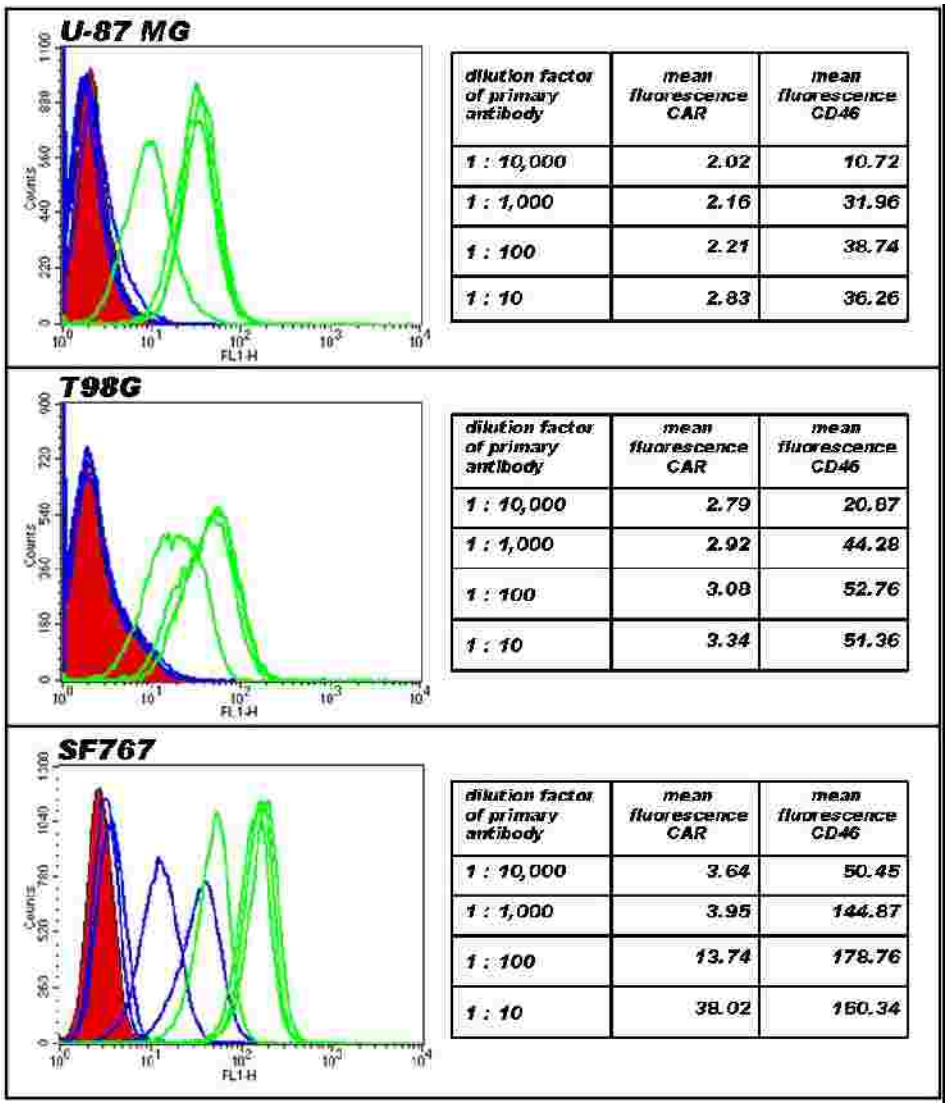
4. Results

4.1 Human GBM cell lines show higher expression of CD46 compared to CAR

A limitation of cancer therapy with adenovirus vectors has been the lack of CAR expression on tumor cells since CAR is the primary receptor of the adenoviral serotype 5 which has been used extensively in adenoviral gene therapy in the past.

For comparison of the impact of the oncolytic vector Ad5/35.IR-E1A/TRAIL on human glioblastoma cell lines, adenovirus serotype 5 (Ad5) and the adenovirus serotype 35 (Ad35) have been used in this study. The capsid of the Ad5/35.IR-E1A construct is derived from the Ad5 capsid combined with the fiber proteins of the serotype 35 which utilizes CD46 instead of CAR as the primary receptor (72, 82-84).

Therefore a first quantification and comparison of the primary viral receptor presentation on human GBM cells had to be conducted to evaluate the potential ability of Ad5/35.IR vectors to infect GBM. Thus, we first compared the expression of CD46 and CAR on the GBM cell lines SF767, T98G and U-87 MG [Figure 13]. The flow-cytometric analysis of all three cell lines showed a higher mean fluorescence after incubation with anti-CD46 antibodies compared to incubation with anti-CAR.
The mean fluorescence after incubation with the anti-CD46 antibody in a 1:100 dilution was >30 in all three cell lines, whereas the mean fluorescence after incubation with anti-CAR did not exceed 4 in case of the cell lines U-87 MG and T98G. Only the cell line SF767 demonstrated comparatively high CAR expression, with a mean fluorescence of 38. However, incubation with anti- CD46 results in a 4-fold higher mean fluorescence and shows that the SF767 cell line also presents higher CD46 levels, as compared to CAR levels. The detectable amounts of the CD46 receptor on the surface of human GBM cell lines correlate with the findings in the human colon carcinoma cell line (LoVo), which was previously successfully transduced with Ad5/35.IR.E1A/TRAIL *in vitro* (106). In contrast to CAR, the expression of the group B receptor, CD46, was found to be upregulated in all tested GBM tumor cell lines.



dilution factor of primary antibody	mean fluorescence CAR	mean fluorescence CD46
1 : 10,000	2.02	10.72
1 : 1,000	2.16	31.96
1 : 100	2.21	38.74
1 : 10	2.83	36.26

dilution factor of primary antibody	mean fluorescence CAR	mean fluorescence CD46
1 : 10,000	2.79	20.87
1 : 1,000	2.92	44.28
1 : 100	3.08	52.76
1 : 10	3.34	51.36

dilution factor of primary antibody	mean fluorescence CAR	mean fluorescence CD46
1 : 10,000	3.64	50.45
1 : 1,000	3.95	144.87
1 : 100	13.74	178.76
1 : 10	38.02	160.34

Figure 13: CD46 and CAR expression on human glioblastoma cells. Monolayer of SF767,U-87 MG, and T98G cells were analyzed for expression of the Ad35 wild-type and Ad5/Ad35.IR vector receptor CD46 and the Ad5 wild-type receptor CAR by surface labeling. Cells were harvested and incubated with anti-CAR or anti-CD46 monoclonal antibodies and a secondary FITC-labeled anti-mouse antibody. The labeled cells were analyzed by flow cytometry. The graphs represent fluorescence after incubation with different dilutions of anti-CAR (blue) or anti-CD46 (green) antibodies compared with an IgG-negative control (red shaded).

4.2 Ad5/35.IR-E1A/TRAIL shows efficient oncolytic effects in human GBM cell lines compared to Ad wild-type

To determine if the abundance of CD46 leads to an infective advantage of Ad35 vectors over Ad5, infectivity and cell killing has been compared with the adenoviral vectors wild-type serotype 5 (Ad5), wild-type serotype 35 (Ad35) and Ad5/35.IR-E1A/TRAIL. In addition Ad5/35.IR-E1A/GFP was added to compare the oncolytic activity of TRAIL in case of the chimeric Ad5/35 vector. In the Ad5/35.IR-E1A/GFP vector, the transgene TRAIL has been exchanged with GFP, thus any observed enhanced oncolysis can be credited to the tumor cell killing abilities of TRAIL.

Human GBM cell lines SF767, U-87 MG and T98G were infected with an MOI of 1 and observed for occurring oncolysis over a time period of 6 days after infection [Figure 14]. In addition T98G were infected with the same vectors using an MOI of 10 after an initial experiment with an MOI of 1 showed only minimal impact on this cell line .

In vitro infection of the human GBM cell lines with an MOI of 1 resulted in a more efficient tumor cell killing of the TRAIL expressing oncolytic vector compared to GBM cells infected with the adenoviral wild-types and the adenoviral construct Ad5/35.IR-E1A/GFP.

The TRAIL expressing vector began to show induction of cell death in 80% of the infected SF767 cells 3 days post infection (dpi) while approximately only 10% - 15% cell death occurred in Ad35 infected cells. Beginning significant cell killing, as compared to mock-infected cells, in Ad35 infected SF767 can be observed after 5 dpi. Ad5 does not have the ability to induce apoptosis in SF767 within the first 6 days after infection with an MOI of 1.

U-87 cells are lyzed by Ad5/35.IR-E1A/TRAIL by up to 90% within 5 days while no complete cell killing can be achieved with wild-type virus or an Ad5/35.IR-E1A construct without TRAIL within 6 days.

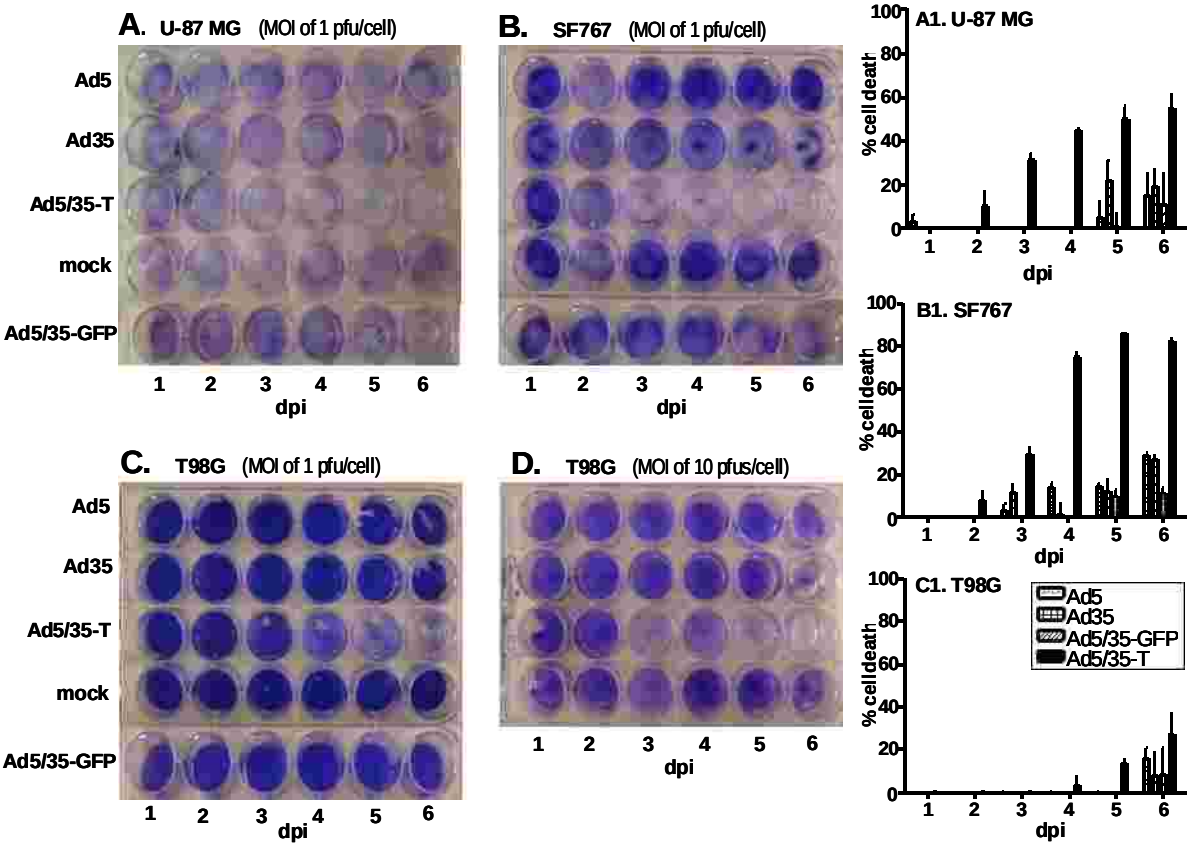


Figure 14: Oncolytic effect of Ad5/Ad35.IR-E1A/TRAIL. Confluent monolayers of the GBM cell lines U-87 MG (A + A1), SF767 (B + B1),and T98G (C + C1) were grown in a 24-well cell culture dish for the crystal violet assay and a 48-well dish for the MTT assay. The cells were infected with Ad5,Ad35, Ad5/Ad35.IR - E1A/GFP (Ad5/Ad35-GFP), or the oncolytic vector Ad5/Ad35.IR-E1A/TRAIL (Ad5/Ad35-T) with an MOI of 1 pfu/cell for each assay. In addition, confluent T98G monolayers were infected with a MOI of 10 pfu/cell (D). One well per viral infection was fixed in 4% PFA every 24 h, and the viable cells were visualized by staining with crystal violet. For the MTT assay, MTT was added to the wells of the infected cell lines everyday. After 3 h incubation, the formazan product was analyzed.

The T98G glioblastoma cell line showed a higher resistance to viral oncolysis in general. Beginning cellular lysis was detectable 4 dpi after infection with Ad5/35.IR-E1A/TRAIL. However only 15% - 20% cell killing can be shown in T98G cells after infection with Ad5/35.IR-E1A/TRAIL, while Ad5 and Ad35 show no detectable cell lysis at the same time point. Infection of T98G cells with an MOI of 10 showed that adenoviral serotypes 5 and 35 are generally capable of inducing cell death in the cell line T98G within 6 days after infection, although with only minimal effect. Ad5/35.IR-E1A/TRAIL on the other hand reached 100% cell lysis capability within 6 days after infection.

The results show that compared to Ad5 wild-type, Ad35 vectors feature a slightly enhanced ability to infect GBM cells and induce cell killing. Expression of the transgene TRAIL results in a significantly enhanced tumor cell killing all three GBM cell lines examined in this study.

4.3 Examination of the observed reduced & delayed cell killing in T98G cells compared to the SF767 and U-87 MG cell lines via Q-PCR

The observed resistance to adenoviral-mediated oncolysis, including Ad5/35.IR-E1A/TRAIL, of the human GBM cell line T98G was further examined via quantitative PCR. In a comparative Q-PCR setting U-87 MG and T98G cells were transduced with an MOI of 100 using Ad5/35.IR-E1A/TRAIL or Ad5/35.IR-E1A/GFP.

Three hours post infection the amount of intracellular viral DNA was quantified, followed by a second quantification 72 hours post infection using primers with binding sites within the viral gene *E1A* and the cellular housekeeping gene *HPRT1* in case of the cells [Figure 15]. Dilutions of ascertained adenoviral genomes and GBM cells were used as a standard for the quantitative PCR.

Analysis of GBM cells 3 hours post infection showed a small number of viral genome copies in U-87 and T98G cells proving that Ad5/35-constructs are generally capable of infecting T98G cells as well as U87-MG *in vitro*. In direct comparison however twice as many viral genomes could be detected on average in U-87 MG cells after infection with Ad5/35- vectors compared to T98G at the same time point post-infection [Figure 15 A].

After 72 hours the gap between detectable viral copy numbers in the two cell lines was greatly increased by 8-10 fold in cells transduced with Ad5/35 vectors [Figure 15, B1 + B2]. This leads to the conclusion that initially reduced viral vector delivery leads to reduced numbers of viral copies in T98G cells which results in detection of less synthesized viral genomes. However the significant difference in detectable viral copies

per cell in a time frame that resembles a complete infection cycle indicates impaired adenoviral DNA synthesis in case of T98G cells as well.

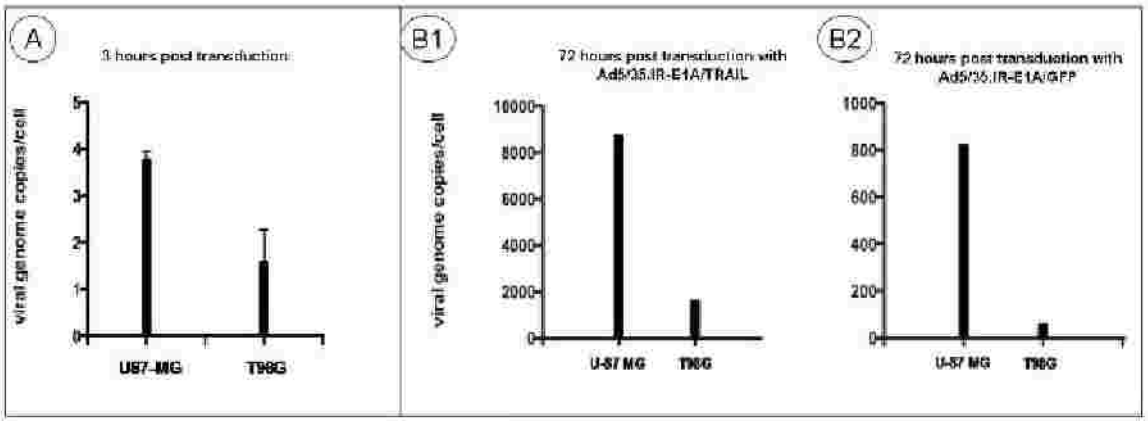


Figure15: Comparative quantitative real time PCR of transduced human GBM cell lines U-87 MG and T98G: U-87 MG or T98G cells were transduced with an MOI of 100 using Ad5/35.IR-E1A/TRAIL or Ad5/35.IR-E1A/GFP. Cellular DNA was detected via the housekeeping gene *HPRT1* and intracellular viral DNA was quantified using the viral gene *E1A*, located within the viral expression cassette. A standard was prepared using dilutions of adenoviral and cellular genomes. Samples were quantified 3 and 72 hours post initial viral transduction.

4.4 A U-87 MG stem-like cell sub-population forms tumor spheres that can be efficiently transduced and lyzed by Ad5/35.IR-E1A/TRAIL infection *in vitro*

It had been proposed that a minor sub-population of cancer stem cells (CSCs) derived from glioblastoma tumor tissue exists in human cell lines available, and may be maintained even in FBS containing medium. CSCs from different tumor cell lines have been isolated either by utilizing the expression of the stem cell marker CD133 or on the basis of their ability to exclude the fluorescent vital dye Hoechst 33342 (107-109). A distinct "sub population" of U-87 MG cells that has the ability to form “non-physiological tumorspheres” *in vitro* has also been found and characterized (110).

We were able to verify the existence of this sub population in the U-87 MG cell line available to us by staining for the stem cell marker CD133 [Figure 16].

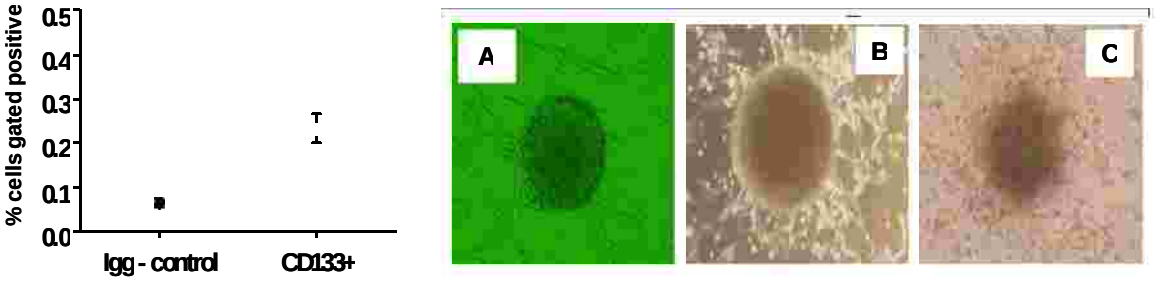


Figure 16: U-87 MG sub-population positive for stem cell marker CD133 in a non CSC-enriched *in vitro* culture and the morphology of *in vitro* tumor stem cell spheres via light microscopy. (A) Primary U-87 MG neurosphere grown in culture "non-physiological" as reported by Shi-Cang Yu et al.(110) were morphologically comparable with our tumorspheres formed from U-87 MG cells in non-selective medium (B). (C) Advanced oncolysis of a U-87 MG sphere 6 days post *in vitro* infection with Ad5/35.IR-E1A/TRAIL at an MOI of 1.

We detected a subpopulation of approximately 0.25% cells expressing the marker CD133 within *in vitro* cell cultures with FBS and a non CSC-enriching setting. Growth of low passage, low density U-87 MG cells in cell culture lead to the *in vitro* growth of tumorspheres morphologically identical to the cancer stem cell neurospheres as reported by Shi-Cang Yu et al. [Figure 16].

In vitro infection of these neurospheres with wild-type adenoviruses result in no visible cell lysis within the tumorspheres 6 dpi while cells infected with Ad5/35.IR-E1A/TRAIL at the same MOI exhibit clearly apoptotic features and cellular lysis of the tumorspheres cells as compared to uninfected, or wild-type infected cells [Figure 17].

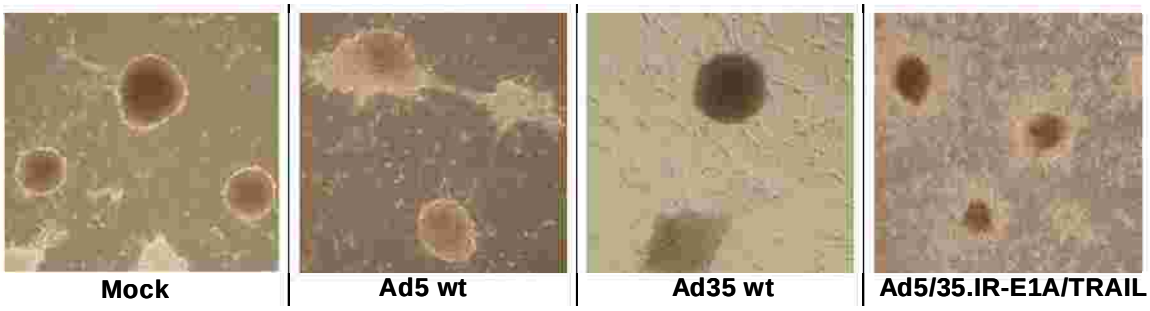


Figure 17: U-87 MG neurosphere infected at an MOI of 1 with wild-type virus serotype 5 (Ad5 wt), wild-type virus serotype 35 (Ad35 wt) or the Ad5/35.IR-E1A/TRAIL vector 6 days post infection. Wild-type infected cells show no signs of significant cell killing. Only Ad5/35.IR-E1A/TRAIL has a significant oncolytic impact on the tumorspheres compared to mock infected cells.

4.5 Ad5/35.IR-E1A/TRAIL shows an enhanced spread in U-87 MG plaque assays compared to wild-type adenovirus

In addition to the observed enhanced cell killing effect *in vitro* we wanted to examine if the shifted tropism of the vector Ad5/35.IR-E1A/TRAIL in combination with the apoptosis inducing transgene leads to an enhanced viral spread in GBM tissue compared to either Ad serotype 5 wild-type or Ad serotype 35 wild-type which features the same receptor but lacks the expression of an oncolytic transgene.

U-87 MG cells were infected with approximately 50 to 100 pfu of each virus and an agarose overlay was added to limit newly generated particles to localized cell-to-cell spread. The resulting plaque development of infected cells was observed over several days [Figure 18].

After 9 days most U-87 MG cells initially transduced with Ad35, featured plaques that were slightly enhanced in size compared to the serotype 5 wild-type which can be attributed to the lack of the primary receptor CAR. The absence of the primary receptor leads to an inhibition of the viral spread in case of Ad5 although it does not inhibit viral transduction completely.

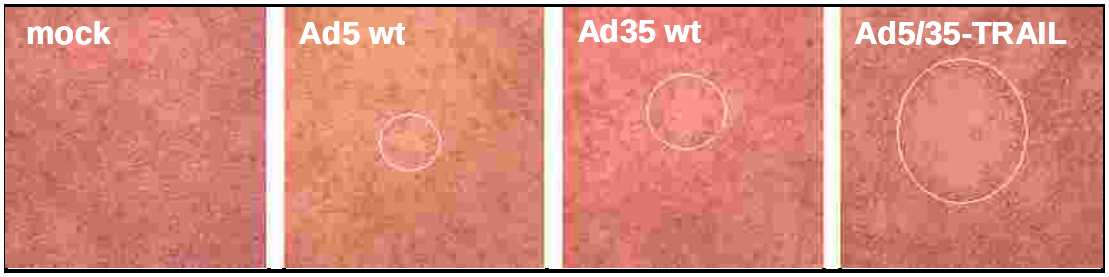


Figure 18: Plaque assay. Confluent U-87 monolayers were infected with 50-100 pfu of wild-type Adenovirus serotype 5 or 35 (Ad5 wt, Ad35 wt) or Ad5/35.IR-E1A/TRAIL (Ad5/35-TRAIL). The cells received a 1% agarose overlay and the plaque development was monitored for 9 days. Plaques resulting from Ad5/35.IR-E1A/TRAIL infection were approximately 3-6 times larger compared to wild-type virus plaques.

However, Ad5/35.IR-E1A/TRAIL showed plaques sized 3-6 times larger compared to those infected with Ad5 or Ad35. This indicates that after human GBM transduction TRAIL expression and resulting apoptosis leads to fast cell lysis and release of *de novo* produced viral particles which, in turn, accelerates the viral spread within a GBM cell monolayer *in vitro*.

This correlates with former reports that TRAIL expressing adenoviral vectors have the ability to release *de novo* produced virus from infected cells *in vitro* and cause a significant bystander effect and verifies that this is also a given feature in human glioblastoma cells (77).

4.6 Ad5/35.IR-E1A/TRAIL mediates enhanced induction of apoptosis in human GBM cells compared to adenoviral wild-type virus.

Cell killing assays did prove that the human GBM cell lines U-87 MG, T98G, and SF767 are sensitive to Ad5/Ad35.IR-E1A/TRAIL. In order to assess if the cell killing can be attributed to TRAIL-mediated apoptosis, infected cells were examined with a TUNEL assay at fixed time points after primary transduction [Figure 19].

The TUNEL assay is an *in situ* cell death detection assay that detects cells undergoing apoptosis by labeling free 3'-OH termini of the cellular DNA fragments that are a result of the DNA fragmentation during the final phase of apoptosis.

In addition to examining the apoptosis-inducing potential of the Ad5/35.IR-E1A/TRAIL, a direct comparison of the effect of the Ad5/35 capsid as a gene delivery vehicle with wild-type serotype 5 was to be tested. For this, it was necessary to compare the vector Ad5/35.IR-E1A/TRAIL with Ad5.IR-E1A/TRAIL which expresses TRAIL in the same manner as Ad5/35.IR-E1A/TRAIL but does not feature the Ad35 fiber and therefore relies on CAR as the primary receptor.

To monitor the apoptotic features of a wild-type virus lacking any oncolytic transgenes, the adenoviral serotype 35 was used. The cell lines were infected with an MOI of 1 pfu/cell and observed for 4 days for virus-mediated induction of apoptosis. We found that 25% to 50% of the cultured human GBM cells from SF767 or U-87 MG were positive by TUNEL assay 4 days after infection with Ad5/35.IR-E1A/TRAIL or Ad5.IR-E1A/TRAIL, whereas wild-type virus showed no significant increase in TUNEL-positive cells compared with mock-infected GBM cultures.

Elevated levels of apoptosis were detectable in T98G cells after infection with Ad5/35.IR-E1A/TRAIL but not Ad35 or Ad5.IR-E1A/TRAIL. Direct comparison of Ad5.IR-E1A/TRAIL and Ad5/35.IR.E1A/TRAIL via TUNEL assay shows that, in SF767 and T98G, 20% to 30% more cells seem apoptosis-positive 4 days after infection with Ad5/Ad35.IR.E1A/TRAIL, whereas this difference can be already observed after 2 days in U-87 MG cells.

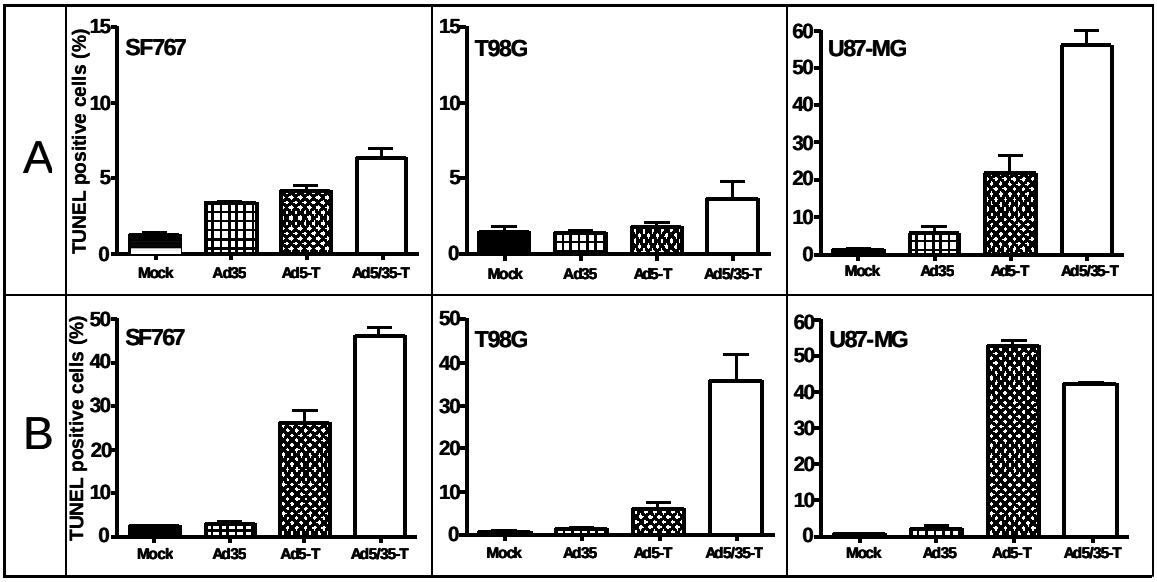


Figure 19: Apoptosis induction in human glioblastoma cell lines SF767, T98G, and U-87 MG after infection with Ad5/Ad35.IR-E1A/TRAIL (Ad5/35-T), Ad5.IR-E1A/TRAIL (Ad5-T), or Ad35 with an MOI of 1 pfu/cell. Wells of each cell line were harvested 48 h post infection (A) and 96 h post infection (B). Induced apoptosis was detected via visualization of occurred DNA fragmentation by FITC labeling of free 3'-OH termini with an *in situ* cell death detection kit. The cells were analyzed using flow cytometry.

4.7 Comparison of the transduction abilities of chimeric adenoviral vectors composed of the serotye 5 capsid with either serotype 35 or serotype 11 fiber protein

Our findings show that the chimeric Ad5/35 vector with the serotype 35 fiber has superior transduction abilities compared to Ad5 wild-type which is most likely due to a high expression of the serotype 35 receptor CD46 on GBM cells.

However we have also observed that the transduction efficacy of Ad5/35 can vary in different human GBM cell lines. The diminished killing of T98G cells by Ad5/35 in combination with the finding that only half the number of viral copies can be found after transduction compared to other cell lines, support this observation.

Recently it has been shown that adenovirus serotype 11, a group B adenovirus, utilizes

CD46 as well as an unidentified receptor glycoprotein which is is abundantly expressed at high levels on human mesenchymal and undifferentiated embryonic stem cells, as well as on human cancer cell lines (111) [Figure 20]

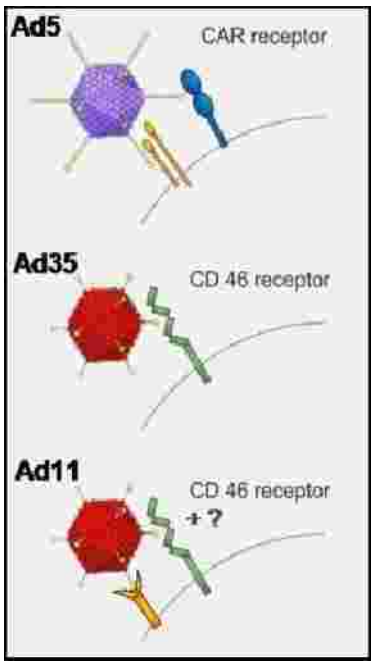


Figure 20: Group III adenoviruses (Ad11p) utilize CD46 alternative target receptor X for binding target cells

Incorporation of fiber protein/knob 11 might improve the transduction efficacy of human GBM cells and most importantly GBM cancer stem cells. Therefore we wanted to compare the transduction abilities of a Ad5/35 vector with a vector composed of the serotype 5 capsid with the fiber protein of serotype 11. For this we transduced the cell lines U-87 MG and T98G
with the chimeric vector Ad5/35 or Ad5/11, both expressing GFP, at MOIs of 0.1 or 1 respectively *in vitro*.

48 hours post transduction the cells were harvested and examined for GFP expression using flow cytometry. Vector Ad5/11 was able to transduce both cell lines successfully and shows a twofold increase of infected U-87 MG and T98G cells compared to Ad5/35 [Figure 21].

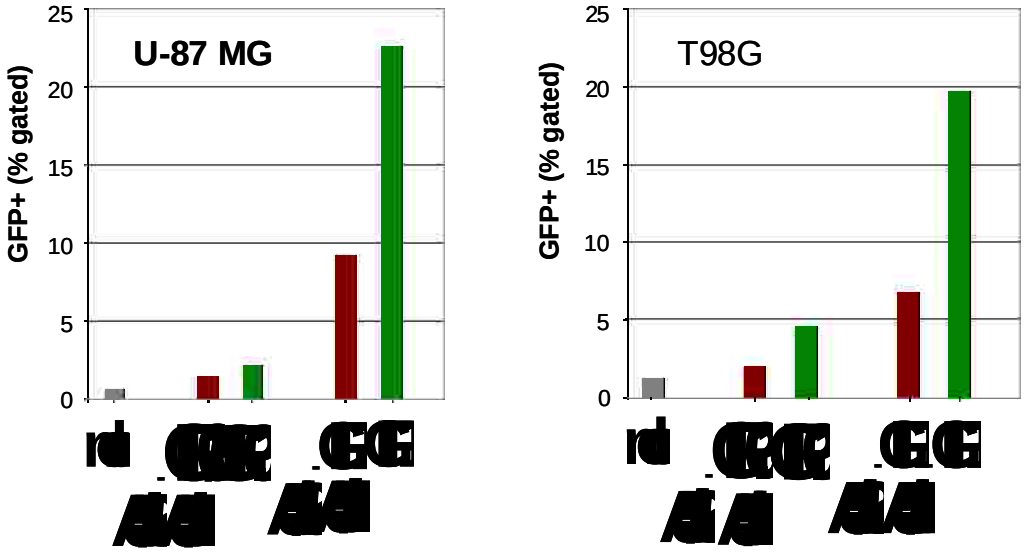


Figure 21: Higher infectivity of Ad5/11 vectors compared to Ad5/35. Human GBM cell lines U-87 MG and T98G were transduced with either Ad5/35- or Ad5/11 vectors expressing GFP at an MOI of 0.1 or 1. Cells were harvested and analyzed for GFP expression via flow cytometry 48 hours post transduction. A two- to threefold increase of successfully transduced cell gated positive for GFP could be observed in case of the Ad5/11 vector.

4.8 *In vivo* test of Ad5/Ad35.IR-E1A/TRAIL by generating a subcutaneous human GBM tumor xenograft model in NOD/SCID mice

For a tumor *in vivo* model human U-87 MG GBM cells were mixed with solubilized basement membrane (Matrigel^{TM}) and subcutaneously injected into the flanks of NOD/SCID mice. This did result in the subcutaneous growth of human GBM tumors of solid and mostly of elliptical shape [Figure 22] .

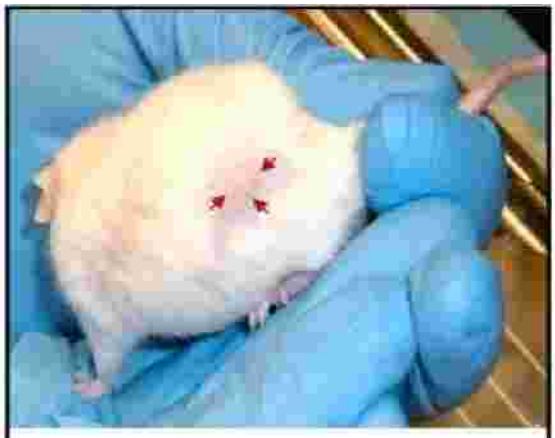


Figure 22: Tumor xenograft model. A NOD/SCID mouse 4 days after subcutaneous injection of U-87 MG cells in Matrigel (red arrows). The tumor grows subcutaniously and is easily accessible for sizing and further manipulation .

The model was used first to evaluate if intratumoral injection of Ad5/35.IR-E1A/TRAIL leads to infection of tumor tissue, viral spread and TRAIL-mediated induction of apoptosis *in vivo*. Therefore Ad5/35.IR-E1A/TRAIL or Ad35 wild-type were injected directly into subcutaneously growing tumor xenografts of U-87 MG cells in NOD/SCID mice.

Two weeks after viral injection the mice were sacrificed and the tumor tissue was extracted, embedded in OCT-media and frozen. Tumor cryosections were then examined for areas of apoptotic tissue around the application site using the TUNEL assay [Figure 23].

Apoptotic tissue areas were found in tumors injected with Ad5/35.IR-E1A/TRAIL and Ad35 wild-type, while tumors injected with PBS showed no significant apoptotic/necrotic areas. Viral capsid hexon proteins were detected in the apoptotic areas using a goat α Ad-hexon capsid protein antibody. Viral capsid protein was found within the apoptotic sections of the dissected tumor tissue in case of Ad5/35.IR-E1A/TRAIL and Ad35 indicating that the induction of apoptosis in these regions was caused by the viral infection. In direct comparison, the tissue samples of the Ad5/35.IR-E1A/TRAIL vectors showed large apoptotic areas with elevated amounts of viral hexon capsid protein in case of Ad5/35.IR-E1A/TRAIL compared to tumor samples injected with Ad35.

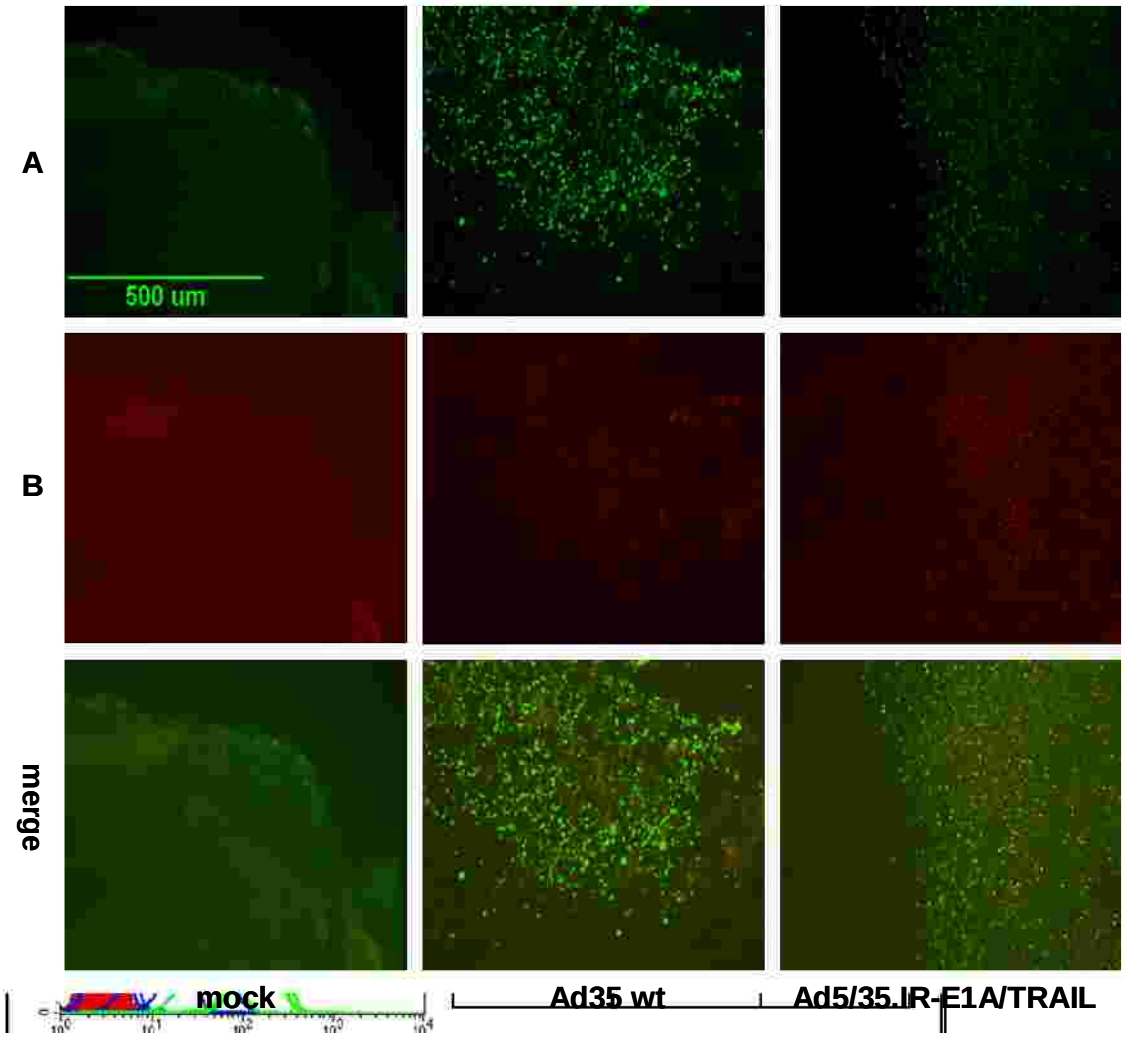


Figure 23: Induction of apoptosis in xenografted GBM tumors after intratumoral (i.t.) Ad5/Ad35.IR-E1A/TRAIL application. U-87 cells (2 x 10^6) were mixed with Matrigel and injected subcutaneously (s.c.) into flanks of NOD/SCID mice. After tumors reached a size of 100 mm^3, 1 x 10^8 pfu of the oncololytic adenovirus Ad5/Ad35.IR-E1A/TRAIL or Ad35 was injected i.t. Mice were sacrificed 2 weeks after viral application. The tumors were embedded in OCT and frozen. Dissected tumor tissue was analyzed for apoptosis/necrotic tissue using an *in situ* cell death detection kit (TUNEL assay, Roche; A) and viral hexon capsid proteins using goat a-adenovirus hexon antibody (B).

4.9 Subcutaneous xenografts in NOD/SCID mice using human GBM U-87 MG treated with Ad5/35.IR-E1A/TRAIL *ex vivo* show a significant growth inhibition

After it was established that Ad5/35.IR-E1A/TRAIL was capable of infecting human GBM cells *in vivo* which resulted in viral spread and induction of apoptosis, the *in vivo* efficiency of Ad5/35.IR-E1A/TRAIL to cause reduction of tumor growth or even tumor recession had to be tested.

In a first experiment U-87 MG cells were infected with Ad5/35.IR-E1A/TRAIL, Ad5/35.IR-E1A/GFP, Ad5 or Ad35 *in vitro* using an MOI of 100 to ensure a 100% infection rate. 1 x 10^6 uninfected U-87 MG cells were mixed with 1% infected cells and injected subcutaneously into NOD/SCID mice. The s.c. growth of the tumors was observed via size measurement every other day after injection of the cells. The size of the tumors containing virus was compared to the tumor growth of 1 x 10^6 untreated U-87 cells [Figure 24].

The tumors containing cells infected with Ad5/35.IR-E1A/TRAIL started to show growth impairment 8 days after subcutaneous injection with 40% less tumor volume compared to the negative control. The average growth of the tumors treated with Ad5/35.IR-E1A/TRAIL remained reduced with approximately 40-50% volume size compared to the untreated tumors for the entire 20 day follow-up after injection. Slight impairment was also detectable in the size of Ad5, Ad35 and Ad5/35.IR-E1A/GFP treated tumors. However,these tumors grew faster compared to Ad5/35.IR-E1A/TRAIL and the growth impairment was not continuous significantly smaller as compared to the mock control in those three groups.

Control tumor size and the size of Ad5/35.IR-E1A/TRAIL treated tumors was analyzed and compared using the Mann Whitney U Test showing that viral tumor growth impairment was significant 8 days after injection ($P < 0.01$, two-tailed test). A significant difference in size among untreated tumors and tumors treated with Ad5/35.IR-E1A/TRAIL was confirmed for the entire 20 day follow-up after injection, while Ad35 and Ad5/35.IR-E1A/GFP treated tumors showed a significantly smaller average tumor

volume only during a period of 4 days ($P < 0.01$, two-tailed test). Ad5 treated tumors showed no significant difference compared to the size of untreated tumors.

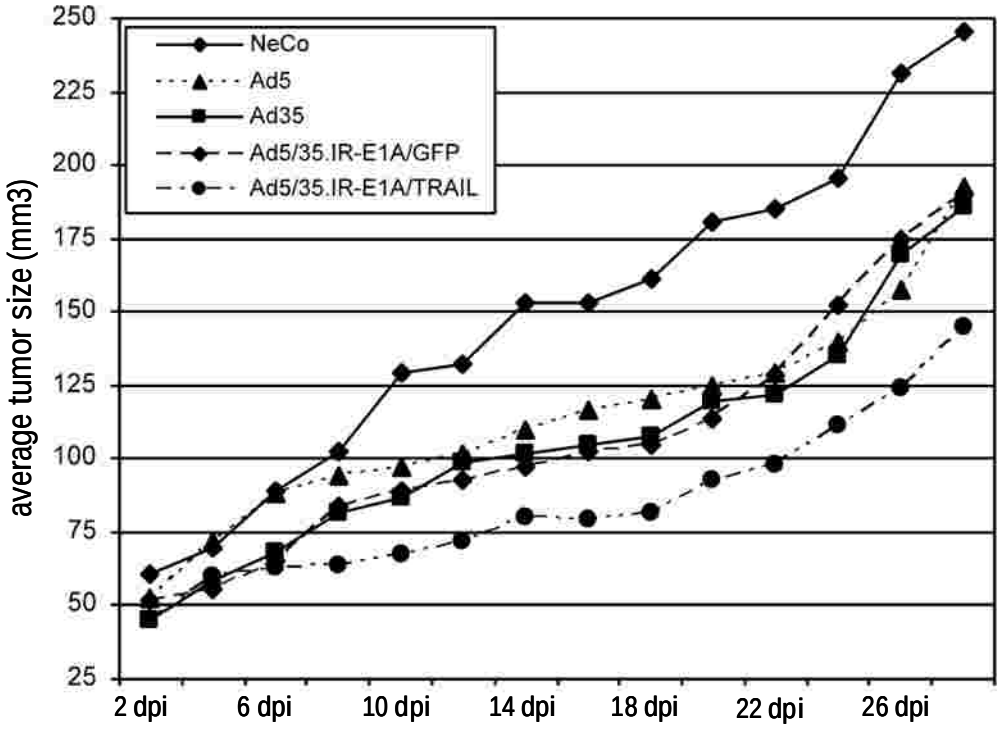


Figure 24: Measurement of the average tumor size after ex vivo virus application and s.c. xenografting in NOD/SCID mice. U-87 MG cells ($1x10^6$) were mixed with 1% cells infected with Ad5, Ad35, Ad5/Ad35.IR-E1A/GFP, or Ad5/Ad35.IR-E1A/TRAIL or uninfected cells as negative control (NeCo) and Matrigel, followed by s.c. injection into the flanks of NOD/SCID mice (n = 10). The tumor growth was monitored using a caliper every other day.

4.10 Intratumoral injection of Ad5/35.IR-E1A/TRAIL into U-87 MG tumor xenografts in NOD/SCID mice *in vivo*

To analyze if direct intratumoral application of Ad5/35.IR-E1A/TRAIL leads to similar effects in regard to tumor growth as observed in an *ex vivo* setting Ad5, Ad35, Ad5/35.IR-E1A/GFP or Ad5/35.IR-E1A/TRAIL virus was injected intratumorally into xenografted U-87 MG tumors in athymic mice and compared to untreated tumors. After s.c. injection of $1x10^6$ U-87 MG cells into both flanks of NOD/SCID the resulting tumor gowth was monitored until the average tumor volume in each group (n = 10) reached 50

mm^3. At this point 1 x 10^8 pfu of Ad5, Ad35, Ad5/35.IR-E1A/GFP or Ad5/35.IR-E1A/TRAIL was injected intratumorally followed by a second injection of 1 x 10^8 pfu 48 hours later [Figure 25].

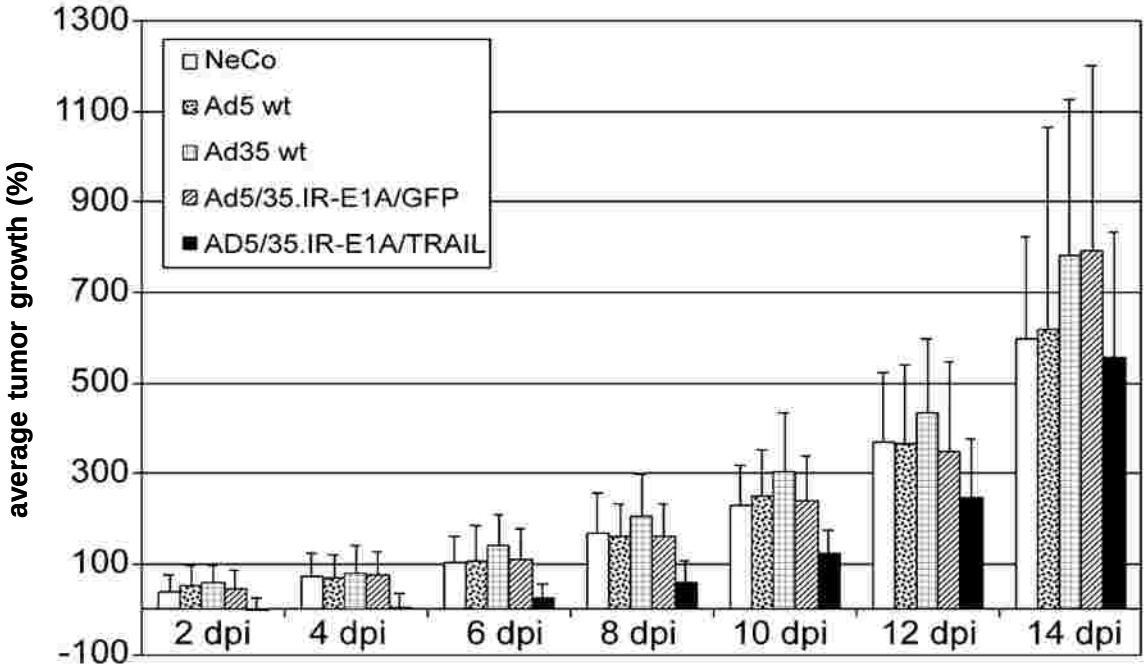


Figure 25: Comparison of tumor average growth after i.t. injections of adenoviral wild-type Ad5, Ad 35, Ad5/Ad35.IR-E1A/GFP, or Ad5/Ad35.IR-E1A/TRAIL into s.c. xenografted U-87 MG tumors. U-87 MG cells ($1x10^6$) in Matrigel were injected s.c. into the flanks of NOD/SCID mice. Tumor growth was monitored using a caliper. After the average tumor volume in each group (n = 10) reached 50 mm^3 (+/-15%), 1 $x10^8$ pfu of Ad5, Ad35, Ad5/Ad35.IR-E1A/GFP, or Ad5/Ad35.IR-E1A/TRAIL or 10 µL PBS as a negative control (NeCo) were injected i.t. Another injection of $1x10^8$ pfu followed 48 h later. The tumor growth was measured every other day using a caliper. Growth of the tumors is illustrated by comparison of the size of each tumor directly after viral treatment (100%) with the size at each following reading point of the tumor. Until day 10 post i.t. injection tumor growth was significantly reduced after Ad5/Ad35.IR-E1A/TRAIL injection ($P < 0.01$, two-tailed test).

While injection with the wild-type virus or the Ad5/35.IR-E1A/GFP virus led to no change in the tumor growth pattern compared to the untreated negative control, Ad5/35.IR-E1A/TRAIL treated tumors grew less than 5% during the first 4 days after the second injection. Furthermore, the injection of Ad5/35.IR-E1A/TRAIL led to an average tumor growth that was significantly lower compared to the tumor growth of xenografts treated

with wild-type virus, Ad5/35.IR-E1A/GFP or untreated tumors until day 10 after viral injections ($P < 0.01$, two-tailed test).

4.11 Human U-87 MG xenografted tumors express CD46 in NOD/SCID mice *in vivo*

To test if CD46 is also expressed at high levels in U-87 MG cells within the tumor *in vivo* human GBM cells were injected subcutaneously in NOD/SCID mice and the tumor xenograft was removed 3 weeks after injection. Tumor tissue was embedded in OCT and frozen. Cryosections were stained using a peroxidase-based visualization kit (EnVision system K1390) [Figure 26]

Sections were incubated with an anti-CD46 (mouse a- human) antibody, an MHC I antibody as positive control or IgG antibody as negative control. As expected, the MHC class 1 antigen was expressed abundantly within the tumor (112) while the mouse IgG Isotype control did not show any non-specific binding. Although the signal strength of the CD46 stained sections showed that MHC class 1 is expressed at higher levels, CD46 is still expressed in clearly detectable levels throughout the entire U87-MG tumor.

Further examination of subcutaneously grown tumors using a lower magnification revealed that the centers of the tumors are largely necrotic, Furthermore staining for factors as CD46 revealed stroma-like areas with excessive amounts of extracellular matrix (ECM) proteins with only few GBM cells included. These stroma structures were found pervading throughout the tumor. This shows that the tissue is not homogenous but that the tumor composition is more likely a center of apoptotic tissue surrounded by dense, invading GBM cells which are in turn divided into sections through areas of ECM proteins.

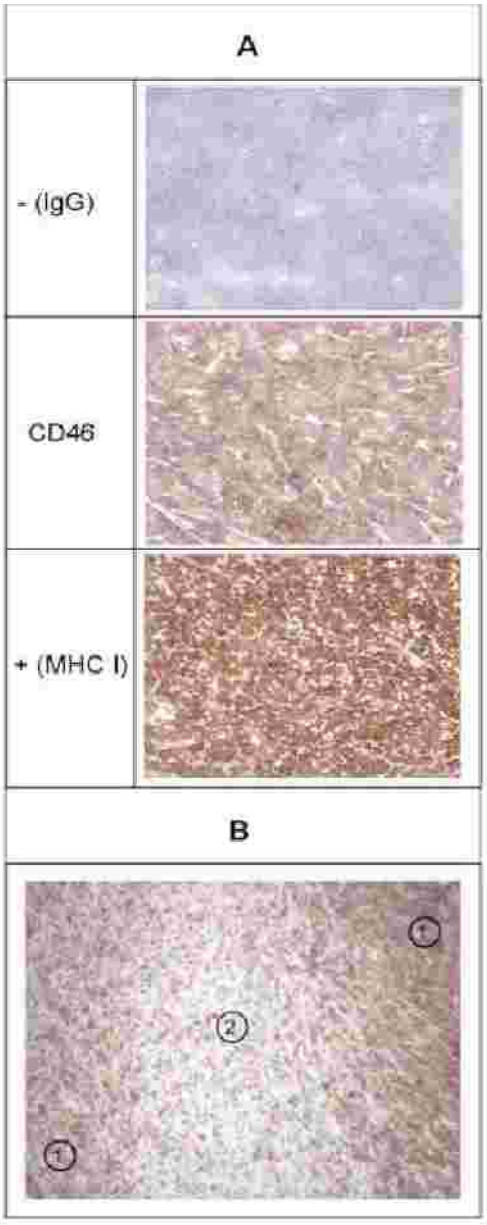


Figure 26: A. U-87 MG tumor sections stained for CD46. OCT embedded cryosections were analyzed for *in vivo* CD46 expression. Sections were incubated with a-CD46 (mouse a- human) antibody, a- IgG as negative control and a- (MHC)I as positive control followed by treatment with chromagen. Staining was performed using Dako "K1390 Envision system, peroxidase" and the slides were counterstained with Gill's (blue).

B. A 10 x magnification of a CD46 stained representive section of GBM tumor tissue. Low magnification reveals a heterologous structure of tumor tissue featuring areas of dense tumor cell layers with abundant CD46 (1) which are pervaded by egions of ECM/stroma protein with only few insular tumor cells (2).

5. DISCUSSION

5.1 Introduction

Here we examined the efficacy of a novel oncolytic adenoviral vector Ad5/35.IR-E1A/TRAIL for the treatment of GBM. We performed a variety of experiments to analyze and compare the effects of Ad5/35.IR-E1A/TRAIL with other adenoviral vectors.

The Ad5/35.IR-E1A/TRAIL primary receptor CD46 was detectable on all three human GBM cell lines. All 3 cell lines showed more expression of the CD46 than the CAR receptor which is used by the Ad5 serotype. [Figure13].

We show that that chimeric vectors were able to transduce all three tested GBM cell lines. Comparison of the two chimeric capsids Ad5/11 and Ad5/35 showed that the transduction efficacy is two fold higher in case of Ad5/11 chimeric vectors in U-87 MG and T98G GBM cell lines [Figure 21]. Our experiments confirmed that utilization of Ad5/35.IR-E1A/TRAIL resulted in an increased transduction rate and oncolytic activity as compared to wild-type virus in all the three GBM cell lines. [Figure 14] .

We found that infection of SF767 and U-87 MG cells with Ad5/Ad35.IR-E1A/TRAIL leads to rapid TRAIL-mediated induction of apoptosis as compared to cells treated with wild-type virus. Specifically U87-MG cells proved to be very susceptible to Ad5.IR-E1A/TRAIL transduction and TRAIL induced apoptosis.

The superior viral oncolytic features of Ad5/35.IR-E1A/TRAIL *in vitro* were also observed in two different experimental *in vivo* settings. Significant tumor growth reduction was observed in case of treatment with Ad5/35.IR-E1A/TRAIL as compared to wild-type adenovirus or Ad5/35.IR constructs without TRAIL expression [Figure24, Figure 25].

5.2 Examination of the primary adenoviral receptor presentation in human GBM

Viral gene therapy is generally limited by the presence of sufficient amounts of the essential viral receptors. Recently the receptor for the adenoviral serotype Ad35 has been shown to be CD46 (77). Examination and comparative quantification of the serotype Ad35 receptor CD46 with the Ad5 wild-type CAR receptor was performed, to shed light on the general surface composition of GBM cells in case of the primary receptors.

The primary receptor levels are of major importance as their surface presentation should correlate directly with viral transduction ability and targeting in vivo while it can be considered advantageous if the expression levels in healthy brain tissue are low to further avoid any viral uptake in non-GBM cells. The serotype 5 primary receptor CAR is expressed during fetal development particularly in brain and muscle (113, 114, 115). Expression is diminishing during the neonatal period and the developing brain has been shown to express increased CAR compared to the adult brain of mice (114). CAR mRNA levels were highest near birth and diminished thereafter. Furthermore CAR can also be detected in epithelial cells of embryonic liver, lung, heart, eye, digestive system, pancreas, kidney, and the submandibular glands (116). A rapid downregulation of CAR occurs at birth, after which CAR is only sparsely detected in most of these tissues as well. CAR is an immunoglobulin-like single-spanning transmembrane intercellular adhesion molecule that has an essential function in development. Besides CAR's role as viral receptor, its physiological and pathological functions remain unclear. It is known that CAR is both a regulator of the Notch signaling pathway (117,118) with roles in embryonic development, and an epithelial intercellular adhesion molecule with reported contributions to mucosal integrity and barrier function (119,120). Furthermore, functional analogies of CAR to E-cadherin or E-selectin have been suggested (116, 121). CAR’s role in tumor engraftment and malignancy and its mechanism underlying this role is still somewhat elusive. Imaging analysis localized CAR in tight junctions of T-84 colon cancer cells (115), or in the adherent junctions in A549 lung cancer cells (116). In case of prostate cancer it has been suggested that high CAR levels diminish with increasing grade of primary prostate cancer while the receptor re-emergences in metastases (122).

But in contrast to this, another study examined breast cancer tissues and demonstrated that elevated CAR expression correlate with an increasing tumor grade (123).

However many groups reported that overall CAR expression seems to be reduced on the cellular surface of malignant tumors of different types of cancers (81, 83+84, 124-126). More importantly it has been shown that mean values of CAR expression in primary GBM tissues are significantly lower as compared to primary lower grade tumors (127). This implies that, in case of GBM, CAR is further down-regulated during tumor progression from low-grade astrocytomas to GBM. Similar findings have been reported for different tumor types and it has been suggested that CAR could be down-regulated due to its adhesive and tumor-suppressive functions (128, 129). This correlates with our observation of general low expression of the CAR receptor in case of all examined GBM cell lines. This does suggest the down-regulation of CAR as a potential tumor suppressor in human GBM. These findings render human GBM as a high grade glioma unattractive for treatment with the commonly used Ad5 vector due to the lack of the primary receptor for this serotype on high-grade glioma.

On the other hand all examined cell lines were strongly positive for CD46, comparable with findings in other tumor cell lines (21). As opposed to CAR, the function of the complement regulatory protein CD46 as a membrane-bound complement inhibitor is well understood. The type I membrane protein is a regulatory part of the complement system and has cofactor activity for inactivation of complement components C3b and C4b by serum factor I, which protects the host cell from damage by complement (130).

CD46 is frequently overexpressed in tumor cells, serving as a mechanism of tumor cell protection against complement-mediated lysis (131+132). Membrane-associated complement regulatory proteins, such as CD46 play an important role in cellular self protection as they have the ability to render cells insensitive to the action of complement. It has been shown that resistance of tumor cells to complement-mediated lysis depends on these complement regulatory proteins which appear to be over-expressed on a variety of tumors and provide tumor cells with extracellular protection. Therefore CD46 can be found in high levels on a variety of tumors including human glioma. In normal brain tissue and the blood–brain barrier CD46 has been reported to be ubiquitous detectable, although only in low levels (133, 1134). This does make CD46 a good

candidate as a primary receptor for an oncolytic virus since a comprehensive source of the primary receptor targets the vector to GBM cancer cells while spreading from the tumor site is impeded by low levels in healthy tissue surrounding the tumor tissue.

Different attempts have already been made to identify viruses that preferentially infect tumor cells that overexpress receptors for virus entry or to genetically engineer viral vectors with those abilities (135). With our Ad5/35.IR vector we reach this goal by utilizing the fiber protein of an adenovirus that binds to CD46 in order to create modified serotype 5 capsids to change the adenoviral vector tropism in order to circumvent the observed CAR deficiency of GBM.

Furthermore it has been shown that vectors utilizing CD46 as cellular receptor, transduce malignant tumor cells like LoVo more efficiently compared to wild-type virus (77). In addition the shifted tropism of the vector Ad5/35.IR-E1A/TRAIL has already been proven to enhance the viral oncolytic effect of the virus *in vitro* (77).

5.3 GBM transduction by Ad5/35.IR-E1A/TRAIL *in vitro* and *in vivo*

In vitro transduction of GBM cell lines with low MOIs showed that chimeric vectors were able to transduce the three tested GBM cell lines. However, since cell lines exhibit different sensitivities to Ad5/Ad35.IR-E1A/TRAIL-mediated cell killing we assume that the transduction ability of the vector does vary. Infection with wild-type Ad5 resulted in no or only little cell killing in GBM monolayers *in vitro*. This was expected because it has been reported that the Ad5 virus does not transduce neural cells effectively (38). Interestingly, we did observe some transduction and cell killing in case of U-87 MG and T98G cells with Ad5 even though we were not able to detect any CAR expression in these cell lines [Figure 13] .

This indicates that the primary receptor expression pattern might not result in a particular difference in case of *in vitro* experiments as even without the primary receptor, serotype 5 capsids seem to be able are able to directly interact with cellular avß3/avß5 integrins and become internalized. This would explain why cell killing can be observed after wild-

type serotype 5 transduction of U-87 MG or T98G cells, even though they do not seem to feature the CAR receptor. This correlates with a previous *in vitro* study showing that primary receptor levels do not translate into correspondingly higher transduction efficiencies in case of Ad5/35 chimeric vectors (136).

An interesting observation supporting this theory has been the inefficient cell lysis of the serotype Ad35 and the vector Ad5/Ad35.IR-E1A/GFP in T98G cells and the reduced cell killing of U-87 MG and SF767 cells compared directly to Ad5/35.IR-E1A/TRAIL [Figure 14]. This does suggest that the increased oncolytic ability of the Ad5/35.IR-E1A/TRAIL vector is not caused by the use of the chimeric Ad5/35 capsid but that the apoptosis-inducing gene TRAIL plays a key role in cell killing of GBM cells after infection with Ad5/35.IR-E1A/TRAIL. *In vivo* tests of viral tumor penetration after *ex vivo* transduction on the other hand showed that the primary receptor is of importance for the viral vector as serotype 5 vectors showed the least effect on tumor growth in both murine xenograft models [Figure 24, Figure 25]. In fact only a vector with the chimeric 5/35 capsid and TRAIL expression was able to show a significant impact after *in vivo* application.

Therefore our findings indicate that the vector Ad5/35.IR-E1A/TRAIL, utilizing the Ad35 fiber protein, features an evident advantage over adenoviral vectors that use the serotype 5 fiber concerning the treatment of glioblastoma cells *in vivo* due to its enhanced ability to infect human GBM cells.

The outcome of comparison between subgroup B fibers of serotype 35 and serotype 11 by comparing Ad5/11 and Ad5/35 chimeric capsids [Figure 21] suggests that it could be beneficial to create and utilize an Ad5/11 oncolytic construct for future studies of the vector as it seems to be the most adequate vector for GBM transduction.

Both serotypes utilize the same receptor CD46 as a high-affinity attachment receptor (137). Although both serotypes have an equivalent CD46 binding efficiency, Ad11 features the additional opportunity to utilize the alternative receptor X, a yet unidentified receptor, via its fiber knob (138). Receptor X is expressed at high levels on human mesenchymal and undifferentiated embryonic stem cells, as well as on human cancer cell lines. It is of particular interest that Ad11 can use two receptors that are both present at high levels on tumor cells which potentially reduces the risk of escape mutants

development by receptor-downregulation. However a recent study showed that Ad5/11 vectors were inferior to Ad5/35 chimeric vectors in pancreatic and breast cancer (139). Furthermore a comparison of transduction abilities from different adenoviral subgroups have proven that even if the particles bind to the same receptor *in vitro*, Ad serotypes prove act differently *in vivo* (140). Therefore it will be necessary to compare directly Ad5/35 chimeric vectors with Ad5/11 constructs in a GBM tumor model *in vivo* to evaluate transduction- and tumor penetration abilities.

5.4 TRAIL mediated oncolysis of GBM by Ad5/35.IR-E1A/TRAIL

Another important feature of the adenoviral vector is the expression of TRAIL. The utilization of TRAIL in different therapeutic approaches has been shown to induce apoptosis in a broad spectrum of different cancer types (141-143).
TRAIL has been shown to be a potent inducer of apoptosis in tumor cells which are far more susceptible to TRAIL than normal cells. Furthermore TRAIL mediated induction of apoptosis is independent of *p53* expression or functionality. This is an important feature as p53 can be inactivated in human GBM tumors (144). TRAIL molecules do not induce severe toxicities as for example expression of the Fas ligand CD95L does which is an additional advantage with regard to the usefulness of the transgene in a clinical setting *(145). In* case of glioblastoma TRAIL has been shown to remain effective as a non-modified ligand in the absence of any neurotoxicity in an *in vivo* rodent model (146). The extensive TRAIL research with mainly promising results in case of GBM treatment indicates that this molecule can make a positive clinical contribution. In fact, translation of the preclinical TRAIL studies into the clinic has started already (147).

TRAIL expression using the Ad5/35.IR-E1A vector has also been proven to lead to effective oncolysis of human colon carcinoma cells in vitro compared to wild-type virus and control vectors (77). In this study our experiments confirmed that Ad5/35.IR-E1A/TRAIL resulted in an increased transduction rate and oncolytic activity in all the three GBM cell lines while Ad5 had no or only little impact on infected GBM monolayers [Figure 14]. The vector showed a similarly enhanced effectiveness in oncolysis as seen in human colon carcinoma cells.

Ad5 transduction resulted in no or only little oncolysis due to its poor transduction abilites (148). At the same time serotype Ad35 and the construct Ad5/35.IR-E1A/GFP showed also efficient cell lysis of T98G cells and only reduced cell killing after infection of U87-MG and SF767. This observation suggests that the elevated oncolytic ability of Ad5/35.IR-E1A/TRAIL is not mainly due to the composition of a serotype 5 capsid with serotype 35 fiber proteins but that the major factor of the efficient cell killing is the expression of the apoptosis inducing transgene TRAIL. We hypothesize that the adenoviral expression leads to intracellular levels TRAIL capable of triggering the apoptosis mechanism eventually. This correlates with the hypothesis by Rieger et al. who found that gliomas preferentially express the agonistic receptors but only small amounts of TRAIL itself. From this they conclude that the presence of ligand and receptor at the cell surface is not enough to result in significant levels of apoptosis in *tumors (149).* However intracellular overexpression of TRAIL ,as it is the case in cells transduced with Ad5/35.IR-E1A/TRAIL, leads to sufficient TRAIL levels to tip the balance and eventually induce apoptosis in the infected human GBM cell.

We also observed a difference in the cell killing kinetics between the different cell lines which might have been caused by different expression levels of the cognate receptor DR4 as described elsewhere (150). In fact multiple factors that lead to TRAIL resistance via upregulation of inhibitors, like expression of anti-apoptotic proteins, have been reported in a variety of different cancer types (151,152).

Since we observed that infection of the fairly apoptosis-resistant cell line T98G with a higher MOI showed improved cell killing abilities of the wild-types and Ad5/35.IR-E1A/GFP but only marginally accelerated oncolysis of the vector expressing the transgene TRAIL, we come to the conclusion that impaired viral transduction is not the main reason leading to the observed resistance. This rather suggests that the oncolytic effect of a TRAIL-expressing viral vector is not proportionally enhanced by the elevation of viral titers. In fact, T98G resistance to TRAIL might be the main reason for the observed effect as it has been shown that the T98G cell line features strong phosphorylation of p68 which mediates resistance to TRAIL by procaspase-8 cleavage inhibition and furthermore causes cell proliferation, and epithelial–mesenchymal transition (EMT) (153).

In situ detection of apoptosis induced in SF767 and U-87 MG cells by Ad5/35.IR-E1A/TRAIL using a TUNEL assay supported the findings from the cytotoxicity assay [Figure19]. Separate studies have shown that administration of human TRAIL induces apoptosis in human GBM cells (154,155),. Here we show via i*n situ* detection of apoptosis using a TUNEL assay that TRAIL expression in an adenoviral context leads to induction of apoptosis in all tested human GBM cell lines. Altogether we observed that infection of SF767 and U-87 MG cells with Ad5/35.IR-E1A/TRAIL leads to rapid induction of apoptosis compared with cells treated with the wild-type Ad35.

These findings show that replication of wild-type adenoviruses alone has only a minor effect on apoptosis induction and cell killing in human GBM cells whereas the additional expression of the transgene TRAIL significantly increases apoptosis. Comparison of the apoptosis inducing features of Ad5/35.IR-E1A/TRAIL and Ad5.IR-E1A/TRAIL show that the switch to the serotype 35 fiber protein generally enhances the transduction efficiency of glioblastoma cells as it was to be expected from the primary receptor levels we found on human GBM.

5.5 Enhanced viral spread of Ad5/35.IR-E1A/TRAIL *in vitro* and *in vivo*

It has been demonstrated that Ad5/35.IR-E1A/TRAIL after completion of virion assembly facilitates the release of de novo produced virus from infected cells in HeLa, 293 and transformed human embryonic kidney cells *in vitro* which indicates the possibility of enhanced viral spread in tumors *in vivo* (77,156).

To test for similar effects in human GBM cells, U-87 MG cells where transduced *in vitro* with wild-type virus and the Ad5/35.IR-E1A/TRAIL vector. We observed that infection of monolayers followed by the addition of an agarose overlay leads also to larger plaque formations compared to the wild-type in U-87 MG cells after Ad5/35.IR-E1A/TRAIL infection [Figure 18]. This indicates a change of the kinetics of the infection cycle in GBM due to the expression of TRAIL. The significantly enhanced spread in tumor cells *in vitro*

in human glioblastoma cells can be explained by the release of *de novo* produced viral particles due to TRAIL-mediated apoptosis comparatively early in the adenoviral infection cycle, which in turn leads to quicker spreading of viral particles.

To examine if the enhanced viral spread of Ad5/35.IR-E1A/TRAIL *in vitro* can also be observed *in vivo* after injections of CD46-dependent Ad35 wild-type virus and Ad5/35.IR-E1A/TRAIL, 1 x 10^8 pfu of the oncolytic adenovirus Ad5/Ad35.IR-E1A/TRAIL or Ad35 was injected intratumorally into subcutaneously xenografted U-87 MG tumors [Figure 23].

The tumor was removed 2 weeks after injection, dissected and analyzed for apoptosis by TUNEL assay and additionally for viral capsid proteins via an anti- adenovirus hexon antibody. Detectable expression of adenoviral capsid proteins and identification of apoptotic cells in the same areas of microdissected tumor tissue via TUNEL assay showed that the viral infection spreads efficiently and that the virus is able to induce apoptosis in infected cells *in vivo*. The large apoptotic areas we found and the detection of higher amounts of viral capsid proteins compared to Ad 35 infected tumor tissue indicates that the TRAIL bystander effect could not only be a detectable factor *in vitro* but also be responsible for a significantly enhanced viral spread and enhanced capability of tumor tissue penetration after application of Ad5/35.IR-E1A/TRAIL in GBM tumors *in vivo*.

5.6 Treatment with Ad5/35.IR-E1A/TRAIL leads to growth inhibition of human GBM U-87 MG xenografts in NOD/SCID mice

The most obvious difference in the oncolytic potential of the vector Ad5/35.IR-E1A/TRAIL compared to wild-type or Ad5/35.IR-vectors that do not express TRAIL was observed using *in vivo* assays. Virus-mediated enhanced oncolysis and spread have already been shown in an *in vivo* model in which a TRAIL expressing adenoviral vector was applied via tail vein infusion in immunodeficient CB17 mice. Those mice were carrying liver metastases induced by LoVo cell infusion which were significantly reduced after application of Ad5/35.IR-E1A/TRAIL compared to the control vectors (77). Therefore we wished to determine if Ad5/35.IR-E1A/TRAIL infection of human GBM

tumors growing *in vivo* leads to intratumoral spread and tumor growth reduction compared to the growth of untreated or wild-type treated tumors.

To test this in a first model we mixed U-87 MG cells with 1% adenovirally pre-transduced U-87 MG cells prior to subcutaneous injection. Only Ad5/35.IR-E1A/TRAIL showed significant growth impairment over a period of 3 weeks [Figure 24]. We conclude that, as already observed *in vitro,* not the adenoviral vector but the viral TRAIL expression is primarily responsible for tumor growth reduction and tumor invasion.

Direct intratumoral injection of viral particles into xenografted solid U-87 MG tumors in NOD/SCID mice *in vivo* support these findings and show an even more distinct difference between wild-type virus, Ad5/35.IR-E1A - constructs lacking TRAIL expression and Ad5/35.IR-E1A/TRAIL. While injection of Ad5, Ad35 and Ad5/35.IR-E1A/GFP into solid subcutaneous tumors showed no effect after intratumoral injection, Ad5/35.IR-E1A/TRAIL exhibited the ability to nearly inhibit further tumor growth for 4 days after viral injection and reduces tumor growth significantly for 10 days [Figure 25]. We propose that the combined E1A expression with TRAIL does lead to significantly enhanced induction of apoptosis in human GBM tumor tissue and that TRAIL expression of the viral Ad5/35.IR construct leads to a comparatively rapid induction of cell death in solid tumors after *in vivo* application, which enables further intratumoral viral spread. These results correlate with the observations we made after infecting cells *in vit*ro. The ability to spread throughout the tumor seems to be crucial for the impact on the growth of GBM tumors after injection *in vivo*.

While a marginal effect of wild-type virus is detectable after an ubiquitous intratumoral application via *ex vivo* mixing of infected and uninfected cells followed by injection, solid GBM tumors seem not to be efficiently penetrated by adenoviruses of the serotypes 5 and 35 after application via intratumoral injection. This supports our theory that the major advantage of TRAIL expressing adenoviral vectors is the rapid induction of cell death via apoptosis together with an advanced capability of tumor tissue penetration after injection. However the tumor inhibiting effect of Ad5/35.IR-E1A/TRAIL dissipates after a time period of approximately 10 days after injection.

5.7 Discussion of factors that could be responsible for the observed short term effect of Ad5/35.IR-E1A/TRAIL after intratumoral injection

Although a significant oncolytic effect after intratumoral Ad5/35.IR-E1A/TRAIL injection can be detected for over a week. This does show that the viral vector is generally capable of infection, replication and transgene expression *in vivo*. However the tumor reducing effect fades away after 3-4 complete viral replication circles and we do not observe a continuing effect of intratumorally build up particles. Several mechanisms can be discussed that could lead to the short term effect of the oncolytic vector:

5.7.1 *In vivo* emerging tumor cells develop resistance to infection with Ad5/35.IR-E1A/TRAIL or TRAIL induced apoptosis impedes viral spread?

One possibility is that a U-87 MG tumor growing *in vivo* shows a different surface membrane expression pattern as monolayers do *in vitro*. We were able to rule this out because dissections of subcutaneously grown tumor tissue did show strong expression of the primary receptor CD46 *in vivo* [Figure 26] , demonstrating that reduced viral effect due to "*in vivo* fading" of the viral receptor is unlikely.

Human GBM has been found to have a multitude of resistance mechanisms that can be developed during gliomagenesis, and the GBM tissue itself becomes highly heterogeneous if growing *in vivo*. As previously discussed in case of the human cell line T98G it has been shown that a strong phosphorylation of p68 mediates resistance to TRAIL (157).

Therefore resistant sub-populations that are less susceptible to TRAIL could emerge and subsequently be not affected by the vector. In fact several further mechanisms that can render a cell less sensitive to TRAIL mediated apoptosis have been shown [Figure 27].
The human inhibitor-of-apoptosis proteins (IAP) binds directly to caspases and inhibits the enzymatic activity that executes the cell death program. IAP gene amplifications and protein overexpression have been found in many tumors including malignant glioma (143,158).

Another caspase inhibitor is the FLICE-inhibitory protein (FLIP) which blocks TRAIL-mediated cell death by inhibiton of caspase-8 activation. In this case the mTOR pathway

mediated alteration of FLIP-mRNA translation leads to the FLIP splice inhibiting variant FLIPs. mTOR in turn is activated by the phosphatidylinositol 3-kinase (PI3K) /protein kinase B (PKB)/Akt. It has been shown that the Akt pathway is highly upregulated in 70% of human gliomas examined (159,160).

Upregulated Akt pathway and/or low levels of pro-caspase 8 in human GBM can lead to TRAIL resistance as factors FLIP and PED/PEA-15 directly compete with procaspase-8 for Fas-associated death domain (FADD) binding. Therefore low levels of procaspase-8 and high levels of the inhibitors PED/PEA-15 and c-FLIP inhibit the formation of the DISC and neutralizes the extrinsic pathway.

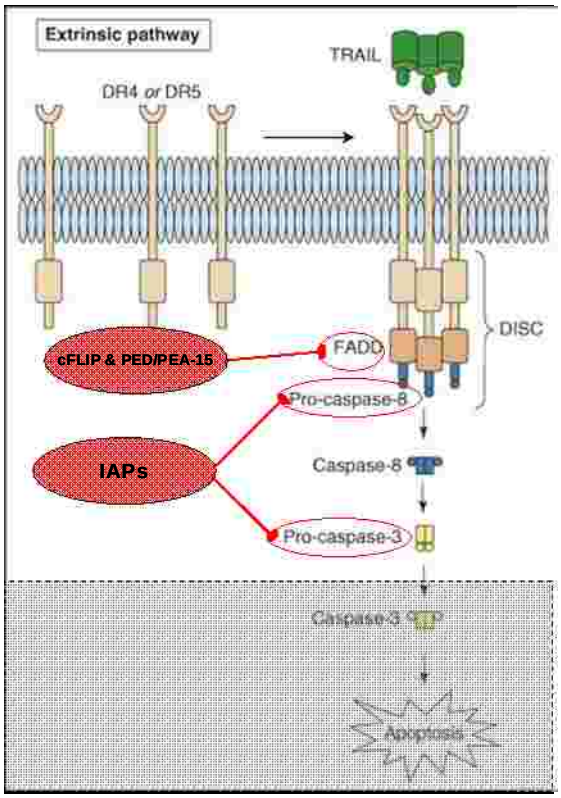


Figure 27: **Human GBM TRAIL resistance mechanisms.** Low levels of procaspase-8 in combination with factors that compete with Fas-associated death domain (FADD) binding disrupts the apoptosis death inducing complex (DISC) formation while inhibition-of-apoptosis proteins (IAPs) bind to pro-caspases and inhibit their enzymatic activation thus stopping the cascade leading to apoptosis.

Although these resistance mechanisms have been previously described we dismissed the possibility of an emerging TRAIL resistance for two reasons. Firstly TRAIL resistance

has been shown in case of T98G, yet the cell line is susceptible for TRAIL mediated induction of apoptosis [Figure 14, Figure 19] as the oncolytic transgene is expressed in high levels intracellular which is most likely to overcome potential cellular adverse effects shifting the balance towards apoptosis. Furthermore we did not see any fast developing resistance in case of subcutaneous tumors where virus was introduced *ex vivo* prior to injection. In this case the oncolytic effect of Ad5/35.IR-E1A/TRAIL had been steady and long term as tumors were still significantly smaller compared to the negative control a month after injection [Figure 24]. Instead, this observation led us to another conclusion:

5.7.2 *In vivo* tumor growth and composition inhibits effective adenoviral spread causing the oncolytic effect to subside

Direct comparison between the long-term effect of *ex vivo* application of Ad5/35.IR-E1A/TRAIL and the intratumor injection of virus sheds light on the possible effect that causes the lack of long-term oncolysis. In case of *ex vivo* application, a mix of uninfected an *ex-vivo* infected cells is injected subcutaneously, causing a significant long term reduction of tumor growth as compared to the mock tumors [Figure 24]. This finding is contradicted by the approach via intratumoral application where a first significant effect after injection diminishes quickly [Figure 25]. The main difference in these two experimental approaches is the form of application of the oncolytic vector. Application of infected and non infected cells leads to a relatively homogeneous spread of Ad5/35.IR-E1A/TRAIL particle producing cells through the resulting tumor. However, viral intratumoral injection exposes only a small section of the tumor to a large dose of virus. In case of the tumors formed with *ex vivo* transduced cells Ad5/35.IR-E1A/TRAIL oncolysis and spreading happens on a multitude of different sites while after injection the main viral load is restricted to the injection site [Figure 28].

Hence, we hypothesize that if Ad5/35.IR-E1A/TRAIL is injected into the tumor the virus is generally able to transduce cells at the injection site leading to the observed oncolytic effect. Our examination of subcutaneously grown tumors revealed that the centers of the tumors are largely necrotic and do not support viral replication while virus is unlikely to diffuse through necrotic areas. Furthermore staining for factors as CD46 revealed stroma-like areas with excessive amounts of extracellular matrix proteins within the tumor [Figure 26]. This shows that the tissue is not homogenous but that the tumor

composition is more likely a center of apoptotic tissue surrounded by dense, invading GBM cells that seem to be divided into sections by large areas of ECM [Figure 26, Figure 29]. We hypothesize that if Ad5/35.IR-E1A/TRAIL is injected into the tumor the virus is able to transduce cells at the injection site leading to the observed oncolytic effect. Followed by this however, virus will reach the observed sites with large amounts of ECM and be incapable to diffuse through these areas. This leads to an end of further tumor penetration and tumor tissue that had been unaffected by Ad5/35.IR-E1A/TRAIL infection is able to reach quickly growth rates that overcome the hampered viral transduction [Figure 29].

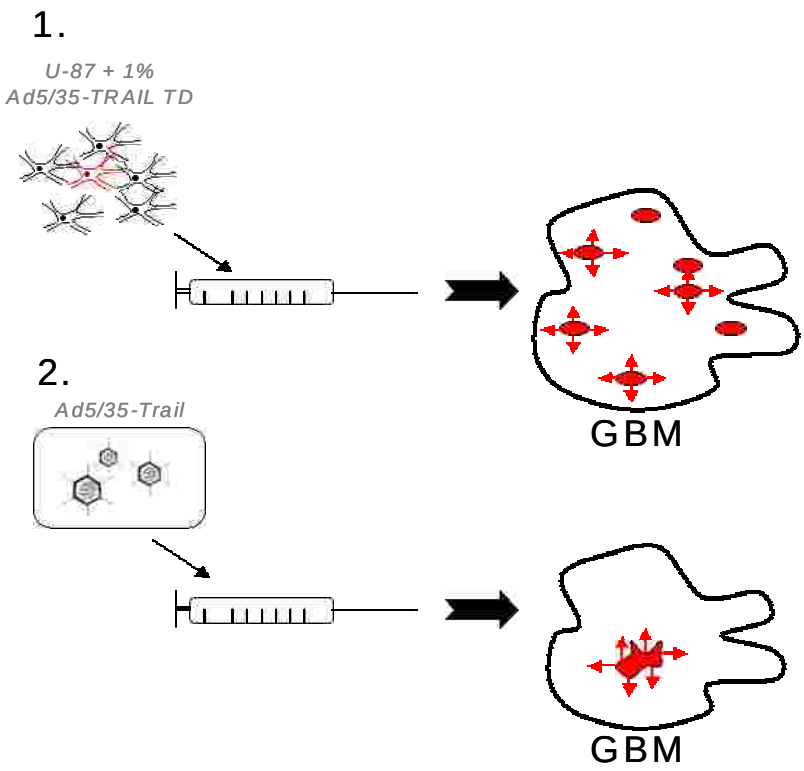


Figure 28: Viral spread in the applied *in vivo* tumor models. (1) Transduced cells are mixed and injected leading to a homogenous spread of virus producing cells within in the emerging tumor. (2) Intratumoral injection leads to a defined spot of viral delivery which is the starting point for viral tumor penetration.

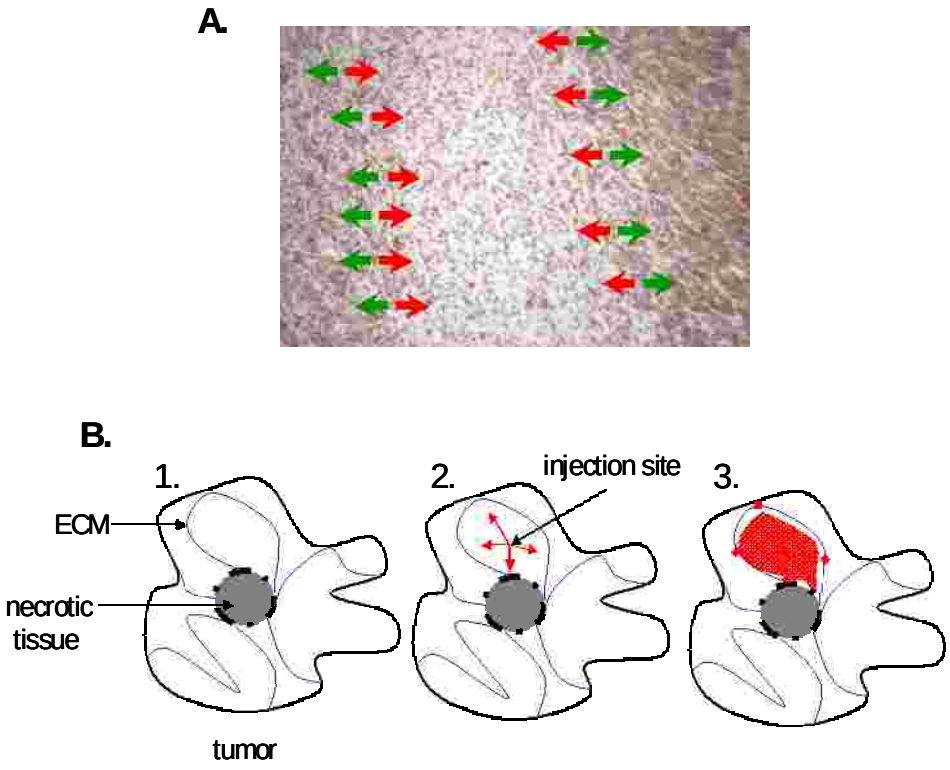


Figure 29: Failure of viral tumor penetration due to intratumoral extracellular matrix stroma structures. (A) U-87 MG tumor sections stained for CD46 at 10x magnification.. Areas of dense tumor cell layer with abundant CD46 green arrow) are disconnected by regions of ECM/stroma protein (red arrows) . (B) Suggested consequence of this observation: subsiding oncolytic effect due to interrupted viral spread by "ECM-compartimentation". (1) Tumor features necrotic core and compartimentation by stroma like sections of ECM protein with only few embedded GBM cells. (2) Ad5/35.IR-E1A/TRAIL injected into the tumor begins to spread and lyses infected cells around the injection site until (3) virus can not diffuse through ECM compartments or the necrotic core stalling further intratumoral viral activity.

5.8 Outlook

5.8.1 Current limitations of oncolytic therapy for GBM

In the 1990s, experiments with selectively targeted vectors for human glioma where performed using retroviral vectors. Limited by low titers and unstable virus particles the use of virus producing cells (VPCs) instead of direct viral injection into the brain had to be used (161,162). Unfortunately, the VPCs proved to be short lived and incapable of

migration which limits their usefulness severely (163). Bystander and tumor transduction rates were considerably lower than observed in preclinical studies (162,164, 165).

Adenoviral vectors on the other hand express transgenes at high levels, can be produced in high titers, and infect both dividing and non-dividing cells. Studies comparing either retrovirus producing cells and replication deficient adenoviral vectors efficiency in transducing human glioma tumors found higher gene transfer efficiency and greater survival times with replication deficient adenoviral vectors (166,167).

However clinical trials showed that the amount of adenoviral particles, that can be administrated safely, is limited. While viral vector amounts below 10^{12} particles showed no systemic toxicity, intratumoral injection of 2×10^{12} viral particles resulted in side effects like confusion, hypoatremia and seizures in patients (168, 169). Unfortunately this limit of administrable vector proved to be a serious handicap and the therapeutic efficiency seen with replication deficient vectors showed that these restrictions were indeed essential as studies transitioned from pre-clinical to clinical trials.

This did cause a resurgence of studies using of oncolytic replicating viral vectors as it became clear that their benefits would outweigh concerns about toxicity to normal tissue which had previously limited their study in humans. Although a variety of viruses, namely herpes simplex virus, adenovirus, influenza virus, vaccinia virus, vesicular stomatitis virus, Newcastle disease virus, poliovirus, and reovirus have all been investigated for clinical oncolytic virus therapeutics, only adenovirus and herpes simplex virus have entered a larger number of clinical trials (170, 171).

Among the replicating adenoviral vectors, ONYX-15 has been used in first clinical trials of glioma. In this vector the E1b region was rendered unable to replicate in cells with normal p53 function as the inactivation of p53 is necessary for viral replication, thus allowing the virus to selectively replicate in cancer cells without p53 activity (171). In a first phase one trial the viral toxicity and MTD in resected glioma patent was examined and a maximum dose was not identified with up to 1×10^{10} pfu being well tolerated (172). No systemic toxicity was observed even with elevated levels of anti-adenoviral antibodies in several patients. It has been encouraging to observe that patients who

failed multiple previous treatments, including surgery, chemo- , and radiotherapy, responded to oncolytic therapy with this vector. For multiple types of tumors however even the clinically extensively tested and optimized ONYX-015 vector type showed only limited effects in patients with refractory head and neck cancer (173). Furthermore it has been shown that once virotherapy is discontinued, patients suffered early relapses. Unfortunately it has to be assumed that similar bleak results will be seen in case of clinical trials with ONYX- 015 and GBM as in a first phase one trial patients median survival was only 6.2 months from recurrence (172) .

The results of the clinical trials show that several further improvements in virotherapy are needed to be combined in one oncolytic vector in order to become a viable treatment alternative for GBM patients in the clinic:
The virus needs the ability to selectively replicate and spread in the tumor mass while safely delivering therapeutic genes to target tissues without causing side effects or systemic toxicity even if the vector is applied in high doses. Ideally a therapeutic gene product needs to be expressed in high levels long term to generate effective, durable responses for the cancer patient. It has already been discussed that simply oncolytic capabilities of a viral vector will not be sufficient but that “armed” therapeutic viruses combining the lytic capability of the virus with the capacity to deliver therapeutic factors are necessary to effectively attack the complexity associated with human tumors (174).

The delivery of an oncogene is in turn limited to the transduction abilities of the oncolytic vector. Since widespread distribution of gene therapeutic products is essential for the efficacy, development of vectors which promote targeted high level transduction efficiency is desired. Alas, current virotherapy vectors are not efficient enough to insure infection of even the majority, much less all, of the tumor cells even after direct intratumoral injection. In fact GBM cell transduction and GBM tumor tissue penetration seems to be the limiting factor of current oncoviral therapy.

In this study we also experienced obstacles in case of tumor penetration with our Ad5/35.IR vector as has been reported elsewhere with other viral agents. Lack of intratumoral spreading can be caused by tumor stroma and has been reported in case of different tumor tissues (175). A GBM tumor growing *in vivo* is not just a collection of cells

but consists of necrotic areas, vascular-, and interstitial extracellular compartments. It seems to be extremely difficult for progeny viral vectors to spread through solid tumor as viral particles are not capable of bypassing intratumoral ECM/stroma structures.
Oncolysis and viral spread is eventually stopped by these structures and instead of progress by broad tumor tissue transduction the infection trickles away. Therefore any impact on tumor tissue can only be detected short term after initial transduction.

5.8.2 Research strategies to overcome limitations of oncolytic virotherapy.

To approach the encountered problems in glioma treatment while further improving oncolytic therapy of human GBM, different approaches can be followed in order to compensate for the drawbacks reported in this study and elsewhere.

5.8.2.1 Invasive tumor penetration using carrier cells

It has been shown that GBM transduction after injection of current virotherapy vectors does not insure infection of the majority of cells. While tumor cells directly at the injection site will most likely be transduced, the decline of this effect away from the actual site seems to be rather exponentially. Therefore intratumoral injection into solid tissue or application into a surgery site is not the ideal form of application as the access to GBM cells with primary virus is very limited.

Therefore enhanced tumor penetration by homing cells has been discussed to be suitable for human GBM. Human bone marrow-derived mesenchymal stem cells (MSCs) have been reported to home specifically into the tumor site and so do transgenic Pmel-1 T-cells (176, 177). Transduced cells can home randomly to the tumor site followed by release of progeny oncolytic viral vectors. Application of homing cells without the necessity of direct intercranial access to the tumor is another advantage of this approach as, unlike direct intratumoral injection, the application frequency could be independent from surgical interventions.

First *in vivo* experiments using a rodent glioma model have proven that stem cell application leads to a significant migratory capacity. Furthermore apoptotic cells were increased more than two-fold in animals treated with MSCs that had the ability to express the agents cytosine deaminase and IFNß against (178).

5.8.2.2 Improvement of tumor penetration through degradation of intratumoral ECM-structures

The creation of intratumoral physical barriers formed by stroma proteins seems to be one of the major factors causing the here and elsewhere reported dismal outcomes of *in vivo* experiments and clinical trials in which human GBM has been treated with oncolytic viral vectors. Degradation of these structures might render the tumor more accessible for virotherapy. This hypothesis has been supported by the finding that direct administration of collagenase/dispase into glioma xenografts leads to an enhanced extent of infection of an adenoviral vector expressing a reporter gene (179). This shows that the degradation of tumor stroma does indeed enhance the intratumoral spread in human GBM and might have a significantly positive impact on GBM virotherapy.

Unfortunately direct intratumoral injection of collagenase it is not a likely option in case of GBM treatment of patients due to the limited access to the tumor site and problematic task of locating the tumor invasion sites during surgery. However a localized expression of an ECM degrading factor might lead to the desired effects. Recently, relaxin (Rlx), a peptide hormone that has the ability to degrade stroma proteins intratumorally, has already been successfully utilized for this purpose *in vivo* (180). Transplantation of mouse HSCs transduced with an Rlx- expressing lentivirus vector has been shown to delay tumor growth in a mouse model of breast cancer. We predict that Rlx mediated degradation of tumor stroma after expression by a targeted adenoviral vector should be feasible for GBM. The expression should break up the intratumoral stroma structures without increasing the toxicity of the treatment due to localized expression, thus rendering the tumor more accessible for oncolytic virotherapy.

5.8.2.3 Targeting human GBM angiogenesis

Angiogenesis, the formation of blood vessels from pre-existing vasculature, has been identified as an essential mechanism in tumor growth (181). Human GBM growth and invasion of healthy tissue relies on this mechanism. Thus, interrupting the process of angiogenesis has been widely discussed as a worthwhile target for GMB therapy lately. Angiogenesis is mainly mediated by proangiogenic growth factors such as vascular endothelial growth factor (VEGF) inducing proliferation, migration and tube formation of endothelial cells (182). To inhibit angiogenesis anti-VEGF antibodies (anti-VEGFab) have been developed. It has been already been shown in a series of clinical trials that anti-VEGF antibodies slow tumor growth by obstruction of tumor angiogenesis. However GBM tumors can adapt to anti-VEGFab application, rendering the tumor re-growth irresponsive to further treatment while tumor growth rate and invasion accelerates (183)
.

However another similar important feature for human GBM angiogenesis is the interaction of endothelial cells with surrounding extracellular matrix (ECM) that is mediated by integrins. Integrins are transmembrane receptors composed of two subunits binding to ECM and base membrane proteins (184). Cilengitide, a cyclic pentapeptide mimicking the Arg-Gly-Asp (RGD) binding site, was identified as a potent and selective integrin antagonist (185, 186) that has been shown to inhibit VEGF and bFGF-induced migration and tube formation *in vitro*.

Cilengitide is capable of inhibiting proliferation and differentiation of endothelial progenitor cells playing an important role in neoangiogenesis in cancer (187).

In a recent phase II clinical study Cilengitide monotherapy is well tolerated and exhibits modest antitumor activity among recurrent GBM patients (188). Another high-affinity ligand for several different integrin heterodimers is contortrostatin, a 13.5-kD homodimeric protein isolated from the venom of the southern copperhead snake (Agkistrodon contortrix contortrix). Contortrostatin has also been shown to inhibit tumor growth and angiogenesis while prolonging survival in a rodent glioma model. Moreover, contortrostatin appears to be well tolerated by the animal and lacks neurotoxic side effects (189). Expression of Cilengitide or contortrostatin by a targeted oncolytic

adenoviral vector may have potential as a novel synergistic therapy for malignant gliomas in the future.

5.8.2.4 Treatment of human GBM via immunotherapy

In the previous years there has been a significant effort for effective immunotherapy strategies for glioma that can be combined with common treatment strategies as more and more data suggested that patients with malignant gliomas feature a heavily impaired immune function. This is mainly due to downregulation of T-cell function by immunosuppressive cytokines inhibiting macrophage function (190) and production of proinflammatory cytokines (191) while regulatory T-cells (Tregs) play an important role in suppressing the immune response to the tumor (192). Thus countermeasures to re-activate the suppressed immune system might be of great benefit for the patient and lead to synergistic effects with standard therapy.

One way to reach this goal is the administration of so called “tumor vaccines” . Those “vaccines” consist of genetically engineered cells that express cytokines like interleukin (IL)-2 and IL-4 or (IFN)? in order to artificially stimulate the growth, differentiation and survival of antigen-selected cytotxic T-cells. While IL-2 and (IFN)? secretion has shown severe toxcicity caused by brain edema, Il-4. expression induced an inflammatory response, leading to tumor regression (193). Even though peripheral tumor vaccination can definitely initiate a systemic immune response against intracranial tumors the large amounts of tumor tissues or autologous tumor cell lines are required to generate gene-modified tumor vaccines of a clinical grade may limit the feasibility of whole tumor vaccination strategies.

Therefore direct delivery of cytokine-genes via cationic liposomes does appear to be the more promising approach. In vivo experiments using nude mice implanted with human glioma cells intracranially or subcutaneously revealed that the local administration of cationic liposomes containing the human *IFNß* gene induced apparent tumor growth reduction, prolonged survival and natural killer (NK) cell activation (194) .
In addition, a similar growth-inhibitory effect was also observed in a syngeneic intracranial mouse glioma model treated with the liposome-mediated murine *IFNß* gene.

This gene therapeutic system induced specific cytotoxic T-cell immunity against mouse glioma and the NK cells (195) Long term local, intratumoral delivery of factors initiate a systemic immune response by a conditionally replicating adenoviral vector like AD5/35.IR instead of single administration of liposomes might improve the observed effect.

5.8.3 Improvement of oncolytic therapy of human GBM with Ad5/X.IR vectors

We wish to focus mainly on two aspects to improve human GBM treatment based on the findings of this study: 1) Specific targeting and oncolysis of tumor progression driving tumor stem cells and 2) enhanced viral spread through intratumoral secretion of a new optimized GMB-targeted transgene. For this further modifications to enhance effectiveness of oncolytic vectors will be necessary.

Recently it has been shown that recurrent GBM can arise from small remaining sub-populations of cancer stem cells (CSCs) which are highly resistant to radiation and chemotherapy, e.g. by featuring high levels of the DNA repair enzyme MGMT, which renders the re-growing tumor mass immune to the available standard treatments (196-198). As it has been shown that primary viral transduction capability is of the essence, we believe it is also of great importance to find or create a viral vector that shows the best transduction capabilities of this recurrency driving source of CSCs. Here we show that chimeric vectors utilizing the fiber protein of serotype 11 have a 2 fold enhanced transduction rate compared to Ad5/35 while elsewhere it has already been shown that serotype 3 has an improved viral tropism (199). We believe it could be of importance to create Ad5/3, Ad5/11 and Ad5/35 vectors for comparison of the CSC targeting and expression abilities in human GBM cells in order to find the chimeric combination with the highest internalization efficiency of GBM tumor stem cells *in vitro* and *in vivo.* A modified capsid providing optimized transduction could be essential for the initial viral impact on an emerging tumor from CSCs since any viral spread after completion of the first completed replication cycle is based on this feature.

Other strategies to overcome the limitations as described in this study could be the improvement of the oncolytic effect. We believe that this could be achieved by either a

more potent apoptosis inducing transgene or a GBM targeted transgene that leads to non-viral mediated GBM cell killing post expression after initial transduction by oncolytic vectors. Viral expression followed by release of oncolytic peptides could enhance the bystander effect of the treatment by GBM cell killing in a non-viral mediated manner while the targeting ensures that the toxicity is not increased by undirected diffusion of the transgene. Intratumoral stroma structures on the other hand, which have been shown to have a negative impact on virus mediated GBM treatment in this study, could prove to be permeable and resolvable by peptides expressed after initial viral transduction.

Another approach to enhance viral tumor tissue penetration could be a change of the adenoviral tropism itself. While a complete viral replication circle takes 2-3 days, TRAIL induced apoptosis has been shown to occur hours after initial transgene expression.
This lead us to the conclusion that the fast induction of TRAIL mediated apoptosis in infected cells causes generation of only few infectious units which in turn causes a limited viral spread after initial transduction. The utilization of a transgene that is as reliable in GBM killing as TRAIL but effects the intracellular viral replication later would be desirable. Another opportunity would be the expression of a transgene that would unfold its oncolytic activity after an additional substrate is applied. The expression of the thymidine kinase of the Herpes Simplex Virus followed by application of Ganciclovir is an example for an extensively examined system for the treatment of a variety of cancers including clinical trials with glioblastoma patients in this way (200, 201). The viral thymidine kinase is capable of converting the non-toxic Ganciclovir by phosphorylation which leads to highly-toxic triphosphates that lead to cell death. Human thymidine kinase, in contrast, with its more narrow specificity, is unable to phosphorylate and activate the prodrug. In this way, only cells expressing the viral kinase are susceptible to the drug. A system like this would allow for “timed” cell killing using a pro-drug and therefore give the opportunity to optimize the best time point of infectious particle release. Maximized amounts of infectious particles might lead to improved viral intratumoral spread and furthermore enhance the long term effect of an oncolytic therapy in human GBM.

5.9 Conclusion

We show that the capsid modification of the Ad5/35.IR-E1A/TRAIL vector leads to improved infection while the expression of TRAIL in the adenoviral context and significantly enhances the ability to induce apoptosis in GBM cells *in vitro* and *in vivo*. Our findings show that the commonly used wild-type serotype 5 adenovirus is inferior to Ad5/35 chimeric constructs. Based on these findings we conclude that Ad5/35.IR-E1A/TRAIL is a better vector system for clinical studies with oncolytic adenovirus vectors. Furthermore, wild-type 5 is commonly occurring in humans leading to a immune response to the viral fiber proteins of potential clinical vectors, while subgroup B Ad vectors do not decrease in the presence of anti-Ad5 antibodies, and seroprevalences of most subgroup B Ads are lower than that of Ad5, indicating that clinical use with subgroup B Ad vectors is unlikely to be hampered by preexisting anti-Ad antibodies

Ad5/35.IR-E1A/TRAIL was able to transduce and lyse U-87 MG cells including the cancer stem cells sub-population. U-87 MG tumorspheres where effectively transduced and showed cell killing after Ad5/35.IR-E1A/TRAIL infection. As human GBM stem-like cells are discussed to be a major factor in recurrence of glioma due to their resistance to standard therapy Ad5/35.IR-E1A/TRAIL could become a useful potential therapeutically approach as the safety of the vector allows it to be introduced after surgery with the goal to eradicate stem-cell like tumor cells that do not respond to standard therapy.

The rapid and efficient apoptosis induction in infected GBM cells and the enhanced intratumoral spread together with a significant growth impairment of tumors after injection compared to adenoviral wild-type virus indicate that the adenoviral vector Ad5/35.IR-E1A/TRAIL is generally a potent new approach for a potential viral gene therapeutic treatment of glioblastoma.

Even though we were able to show that Ad5/35.IR-E1A/TRAIL effectively spreads in GBM tissue after injection the effect on tumor growth on human solid GBM was only short term. We believe that the structure of GBM is responsible for the interruption of viral intratumoral penetration. Hence further improvements will be necessary to maximize the oncolytic effect.

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SUMMARY

Despite a variety of treatment strategies including surgery, radiation therapy and chemotherapy, prognosis of patients with Glioblastoma multiforme (GBM) has remained poor. As there is need for novel and improved therapeutic approaches we tested an engineered chimeric adenoviral vector for the efficient treatment of GBM. The vector combines several aspects that enhance treatment of malignant disease, such as the adenovirus serotype 5 (Ad5) capsid with the adenovirus serotype 35 (Ad35) fiber protein which binds human CD46 instead of the underrepresented Ad5 receptor CAR. The vector replicates specifically in tumors due to deletion for all E1A and E1B genes combined with an expression cassette modification allowing for expression of the transgenes AdE1A and the TNF-related apoptosis-inducing ligand (TRAIL) in case of tumor specific homologous recombination. The transgene AdE1A allows for efficient production of progeny virus while TRAIL has been shown to induce apoptosis in a variety of human cancer tissues including GBM. Initially we confirmed surface expression of CD46 in human GBM cell lines SF767, T98G and U-87 MG and efficient infection with Ad5/35.IR-E1A/TRAIL. Additionally, Ad5/35.IR-E1A/TRAIL showed enhanced *in vitro* tumor cell killing in all three human GBM cell lines relative to wild-type virus or Ad5/35.IR-E1A- vectors without TRAIL. TUNEL analysis of Ad5/35.IR-E1A/TRAIL infected cells verified that the mechanism of i*n vitro* cell death is apoptosis. Examination of Ad5/35.IR-E1A/TRAIL oncolytic abilities in an *in vivo* tumor model using human GBM xenografts in NOD/SCID mice showed significant inhibition of tumor growth. Also, detection of adenoviral proteins using an Ad-hexon capsid protein antibody showed efficient spread of the vector after intratumoral injection. Areas positive for adenovirus capsid proteins were also positive for TUNEL staining, confirming that the virus induces apoptosis in infected cells after *in vivo* injection. Viral intratumoral injection lead to significant reduction of solid tumor growth but due to impeded intratumoral spread of progeny virus the effect proved to be short-lived. Thus, our study demonstrates that the use of Ad5/35.IR-E1A/TRAIL vectors offers a potential treatment for human GBM and indentifies further modifications necessary for an effective therapeutic strategy.

ZUSAMMENFASSUNG

Trotz einer Vielzahl von Behandlungsstrategien wie Chirurgie, Bestrahlungs- und Chemotherapie ist die Prognose fuer Patienten mit Glioblastoma multiforme (GBM) derzeit sehr schlecht. Daher sind neue, verbesserte Behandlungsansätze dringend notwendig. Hierzu wurde in dieser Arbeit ein neu entwickelter, chimärer adenoviraler Vektor für die Behandlung von GBM getested. Der Vektor kombiniert verschiedene Aspekte, die die Behandlungsaussichten von malignen Erkrankungen mit Hilfe von viraler Therapie verbessern könnten. Hierzu zählt der Austausch der Wildtyp-Serotyp 5 (Ad5) Fiberproteine mit denen vom Serotyp 35 (Ad35). Ad35 Fiberproteine binden an humanen CD46 als Ligand, während Wildtyp Ad5 am Rezeptor CAR bindet. Der Rezeptor CAR ist auf Krebszellen meist unterrepresentiert. Zusätzlich hat der Vektor die Fähigkeit sich spezifisch in Tumorzellen zu replizieren. Dies geschieht durch die Deletion aller E1A und E1B Gene in Kombination mit dem Einsatz einer Expressionskassette, die nach tumorspezifischer homologer Rekombination aktiviert wird und die Transgene AdE1A und „TNF-related apoptosis inducing ligand“ (TRAIL) exprimiert. AdE1A ermöglicht effiziente intratumorale Produktion von neuen viralen Partikeln, während TRAIL die Fähigkeit besitzt, in Krebszellen Apoptose auszulösen. Zunächst haben wir die Oberflächenexpression von CD46 in den humanen GBM Zelllinien SF767, T98G und U-87 MG nachgewiesen, gefolgt vom Nachweis effizienter *in vitro* Infektion der Zellen mit dem Vektor Ad5.35.IR-E1A/TRAIL. Der Vektor löste stärkeres Zellsterben in allen Zellinien im Vergleich zum Wildtyp oder Ad5.35.IR-E1A ohne TRAIL aus. Analyse mittels TUNEL-Test verifizierte, dass Apoptose-Vermittlung tatsächlich der Auslöser der beobachteten Abtötung der Krebszellen ist. Untersuchung der onkolytischen Fähigkeiten von Ad5.35.IR-E1A/TRAIL *in vivo* mit Hilfe eines GBM Xenograft-Modells in NOD/SCID Mäusen zeigte signifikante Inhibition des Tumorwachstums. Detektion von adenoviralen Protein mit einem Antikörper, welcher gegen das Kapsidprotein gerichtet ist, zeigte effiziente virale Ausbreitung im Tumorgewebe. Bereiche in denen virale Kapside nachgewiesen werden konnten wurden ebenfalls mittels TUNEL-Färbung positiv auf Apoptose getestet. Dies legt Nahe, dass das Virus in der Lage ist, sich *in vivo* nach Applikation im Gewebe auszubreiten und Apoptose auszulösen. Intratumorale Injektion führte zu signifikanter Verminderung des

soliden Tumorgewebes. Allerdings war dieser Effekt nur über einen kurzen Zeitraum zu beobachten. Es besteht die Möglichkeit das dies an einer behindertern intratumoralen Ausbreitung von neugebildeten viralen Partikeln liegt. Unsere Studie demonstriert, dass die Verwendung des viralen Vektors Ad5.35.IR-E1A/TRAIL eine neue onkolytische Behandlungsstrategie für humane GBM Tumore eröffnet. Des weitern schlagen wir Vektor-Modifikationen vor, die nötig sind um eine potentielle Therapie effektiver zu gestalten.

Publications

? Bonig H, Priestley GV, **Wohlfahrt M**, Kiem HP, Papayannopoulou T. Blockade of alpha6-integrin reveals diversity in homing patterns among human, baboon, and murine cells. Stem Cells Dev. 2009 Jul-Aug;18(6):839-44.

? Wang H, Cao H, **Wohlfahrt M**, Kiem HP, Lieber A.Tightly regulated gene expression in human hematopoietic stem cells after transduction with helper-dependent Ad5/35 vectors. Exp Hematol. 2008 Jul;36(7):823-31. Epub 2008 Apr 18.

? Trobridge GD, Beard BC, Gooch C, **Wohlfahrt M**, Olsen P, Fletcher J, Malik P, Kiem HP.Efficient transduction of pigtailed macaque hematopoietic repopulating cells with HIV-based lentiviral vectors. Blood. 2008 Jun 15;111(12):5537-43. Epub 2008 Apr 3.

? **Wohlfahrt ME**, Beard BC, Lieber A, Kiem HP. "A capsid-modified, conditionally replicating oncolytic adenovirus vector expressing TRAIL Leads to enhanced cancer cell killing in human glioblastoma models."Cancer Res. 2007 Sep 15;67(18):8783-90. (see appendix)

Meeting presentations

? Taylor JA, Trobridge GD, Wilks AN,Chien S, **Wohlfahrt ME**, Kiem HP, Becker PS (2008) Optimizing lentiviral transduction of hematopoietic progenitors for Fanconi Anemia complementation group A (poster presentation) Annual Meeting of the American Society of Gene Therapy in Boston, Massachusetts, USA

? **Wohlfahrt ME**, Beard BC, Lieber A, Kiem HP (2007) "Enhanced Tumor Cell Killing in a Human Glioblastoma Xenograft Model with TRAIL Expressed from an Engineered Adenovirus Vector (oral presentation) Annual Meeting of the American Society of Gene Therapy in Seattle, Washington, USA.

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Thank you!

Curriculum Vitae

Martin Wohlfahrt
*22.12.1975 in Bielefeld

Familienstand: verheiratet, keine Kinder

Schule

1986 – 1995	Gymnasium des Schulzentrums Werl-Aspe, Bad Salzuflen
1995	Abschluss mit dem Abitur

Zivildienst

07/1995 – 08/1996	Abteilung für Jugendschutz und Jugendpflege der Stadt Bad Salzuflen

Studium

10/1996 – 10/2002	Philipps-Universität Marburg, Studium der Biologie
11/2001 – 08/2002	Diplomarbeit am Institut für Virologie der Philipps- Universität Marburg in der Arbeitsgruppe von Herrn Prof. Dr. Radsak mit dem Thema „Charakterisierung einer Glykoprotein B-Mutante des Humanen Cytomegalievirus“
10/2002	Abschluss des Studiums; Diplom mit der Note „gut“ (1,8)

Praktika während des Studiums

08/1996 - 09/1996	Praktikum in der Diagnostik des Instituts für medizinische Mikrobiologie und Hygiene in Marburg
08/1999 – 09/1999	Praktikum am Institut für Virologie der Philipps- Universität Marburg in der Arbeitsgruppe von Herrn Prof. Dr. Wolfgang Garten. Thema: „Expression und Aufreinigung der Proteine GP-1 und GP-2 des Lassavirus“
10/2000 – 01/2001	Praktikum im Rahmen des Studiums am Institut für Virologie der Philipps- Universität Marburg in der Arbeitsgruppe von Herrn Prof. Dr. Radsak . Thema: „Nachweis und Isolierung einer Mutante des Humanen Cytomegalievirus mittels selektiver PCR“

01/2001 – 03/2001	Praktikum im Rahmen des Studiums am Institut für Virologie der Philipps- Universität Marburg in der Arbeitsgruppe von Herrn Prof. Dr. Klenk. Thema: „Generierung von rekombinanten Acylierungsmutanten des KP- Virus mittels Transfektion“
	Tätigkeiten nach dem Studium
11/2002 – 01/2003	Anstellung als wissenschaftlicher Mitarbeiter in der Arbeitsgruppe von Herrn Prof. Dr. Radsak am Institut für Virologie der Philipps- Universität Marburg zwecks weiterführender Forschung am Diplomarbeitsthema
	Doktorarbeit
02/2003 – 11/2003	Anstellung als Doktorand bei der Firma Custos Biotechnologie GmbH. Thema: “Generierung adenoviraler Shuttlevektoren für die gentherapeutische Behandlung von Lebermethastasen“. Vorzeitig beendet auf Grund der Insolvenz der Firma Custos
seit 11/2004	Anstellung als wissenschaftlicher Mitarbeiter im Fred Hutchinson Cancer Research Center in Seattle/U.S.A. in der Arbeitsgruppe von Prof. Dr. Hans-Peter Kiem. Begin der Doktorarbeit mit dem Thema “Analysis of a capsid-modified and conditionally replicating, oncolytic adenoviral vector as a novel treatment for human glioblastoma multiforme.”

Erklärung

Ich versichere, dass ich meine Dissertation

“Analysis of a capsid-modified and conditionally replicating, oncolytic adenoviral vector as a novel treatment for human glioblastoma multiforme.”

Selbstständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

_____ _____

Ort/Datum Unterschrift (Vor- und Zuname)