"SUICIDE" GENE THERAPY FOR MALIGNANT CENTRAL NERVOUS SYSTEM TUMORS

"Zelfmoord" Gentherapie voor maligne tumoren van het centrale zenuwstelsel

Proefschrift

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM OP
GEZAG VAN
DE RECTOR MAGINIFICUS

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EN VOLGENS BESLUIT VAN HET COLLEGE VOOR
PROMOTIES

DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN
OP
WOENSDAG 20 MEI 1998 OM 11.45 UUR

door

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Het onderzoek dat in dit proefschrift wordt beschreven, is uitgevoerd binnen de afdeling neurochirurgie van het Academisch Ziekenhuis Rotterdam en Introgene bv Leiden, in samenwerking met de afdeling neuro-oncologie van de Dr. Daniël Den Hoed kliniek (Rotterdam), afdeling medische biochemie van de Rijksuniversiteit Leiden en afdeling kindergeneeskunde van het Academisch Ziekenhuis Leiden.
Het onderzoek werd financieel ondersteund door het Koningin Wilhelmina Fonds en de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (N.W.O.)

The known is finite
The unknown is infinite
Intellectually we stand on an island in the
midst of an illimitable ocean of inexplicability
Our bussiness in every generation is to reclaim a little more land

T.H. Huxley (1887)

Aan mijn ouders

Contents

Introduction

Despite development in surgical techniques, chemotherapy and radiotherapy, most malignancies of the central nervous system are still devastating tumors with a poor prognosis. For example, median survival of patients with malignant gliomas (astrocytoma, oligodendroglioma or mixed type) is roughly 12 months and only 5 % of the patients survive more than 5 years after diagnosis (1, 2). Fifty % of astrocytomas are typed as glioblastoma multiforme, the most malignant form of glioma (3). Glioblastoma account for 15-23 % of all intracranial tumors and have a very poor median survival of 6 months with conventional therapy (3-7). Metastases account for 15-30 % of all intracranial neoplasm's and develop in 25 to 30 % of all cancer patients (8-10). The overall median survival time after surgery followed by radiation therapy in solitary metastatic lesions ranges from 9 to 23 months, depending on the type of primary cancer (10-15). The prognosis in patients with multiple metastases, however, is much worse (15). Lepto-meningeal metastases from solid tumors are of increasing importance in neuro-oncology, because of the increasing frequency and the severe neurologic disability it causes (16, 17). About 0.8 to 8 % of patients with cancer develop leptomeningeal metastases and median survival in these patients after radio- and chemotherapy ranges from 7 to 24 weeks (9, 10, 18).

The response rate to standard therapy in Central Nervous System (CNS) cancer is limited for several reasons. First, invasive growth and the presence of multiple tumor locations limit the feasibility of total resection in cerebral and spinal cancer (19). Second, radiotherapy and chemotherapy are limited in their use due to relative resistance of most tumors and side effects in surrounding brain tissue (20, 21). Refinements in neurosurgical technique, radiation therapy (including brachytherapy and stereotactic radiosurgery) and conventional chemotherapy are therefore unlikely to provide dramatic improvement in tumor control and survival. New approaches should overcome toxic side effects of current regimens, control tumor growth and increase survival in patients.

Gene therapy is a recently developed strategy which in theory might fulfill these aims. Gene therapy involves the delivery of a therapeutic gene to target cells or tissue were it has to be transcribed to messenger RNA (mRNA) and finally should result in the presence of a therapeutic protein. The delivery of a therapeutic gene should correct genetic abnormalities or provide new functions in a cell. Gene therapy strategies are being devel-

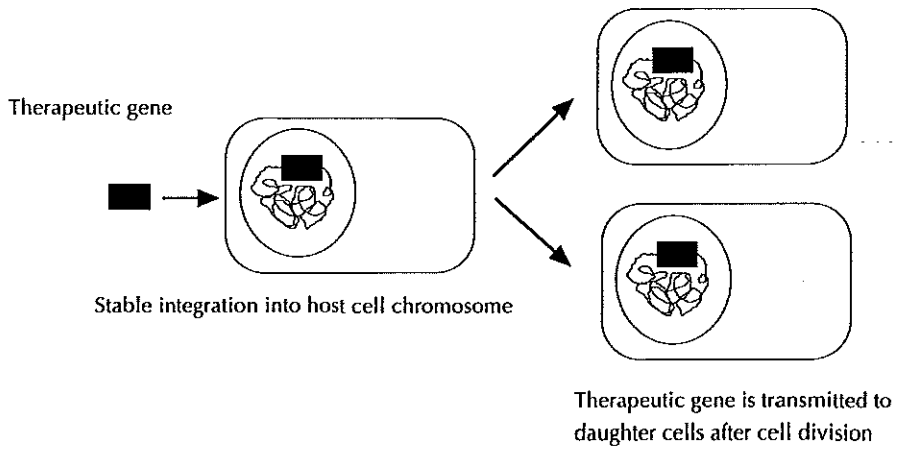
oped for the treatment of inherited diseases (e.g. inborn errors of metabolism, Duchennes disease, Cystic Fibrosis), acquired diseases (e.g. AIDS) and show increasing potential for the treatment of several malignancies (22). In gene therapy approaches for cancer, genes have been developed which after transfer in malignant cells will increase its susceptibility to any form of destruction or will restore normal growth pattern. They include genes encoding for cytokines (e.g.: TNF, GM-CSF, IL-2, IL-4), tumor suppressor genes (p53) and "sensitizing" genes (e.g.: suicide genes encoding Thymidine Kinase (see chapter 2), Cytosine Deaminase, Cytochrome P450). Theoretically, intracellular introduction of genes coding for cytokines will increase immunological response to the tumor cell. Transfer of tumor supressor genes into the nucleus on the other hand, will restore normal cell functions. Sensitizing genes encode for proteins which form a tumor cell specific toxic substrate after administration of a non-toxic drug. In other approaches, tumor neovasculature is targeted in order to obtain growth inhibition. The aim in these "genetic" approaches is that tumor cells are specifically killed, while normal surrounding healthy tissue is spared. This could have some significant advantages over current treatment modalities (radio-, chemotherapy) for the treatment of CNS tumors. Although gene transfer methods need further improvement, data from recent clinical trials using gene therapy for the treatment of glioblastoma and brain metastases have revealed regression in tumor volume (23, 24). The increased interest in this promising therapy has generated many experimental data to further improve gene therapy strategies and gene delivery systems. In this thesis we will present and discuss advantages and limitations of different genetic transfer methods and elaborate on suicide gene therapy strategies for CNS cancer therapy.

CHAPTER 1

Methods of Gene Transfer

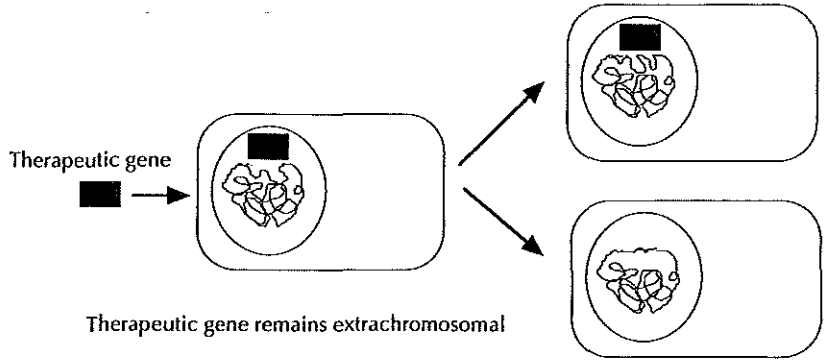
Methods of gene delivery by vehicles or vectors to target cells and tissue have been developed ranging from direct injection of naked DNA to the delivery via recombinant viruses (25). Although each technique has its own advantages and limitations, low toxicity, safety and efficient gene transfer are the main concerns of every method. After DNA is delivered to the nucleus, either integration or non-integration will take place depending on the transfer system used (25). Theoretically, following stable integration of the transgene into the genome of the target cell, the gene will consequently pass to all daughter cells after replication. E.g., integration (intrachromosomally) of therapeutic genes into bone marrow stem cells, may result in correction of all progeny and result in life-long correction of the disease (Fig 1).

Figure 1 Intrachromosomal expression



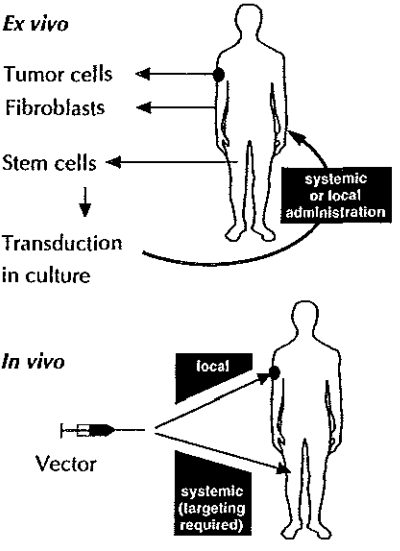
This can be achieved by using retroviral vectors. After gene transfer by delivery methods which don't result in stable integration of the gene, for instance adenoviral vectors, the non-integrated extrachromosomal genes will be 'diluted out' if cell division takes place (Fig. 2).

Figure 2 Extrachromosomal expression



Extrachromosomal transgenes will therefore exert only transient expression in a mitotically active cell population. Depending on the strategy used, methods aiming at short or long lasting gene expression can applied. Genetic engineering of cells can take place *ex vivo* and *in vivo* (26) (Fig. 3).

Figure 3 Gene therapy – procedures



In the *ex vivo* approach, cells are aspirated or surgically removed, cultured, irradiated if necessary, and returned to the patient after gene transfer. This method is currently used in cancer vaccination studies and for transduction of hemopoietic cells and hepatocytes. The *in vivo* approach involves the direct introduction of the vector into cells or tissues. Gene delivery to mammalian cells can be accomplished by non-viral and viral methods (Table 1). Both methods have their advantages and disadvantages (Table 2). The different vectors will be discussed shortly.

Non-viral gene delivery methods:

Naked DNA

Purified genes or recombinant DNA can be administered to various cells and tissues *in vitro* and *in vivo* (27-38). After transfer to the nucleus, the administered DNA will reside mainly extrachromosomally where translation and transcription can take place. Purified naked DNA can be transferred *in vitro* by calcium-phosphate transfection, electroporation or microinjection (25, 39). Naked DNA *in vivo* is delivered by direct injection, needle-free injection devices or by the 'gene gun' in which DNA coated microparticles are bombarded under high pressure into tissues (40, 41). DNA delivery to arterial endothelium has been demonstrated with an intravasive hydrogel impregnated balloon catheter (42). Although direct DNA administration provides an easy, safe method and transfer of large gene constructs can also be achieved, transfer efficiency and expression levels are relatively low. Enhancement of DNA delivery *in vivo* can be achieved by preinjection of the target tissue with hypertonic sucrose or myotoxins (35, 43, 44).

Ligand mediated transfer

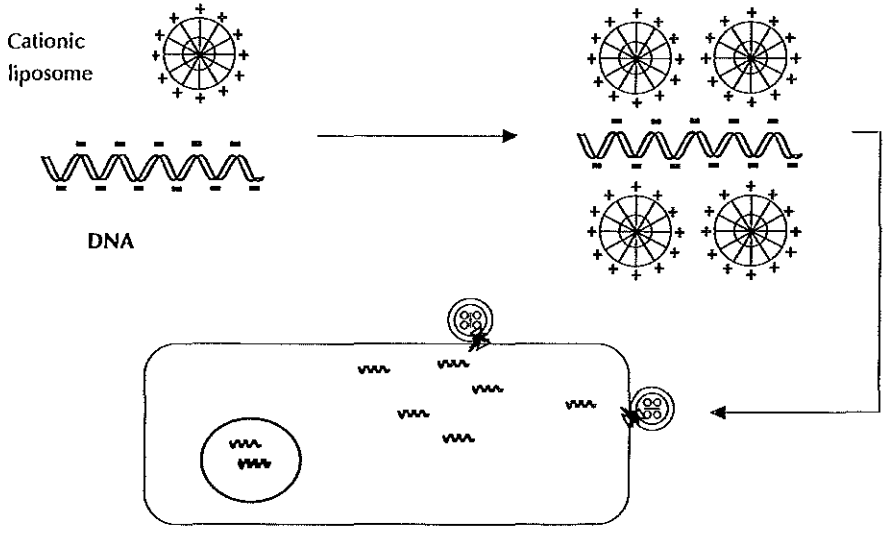
Gene transfer efficiency can be enhanced by attaching the transgene to a ligand capable of binding to a specific target cell receptor. This can be achieved by linking DNA to a covalently bound ligand-polylysine complex (45-54). After administration, the ligand interacts with specific receptors on the target cell, leading to receptor mediated internalisation of the ligand-gene complex into the cell. In addition, aspecific ligands with specific receptor affinity like transferrine (targeting hematopoietic cells, pulmonary epithelium, 48, 49, 52), asialo-orosomucoid (hepatocytes and heptoma cells, 45, 46, 55 , 56), surfactant B (lung, 54), anti-thrombomoduline (lung, 50), tris-galactosil (liver, 55), and folate (tumor cells, 51) have been developed to target gene transfer into desired tissue.

Cationic lipids and liposomes

Lipids are known to cross cell membranes very efficiently and are therefore used as vectors to deliver DNA to the cell. Cationic lipids are able to form complexes via ionic interaction with negatively charged DNA (57) (Fig. 4). These cationic liposome-DNA complexes have been demonstrated to deliver several genes to tissues *in* and *ex vivo* (33, 57-78). The cationic liposome-DNA complex fuses with the plasma membrane, after which the complex is transported via endosomes to the nucleus were it will mainly reside extrachromosomally (Fig.4). Liposome-DNA complexes provide relatively high gene delivery efficiency and are used in several clinical trials for cancer and cystic fibrosis (79).

For *in vivo* use, the complex has been administered intravenously for systemic application, by aerosol to reach lung tissue or via intravascular ballon catheters to reach endothelial cells at specific location (33, 57-90).

Figure 4 Infection with cationic liposomes



Endosomal release enhancement

The rapid degradation of DNA within the cytoplasmatic endosome after endocytosis is a major limitation for non viral gene delivery. Methods to enhance release from the endosome before these organelles fuse with intracellular lysosomes may increase gene transfer efficiency to the nucleus. Since adenoviral particles are known to induce endosomal degradation

during adenoviral infections, addition of these particles to the DNA has resulted in increased gene transfer efficiency into mammalian cells (91). The adenoviral particle-induced endosomal degradation resulted in the release of the co-internalized DNA into the cytoplasm of the cell (92). The addition of adenoviral particles together with complexes for ligand mediated transfections has shown to increase gene delivery 100 to 1000 fold (51, 52, 93-99). In other gene delivery methods, virusparticles are attached via polylysine (100, 101) or a streptavidine-polylysine complex (102) to the DNA, thereby also increasing endocytosis into the cell. Increased endocytosis can also be achieved by binding Influenza virus hemagglutin to the complex (103). Due to the large size of the adenoviral-DNA complex and relative high concentration of adenovirus needed for effective transfer in vivo, this method has not been employed effectively *in vivo* yet.

Polymers

Polymers are used to enhance tissue specific bioavailability and delivery of genes to target cells. In addition to cationic lipids, cationic dendrimer-DNA complexes have been developed which exhibit efficient gene delivery in several cells in *vitro*(104). Addition of several peptides to the dendrimer-DNA complex has further enhanced delivery by increasing endosomal release (104).

Table 1 Most commonly used methods of gene transfer

Non-viral gene transfer methods
naked DNA (administration by: CaP-transfection,
electroporation, microinjection or gene gun)
ligand mediated
cationic lipids and liposomes
endosomal release enhancement
polymers
Virus mediated
Retrovirus
Adenovirus
Adeno-associated virus
Herpes Simplex virus
other viruses

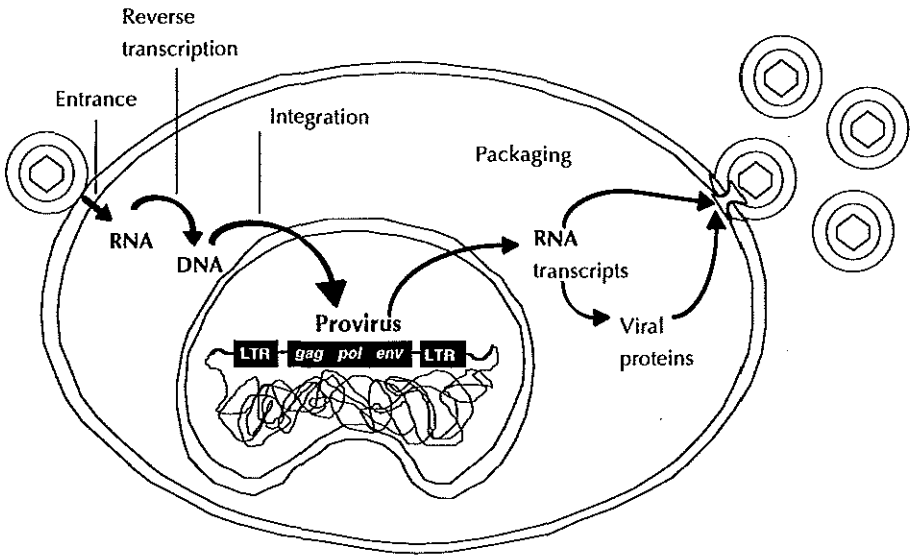
Viral gene delivery methods:

Several recombinant viruses have been developed to deliver genes to target cells. These recombinant viral vectors make use of the natural ability of viruses to infect host cells. Recombinant viruses are very effective in gene delivery and are therefore promising vectors. The wild-type cycle of each virus is discussed, followed by the basic principle of recombinant construction.

Recombinant Retrovirus

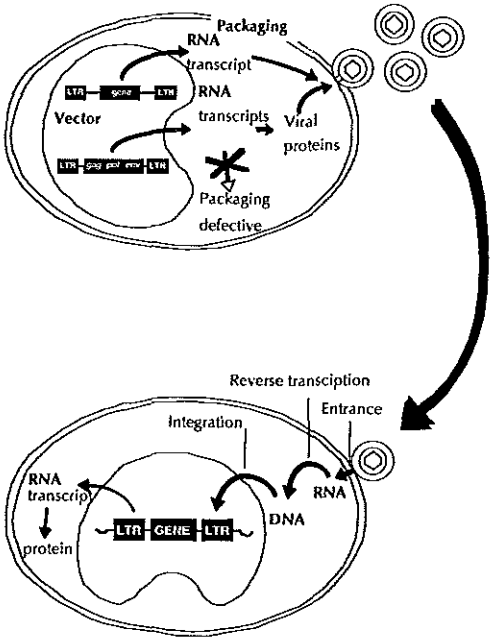
Retroviruses are diploïd positive strand RNA viruses. After infection of the target cell the RNA, is transcribed to DNA by reverse transcriptase which is carried as a protein in each viral particle (105). During replication of the host cell, the pro-viral DNA enters the nucleus were it integrates randomly into the host genome. Subsequently, via normal cellular transcription and translation processes, spliced versions of the mRNA copies are generated which encode for the retroviral proteins gag, pol and env. After assembling the diploïd positive strand RNA, the virusparticles is bud off from the cell, starting a new wild-type life cycle (105) (Fig.5).
The retroviral vectors most commonly used for gene therapy are derived from Murine Leukemia Virus (MuLV). The virus genome consists of 9200 base pairs (=9.2 kb) with a central region (8 kb), encoding for retroviral proteins (gag, pol, env), which can be replaced by recombinant genes (106-108). If the gag, pol and env genes are replaced by a recombinant gene, the virus is still able to integrate the therapeutic gene into the DNA of the target cell. However, since the genes encoding viral proteins are missing, the infected cell is unable to generate new viral particles. Retroviruses lacking the gag, pol and erv gene are so called 'replication defective viruses'. The retroviral genome contains long terminal repeats (LTR's) which are specific sequences on both ends of the genome and are responsible for gene expression (promoter) and gene integration (Fig.6).

Figure 5 The retrovirus life cycle



The recombinant virus is generated with the help a so-called packaging cell line. The vector is transduced into a cell line (packaging cell line) which has been engineered to produce the necessary retroviral gag, pol and env proteins RNA transcripts that encodes for the complete retroviral genomes (109-121) however cannot be packaged in the viral particles (Fig.6). In packaging cell lines that produce viruses that can infect tumor cells the MuLV genes gag and pol are combined with env from another strain (4070A), to change the ecotropic envelope into an amphtropic envelope, which allows infection of human and other mammalian cells. This packaging or vector producing cell line (VPC) is capable of excreting replication defective retroviral vectors without giving rise to replicative competent retroviruses (RCR). The replication defective retroviral vector is able to tranduce target cells which eventually will lead to trangene expression (Fig.6). Although generation of RCR has been reported, most existing packaging cell lines today do not result in generation of RCR and are therefore suitable for human use (111, 114, 117). Titers varying from 10^4 to 10^7 virus particles/ml can be achieved depending on different techniques and packaging cell lines used (119, 120, 122, 123). After purification of the medium, cell free batches of retroviral vectors can be obtained.

Figure 6 Recombinant retrovirus life cycle



Alterations in binding sites by attaching or incorporating ligands on the envelope of the retroviral vector have increased target cell specificity (124-127). Another approach to target a vector to tissue is the use of specific expression enhancers and/or promoters (128, 129). Retroviral vectors are mainly used to transduce cells *ex vivo* (130, 131) because of their relative instability *in vivo*. Efficient transduction in several tissues was only observed after direct vector injection with high titers (132-135). Although limited survival of producer cells following *in vivo* inoculation has been observed, direct injection of these cells into tumor tissue has resulted in some improvement in gene delivery as compared to purified virus (136) .

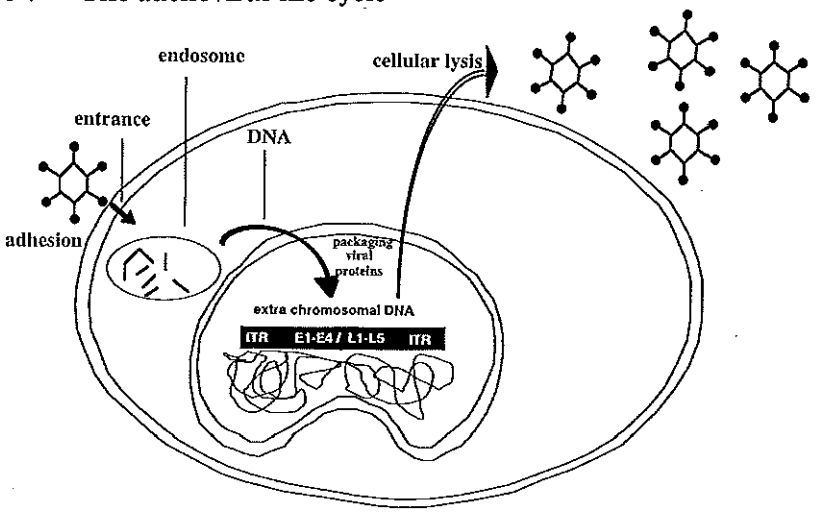
Retroviral vectors are only able to transduce dividing cells to achieve stable integration of the delivered gene. This may be an advantage in treating patients with genetic or chronic diseases, but also carries the risk of insertional mutagenesis when the viral derived DNA is randomly integrated into onco- or onco-suppressor genes. Although very unlikely, introduction of a gene into the germ line can theoretically also occur during application *in vivo* (108, 137-139). Furthermore, RCR which could be generated in

VPC's through recombination has been shown to induce hemopoietic malignancies in rodents and immunosupressed primates in certain conditions (138). The MuLV derived retrovirus however is not known to induce any pathology in humans and is considered to be a safe vector (140). Clinical trials have thusfar not shown any side effects due to the application of retroviral vectors (111, 114, 117).

Recombinant Adenovirus

Wild-type adenoviruses are non-enveloped double stranded DNA viruses which attach with their fiber proteins to permissive cells (141). After adsorption, the virus is taken up in a cytoplasmic endosome were it is dissociated and "uncoated" (142-144). The double stranded viral DNA is then transported to the nucleus where translation to mRNA takes place. After translation, the mRNA is translated to viral proteins. The viral DNA is not integrated into the host cell genome. Finally, after reassembling in the nucleus, the newly generated adenoviruses are released by cellular lysis (145) (Fig.7). The viral DNA genome of the wild type adenovirus is 36 kb long with inverted terminal repeats (ITR), which are specific DNA sequences on each side of the genome. These repeats are responsible for packaging the viral DNA into capsids (146).

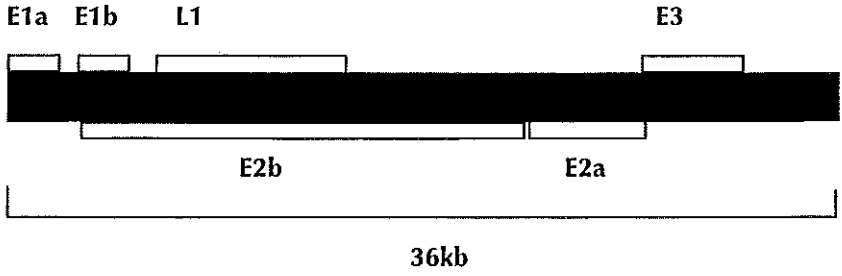
Figure 7 The adenovirus life cycle



The genome is divided in early (E1-E4) and late regions (L1-L5) which encode for viral proteins. The E1 region is responsible for activation of

other early genes and thereby for replication of the virus (147). The E3 region encodes for proteins which block cell surface expression of viral proteins thereby diminishing immune response (148). The E2, E4 and late region proteins have several functions in transcription, gene regulation and virus replication (147, 149-151) (Fig.8).

Figure 8 Topography of the adenovirus genome

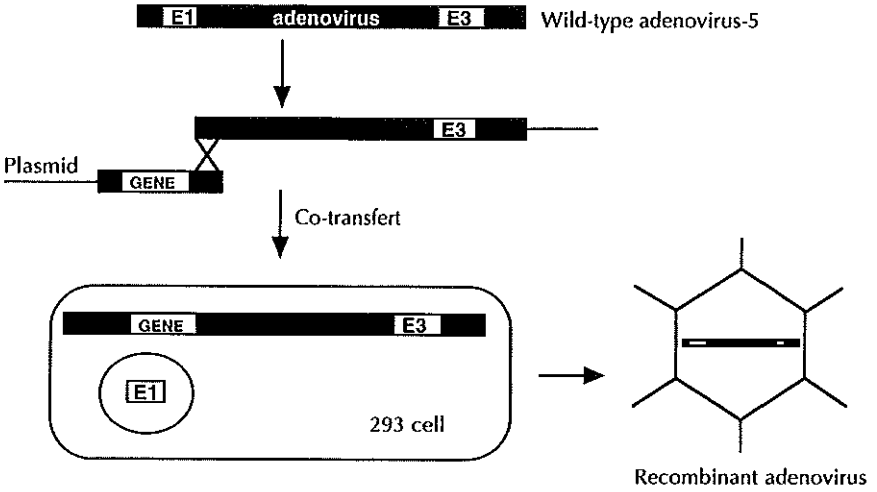


E1a/E1b: transcription regulation
E2a/E2b: synthesis of viral replicative functions
E3: prevention of cytolysis by CTL and TNF
E4: involved in DNA replication, late gene expression and host-cell shut-off.
L1: encode majority of virion capsid proteins

Recombinant adenoviruses used for most gene therapy studies, including the studies reported in this thesis, are derived from the subgroup C adenoviruses types 2 and 5, which are known to induce self limiting, mild respiratory tract infections (145, 152). These viruses have been extensively studied and the complete sequences of both viruses have been determined (153).
Recombinant adenoviruses are constructed by replacing the E1 region by a therapeutic gene (154-156). Deletion of the E1 region allows for up to 3.2 kb of foreign DNA to be cloned into the adenoviral vector. Briefly, the gene of interest with the promotor of choice is constructed into a bacterial plasmid with adenoviral sequences on both sides. Subsequently, the plasmid is linearised and cotransfected with a linearised wild type adenovirus genome into a cell line, e.g. human embryonic kidney cell line (293), harbouring the complementary E1 region (157, 158). The linearised plasmid and the wild type adenovirus contain specific complementary base pairs at one end after linearisation. After transfection, intracellular homologous recombination will generate infectious viruses containing the wild type adenovirus genome with the E1 region replaced by the gene of interest

(Fig.9). In addition, the E3 region can also be deleted to create more space for insertional genes (159). However, increased host cell immune response was observed after administration of E3-deleted vectors *in vivo* (160).

Figure 9 Generation of recombinant adenovirus



A transduced cell line e.g. a human embryonic kidney cell line (293), harbouring the complementary E1 region, is used to generate recombinant adenoviruses (157, 158). E1 deleted recombinant adenovirus is able to replicate within these cells after infection and are released into the culture medium after cellular lysis (Fig.10). The virus can then be obtained after purification and ultracentrifugation (155) (Fig.10). Cell infection with recombinant adenovirus leads to transgene expression without formation of newly generated adenoviruses, since the E1 region is deleted. Recombinant adenovirus has the advantage that it is stable, a cell free virus can be obtained to administer directly via multiple routes (*ex vivo*, aerosol, intracranial, i.m., i.v.) (155, 157-159), and high titers (10^{11}-10^{12} IU) can be obtained. The virus can infect a broad range of dividing and non-dividing cells (145) and insertional mutagenesis or integration into the germ line is unlikely because the DNA is not integrated into the host's cell genome. Although high expression levels can be achieved, the expression of genes is usually transient due to host immune responses or extrachromosomal 'gene dilution' in a dividing cell population.

Figure 10 Preparation and propagation of recombinant adenovirus

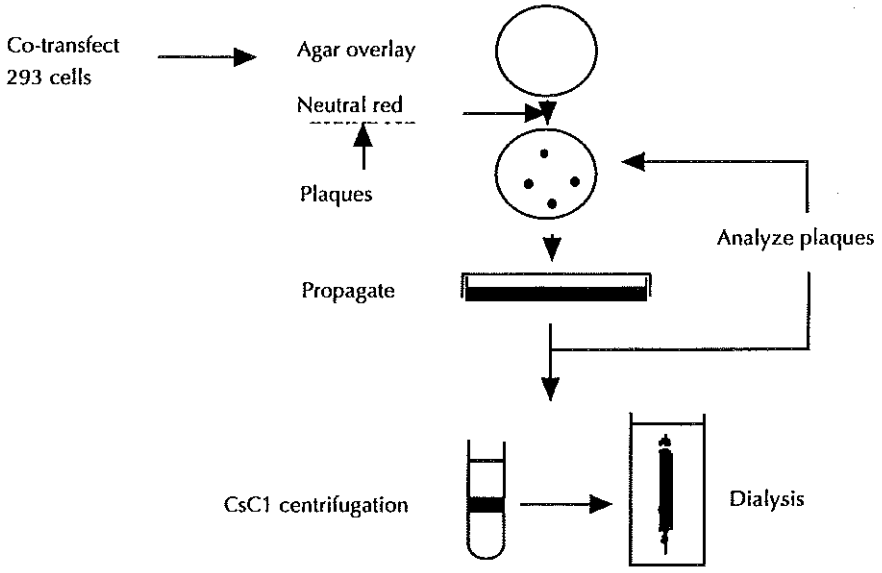
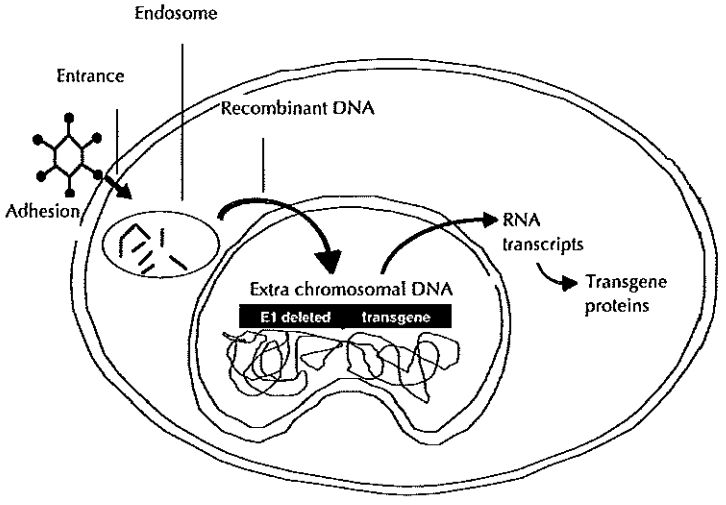


Figure 11 Recombinant adenovirus life cycle



A limitation in the use of these vectors could be the preexisting immunity to adenoviruses types 2 and 5 in the general population. Also repeated injections may lead to direct complement mediated inactivation after two or more administrations (161). Another disadvantage may be the immune response to viral proteins expressed on the hosts cell surface after infection (162). Future additional modifications and deletions in regions of the virus genome should increase space for larger inserts and decrease immune response directed to cell surface expressed adenoviral proteins (162-165). However, complex cell lines or helper virus containing all complementary essential viral proteins will be necessary for their generation.

Recombinant Herpes Simplex Virus

The herpes simplex virus 1 (HSV-1) is a double strand, enveloped, non-integrating DNA virus of 152 kb. After transcription in the nucleus and translation in cytoplasm, the wild-type virus is released by fusion of transport vesicles with the cell membrane (166). The virus is a common pathogen in humans which occasionally can induce encephalitis (166). Most of the viral functions are known and the complete genome has been sequenced (167). The wild-type genome consists of two identical genetic sequences (U1 and U2) bound by inverted repeats (a' and a, b' and b, c and c') (167). Most viral genes encode for cytotoxic proteins which partly cause cell death during virus replication.

Recombinant, replication incompetent HSV-1 viruses have been constructed in which many of "non-essential cytotoxic" genes have been deleted or mutated to decrease virulance of the virus (168-175). Recently, mutant viruses, which are able to replicate in dividing but not in non-dividing cells, have been developed for cancer gene therapy (175). Recombinant HSV-1 can be generated by transfecting a plasmid containing the transgene, replication and encapsidation sequences of the virus into a HSV-1 infection permissive cell. After superinfection with (mutant replication defective) HSV-1 helpervirus, packaged vectors containing the transgene, and replication defective HSV-1 virus are produced after recombination in a 0.1 to 1.0 ratio (172-176). Another approach for generation of recombinant HSV-1 viruses has similarities with production of adenovirus vectors (177-180). Essential genes deleted from the virus genome is supplied in a producer cell. After superinfection with helper virus, recombinant HSV-1 can be obtained. With these methods titers varying from 10^8 to 10^9 cfu/ml can be achieved. The vector can be administered *ex vivo* or by direct injection *in vivo* (173, 175, 180-197). Recombinant Herpes Simplex virus is relatively stable and has a large capacity to

accommodate therapeutic genes. After infection, the transgene is not integrated and the virus is able to infect dividing and non-dividing cells. The transgene expression, however, is transient and only moderate expression levels can be attained. At present, the virus is of limited value for gene transfer due to the cytotoxicity of the viral proteins in mammalian cells. The vector further habours the risk of wild-type virus generation and reactivation of pre-existent latent viruses after *in vivo* application.

Adeno-associated virus

The adeno-associated (AAV) single stranded DNA virus is a 'defective' parvovirus with a 4.7 kb genome (198). The wild type virus integrates after infection into the host's cell genome where it remains in a latent state (199-204). Activation of the virus will only occur after superinfection of the cell with (helper) adenovirus or herpes simplex virus (205-210). The virus is not associated with any human disease and is therefore, a safe vector for application in humans (211). AAV contains two genes, cap and rep which encode the viral proteins. The protein coding domain is flanked by two inverted terminal repeats (ITR's) (212-221).

In a recombinant AAV plasmid, the protein coding domain is replaced by a therapeutic gene. (222-225). The complementary rep and cap genes are provided by another plasmid. For production of recombinant AAV, both plasmids are transduced into a permissive cell. After infection with helper adenovirus, recombinant AAV's are produced and released during cell lysis. Newly generated vectors can be concentrated and purified on cesium chloride gradients. This packaging system gives rise to titers ranging from 10^{10}-10^{13} infectious particles/ml.

The virus can be administered ex vivo or by direct injection and is able to transduce a large population of dividing and quiescent cells (225-227). The virus is stable and probably remains episomal in non-dividing cells and integrates after the cell enters the S phase (224). For unknown reasons, the wild type virus has a preference for an integration site in one region of chromosome 19 (228, 229). Unfortunately the recombinant virus does not have this preference. The small genome and the limited space for insertional genes (4.7 kb) are major drawbacks in this vector system. Preexisting immunity in 40 to 80 % of adults, probable presence of wild-type AAV in the host and possible genetic alteration in infected cells could be other disadvantage when considering the use of these vectors in humans (230, 231).

Other viruses

Vaccinia vectors have been developed for vaccination purposes and as gene transfer vehicle (232-235). The Vaccinia virus is a 186 kb DNA virus which replicates in the cytoplasma and can infect dividing and non-dividing cells (236). The present vectors are able to carry at least three insertional genes and there is apparently no size limit for the insert. Relatively high levels of transgene expression can be achieved with a duration of about 1 to 4 weeks. The high immunogenecity of the virus makes repeated injection problematic but on the other hand an ideal candidate for vaccination studies. Vaccinia vectors encoding the HIV-1 envelope have been used in clinical trials with apparently no severe side effects (237, 238). The backbone of the HIV-1 virus itself is an interesting candidate for gene therapy in AIDS and other stem cell disorders because of the natural tropism for CD4-positive cells (239).

Other viral vectors under development concern Sindbis/Semliki forest virus (240), poliovirus (241), hepatitis B virus (242), influenza virus (243), and the MPSV virus (244).

Table 2 Comparison of gene transfer systems

Vector	Advantage	Disadvantage
retrovirus	stable integration	dividing cells only producer cells necessary
adenovirus	high transduction efficiency high titers	pre-existing immunity
Herpes Simplex Virus	replication in dividing tumor cells	neurotoxicity
AAV	stable integration	low titers
non viral	safety profile	low efficiency in vivo

CHAPTER 2

Suicide Gene Therapy for Malignant CNS Tumors: Review

Introduction

Several experimental strategies are being employed in gene therapy for CNS tumors. Genes are delivered to malignant cells or tumor vascularisation to induce cell kill or growth control. They include genes encoding for sensitizing enzymes (enzymes which can convert non toxic drugs into toxic drugs), cytokines and oncogene suppression proteins. Also combinations of genes are used to obtain an anti-cancer effect. Most of the transgenes are delivered via viral vectors, although in vaccination studies also cationic liposomes are also used as vehicles. The vectors are usually injected directly into the tumor mass or intrathecally in the cerebral spinal fluid (CSF). Recently, investigators have transduced experimental rat brain tumors via intra-arterial (carotid artery) delivery of the vector in combination with mannitol to open the blood brain barrier (195, 245). This method has the advantage that multiple intracerebral tumor foci can be transduced. Leptomeningeal metastases can be transduced effectively by intrathecal application of the vector (246, 247). The vehicle is transported via the CSF flow to the total neuraxis, where transduction of tumor foci takes place. In this chapter, the literature is reviewed for experimental suicide and other drug sensitizing gene therapy studies in the treatment of CNS tumors.

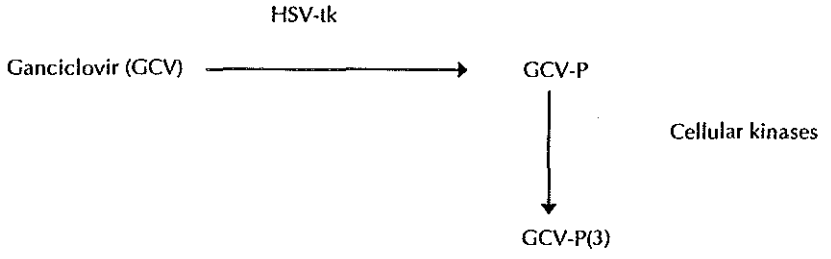
Drug sensitivity

Intracellular delivery of enzymes that mediate the formation of a cytotoxic drug from a non-toxic pro-drug is a pharmacological method which has been used for many years. This basic principle is now also used for gene therapy strategies. A vector with a transgene encoding such an enzyme can be delivered to the target cells. Expression of the vector will result in intracellular production of a 'drug sensitizing enzyme'. Delivery of a non-toxic drug to the transduced cells will consequently result in the production of a cytotoxic drug which kills the cell. Based on this, this form of

gene therapy is also referred to as "suicide gene therapy". The localised release of toxic drugs in this approach has the advantage that systemic toxicity can be circumvented. The Thymidine Kinase suicide gene (TK) and several other genes encoding drugs sensitizing enzymes used in experimental brain tumor therapy will be discussed.

Herpes Simplex Thymidine Kinase/GCV System
The prototype of suicide genes is the Thymidine Kinase suicide gene (TK). The gene is derived from the Herpes Simplex virus and encodes for the enzyme thymidine kinase (248). This enzyme enhances the phosphorylating of nucleoside analogues like non-toxic ganciclovir (GCV) or acyclovir, which can be administered intravenously, into a mono-phosphate. The mono-phosphate is converted by intracellular enzymes into a GCV-triphosphate which is a nucleoside analogue. During DNA replication, the nucleoside analogue is incorporated instead of the natural substrate deoxyguanosine triphosphate (249) (fig. 1).

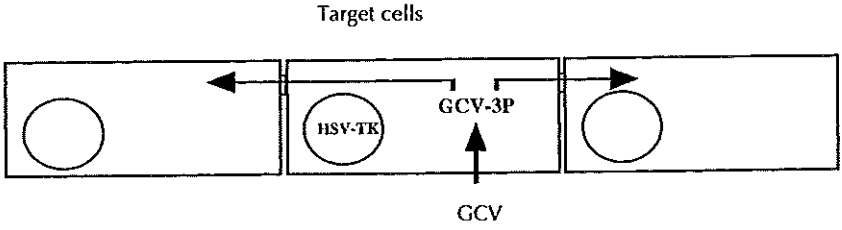
Figure 1 Suicide TK-gene transfer: principle



This introduction prevents DNA elongation by inhibiting DNA polymerase which eventually results in cell death during replication. As the nucleoside analogue is only incorporated during cell division, non dividing cells are not affected by this mechanism. CNS malignancies are therefore ideal candidates for this approach, since the majority of brain cells surrounding these tumors are non-dividing. The efficiency of this approach has been mainly subscribed to a so-called "bystander effect" (250). It has been demonstrated that only 3 to 10 % of cells *in vitro* and 10 to 70% of cells *in vivo* transduction is necessary for 100 % tumor cell kill (251-253). Other *in vivo* experiments showed tumor disappearance with a mixture of 90 % untransduced and 10 % transduced cells after GCV administration (136, 254). Due to this bystander effect, not all malignant cells have to be transduced with the TK gene to be eredicated after GCV treatment.

Several mechanisms have been described to explain this "bystander effect". Cell-cell interactions or "gap-junctions" between transduced and non transduced cells seemed to be important for intercellular transport of toxic products (248, 251, 253-256) (Fig. 2). Cells killed by the HSV-TK/GCV mechanism undergo apoptosis. Endocytosis or phagocytosis of apoptotic vesicles by untransduced cells has also been shown to result in death of untransduced cells (251, 253). An additional role *in vivo* has been subscribed to the killing of endothelial cells of the tumor vascular system. Disruption of the blood supply to the tumor will eventually result in cell kill and necrosis (257). An enhancement of an immune response due to expression of foreign genes (HSV-TK) has also been suggested to contribute to the anti-tumor response *in vivo* (193, 258).

Figure 2 Bystander effect



Vectors used for HSV-TK/GCV system

Retrovirus vectors employing the HSV-TK/GCV system

The use of a retrovirus has the advantage that only proliferating cells are transduced with the TK transgene which decreases possible toxicity or gene delivery in tissue directly surrounded by tumor. Marker gene studies have demonstrated that direct injection of retroviral vectors in experimental rat brain tumors resulted in a low transduction efficiency of<0.1% (259). This has been partially subscribed to the instability of the vector *in vivo*. Although direct intratumoral injection of retrovirus vector producing cells increased the transduction efficiency to 10 %, producer cell survival *in vivo* is rather low (5-7 days) (259, 260). Survival of the producer cells could be enhanced in immunocompromised animals and in animals treated with dexamethasone and was most probably caused by the suppression of an anti-immune response against these cells (261, 262). Retroviral vectors harbouring the TK gene have been studied *in vitro* and *in vivo* in Fischer rats bearing 9L gliosarcoma cells as solid brain tumors (136, 193, 258, 261, 263-265) and as leptomeningeal metastases (247, 266). Generally, increased survival or decreased tumor growth indicated

the effectiveness of the HSV-tk/GCV system in these studies (Table 1, 2). There was some variation in the response between the viruses used and animal survival (Table 1, 2). This may be related to differences in retrovirus titers used, number of injected producer cells, immunosuppression by corticosteroids, the volume of the tumor at the beginning of therapy, GCV dosages administered, and length of GCV treatment. Tumor regression using the HSV-tk/GCV system has also been observed in nude mice bearing subcutaneous HSV-TK transduced tumors (267).
No significant toxicity was observed after intracerebral or intrathecal injection of retroviral HSV-TK producer cells in rats and non-human primates (268-270).
A major disadvantage in the use of retroviral vectors is the relative large amount of producer cells that has to be injected to obtain a therapeutical effect in animal models (136, 261, 271). This could lead to volume constraints and CSF flow disturbances in a clinical situation. Other drawbacks could be the immunogenic properties of the producer cell, the relative low titers of the recombinant retrovirus, and the fact that these vectors cannot infect quiescent, non-dividing, tumor cells.

Adenovirus vectors employing the HSV-TK/GCV system
Adenovirus vectors have the advantage over retroviral vectors that high titers can be obtained and that both dividing and non-dividing tumor cells can be infected. The vectors have been studied in experimental brain tumors (264, 272-276), and leptomeningeal metastases (246) in immunocompetent rat models. Additionally, the vector was also tested in nude mice carrying C6 brain tumors (277). In these experiments, tumor cells were injected stereotactically into the brain. When tumor growth was assessed, adenovirus vectors containing the HSV-TK gene were injected directly into the tumor. Since the adenoviral infection process only takes several hours and the transgene is not integrated and almost immediately expressed, GCV was administered already after 12 to 24 hours. Excessive 'dilution' of the extrachromosomal transgene by cell division is thus prevented by generating immediate cell kill.
Prolonged survival or tumor regression in rats with brain tumors (264, 272-277) or leptomeningeal metastases (246) was observed by several investigators using the adeno-TK/GCV system (table 1, 2). Variation in animal survival between the different observers was most probably caused by the differences in amounts of injected adenoviral particles, tumor volume at the beginning of therapy, immunosuppression, GCV dosages administered, and length of GCV treatment (table 1, 2).

Significant toxicity was not observed in any of these experimental models after intratumoral or intrathecal administration of the adeno-TK virus although some normal brain cells were also transduced by adenoviral vectors (158, 245, 278-285). However, normal brain cells are not affected by the HSV/TK system because of their a-mitotical behavior. Direct intracerebral injection of adenoviral vectors in rat brains demonstrated that titers higher than 10^8 pfu's recombinant adenoviruses were able to induce inflammation (158, 278-280). Others however, reported already significant immune reactions in rodents after intracerebral injection of 10^6 adenovirus particles (286). Differences in titer measurements and the presence of replication competent adenovirus may eventually explain these different findings. In another experiment, Byrnes at al. (161) demonstrated that gene expression after adenoviral gene transfer persisted in the brain for at least 2 months, without inducing chronic inflammation. However, subsequent systemic exposure of the virus was able to induce a cell-mediated immune response in the CNS.

Only limited neurotoxicity after intracerebral injection of an recombinant adenovirus in the brain was reported in non-human primates by several investigators (281, 282). Goodman et al. (287), however, reported severe toxicity in the brain of a baboon after adeno-tk/GCV treatment. Especially primates treated with high-dose ($1{,}5x10^9$ pfu's) adeno-tk and subsequent GCV treatment died or became moribund. Necrosis was found at the injection sites. Intracerebral injection of lower dosages ($7.5x10^7$ pfu's) of adeno-tk resulted in minor lesions like cystic cavities or microscopic foci of necrosis at the injection site. It was suggested that that the immunogenic adenoviral proteins were partially responsible for the pathological changes. Severe toxicity found in baboons treated with high dosage of adeno-tk was not observed.

Herpes Simplex vectors employing the HSV-TK/GCV system

Recombinant Herpes Simplex vectors have been proved to be effective in experimental brain tumor therapy (180-182, 191, 192, 194, 288-290). The recombinant Herpes Simplex vectors used for the experimental treatment of brain tumors are constructed by inducing one or more mutations in viral genes (e.g. thymidine kinase (195), ribonucleotide reductase (291), dUTPase (194) encoding for proteins for replication or neurovirulence. These proteins (thymidine kinase or ribonucleotide reductase) necessary for replication of the Herpes Simplex virus also occur in mammalian (tumor) cells and are upregulated during normal cell mitosis. During cell division the mutated virus can obtain the complementary protein from the infected cell. This will lead to viral replication and sub-

sequent cell death. The use of this virus for CNS malignancies could therefore have some major advantages over other vectors since propagation of new viruses is restricted to dividing cells (292).
First generation recombinant Herpes Simplex derived vectors were mutated in the thymidine kinase gene and established an anti-tumor effect in several experimental brain tumor models (175, 192, 288). However, the virus was associated with encephalitis, especially in immuno-incompetent animals (290). Some decrease in neurovirulence could be obtained by other mutation of the virus genome (181, 191, 290, 293).
HSV mutants with an intact thymidine kinase gene have shown an effective anti-tumor response to experimental rat brain tumors (180, 182, 294) after GCV administration (Table 3). This thymidine kinase intact mutant has the advantage over TK-negative vectors that the virus can be killed at any time during therapy after administration of GCV. Recently, second generation HSV viruses with multiple mutations but with intact thymidine kinase gene have been developed. The virus showed reduced neurovirulence and has also the advantage that the risk of wild-type generation is reduced (194).
Intra-arterial (carotid artery) application of the virus combined with opening of the blood brain-barrier by mannitol (195) or bradykinin (260) has shown HSV transgene delivery to experimental brain tumors *in vivo*. The aim of these studies was to deliver the vector selectively to one or more tumor foci within the brain which is difficult to achieve with direct intratumoral injection. Despite promising results in experimental animal models, the vector still causes mortality in treated animals (195) and severe immune reactions and cytotoxicity in mammalian cells (291) and CSF (292). The potential risk of wild-type HSV regeneration and activation of latent HSV-1 viruses when single mutated vectors are injected are other problems to overcome before human trials are considered.

Adeno-associated virus (AAV) vectors employing the HSV-TK/GCV system
Recombinant AAV vectors harbouring the TK gene have mediated efficient gene delivery in an human medulloblastoma model, dissiminated in the CSF of nude rats (295). Survival or tumor regression have not yet been reported.
AAV vectors have the advantage that they are able to infect quiescent (296) as well as dividing cells (225-227). Administered GCV will consequently kill all TK transduced tumor cells whenever they start to proliferate. The low titers that can be obtained after isolation of the virus are still a major problem to overcome for efficient gene transfer *in vivo*.

Other genes inducing drug sensitivity:

Cytosine deaminase/5-fluorocytosine system
The fungal and bacterial enzyme cytosine deaminase (CD) is able to convert the non-toxic 5-fluorocytosine (5-FC) into the toxic metabolite 5-fluoro-uracil (5-FU) which is frequently used as a chemotherapeutic drug (297). 5-FU inhibits RNA and DNA synthesis, resulting in the killing of dividing and non dividing tumor cells. It has also been demonstrated that the system has a strong "bystander effect" (298).
Adenovirally delivered CD to 9L rat gliosarcoma cells has been demonstrated to kill tumor cells effectively *in vitro* after 5-FC administration (299). Effectivity *in vivo* and toxicity to surrounding non-dividing brain tissue has to be assessed.

Cytochrome P450 2B1/cyclophosphamide system
The liver specific P450 cytochrome enzyme is able to convert the anticancer drug cyclophosphamide into the metabolites acrolein and toxic phophoramide musterd (300, 301). These metabolites cause DNA interstrand crosslinkages regardless the phase of the cell cycle. Cell kill is consequently induced in mitosis (302). Cyclophosphamide has been used as an chemotherapeutic drug in brain tumor therapy unsuccessfully caused by poor penetration of the blood brain barrier and severe side effects (303).
To increase sensitivity of cancer cells to cyclophosphamide, the cytochrome P450 gene was delivered retrovirally into C6 rat brain tumor cells (304). A 40-fold increase in cell kill was observed *in vitro* after administration of cyclophosphamide. Direct delivery of recombinant retrovirus harbouring the P450 gene into established brain tumors (C6) in nude rats followed by cyclophosphamide treatment resulted however in low antitumor response (304) and was probably due to the low transduction efficiency of retrovirus *in vivo*. *Ex vivo* P450 transduced 9L rat gliosarcoma cells on the other hand, demonstrated growth inhibition subcutaneously and intracerebrally in rats after intra-peritoneal cyclophosphamide injections (305).

The Escherischia coli guanine-phosphoribosyltransferase/6-thioxanthine system
The enzyme guanine-phosphoribosyltransferase (GPT) mediates the conversion of 6-thioxanthine (6-TX) into 6-thioxanthine monophosphate. The 6-thioxanthine monophosphate is metabolized by cellular enzymes into 6-thioguanine monophosphate which inhibits cellular DNA synthesis (306). Following retroviral delivery of the gene encoding for GPT, C6

glioma cells were effectively killed after 6-TX treatment *in vitro* (307). *In vivo* experiments are in progress.

Clinical trials

Clinical trials using gene therapy for malignant brain tumors have been conducted or are being employed at present in North America and Europe. Next to patients with primary brain tumors and secondary metastasis from systemic cancer such as melanoma, lung and breast cancer also pediatric patients with progressive or recurrent primary supratentorial malignant brain tumors are or have been randomized for phase I/II clinical studies (23, 79, 263, 269, 308-311). In the first clinical study using the HSV-tk/GCV system, retroviral producer cells were injected stereotactically via multiple injections into the tumor followed by i.v. GCV treatment (269). The first published results of this study showed that gene transduction efficiency into tumor tissue was very low, although it has been reported that about 50 % of the patients showed significant tumor regression on MRI in the early period after treatment (23). The tumor regression may result from either the bystander or an immune effect. Some patients demonstrated transduction of peripheral blood lymfocytes and in all patients a minimal antibody titer against the producer cells was observed (23). Two patients in the study suffered from a preoperative hemorrhage. Effects of the second clinical study (308) has reported meningeal inflammation, pancytopenia, seizures and severe headache as side effects. Increase of survival in patients with brain tumor, has not been reported yet in both studies.
In a phase 1 clinical trial for the treatment of leptomeningeal metastases, retroviral producer cells were injected intrathecally (312) followed by intravenously GCV treatment. This study was temporarily closed after transient meningeal inflammation in the first patient.
Adenoviral vectors have gained importance as a HSV-tk vehicle in cancer gene therapy because its advantages over retroviral vectors. Several centers are already using or starting to use the vector in clinical trials for malignant brain tumor therapy (79, 311, 313).

Conclusions and Future Prospects

Effective *in vivo* brain tumor therapy is at present restricted to viral vectors. Recombinant retroviruses have shown in phase I/II studies to be a

relative safe vector. In addition, tumor regression using the HSV-tk/GCV system was observed in human brain tumors. However, low retroviral titers, low transduction rates, the non-infectibility of quiescent tumor cells and the use of producer cells however, are still major problems to overcome. Studies with recombinant adenoviruses will demonstrate the possible potential in CNS malignancies. They are able to infect a wide array of quiescent and dividing tumor cells and the chances of mutagenesis are low. Gene transfer is efficient and the virus can be applied immediately to the tumor. Other recombinant viruses like HSV-1 and AAV vectors are theoretically ideal vectors for cancer therapy but need further development.

The method of vector application will play an crucial role in the effectiveness of experimental gene therapy in brain tumors. Direct intratumoral injection has limitations in large, "spreading", tumors like glioblastoma. In future trials vectors will be administered post-operatively in the tumor-bed after bulk resection (313). Since malignant brain tumors like glioblastoma usually reoccur at the resection site, regrowth could theoretically be delayed or prevented. However, tumor progression at distant sites is not prevented. Arterial administration in combination with opening of the blood brain barrier may also be of value in future gene therapy treatment of CNS malignancies (195). Targeting of tumor neovascularisation has the potential for effective anti-cancer strategy, but needs further development.

Treatment for leptomeningeal metastases by means of gene therapy has shown to be effective after direct intrathecal administration of the vector or vector producing cells (246, 247, 266). Intrathecal injection of retrovirus producer cell in humans however, has shown meningeal inflammation (312). Toxicity after intrathecal injection of other vectors has to be evaluated further in clinical trials.

Limitations seen in clinical trials have to be adequately addressed before beneficial clinical effects can be expected. Although effective gene transfer can be achieved *in vivo*, the immune response against several recombinant viruses may result in severe side effects. Construction of vectors which will decrease immune response to viral proteins will be necessary.

Table 1 Studies of viral mediated HSV-tk transfer into intracerebral tumors in rats followed by GCV administration

investigator	tumorcell/rat	number of 9L cells	virus or producer-cell adm. (days)	vector	titer	number producer cells	start GCV (days after virus injection)	GCV dose (mg/kg/day)
Culver et al. ref.136)	9L/Fischer	4×10^4	5	retrovirus	ND	3×10^5	5	300
Ram et al. (ref. 261)	9L/Fischer#	4×10^4	7	retrovirus	0.5×10^6 IU/ml	1.8×10^5	7	30
	9L/Fischer#	4×10^4	7	retrovirus	0.5×10^6IU/ml	3×10^5	7	30
	9L/Fischer#	4×10^4	7	retrovirus	0.5×10^6IU/ml	5×10^5	7	30
Barba et al. (ref.271)	C6-TK**/Fischer	2×10^5	0	–	–	–	3	50
	9L/Fischer	2×10^5	mix*	–	–	9L-TK	3	50
Barba et al. (ref.193)	9L/Fischer	2×10^5	3	retrovirus	ND	2×10^5	7	50
	9L/Fischer	2×10^5	3	retrovirus	ND	2×10^6	7	50
Tappscott et al. (ref.258)	9L/Fischer	4×10^4	5	retrovirus	1×10^6IU/ml	3×10^6	5	90
	9L/Fischer	4×10^4	5	retrovirus	1×10^6IU/ml	3×10^6	5	30
	9L-TK**/Fischer	4×10^4(9L-TK)	5	–	–	–	5	90
Rainov et al. (ref.265)	9L/Fischer	1×10^5	4	retrovirus	7.5×10^5IU/ml	5×10^5	7	60
Izquerdo et al. (ref.263)	C6/Wistar#	5×10^5	?	retrovirus	1×10^4cfu/ml	1×10^6	7	30
Perez-Cruet et al.(ref.272)	9L/Fischer	1×10^4	8	adenovirus (RSV pr.)	1.2×10^9pfu	–	1	100
	9L/Fischer	1×10^4	8	adenovirus (RSV pr.)	1.2×10^9pfu	–	1	20/40/100/160/200/300
Ross et al.(ref.273)	9L/Fischer	1×10^5		adenovirus	1.6×10^{11}IU/ml	–		30
Chen et al. (ref.277)	C6/nude mice	1×10^4	8	adenovirus (RSV pr.)	3×10^8	–	8	250/200
Colak et al. (ref.275)	MAT-B##/Fischer	1×10^4	7	adenovirus (RSV pr.)	1.7×10^6IU	–	1	100
Vincent et al.(ref.264)	9L/Fischer	4×10^4	3	retrovirus	5×10^4 IU/ml	5×10^4	2	30
	9L/Fischer	4×10^4	3	adenovirus (MLP pr.)	5×10^6IU	–	2	30
	9L/Fischer	4×10^4	3	adenovirus (MLP pr.)	1×10^6IU	–	2	30
	9L/Fischer	4×10^4	3	adenovirus (MLP pr.)	1×10^7IU	–	2	30
	9L/Fischer	4×10^4	3	adenovirus (MLP pr.)	1×10^8IU	–	2	30
Maron et al. (ref.276)	C6/Wistar	1×10^4	5	adenovirus (RSV pr.)	2.5×10^8IU	–	0,5	50
Boviatsis et al. (ref.180)	9L/Fischer	4×10^4	5	Herpes Simplex Virus	2×10^7pfu	–	7	15

Table 1 *(Continuation)*

investigator	days of GCV treatment	50% survival with GCV (days)	50 % survival controls (days)	histology	long-term survival
Culver et al. (ref.136)	5	ND	ND	78.5% no tumor at end of GCV treatment	ND
Ram et al. (ref. 261)	14	ND	ND	38 % no tumor (28 days after tumor injection)	ND
	14	ND	ND	72 % no tumor (28 days after tumor injection)	ND
	14	ND	ND	83 % no tumor (28 days after tumor injection)	ND
Barba et al. (ref.271)	14	>90	>90 (C6 cells rejected)	significant smaller (17 days after tumor implantation)	100% (C6 cell rejection)
	14	>90	0	100%, 70% and 50% 9L-TK cells: complete tumor regression (17 days after tumor implanation)	100% > 90 days (100% 9L-TK tumors)
Barba et al. (ref.193)	7 or 14	30	22	40% complete or near complete tumor regression (17 days after tumor implantation)	22% > 90 days
	7 or 14	30	22	40% complete or near complete tumor regression (17 days after tumor implantation)	22% > 90 days
Tappscott et al. (ref.258)	5	32	35	ND	0%
		34	35	ND	0%
	5	47	81	ND	0%
Rainov et al. (ref.265)	10	36	20	ND	0%
Izquerdo et al. (ref.263)	14	ND	ND	no tumor on MRI 2 weeks and several months after treatment	>8 months
Perez-Cruet et al.(ref.272)	6	>120	20	ND	75% > 120 days
	6	ND	ND	no tumor in rats treated with 160, 200 and 300 mg/kg/day GCV (20 days after tumor implantation)	ND
Ross et al.(ref.273)	14	35	25	ND	0%
Chen et al. (ref.277)	6	-	-	500 fold tumor cell reduction	ND
Colak et al. (ref.275)	6	16	27	no tumor in treated rats16 days after tumor injection	0%
Vincent et al.(ref.264)	10	28	17	ND	20% > 80 days
	10	68	18	ND	25% > 80 days
	10	26	18	ND	10 % > 80 days
	10	18	18	ND	0%
	10	16	18	ND	0%
Maron et al. (ref.276)	14	70	22	28-fold tumor volime reduction 20 days after tumor implantation	37.5% > 100 days
Boviatsis et al. (ref.180)	7	35	22	50% no tumor	50% > 90days

* a mix of different ratio's of parental tumor cells and TK-transduced tumor cells was injected into the tumor

** TK-transduced tumor cells

\# rats treated with dexamethason

\#\# Mat-B: rat mammary adenocarcinoma

RSV pr. = Rous Sarcoma Virus Promotor

MLP pr. = Major Late Promotor

Table 2 Studies of viral mediated HSV-tk transfer into lepto meningeal metastases in rats followed by GCV administration

investigator	tumorcell/rat	number of 9L cells	virus or producer-cell adm. (days)	vector	titer	number producer cells	ratio tumor cell\ producer cells	start GCV (days after virus injection)
Ram et al. (ref.247)	9L/Fischer	8×10^4	directly***	retrovirus	ND	8×10^4	1\1	7
Vrionis et al. (ref.266)	W256/Sprague-Dawley	1×10^5	mix*	retrovirus	5×10^6 cfu/ml	1×10^5	1\1	3
	W256/Sprague-Dawley	0.18×10^4	mix*	retrovirus	5×10^6 cfu/ml	1.82×10^5	1\10	3
	W256/Sprague-Dawley	1×10^5	mix*	–	1×10^4 cfu/ml	1×10^5W56-VP-TK+##	1\1	3
	W256/Sprague-Dawley	0.18×10^4	mix*	–	1×10^4 cfu/ml	1.82×10^5W56-VP-TK+##	1\10	3
	W256/Sprague-Dawley	1×10^5	mix*	–	–	1×10^5W256-TK+	1\1	3
	W256/Sprague-Dawley	0.18×10^4	mix*	–	–	1.82×10^5W256-TK+	1\10	3
	W256/Sprague-Dawley	4×10^3	mix*	–	–	1.96×10^5W256-TK+	1\50	3
	W256-TK**/Sprague-Dawley	2×10^5	0	–	–	–	–	3
	W256-TK**/Sprague-Dawley	1×10^7	0	–	–	–	–	3
	W256-VP-TK#/Sprague-Dawley	2×10^6	0	–	1×10^4 cfu/ml	–	–	3
	W256/Sprague-Dawley	5×10^4	3	–	–	W256-TK+	–	4
	W256/Sprague-Dawley	5×10^4	3+5+7 (3x)	–	–	W256-TK+	–	4
Vincent et al. (ref.246)	9L/Fishcher	4×10^4	3	adenovirus (MLP)	1 x 108 pfu's	–	–	1

Table 2 *(Continuation)*

investigator	GCV dose (mg/kg/day)	days of GCV treatment	50% survival with GCV (days)	50 % survival	histology controls (days)	long-term survival
Ram et al. (ref.247)	30	14	21	14	tumor regression (7 days after tumor implantation)	0%
Vrionis et al. (ref.266)	30	14	27	17	ND	18% >120 days
	30	14	24	17	ND	15 % >120 days
	30	14	34	17	ND	18% >120 days
	30	14	27	17	ND	11% >120 days
	30	14	40	17	diminution in tumor cell population with time (20 or 27 days after tumor implanation)	50% >120 days
	30	14	25	17	diminution in tumor cell population with time (20 or 27 days after tumor implanation)	40 %>120 days
	30	14	31	17	diminution in tumor cell population with time (20 or 27 days after tumor implanation)	42% >120 days
	30	14	45	20	ND	50%>120 days
	30	14	>120	20	ND	80% > 120 days
	30	14	50	20	ND	50% > 120 days
	30	14	17	12	ND	0%
	30	14	27	12	ND	0%
Vincent et al. (ref.246)	30	10	22	19	ND	0%

* a mix of different ratio's of parental tumor cells and TK-transduced tumor cells was injected into the tumor

** TK-transduced tumor cells

*** producer cells were injected directly after the tumor cells

\# rats treated with dexamethason

\## tumor vector producer cells

RSV pr.= Rous Sarcoma Virus promotor

MLP pr.= Major Late Promotor

Aims of this thesis

- To asses transgene expression in human or rat brain tumor cells *in vitro* after infection with recombinant adenovirus.
- To asses transgene expression in rat brain tumors and leptomeningeal metastases *in vivo* after infection with recombinant adenovirus.
- To investigate human or rat brain tumor cell survival *in vitro* after infection with recombinant adenovirus containing the HSV-tk and subsequent Ganciclovir treatment.
- To investigate rat survival with experimental brain tumors or leptomeningeal metastases after infection with recombinant adenovirus containing the HSV-tk gene and subsequent Ganciclovir treatment.
- To compare rat survival with experimental brain tumors after treatment with either recombinant adenovirus or retrovirus producer cells containing the HSV-tk gene and subsequent Ganciclovir treatment.
- To investigate the differences in transgene expression and tumor cell kill *in vitro*, using recombinant adenoviruses containing either an MLP or CMV promotor.
- To investigate the differences in rat survival with brain tumors or leptomeningeal metastases *in vivo*, using recombinant adenoviruses containing either an MLP or CMV promotor.
- To asses the histopathology in the rat brain after intracerebral injection of adenoviral vectors harbouring the HSV-tk gene and subsequent GCV administration.
- To asses the systemic spread of recombinant adenovirus after intracerebral injection.

References

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CHAPTER 3

Herpes Simplex Virus Thymidine Kinase Gene Therapy for Rat Malignant Brain Tumors

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Human Gene Therapy. 1996;7:197-205.

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Abstract

Transfer of a herpes simplex virus derived thymidine kinase (HSV-tk) gene into brain tumor cells and subsequent Ganciclovir (GCV) treatment has been shown by others to be an effective treatment in rats with intracerebrally inoculated 9L gliosarcomas. Mechanism of action and reproducibility are however still a matter of debate. We have used the same model to test the therapeutic effects of both retrovirus and adenovirus mediated transfer of the HSVtk gene followed by GCV treatment. Survival time of rats with intracerebral 9L tumors was significantly prolonged after a single administration of adenovirus carrying a HSV-tk gene as compared to controls. Retrovirus mediated gene transfer also resulted in significantly prolonged survival time when recombinant retrovirus producing cells were transplanted. Direct injection of the recombinant retrovirus, HSVtk expressing cells, virus producing cells without GCV administration and recombinant retrovirus-LacZ or IL-2 producing cells did not result in tumor cell kill. In the present study, no significant difference in survival of 9L brain tumor carrying rats was found after treatment with adenovirus as compared to retrovirus mediated HSVtk mediated gene transfer and subsequent GCV treatment.

Introduction

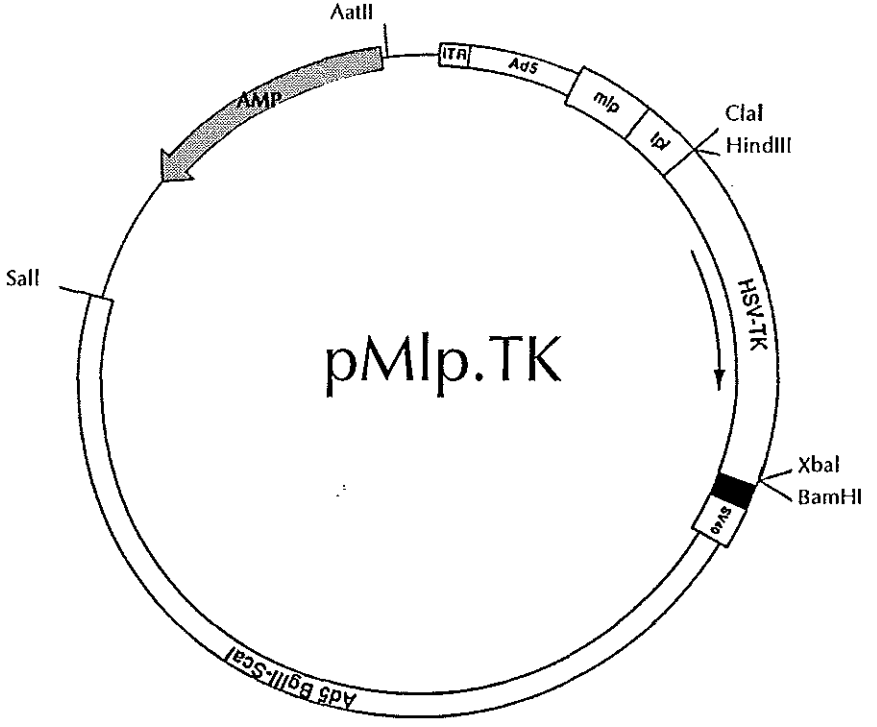
Despite surgery, radiation therapy and chemotherapy, the prognosis of glioblastoma in humans has not substantially been improved in the last decades. The overall median survival time of patients with malignant astrocytomas is approximately 10 months. Two years after diagnosis only 10 % of the patients is alive (33, 35, 18, 6). A new approach for the treatment of brain tumors may be application of virus-mediated transfer of the Herpes *simplex* virus thymidine kinase (HSV-tk) gene into tumor cells (10). Expression of the HSV-tk gene renders dividing cells sensitive to GCV. GCV is converted into triphosphate-GCV, a chain terminator of DNA synthesis. (22, 23). A promising aspect of this form of gene therapy, designated as the "bystander effect", is that after GCV treatment more tumor cells are killed than those that are transduced with HSV-tk (39, 10, 31, 7, 19). Non-dividing cells that have been transduced with the HSV-tk gene are not killed by GCV. As brain tumors are embedded in essentially non-dividing brain tissue, these tumors are good candidates for this therapy. Other investigators showed improved survival and decreased tumor size of rats carrying intracerebral transplants of glioma (9L) cells after treatment with either a recombinant retroviral vector or adenoviral vector containing the HSV-tk gene followed by GCV therapy. (10, 26, 31, 9). The mechanism of action is still incompletely understood. TK-transduced tumor cells for example, may increase immunogenecity (41) and destruction of proliferating TK-transduced tumor vasculature after GCV administration influences tumor growth (32).
In the present study, the effects of recombinant retrovirus and adenovirus mediated transfer of the HSV-tk gene into brain tumor cells followed by treatment with GCV have been studied.

Material and Methods

Tumor cells
The 9L rat gliosarcoma brain tumor (a kind gift from Dr. K.M. Hebeda, Dept. Experimental Neurosurgery, Free University Hospital Amsterdam, The Netherlands) was induced by N-methylnitrosurea in Fischer 344 rats and characterised by Weizsaecker et al., '(44). The U251 human glioma cell line (29, 37) was obtained from Dr. C.H. Langeveld (Dept of Pharmacology, Free University Hospital Amsterdam, The Netherlands). Tumor cells were cultured in Dulbecco's modified Eagle's medium supplemented with penicilline (100IU/ml; Gibco), streptomycine (50

mg/ml; Gibco), 10% fetal calf serum and non-essential amino acids. The cells were grown at 37°C; 5% CO_2 in 80 or 175cm^2 flasks (Nunclon).

Figure 1 Physical map of pMLP.TK, the plasmid that was used to generate the recombinant adenovirus IG.Ad.MLP.TK.



Ad5: nucleotides 1-458 of the adenovirus type 5 genome
mlp: major late promotor, **tpl**: tripartite leader sequence. The mlp and tpl are derived from adenovirus type 2
HSV-tk: Herpes Simplex Virus type 1 thymidine kinase
SV 40: Simian Virus 40 poly-adenylation sequence (nt 2533-2668 of the SV 40 genome)
***Bg*III-ScaI fragment of adenovirus type 5**: nt 3328-6092 of the adenovirus type 5 genome
AMP: Ampicillin resistance gene

Recombinant vectors:

1. Adenoviral vectors

The Herpes Simplex Virus type 1 thymidine kinase sequences were derived from plasmid pHA140 (gift from Dr. A. Berns, Netherlands Cancer Institute, Amsterdam), by PCR amplification using primers TK-1 (5'-CTCTAAGCTTGAAGCGCGCGTATGGCTTCG-3' and TK-2 (5'-ACACTCTAGAGTGTTTCAGTTAGCCTCC-3').

The TK sequences were cloned after digestion with restriction enzymes *Hind*III and *Xba*I and ligated into pSP65 (Promega) and sequenced completely. As compared to the published sequence (20) 3 differences exist: on position 16 (G instead of T), 126 (T instead of C), 267 (A instead of G). Recombinant adenoviruses harbouring the HSV-tk (IG.Ad.MLP.TK) were made from plasmid pMLP.TK (Fig. 1) in which HSV-tk expression is under the direction of the adenovirus derived major late promoter and transcription is terminated by the SV40 poly-adenylation signal.

A similar construct harbouring the firefly luciferase (luc.) gene (45) was made by replacement of HSV-tk in pMLP.TK by luc. Recombinant viruses were generated by co-transfecting 293 cells with *Sal*I linearized pMLP.TK and the large *Cla*I fragment of wild-type Ad5 DNA. Recombinant viruses were plaque purified twice, propagated and titrated according to standard procedures (30). Ad.RSVβgal recombinant adenovirus harbouring the *Escherichia coli lacZ* gene encoding β–galactosidase and preceded by the nuclear localisation signal (38) was obtained from Dr. M. Perricaudet (Institut Gustave Roussy, Villejuif, France). Titers determined by end point titration of the virus batches ranged from $1x10^{10}$ to $1x10^{11}$ IU.

2. Retroviral vectors

pLXSN (21) was digested with *Bam*HI, blunted with Klenow enzyme and digested with *Nhe*I. The 1452 bp *Nhe*I/*Bam*HI fragment was ligated to the 98 bp *Stu*I/*Nhe*I fragment from pLNSX (21) in a three-part ligation with pUC19 vector backbone obtained after digestion of pSFG-tpa (Kindly provided by I. Riviere and R. Mulligan, Whitehead Inst. for Biological Research, Cambridge, MA) with *Nhe*I. The resulting construct, pLec, was digested with *Xho*I, blunted with Klenow and subsequently digested with BamHI. After dephosphorylation using calf intestine phosphatase, this fragment was ligated to a fragment containing the coding region of the Herpes simplex virus type 1 thymidine kinase gene (HSV-1 tk) obtained from pMLP.TK by digestion with *Hind*III, blunting with Klenow and digestion with BamHI. The resulting viral vector was named pLTk.

pLTk was linearised with *Bsi*WI, partially digested with *Eco*RI and dephosphorylated with CIAP. The 5' part of the tk gene was then reintroduced as a *Eco*RI/*Bsi*WI pcr fragment obtained after amplification of the 5' part of the tk gene in the vector TNFUS69 (36) using the primers TKkozUp: 5'-CGGAATTCGCCGCCACCATGGCTTCGTACCCC-GGCCACAG-3' and TkDo-1: 5'-CGGCTCGGGTACGTAGACGA-TATCG-3' followed by digestion with *Eco*RI and *Bsi*WI. The resulting retroviral construct, named pLTKkoz only contains the coding sequences of the HSV-1 tk gene with an optimized Kozak sequence (16) around the start codon. The *Nhe*I fragment from pLTKkoz was introduced into the unique *Nhe*I site from pSK/ZipÆMo+PyF101 to create construct pIGTk. pSK/ZipÆMo+PyF101 has been generated by subcloning of the ClaI-*Eco*RI fragment from pZipDMo+PyF101(N-) into the pBluescript (Stratagene) vector. pZipDMo+PyF101(N-) is described in Valerio et al., '(1989). PA317 packaging cells were co-transfected with pIG-TK and an expression construct containing the neo^r gene by $CaPO_4$ coprecipitation. Individual colonies were isolated and tested for virus production by supernatant infection of TK- Rat-2 fibroblast cells. Supernatant harvested for 2 hours at 37°C from a 75% confluent culture of the cell clone used in this study contains $5x10^4$ pfu's/ml. No replication competent retrovirus was detected in a marker rescue assay. A TK positive cell line, designated TK+RV- that did not produce retroviral vectors was generated by co-transfection in PA317 cells of a non-viral expression construct, harboring the HSV-tk gene under control of the murine phosphoglycerate kinase promoter. Both the TK+RV- non producer cell line and the IG-RV-TK virus producer cell line were killed 100% by GCV concentrations as low as 0.1mM.

A retroviral *Lac*Z producer cell line was kindly provided by Dr. R.C. Hoeben (Lab. of Mol. Carc, Univ. of Leiden, The Netherlands). This retroviral vector was made according to procedures described in Hoeben et al., (1991).

A PA317 packaging cell line producing retrovirus encoding human IL-2 was constructed by standard procedures (34), using cDNA encoding for human IL-2 (cDNA was kindly provided by Dr. Schrier, Depart.of Clin. Oncol. Acad. Hosp. Leiden, The Netherlands). The gene was ligated into the pMFG retroviral construct digested with *Nco*I / *Bam*H1 (12). Infection of a rat lung tumor cell line with the IL-2 virus resulted in succesful gene transfer into more than 25 % of the cells. The IL-2 activity was measured by a bioassay. The IL-2 producing cell clone had a maximum activity of 200 U/ 24 hours/ 10^5 cells.

In vitro studies
The 9L rat glioma and human U251 glioma cells were plated in 24 well culture dishes (Costar) at a density of 10^5 cells/ well. Triplicate wells were infected with Ad.RSVβgal at multiplicity of infections (m.o.i.) of 0, 1, 10 and 100. Three days after infection, duplicate wells were used to measure *LacZ* gene expression by assessing β–galactosidase activity as described by Norton at al. (1985). Three days after infection, remaining wells were stained with X-Gal (27). Killing efficiency of the suicide gene transfer was tested according to Chen et al., (9). 9L rat glioma and human U251 glioma cells were plated in 24 well culture dishes (Costar) at a density of 10^4 cells/ well and infected with IG.Ad.MLP.TK at m.o.i. 0, 1, 10 and 100. Twenty-four hours later, the infected cells were treated with GCV or PBS at a concentration of 10 mg/ml. After 72 hours, the surviving cells in quadruplet wells were trypsinised and surviving cells determined by trypan blue exclusion method, were counted.

In vivo studies
Fischer 344 rats, weighing 200-400 grams, were randomised, anesthetized with ether and placed in a stereotaxic frame. A burr hole was made 1 mm in front of the bregma and 2 mm lateral of the midline. $4x10^4$ 9L rat- gliosarcoma cells in 1 µl of Hank's buffered saline were injected by a microliter syringe (27 gauge needle; Hamilton) in the left forebrain, 4 mm ventral of the skull. The cells were injected over a period of 2 minutes. The needle was slowly retracted and the burr hole was closed with bonewax (Braun). The skin was closed with 9 mm autoclips. The same procedure and coordinates were subsequently used to inject recombinant adenovirus. A volume of 10 µl was infused over 5 minutes along the needle track (1 µl per 0.5 mm) starting 1 mm deeper than the injected tumor cells. Ad.RSVβgal was injected 6 days after tumor inoculation in dosages of $2.5x10^5$, $2.5x10^7$,and $2.5x10^9$ pfu 's (n=6 /group) in a volume of 5 µl. PBS (5 µl) was injected into the tumor as control (n=6). 3 days after tumor cell implantation different amounts of IG.Ad.MLP.TK or IG.Ad.MLP.luc as control were injected into the tumor in 8 different groups of rats; 5.10^8 (n=6), 10^8 (n=16), 10^7 (n=10) and 10^6 (n=10) IU of IG.Ad.MLP.TK and 5.10^8 (n=7), 10^8 (n=12) IU of IG.Ad.MLP.luc. It was calculated that the different groups treated at 3 days with IG.Ad.MLP.TK and IG.Ad.MLP.luc were infected with an m.o.i. of 5000, 1000, 100, 10 and 5000, 1000 respectively, by assuming a population of 10^5 tumor cells at the respective time points of virus inoculation, based on a cell doubling time of 18-20 hours (44). Fourty eight hours after the injection of the

virus, the rats received twice a day 15 mg/kg ganciclovir (Syntex) or PBS intraperitoneally for ten days (Fig. 4).
After death, the brain tumors were dissected from surrounding tissue and weighed. To examine the *LacZ* expression at different time points, three animals per group were sacrificed on day 5 and day 11 after virus injection. The rats were perfused via the left ventricle with ice cold 4% paraformaldehyde, the brains were removed and postfixed in the same solution. The brain was cut in 2 mm sections, washed thoroughly with PBS and stained with X-Gal for 3 hours at 37°C, essentially as described by Bout et al., 1994. After staining and photography the samples were embedded in paraffin, cut into 2 mm sections and counterstained with hematoxilin, phloxin and saffrane.
For the study of retroviral vectors, 3 days after tumor cell implantation, $5x10^6$ IG-RV-TK producer cells were infused over 15 minutes into the tumor (n=10) in a volume of 20 ml along the needle tract. Control animals were injected with PBS (n=5), $5x10^6$ retrovirus *LacZ* producer cells (n=5), $5x10^6$ TK+RV- cells (n=5), supernatant of IG-RV-TK producing cell line (n=5) and $5x10^6$ retrovirus IL-2 producer cells (RV-IL2; n=5), all in a volume of 20 µl. From eight days after tumor cell implantation the rats were treated twice a day with 15 mg/kg GCV intraperitoneally for ten days. A control group was treated with PBS instead of with GCV following injection of HSV-tk virus producer cells (n=5). Survival data of the adenovirus and retrovirus injected rats were presented as Kaplan – Meier plots (15). The log rank test was applied for statistical analysis of survival data.

Results

In vitro results
After infection *in vitro* with Ad.RSVβgal at an m.o.i. of 100, almost all U251 tumor cells and approximately half of 9L tumor cells were positive for *LacZ* at 3 days after infection. (fig. 2a, 2b – appendix). Following infection with an m.o.i. of 10 and 1 only a few transduced tumor cells were observed. Non transduced tumor cells (controls) did not show blue precipitates in the nucleus, but there was a slight blue staining in the cytoplasma of glioma cells, probably caused by endogenous β-galactosidase (data not shown). Dead cells, morphological changes or other cytopathic effects were not observed at an moi of 100 in both tumor cell types. β-galactosiase activity in the human tumor cell line at an m.o.i. of 100 was higher than in the rat tumor cells (Table 1). This was in agreement with

the experiment (Fig.2 – appendix) where the number of blue cells was higher in U251 than in 9L tumor cells. Cultured human U251 glioma cells were more sensitive to infection with IG.Ad.MLP.TK and subsequent GCV treatment than the rat tumor cells (Table 2.). Only 5 % of the U251 tumor cells survived at m.o.i. 100 as compared to 40 % of the rat glioma cells.

Table 1 β–Galactosidase Activity in Cultured Glioma Tumor Cells following infection with Ad.RSVbgal

Tumor cell	M.O.I. 0	M.O.I. 1	M.O.I. 10	M.O.I. 100
U251	0	2.4	1.1	52.2
9L	0	0.7	1.1	6.3

The table shows the mean values (n=2) of β–galactosidase activity/ mg protein (x1000) at different m.o.i's and different tumor cell cultures in vitro 72 hours after infection with Ad.RSVβgal. The β–galactosidase activity per well was corrected for background activity produced by the tumor cells. Cultured human U251 and rat 9L gliosarcoma were infected with AdRSVβgal at different m.o.i.. M.o.i was calculated assuming a cell population of $3x10^5$ cells at confluency.

Table 2 Glioma Cell Survival (in %) after Infection with IG.Ad.MLP.TK and Subsequent GCV or PBS Treatment

Tumor cell	Treatment	M.O.I. 0	M.O.I. 1	M.O.I. 10	M.O.I 100
9L	PBS	100	87	79	84
9L	GCV	79	67	65	40
U251	PBS	100	115	71	67
U251	GCV	81	29	16	5

The table shows the mean percentages (n=4) of glioma cell survival after infection with IG.Ad.MLP.TK and subsequent GCV or PBS treatment. 9L rat glioma and human U251 glioma cells were plated in 24 well culture dishes at a density of $1x10^4$ cells/ well and infected with IG.Ad.MLP.TK at m.o.i. 0, 1, 10 and 100. Twenty-four hours later the infected cells were treated with GCV or PBS at a concentration of 10 mg/ml. After 72 hours the surviving cells in quadruplet wells were trypsinised and counted by trypan blue exclusion method.

Adenoviral marker gene studies

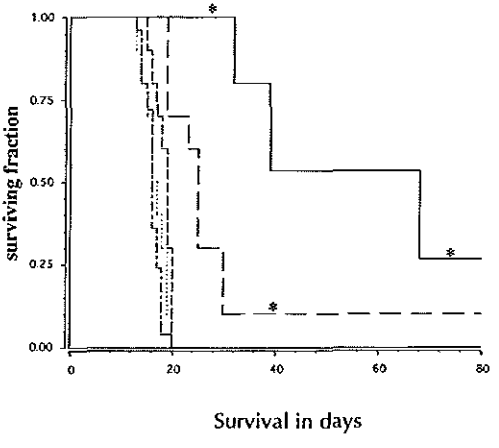
Five days following infection of the intracerebral brain tumor, brain sections showed *LacZ* staining of the tumor region. The blue precipitates were mainly localised in tumor regions adjacent to normal brain tissue (Fig. 3a – appendix).

Tumors injected with PBS did not show any blue precipitates (data not shown). At 11 days after infection, a few blue patches were still seen in tumors injected with $2.5x10^9$ IU. Tumors injected with PBS or with lower amounts of virus ($2.5x10^7$ and $2.5x10^5$ IU) showed no blue precipitates at this time point. The latter was probably due to a 'diluting' effect in the dividing tumor cell population. Microscopical examination of in vivo transduced tumors, revealed blue precipitates in the nucleus of the malignant cells. A large proportion of the tumor cells expressed β–galactosidase (Fig. 3b – appendix). Brain cells adjacent to the tumor showed also some blue nuclear precipitates, but no dead cells, no mophological changes and no other cytopathic effects were observed (Fig. 3c – appendix).

In vivo gene therapy

Rats treated with IG.Ad.MLP.TK at an m.o.i of 5000 and 1000 showed significantly prolonged survival time as compared to the rats given IG.Ad.MLP.luc or IG.Ad.MLP.TK without GCV treatment (log rank test, $p<0.01$, Fig. 4). Two animals in the group treated with m.o.i 5000 and 1 animal in the group treated with m.o.i 1000 died of superficial leptomeningeal tumor (Fig. 4) which was caused by spill of tumor cells through the burr hole. Intracerebral tumors were not present in these rats. In addition, rats treated with IG.Ad.MLP.TK at an m.o.i. of 100 survived 18.3 days on the average (Fig. 4) as compared to 15.7 days for the control group. The prolonged survival time in this treated group was significant as compared to controls (log rank test, $p<0.05$). Survival of rats treated with 10^6 pfu's (m.o.i.of 10) was not significantly different from controls (log rank test, $p>0.05$).

Figure 4



Kaplan-Meier survival curves of rats with brain tumor treated with single doses recombinant adenovirus and subsequent GCV administration. Intracerebral injection of tumor cells at day 0. 3 days after tumor cell implantation different amounts of IG.Ad.MLP.TK(TK) or IG.Ad.MLP.luc.(luc.) as a control were injected into the tumor in different groups (1 to 8) of rats (n=61) . Fourty eight hours after virus injection 15 mg/kg GCV (G+) or 1ml PBS (G-) was administered i.p. twice daily for ten days.

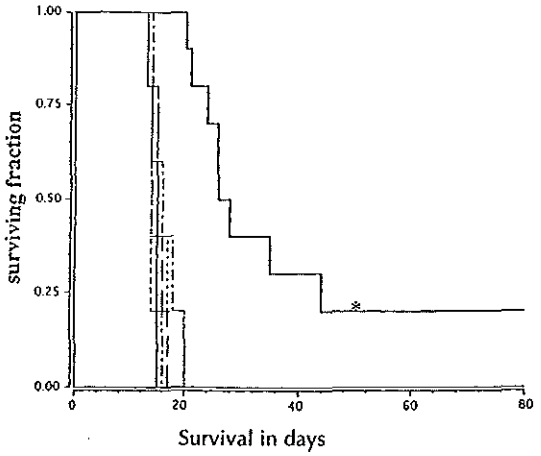
1. TK/G+, 5.10^8 IU, n=6 (_____).
2. TK/G+, 1.10^8 IU, n=10 (__ __).
3. TK/G+, 1.10^7 IU, n=10 (--------).
4. TK/G+, 1.10^6 IU, n=10 (.....).
5. luc./G+, 5.10^8 IU, n=7 (__ __ __).
6. luc./G+, 1.10^8 IU, n=6 (__ __ __).
7. luc./G-, 1.10^8 IU, n=6 (__ __ __).
8. luc./G-, 1.10^8 IU, n=6 (__ __ __).

Rats in groups 5 to 8 were not significantly different from each other in survival time ($p>0.05$; log rank test) and are represented as one group (__ __ __) in the Kaplan-Meier survival curve. The rats in groups 1, 2 and 3 lived significantly longer than all control groups ($p<0.01$, $p< 0.01$, $p<0.05$ respectively, log rank test). Three rats died of superficial leptomeningeal tumor (*). These are censored in the survival analysis.

In all animals that died, the tumor weight was determined. Tumors inoculated with IG.Ad.MLP.TK did not differ in mean weight (0.322 mg +0.81 mg) from tumors injected with IG.Ad.MLP.luc. (0.318 mg + 0.63 mg). This shows that death ensues when the tumor mass has reached a certain volume. It was calculated that the brain tumors at the time of death contain approximately $3.2x10^8$ cells (assuming that 10^9 cells weigh 1 gram). As $4x10^4$ cells were inoculated, the number of tumor cells in untreated rats increased approximately 10^4 times in 16.7 days, indicating a tumor doubling time of approximately 1 day if all tumor cells are dividing. The shortest survival time among rats treated with IG.Ad.MLP.TK with an m.o.i of 5000, 3 days after tumor cell inoculation was 32 days. At the start of the GCV therapy the tumor contained + 10^5 cells (3 cell divisions). The number of tumor cells after the end of GCV therapy at day 15, was extrapolated to be 10^4, indicating that at least 1 log cell kill occurred. The rat that died at 68 days from the brain tumor must have had between 1-10 viable tumor cells present after GCV therapy, which corresponds 4 to 5 log cell kill.

Rats inoculated with a IG-RV-TK producing cell line followed by GCV treatment survived significantly longer than any of the 5 control groups ($p<0.01$, log rank test; average survival time control groups: PBS: 15.2 days; retrovirus *Lac*Z producer cells: 14.4 days; TK+RV- non-producer cell line: 14.0 days; supernatant IG-RV-TK producer cell line: 16.6 days; IG-RV-TK producer cell line without GCV administration: 16.2 days; IG-RV-hIL 2 producer cell line: 14.6 days; Fig. 5).

Figure 5 Kaplan-Meier survival curves of rats with brain tumor treated with single doses recombinant retrovirus producer cell lines and subsequent GCV administration.



Intracerebral injection of tumor cells at day 0. IG-RV-TK was injected 3 days after tumor cell inoculation. One group was injected with IG-RV-TK producer cells (n=10)(_____). The controls (n=5/ group) were injected with PBS (_____), RV-*Lac*Z producer cells (--------) TK+RV- non producer cells (__ __), supernatant of IG-RV-TK producer cells (__ _ __) and RV-IL2 producer cells (__ - - __). One group was injected with IG-RV-TK producer cells without subsequent GCV treatment (- - - - - -) . Five days after virus injection 15 mg/kg GCV was administered i.p. twice daily for ten days. IG-RV-TK treated rats lived significantly longer than controls ($p<0.01$; log rank test). One rat died of superficial leptomeningeal tumor (*). This rat is censored in the survival analysis.

Eight rats in the group treated with IG-RV-TK producer cells died from their brain tumor. One animal with a survival time of 50 days died of superficial leptomeningeal tumor which was caused by spill of tumor cells through the burr hole. No intracerebral brain tumor was present. Using the same cell population kinetics as above, it appears that also after treatment with IG-RV-TK producing cells 1 to 5 log cell kill was achieved. Although the median survival time (26 days) was less than after treatment with 5.10^8 IU IG.Ad.MLP.TK (39 days) there was no significant difference in mean survival time between the different treated groups (log rank test, $p>0.05$).

Discussion

Our study shows an efficient adenovirus-mediated gene-transfer into rat and human brain tumor cells *in vitro* at an m.o.i of 100. At this m.o.i, human glioma cells showed a higher level of *LacZ* expression and β-galactosidase activity after Ad.RSVβgal infection as compared to rat tumor cells (Fig. 2a, 2b – appendix and Table 1). The *in vitro* data also showed that human U251 tumor cells are more sensitive to IG.Ad.MLP.TK / GCV treatment than 9L rat tumor cells (Table 2).We have no satisfactory explanation for this phenomenon, but is most likely caused by more efficient adenovirus mediated gene delivery to human cells. Effective adenovirus-mediated marker gene-transfer in rat brain tumors in vivo was only achieved after injection of $2.5.10^9$ Ad.RSVβgal IU. The amount of *LacZ* positive tumor cells in vivo decreased dramatically in time indicating a 'diluting effect' of extra- chromosomal adenoviral DNA in a rapidly dividing cell population. These results are in agreement with recently published data of Badie et al.(2) and Boviatsis et al., (43). This indicates that maximum tumor cell kill in vivo after transduction with IG.Ad.MLP.TK will only take place if GCV treatment has already commenced when the thymidine kinase gene is expressed. In contrast, after retroviral gene transfer, stable integration of the recombinant gene in the tumor cell DNA will occur and will be retained in all progeny. However, the therapeutic efficiency of thymidine kinase gene therapy with retrovirus has been questioned by Wei et al., '(1994), since retroviral integration of the recombinant gene in tumor cells is dependent on cell division and the majority of cells in human malignant brain tumors remain in G0 (resting phase) at any one time (24, 46). These arguments can not hold for the treatment with adenoviral vectors which are also capable of transducing non-dividing cells. Whenever such transduced tumor cells enter the S phase of the cell cycle, cell death will occur as long as GCV is provided.

Injection with a m.o.i. lower than 100 resulted in ineffective transduction and tumor cell kill *in vitro* in both tumor cell cultures. Our in vivo experiments indicate that an m.o.i. of at least 100 is necessary for effective tumor cell kill following GCV treatment (Fig. 4). Chen et al., (9) showed that adenoviral TK transduced gene expression in the C6 rat glioma cell line *in vitro* continued to increase at multiplicity of infections higher than 125, where 100 % transduction was achieved using the *LacZ* gene. It can be expected that the amount of adenovirus that can be injected in the tumor will be limited by direct toxicity for surrounding tissue. Intratumoral injection of 2.5 $x10^9$ IU's resulted in no cell death, no morphological

changes and no other cytopathic effects in the surrounding brain tissue measured after 5 days, using light microscopy. Akli et al., '(1993) found that four days after direct intracerebral injection of more than 10^7 IU of adenovirus resulted in detectable cytopathic effects in brain tissue. Our *in vivo* results showed that the distribution of injected virus is predominantly limited to the tumor and that only small areas of the surrounding brain tissue were infected. Although the virus was directly injected into the tumor, spread through the loose incoherent tumor tissue is apparently more effective than within brain tissue where fiber tracts or the glia cells may prevent their diffusion (17, 1, 3, 11).

The prolonged survival time of rats treated with IG.Ad.MLP.TK and IG-RV-TK as compared to control rats demonstrated the effectivity of IG.Ad.MLP.TK and IG-RV-TK in this brain tumor model. Our data show also that rats injected with IG-RV-TK producer cells live significantly longer than control animals, including controls injected with IG-RV-TK producer cells without GCV treatment. This is in contrast to recently published data by Tapscott et al., (41) who reported that GCV selection is not required for tumor cell kill, implicating an immunologic mechanism induced by TK-transduced brain tumor cells. Immunogenicity of in vivo retroviral TK-transduced tumor cells was not observed by us, even after in vivo transduction with RV-hIL-2 producer cells.

With both recombinant viruses, a tumor cell kill was calculated of between 1 and 5 log. The broad range in tumor cell kill in the different animals suggest that virus delivery varied widely. Although the viruses were injected at the same stereotactic coordinates as the tumor cells, not all tumor cells were transduced. Methods of virus delivery to the tumor have to be improved.

In the present study, no significant difference in survival time (log rank test; $p>0.05$) was observed after treatment with IG.Ad.MLP.TK as compared to IG-RV-TK producer cells, indicating that both methods may be clinically useful. Other factors may favor the use of adenoviral vectors in clinical studies. Recombinant adenoviruses have the advantages that high titers can be obtained and that the cell free virus can be administered in vivo. Recombinant adenovirus also infects non-dividing tumor cells (resting phase) and consequently kill these cells in the S phase of the cell cycle when GCV is provided. A disadvantage may be toxicity of adenovirus for normal brain at high m.o.i., although in our study no adverse effects were seen.

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CHAPTER 4

Treatment of Leptomeningeal Metastases in a Rat Model using a Recombinant Adenovirus containing the HSV-tk gene

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J. Neurosurg. 1996;85:648-654.

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Abstract

We constructed recombinant adenoviral vectors to investigate its potential for gene therapy treatment of leptomeningeal metastases. Several human cell lines derived from tumors occurring as leptomeningeal metastases infected in vitro with recombinant adenovirus containing the luciferase gene, (IG.Ad.MLP.luc.) showed high levels of expression. When these human tumor cell lines were infected in vitro with recombinant adenovirus harbouring the HSV-tk gene (IG.Ad.MLP.TK), they were highly sensitive to killing by GCV. Transduction efficiency of leptomeningeal tumor cells in vivo was assessed by injecting 9L rat brain tumor cells into the cerebrospinal fluid via the cisterna magna of Fischer rats. After three days, recombinant adenovirus containing the LacZ reporter gene (IG.Ad.MLP.LacZ) was injected via the same route. Six days after tumor cell injection expression of the reporter gene was observed in tumor cells along the total neural axis. Subsequently, rats with leptomeningeal metastases were treated 3 days after tumor cell injection with IG.Ad.MLP.TK. The next day, ganciclovir was injected i.p. for ten days. Rats that developed neurological symptoms were killed instantly. The symptom free latency of every rat was determined. Rats treated with IG.Ad.MLP.TK and subsequent GCV had significantly longer ($p<0.01$) symptom free latency than all control groups.

This study demonstrates the feasibility and efficacy of this therapeutic approach in a rat model. Clinically, it should be employed in the palliative treatment of patients with lepto-meningeal metastases.

Introduction

The prognosis of patients with malignant leptomeningeal metastases is generally poor. Without treatment, the rapidly proliferating malignant cells in the circulating cerebro spinal fluid (CSF) cause a large diversity of disabling neurological symptoms and the average survival without treatment is reported as 4-6 weeks (15,19,33). Palliative treatment of leptomeningeal metastases aims at long lasting neurologic improvement. However, the number of effective drugs that can be administered directly in the subarachnoid space is small and systemic administration is often limited by drug resistance and toxicity (11). Whole neuroaxis radiotherapy may cause severe bone marrow depression which may therefore limit future additional chemotherapy treatment. New treatment modalities are needed for this progressive fatal neurological illness.

A promising approach for treatment of local tumors is the transfer of the Herpes Simplex Virus thymidine kinase gene (HSV-tk) into tumor cells, which renders these cells highly sensitive to the toxic effects of ganciclovir (GCV) (7,16,17). GCV is phosphorylated by tk-transduced cells which interferes with DNA synthesis during cell replication. Since only dividing cells are killed by this mechanism, central nervous system (CNS) tumors are good candidates for this approach. We and others have already shown the effectivity of "suicide" gene therapy in a rat brain tumor model using recombinant adenovirus (6,21,31), Herpes Simplex virus (3) or retrovirus (7,20,24) harbouring the HSV-tk gene. Since leptomeningeal metastases are surrounded by essentially non-dividing tissue, they are also another possible candidate for this treatment. Theoretically, injection of recombinant virus into the CSF could transduce multi-focal tumor cells or masses throughout the CNS. Direct injection of the viral vector into the subarachnoid space is clinically feasible and high concentrations of GCV in the CSF can be achieved by systemic administration.

Ram et al (25) have shown the therapeutic potential of direct injection of retroviral-tk vector-producer cells in the CSF of rats with leptomeningeal neoplasia. Recombinant adenovirus has the advantage that high titers can be obtained and that the cell free virus can be administered directly *in vivo*. Since no producer cells are injected, the risk of CSF obstruction by clustering cells can be circumvented. In contrast to retrovirus, recombinant adenovirus also infects non-dividing, quiescent, tumor cells and consequently kills these cells upon their entry into the S phase of the cell cycle when GCV is provided.

In this study, we investigated the feasibility of treating leptomeningeal metastases. The sensitivity of malignant human tumor cells, known to

metastasize into the leptomeninges was assessed *in vitro* using a recombinant adenovirus containing the HSV-tk gene. Subsequently, distribution and viability of the adenovirus in CSF, and treatment effectivity in an immunocompetent rat leptomeningeal tumor model was tested.

Materials and methods

Adenoviral vectors

Recombinant adenoviruses harbouring the HSV-tk gene (IG.Ad.MLP.-TK) were made from plasmid pMLP.TK in which HSV-tk expression driven by the adenovirus derived major late promoter (14) and transcription is terminated by the SV40 poly-adenylation signal. The generation of IG.Ad.MLP.TK was described elsewhere (31). Similar adenovirus constructs containing the firefly luciferase gene (luc.) or nls.*LacZ* gene (Escherischia coli *LacZ* gene preceeded by the nuclear localisation signal) were made. The luc cDNA was derived from pRSV.luc (9). pRSV.luc was digested with *Hind*III and SspI, to excise the luc sequence, that was cloned in the multiple cloning site of pBluescript (BS) (Stratagene). Subsequently, the *Hind*III and BamHI cloning sites of pMLP.TK were used to replace the tk cDNA by the luc sequence derived from pBS.luc.

The nls.*LacZ* gene was excised from L7RHbgal (11) with AvrII and BamHI and ligated together with a *Hind*III – XbaI linker sequence into pMLP10 that was digested with *Hind*III and BamI. The new construct was named pMLP.nls.*LacZ*/-Ad. The BglII – ScaI fragment (nt. 3328 – 6092) from adenovirus type 5 was ligated into pMLP.nls.*LacZ*/-Ad after digestion with BamHI and NruI. The resulting construct was pMLP.nls.*LacZ*.

Recombinant viruses were generated by co-transfecting 293 cells with *SalI* linearized pMLP.TK/luc/nls.*LacZ* and the large ClaI fragment of wild-type Ad5 DNA. Recombinant viruses were plaque purified twice, propagated and titrated according to standard procedures (23). Virus titers determined by end point titration ranged from $1x10^{10}$ to $1x10^{11}$ IU. The respective resulting recombinant viruses were named IG.Ad.MLP.TK, IG.Ad.MLP.luc and IG.Ad.MLP.*LacZ*.

Tumor cells

Tumor cells were cultured in Dulbecco's modified Eagle's medium (9L, U251, A549, 518 A2)(Gibco) or RPMI 1640 (GLC-1) (Gibco) supplemented with penicilline (100 IU/ml; Gibco), streptomycine (50 mg/ml; Gibco), 10% fetal calf serum and non-essential amino acids. GLC-1 was

cultured in RPMI 1640 (Gibco) The cells were grown at 37°C; 5% CO_2 in 80 or 175cm^2 flasks (Nunclon).

Efficiency of gene transfer in vitro
The rat glioma (9L) and human tumor cells were plated in 24 well culture dishes (Costar) at a density of 10^4 cells/well. Cells in triplicate wells were transduced with IG.Ad.MLP.luc at multiplicity of infections (m.o.i.) of 0, 10, 100 and 1000. Seventy-two hours after infection cells were trypsinized, centrifuged and resuspended in lysis buffer (100 mM Na-Phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 % Triton X-100, 15 % glycerol). After centrifugation the luciferase activity was determined in the supernatant by measuring integrated light emission for 10 seconds in the presence of 25 mM ATP and 1mM luciferin (9) with a MGM Optocom II luminometer. Activity was presented as light units (LU) per mg of soluble protein (LU/mg). The protein concentration was determined by the BIO-RAD DC protein assay kit (Bio-Rad Laboratories).

Killing efficiency in tumor cells in vitro
Killing efficiency of GCV after TK-gene transfer was tested according to Chen et al.(6). The rat glioma and human tumor cells were plated in 24 well culture dishes (Costar) at a density of 104 cells/well and infected with IG.Ad.MLP.TK at m.o.i. of 0, 10, 100 and 1000. Twenty-four hours later, the infected cells were treated with GCV (Syntex) at a concentration of 10 mg/ml or PBS (control). After 72 hours, the surviving cells in triplicate wells were trypsinised and determined by trypan blue exclusion method. To evaluate killing efficiency of IG.Ad.MLP.TK/GCV treatment over time, 9L tumor cells and U251 tumor cells were plated in 24 well culture dishes (Costar) at a density of 10^4 cells/well and infected with IG.Ad.MLP.TK at m.o.i.100 and treated with GCV (10 mg/ml) or PBS (control). The surviving cells were determined as described above every 24 hours for 6 days.

Leptomeningeal tumor model
A modified version of the 9L rat syngeneic leptomeningeal metastases tumor model was developed (13). Fischer F344 rats were anesthetized with ether, shaved suboccipitally and placed in a stereotactic frame with the skull in flexion. $4x10^4$ 9L rat tumor cells in a volume of 30 µl using a 0.1 ml syringe were injected through the skin, dorsal neck muscles and dura mater directly into the CSF of the cisterna magna. The right position

of the needle tip prior to cell injection was affirmed by positive CSF aspiration.

In vivo studies

Male Fischer 344 rats weighing 250-300 grams were anesthetized with ether and injected with $4x10^4$ 9L tumor cells suboccipitally as described above. After 3 days, rats were injected via the same route with 1.10^8 pfu's IG.Ad.MLP.*Lac*Z (n=3) , 2.10^9 IU IG.Ad.MLP.TK (n=25) or phosphate buffered saline (PBS, n=22, control) in a volume of 30 µl. To examine *Lac*Z expression in leptomeningeal tumor cells, rats injected with IG.Ad.MLP.*Lac*Z and PBS (n=2) were sacrificed 6 days after tumor injection. The rats were perfused via the left cardiac ventricle with ice cold 4 % paraformaldehyde, the brains and spinal cord removed and postfixed in the same solution. The brain and spinal cord were cut into 2 mm sections, washed thoroughly with PBS and stained overnight with X-Gal at 37°C for histochemical detection of transduced cells. After staining the sections were embedded in paraffin, cut into 2 µm sections and counterstained with hematoxilin, phloxin and saffrane.

Rats injected with either IG.Ad.MLP.TK or PBS received twice a day intraperitoneal injections of GCV (15 mg/kg, Syntex, n=15) or PBS (n=30) for 10 days. Rats were observed daily and killed instantly when moribund or when neurological symptoms such as paralysis of the legs, unsteady gait or incontinence developed. The symptom free period of every rat was calculated starting with the injection of tumor cells on day 0. The symptom free latency of each group was presented in a Kaplan-Meier plot (12). The log rank test was applied for statistical analysis of symptom free latency data.

Sources of supplies and equipment

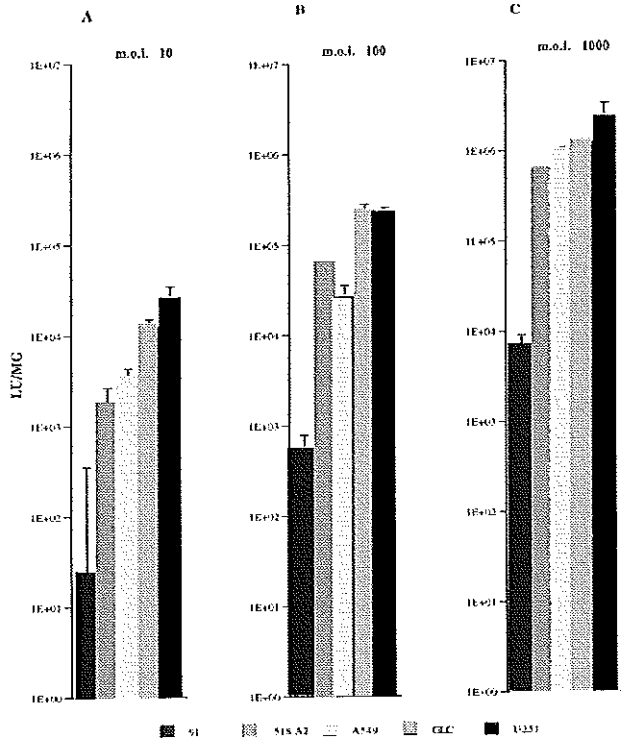
The 9L rat gliosarcoma brain tumor (a kind gift from Dr. K.M. Hebeda, Dept. Experimental Neurosurgery, Free University Hospital Amsterdam, The Netherlands) was induced by N-methylnitrosurea in Fischer 344 rats and characterised by Weizsaecker et al., '(34). The U251 human glioma cell line (22,28) was obtained from Dr. C.H. Langeveld (Dept. of Pharmacology, Free University Hospital Amsterdam, The Netherlands). The A549 human lung carcinoma cell line (10) was purchased from Biowhittaker (Brussel, Belgium). The GLC-1 human small cell lung cancer (SCLC) cell line (8) was kindly provided by Prof. Dr. L de Ley (Dept. of Clin. Immunology, State University Groningen, The Netherlands) and the 518 A2 human melanoma cell line (27 by Dr. P Schrier (Dept. of Clin. Oncology Academic Hospital, Leiden, The Netherlands).

Results

In vitro experiments:

Gene transfer and killing efficiency
The efficacy of adenovirus mediated gene transfer to human and rat tumor cells was determined by infecting the cells with IG.Ad.MLP.luc. Luciferase-activity was detectable in all tumor cells infected at m.o.i. of 10, 100 and 1000. In all cell lines tested luciferase activity increased with the m.o.i. (Fig. 1).

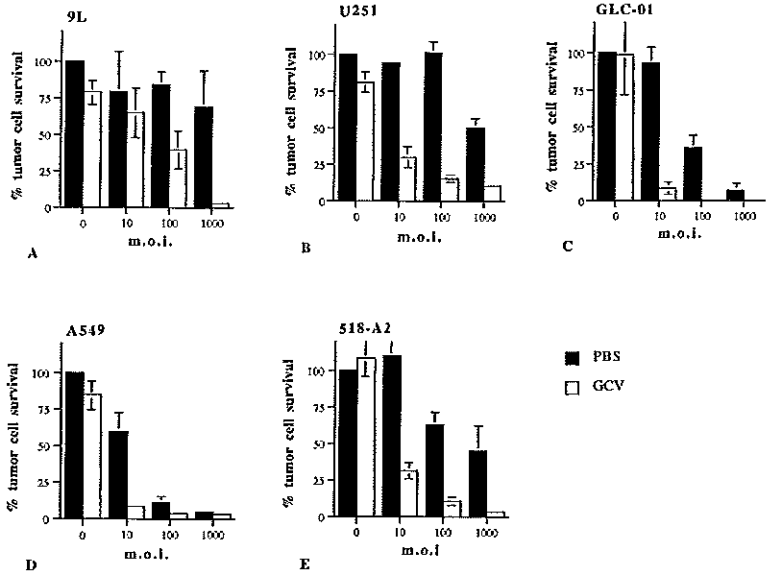
Figure 1 Luciferase activity of tumor cells (9L, 518 A2, A549, GLC-01, U251) transduced *in vitro* with IG.Ad.MLP.luc



Tumor cells were plated at a density of $1x10^4$/well and infected with IG.Ad.MLP.luc. at m.o.i. of 1, 10 and 1000. Seventy-two hours after infection, cells were lysed and luciferase activity was determined. Activity was presented as light units (LU) per mg of soluble protein (LU/mg). A. m.o.i. 10. B. m.o.i. 100. C. m.o.i. 1000.

Human tumor cells infected with IG.Ad.MLP.luc. showed a 10 to 100 times higher luciferase activity as compared to the rat 9L tumor cells. The highest activity was detected in the U251 glioma and GLC-01 small cell lung carcinoma cells.
Cultured tumor cells infected with IG.Ad.MLP.TK were killed effectively within 72 hours after treatment with GCV (Fig. 2).

Figure 2 Survival percentage of tumor cells infected *in vitro* with IG.Ad.MLP.TK and subsequent treated with GCV or PBS

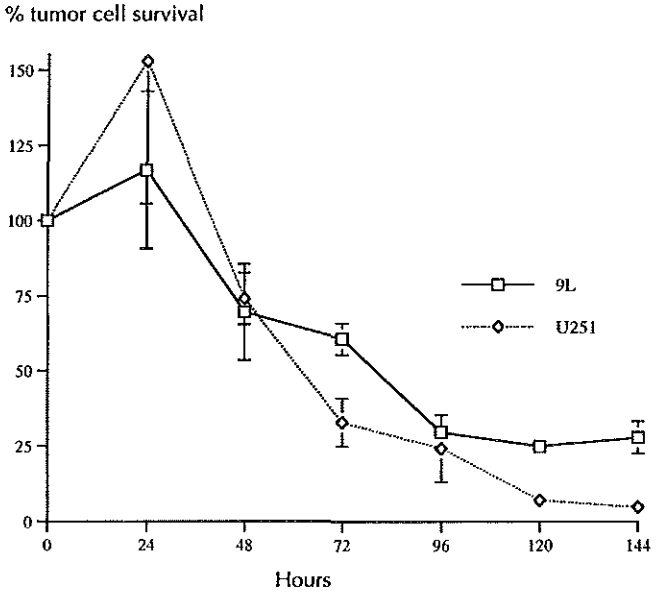


Tumor cells were plated at a density of $1x10^4$/well and infected with IG.Ad.MLP.TK. at m.o.i. of 10, 100 and 1000. Twenty-four hours later the infected cells were treated with GCV at a concentration of 10 µg/ml or PBS. After 72 hours, the cells in triplicate wells were trypsinised and surviving cells counted by trypan blue exclusion method. A. 9L rat glioma cells. B. U251 human glioma cells. C. GLC-01 human small cell lung carcinoma cells. D. A549 human non-small cell lung carcinoma cells. E. 518 A2 human melanoma cells.

The cell kill continued to increase after 72 hours as shown in the 9L and U251 tumor cells (Fig. 3). The human tumor cells were more sensitive to the TK/GCV treatment than the 9L rat tumor cells. At m.o.i. of 10, 100 and 1000, mean cell survival after 72 hours ranged from 8.5 % -31 %, 3.7

% -15.5 % and 0 – 10.2 % respectively in human tumor cells as compared to a 64.8 %, 39.6 % and 3.7% in rat glioma cells.

Figure 3 tumor cell survival



Survival with time percentages of 9L rat glioma and U251 human glioma tumor cells *in vitro* infected with IG.Ad.MLP.TK and subsequent GCV treatment. Tumor cells were plated at a density of $1x10^4$/well and infected with IG.Ad.MLP.TK. at an m.o.i. of 100 and treated with GCV at a concentration of 10 µg/ml or PBS. For 6 days, every 24 hours, cells in triplicate wells were trypsinised and surviving cells were counted by trypan blue exclusion method.

In vivo experiments:

Tumor model

Rats inoculated with $4x10^4$ 9L tumor cell developed symptoms between 16 and 19 days after tumor inoculation. The symptoms ranged from paralysis of one or more legs, unsteady gait, very poor general or moribund condition. Rats died within 24 hours after onset of the symptoms. Microscopic examination of the CNS 6 days after tumor inoculation showed infiltration of tumor cells in meninges of cerebrum, cerebellum and along the spinal cord. The largest tumor mass was usually observed near the injection site. No infiltration of normal brain tissue or spinal cord was observed (data not shown).

Adenoviral marker studies

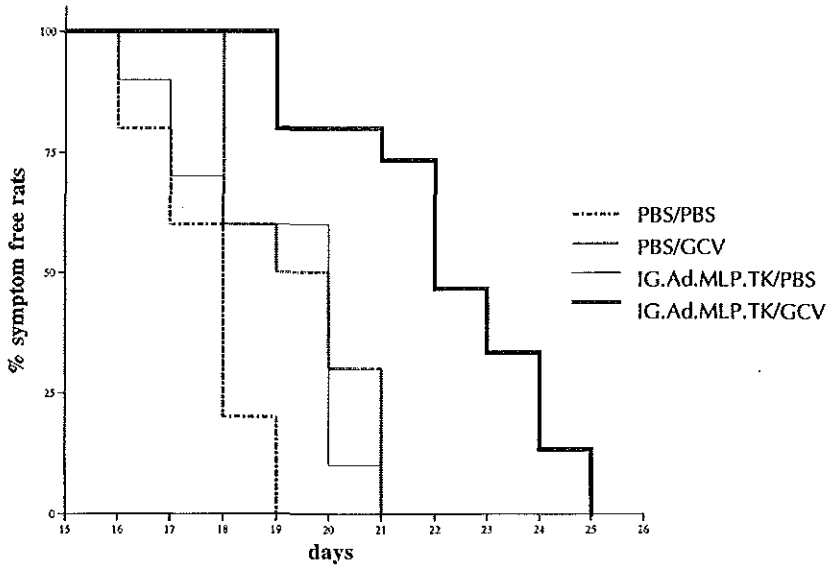
Microscopic examination of sacrificed rats inoculated with IG.Ad.MLP.-*ac*Z showed expression of the reporter gene in tumor cells all along the neural axis. Transduced tumor cells were observed between the meninges and surface of cerebrum, cerebellum and spinal cord (Fig. 4a,b,c – appendix). Most transduced cells were observed in the tumor mass near the injection site. Few ependymal cells surfacing the cerebrospinal fluid cavity showed also some blue nuclear precipitates, but no morphological changes or other cythopathic effects were observed (Fig. 4d – appendix). No *Lac*Z staining was observed in the brain tissue or myelum adjacent to transduced tumor cells. Choroid plexus was stained blue after X-Gal staining in both treated and control animals (PBS), which was probably caused by a high endogenous ß-galactosidase activity. No further blue staining was observed in the CNS of control animals.

In vivo therapy

Animals injected with recombinant adenovirus did not show any clinical or neurological signs related to vector administration. Rats treated with IG.Ad.MLP.TK and GCV showed a significantly prolonged symptom free period as compared to control rats (log rank test, $p<0.01$)(Fig. 5). Control rats had a mean symptom free period (days) of 17.6±1.0 SD (PBS/ GCV-), 19.4±1.3 SD (PBS/GCV+), 18.9±1.7 SD (IG.Ad.MLP.-TK/GCV-), as compared to 22.2± 2.0 SD of the treated group.

The symptoms of control rats ranged from paralysis of one or more legs, unsteady gait or very poor general or moribund condition. This was in contrast to the treated (IG.Ad.MLP.TK/GCV+) rats which developed incontinence and/or paralysis of one or more hind legs without the more generalized detoriation seen in the control animals. In 26 % of the treated rats, incontinence was accompanied by hematuria. Macroscopic examination of the CNS of control animals showed superficial massive tumor growth in the cisterna magna, compressing cerebellum, brain stem and medulla oblongata. Superficially growing small tumor nodules were observed all around brain tissue and in the thoraco-lumbar region. In treated rats macroscopic tumor was only observed in the thoraco-lumbar region which compressed the lower parts of the myelum and cauda equina. Rats with incontinence had large 'retention' bladders sometimes filled with blood. Hematuria was probably caused by the increase in intravesical pressure, thereby causing disruption of blood vessels in the bladder wall.

Figure 5 Symptom free rats



Kaplan-Meier curves of rats with established lepto-meningeal tumors treated with recombinant adenovirus and subsequent GCV administration. Rats (n=45) were injected intrathecally with $4x10^4$ 9L tumor cells. After 3 days, rats were injected via the same route with 2.10^9 IU IG.Ad.MLP.TK or PBS. Twenty-four hours after tumor cell injection 15 mg/kg GCV or 1 ml PBS was administered i.p. twice a day for ten days. Rats were killed instantly when moribund or if neurological symptoms developed. Rats treated with IG.Ad.MLP.TK and subsequent GCV had significantly longer ($p<0.01$) symptom free latency than all control groups (PBS/GCV-, PBS/GCV+ and IG.Ad.MLP.TK/PBS).

Discussion

Our in vitro results show that adenovirus mediated gene transfer and gene expression in various tumor cells known to metastasize to the leptomeninges is very efficient (Fig. 1). The data show that the amount of gene expression is directly related to increased m.o.i. (Fig. 1). Tumor cell killing efficiency after TK/GCV treatment was also dependent on m.o.i. (Fig. 2), indicating that gene expression levels are an important parameter for cell kill. The in vitro data also demonstrate that a more efficient transfer and expression of genes can be obtained in human tumor cells than in 9L rat tumor cells (Fig. 1). The average luciferase activity measured in hu-

man tumor cells after IG.Ad.MLP.luc infection was 100 times higher as compared to rat tumor cells (Fig. 1). Human derived, malignant cells were also more sensitive to killing by IG.Ad.MLP.TK and subsequent GCV treatment than 9L tumor cells (Fig. 2,3). This observation is in agreement with our previous experiments (31) and is most likely caused by a more efficient adenovirus mediated gene expression or delivery to human than to rat tumor cells. However, gene expression levels may not be the only relevant factor as tumor cell killing efficiency in vitro did not always correlate with expression levels of the luciferase gene. U251 glioma tumor cells for instance, showed about the same expression levels of luciferase after 72 hours as in the GLC-1 Small cell lung carcinoma cells. The latter, on the other hand, were more effectively killed after IG.Ad.MLP.TK/GCV treatment (Fig. 2). This is most likely explained by the so called "bystander effect", the phenomenon that more tumor cells are killed than are transduced with the HSV-tk gene (3,7,30). Because cell-cell interaction and gap-junctions play an important role in the bystander effect (1), it is not unlikely that GLC-1 small cell lung carcinoma cells, which grow in three dimensional non-adhering large floating aggregates (8), are killed more effectively than U251 mono-layer cell cultures. In addition, cell cycling time or other factors may also play a role.

In this study, we used a simplified version of the 9L rat syngeneic leptomeningeal metastases tumor model. Instead of introducing a permanent catheter into the upper thoracic subarachnoid space via the cisterna magna, as described by Kooistra et al.(13), we have chosen to inject the tumor cells directly into the cisterna magna to avoid any damage to the CNS, CSF flow obstruction or other problems caused by the catheter. With the skull in flexion, injections of tumor cells into the cisterna magna of Fischer rats after positive CSF aspiration is accurate and fairly easy. This modified model proved highly reproducible in assessing tumor growth as all untreated rats developed neurological symptoms between day 16 and day 19, with an average of 17.6 days. Although a tumor mass was found near the injection site as demonstrated in the 'catheter studies', histological examination of CNS showed that a substantial number of tumor cells formed multiple foci along the total neural axis (Fig.4 – appendix), indicating that injected tumor cells were transported effectively along the CNS. Furthermore, the model showed to be very useful to demonstrate the therapeutical efficacy of a single treatment therapy. A similar and reproducible model has been described before in nude rats using H-146 SCLC tumor cells (18).

The in vivo marker studies show that recombinant adenovirus can transduce tumor cells at a distance from the injection point in a relatively large

closed compartment (Fig. 4 – appendix). Thusfar, recombinant adenovirus has been used in most studies for direct injection into tissue or tumor mass (2,5,21,31). Gene transduction into established lepto-meningeal tumor cells using retrovirus producer cells or recombinant adenovirus has been published before (25,32), but the majority of transduced cells in these studies were in the vicinity of the implanted catheter tip. Our study demonstrates that the adenovirus can be transported effectively within the CSF, without inducing clinical or neurosurgical symptoms and is not immediately inactivated by humoral or cellular factors. Only few ependymal cells surfacing the CSF were transduced using IG.Ad.MLP.*Lac*Z (Fig. 4B). It is not expected that transduction of ependymal cells or other non replicating cells in the CNS with 'suicide genes' will lead to cell kill after administration of GCV because they are relatively mitotically inactive.
Microcopically, no morphological and no cythopathic effects were observed in the CNS. This is in agreement with other data, in which recombinant adenovirus was directly injected into brain tissue (30). Whether strong immune responses occur in the relatively "immune privileged" CNS after recombinant adenovirus injections, is still a matter of debate (4,6,29,31).
As the mechanism of transduction by retrovirus and adenovirus are different (31), it is not possible to compare transduction efficiency we obtained with those reported after injection of retrovirus producing cells (25).
The effectivity and safety of a single treatment with IG.Ad.MLP.TK and subsequent GCV administration in rats with established lepto-meningeal metastases is demonstrated in this study (Fig. 5). The study also shows that the therapeutic effect is tk/GCV dependent and not related to an immune reaction against virus transduced cells (Fig. 5). Rats did not show any clinical or neurological signs related to the vector and/or GCV administration, demonstrating the clinical feasibility of this approach. Since higher expression and cell kill are observed in human as compared to rat tumor cells (Fig. 1,2,3), it is to be expected that the therapeutic effect in a clinical situation will be stronger than demonstrated in this model. Furthermore, leptomeningeal metastases in humans are not as aggressive as demonstrated in this model and relatively higher amounts of recombinant adenovirus can theoretically be administered intrathecally in humans as compared to the limited injection volume in rats.
The symptomatology and macroscopic tumor location in the different groups at death show that developing tumors at a certain distance from the injection site can be killed effectively and that systematically administered GCV can penetrate into leptomeningeal tumor cells. As tumor cells in the CSF of patients with lepto-meningeal metastases can circulate

through the CSF causing new tumor nodules it is an important observation that non-adhering tumor cells were also killed effectively with this method as measured *in vitro* (Fig. 2B). The study demonstrates that recombinant adenovirus can be administered safely intrathecally without inducing acute neurological or other clinical symptoms. The effectivity of gene therapy for leptomeningeal metastases has been shown earlier using retrovirus-tk producer cells (25). Although both methods may be clinically useful, the use of recombinant adenovirus has a few advantages over injection of retrovirus producer cells. High titers of purified cell free virus can be injected directly intrathecally, thereby limiting the volume of administered fluid. The number of retrovirus-tk producing cells that can be injected into the CSF is limited because of volume constraints and injected cells can theoretically cause CSF obstruction problems. Clinical studies with retroviruses have been complicated by severe immune reactions against the producer cells (26). Furthermore, retroviral integration of the recombinant tk-gene is completely dependent on mitotically active cells whereas recombinant adenovirus can also infect non-dividing 'quiescent' tumor cells and consequently kill these cells in the S phase of the cell cycle when GCV is provided.

Conclusions

Our results show therapeutic efficiency and feasibility of recombinant adenovirus harbouring the HSV-tk gene in leptomeningeal metastases. Clinical application of this approach is considered feasible.

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CHAPTER 5

Preclinical testing of recombinant adenoviral HSV-tk gene therapy for CNS malignancies

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Neurosurgery. 1997;41:442-452.

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Abstract

Objectives:
Adenoviral gene transfer and killing efficiency using the TK/GCV mechanism was evaluated in human cancer cells occurring as CNS tumors. Effectivity of this approach was tested in vitro and in experimental models for brain tumor and leptomeningeal metastases in rats in vivo. Recombinant adenoviruses with different promoters were compared.

Methods:
Adenoviral vectors harbouring a marker (LacZ) or a Thymidine Kinase (TK) gene were constructed. Transcription of genes was under the control of either the Adenovirus type 2 major late promoter (MLP) or the human cytomegalovirus (CMV) immediate early gene promoter. LacZ expression and GCV killing efficiency after TK gene transfer in several human tumor cells was evaluated in vitro. The 9L-rat brain tumor and -leptomeningeal metastases model were used to determine the effectivity of adeno-TK and subsequent GCV treatment in vivo. MLP and CMV containing adenoviral vectors were compared.

Results:
Gene expression and tumor cell kill was very efficient in all human tumor cell lines tested. The adenovirus containing the CMV promoter showed cytopathic effects in cultured tumor cells at high moi, but also higher cell killing efficiency after TK/GCV treatment as compared to the MLP promoter. Although both the MLP and CMV vectors showed a significant dose dependent therapeutic effect, animals treated with recombinant adenovirus containing the CMV promoter showed significantly longer survival time (brain tumors) or symptom free period (leptomeningeal metastases).

Conclusions:
This study demonstrates the therapeutic efficiency and feasibility of the TK/GCV approach in experimental brain tumors and leptomeningeal metastases. It also demonstrates that the promoter driving the transgene in an adenoviral vector influences toxicity and efficiency of treatment.

Introduction

Despite extensive surgery, high dose radiation and chemotherapy, minimal improvement in survival has been achieved in most Central Nervous System (CNS) malignancies over the last few decades. Glioma, cerebral and leptomeningeal metastases remain neoplasm's with very poor prognosis (19,20,24,34). The current regimens induce severe side effects and are therefore restricted in their use. New treatment modalities are needed which increase survival without inducing such severe toxic reactions., 'Suicide gene therapy' has been shown to be an effective and relatively safe new approach for treatment of experimental brain tumors and leptomeningeal metastases (6,10,11,39,40). First, Retrovirus (11,25,31), Herpes simplex virus (6,9), and later, Adenovirus (10,27,40) vectors have been developed to deliver the Herpes simplex thymidine kinase (HSV-tk) 'suicide' gene to brain tumor cells. Expression of the HSV-tk renders dividing cells sensitive to ganciclovir (GCV). HSV-tk converts GCV into a toxic phosphate which acts as a chain terminator of DNA synthesis, which will eventually lead to cell death (21,22). Since only dividing cells are killed selectively while sparing normal surrounding healthy tissue, treatment of CNS malignancies by suicide gene therapy could have advantages over current treatment modalities (radio-,chemotherapy). Adenoviral vectors have the advantage that high titers can be obtained, and cell free virus can be administered *in vivo*. Adenovirus does not integrate into the genome, thereby reducing the risk of insertional mutagenesis. The vector is also able to infect non-dividing quiescent tumor cells and subsequently kill these cells upon their entry into the cell cycle when GCV is provided. Several investigators (10,27,37,39,40) have demonstrated that treatment of experimental glioma and leptomeningeal metastases with adenovirus vectors harbouring the HSV-tk gene was not associated with toxicity. Recombinant adenoviruses thus have potential to be used as vectors in 'suicide gene therapy' of CNS malignancies in humans. However, adenoviral gene expression and tumor cell killing effectivity using the TK/GCV mechanism in human glioma cells has not been given much attention yet in literature. Since the adenoviral transgene is not integrated into the genome, rapidly dividing tumor cells can significantly decrease transgene expression. When clinical use is considered, more fundamental research concerning interaction of tumor cell rate division and killing efficiency should be investigated.

In this study, we investigated the feasibility of suicide gene therapy in several human tumor cell lines occurring within the CNS as malignancies using adenoviral vectors. First, we have used vectors harbouring the *LacZ*

gene to investigate transduction efficiency *in vitro* in several human tumor cells known to occur as glioblastoma, brain metastases or leptomeningeal metastases. Subsequently, tumor cell kill after treatment with recombinant adenovirus carrying HSV-tk and subsequent GCV administration was tested. Treatment effectivity *in vivo* was assessed in immuno-competent rat models for brain tumors and leptomeningeal metastases. Finally, the potential of different promoters was investigated. Adenoviral vectors harbouring either the Adenovirus type 2 major late promoter (MLP) or the human cytomegalovirus (CMV) immediate early gene promoter were constructed. We compared cytopathic effects of the vectors only on cultured cells and cytotoxicity of vector administration and GCV treatment in vitro. Furthermore, effectivity of the treatment of tumors was assessed in vivo.

Materials and methods

In vitro studies:

Adenoviral vectors

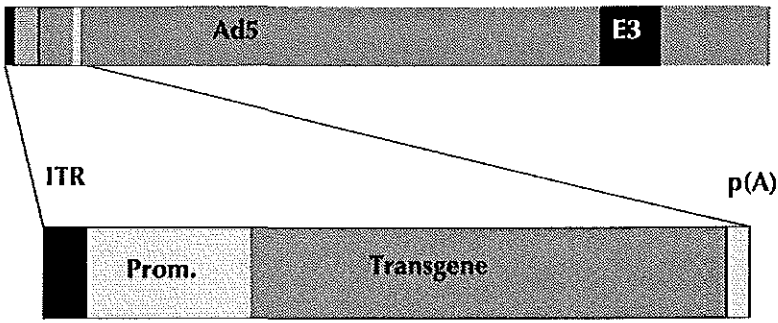
Recombinant adenovirus containing the TK gene driven by the MLP promoter was derived from plasmid pMLP.TK in which HSV-tk expression is driven by the adenovirus-derived major late promoter and transcription is terminated by the SV40 polyadenylation signal. A similar adenoviral construct containing the nls.*LacZ* gene (Escherischia coli *LacZ* gene preceded by the nuclear localisation signal) was made (pMLP.*LacZ*)

Recombinant adenovirus containing the TK gene driven by the CMV promoter was made from the pCMV.TK plasmid in which the HSV-tk expression was under the control of the CMV promoter. SV40 RNA splicing signals (180 bp) containing the late viral protein gene 16s/19s splice donor and acceptor signal are in between the promotor and transgene. These sequences were isolated from pCMV nls/*LacZ* (12). The Escherischia coli *LacZ* marker gene, preceded by a nuclear location signal, was cloned by replacing TK in pCMV.TK by *LacZ* in a plasmid similar to pCMV.TK called pCMV.nls.*LacZ* .

The adenoviral vectors were generated by co-transfecting 293 cells with *Sal*I linearized plasmids (pMLP.TK/*LacZ* or pCMV.TK/*LacZ*) and the large ClaI fragment of wild type Ad5 DNA. The E3 region was retained in all adenovirus vectors. Recombinant viruses were plaque purified twice, propagated and titrated according to standard procedures (28). Virus titers determined by end point titration on 911 cells ranged from

$1x10^{10}$ to $1x10^{11}$ Infectious Units (IU) The resulting recombinant viruses were named IG.Ad.MLP.TK, IG.Ad.CMV.TK, IG.Ad.MLP.*LacZ* and IG.Ad.CMV.*LacZ* respectively (Fig.1). The generation of IG.Ad.MLP.-TK, IG.Ad.CMV.TK, IG.Ad.MLP.*LacZ* and IG.Ad.CMV.*LacZ* have also been described in detail elsewhere (13,39,40).

Figure 1



Map of the constructed recombinant adenoviral vectors. Shown are the Human Ad 5 DNA containg the expression cassete. The cassette (enlarged) contains either the Adenovirus type 2 major late promoter (MLP) or the human cytomegalovirus (CMV) immediate early promoter. The promoter is preceded by an inverted terminal repeat, followed by followed by either the nls*LacZ*, (Escherischia coli *LacZ* gene preceeded by the nuclear localisation signal) or Herpes Simplex derived Thymidine Kinase (TK) suicide gene. Transcription is terminated by SV40 poly-adenylation sequences.
Prom.: ether the CMV or MLP promotor. **Transgene:** either TK or nls*LacZ* gene.
Ad 5: human adenovirus DNA. **ITR:** inverted terminal repeat. **p(A):** SV40 poly-adenylation sequences.

Tumor cells

The 9L rat gliosarcoma brain tumor (a kind gift from Dr. K.M. Hebeda, Dept. Experimental Neurosurgery, Free University Hospital Amsterdam, The Netherlands) was induced by N-methylnitrosurea in Fischer 344 rats and characterised by Weizsaecker et al.(42). The U251, D384 and LW5 human glioma cell line (27,35) were obtained from Dr. C.H. Langeveld (Dept. of Pharmacology, Free University Hospital Amsterdam, The Netherlands). The A549 human lung carcinoma cell line (13) was purchased from Biowhittaker (Brussels, Belgium). The GLC-1 human small cell lung cancer (SCLC) cell line (10a) was kindly provided by Prof. Dr.

L de Ley (Dept. of Clin. Immunology, State University Groningen, The Netherlands) and the 518 A2 human melanoma cell line (35) by Dr. P Schrier (Dept. of Clin. Oncology Academic Hospital, Leiden, The Netherlands).
Tumor cells were cultured in Dulbecco's modified Eagle's medium (9L, U251, A549, 518 A2)(Gibco) or RPMI 1640 (GLC-1) (Gibco) supplemented with penicilline (100 IU; Gibco), streptomycine (50 mg/ml; Gibco), 10% fetal calf serum and non-essential amino acids. GLC-1 was cultured in RPMI 1640 (Gibco). The cells were grown at 37°C; 5% CO_2 in 80 or 175cm^2 flasks (Nunclon).

Tumor cell doubling time
To determine the doubling time of human glioblastoma cells in vitro, U251, D384 and LW5 tumor cells were plated in 24 well culture dishes (Costar) at a density of 10^4 cells/well. Every 24 hours for 6 days, quadruplet wells were trypsinised and living cells were counted by trypan blue exclusion method. Doubling time of the tumor cells were determined by the following formula; $\mathbf{n = \ln (p/x) / \ln 2}$ (14,34); where n= number of cell divisions; p=number of cells after n divisions; x = initial number of cells, assuming that no cells are lost. Tumor cell doubling time was assessed by dividing the duration of the experiment (=6 days =144 hours) by the number of cell divisions. Statistical analysis (Student-t test) was performed on the data to determine the significance of the observed differences in doubling time.

Efficiency of gene transfer in vitro
The rat glioma and human tumor cells were plated in 24 well culture dishes (Costar) at a density of 10^4 cells/well. Cells in triplicate wells were infected with IG.Ad.MLP.*Lac*Z or IG.Ad.CMV.*Lac*Z at multiplicity of infections (m.o.i.) of 0, 10 or 100. To asess β-galactosidase activity, triplicate wells infected with *Lac*Z-adenovirus were stained with X-Gal[38] seventy-two hours after infection and examined microscopically.

Killing efficiency in vitro
Killing efficiency of GCV after TK-gene transfer was tested as described before (39,40). The rat glioma and human tumor cells were plated in 24 well culture dishes (Costar) at a density of 10^4 cells/well and infected with IG.Ad.MLP.TK or IG.Ad.CMV.TK at m.o.i. 0, 10, 100 and 1000. Twenty-four hours later, the infected cells were treated with GCV (Syntex) at a concentration of 10 μg/ml or PBS (control). 48 hours after GCV administration, the cells were trypsinised and living cells were

counted by trypan blue exclusion method. Statistical analysis (Student t-test) was performed on the data to answer the following questions: 1. Are TK/GCV treated tumor cells killed more effectively than TK/PBS (controls) treated cells? 2. Is killing efficiency using the GCV/TK mechanism in human tumor cells dependent on the promoter used (CMV versus MLP)? 3. Is TK/GCV tumor cell killing efficiency dependent on doubling time of human glioma cells in vitro?

In vivo studies:

Brain tumor
Fischer rats (n=90) weighing between 250-350 grams were anesthetized with ether and placed in a stereotactic frame. A burr hole was made 1 mm in front of the bregma and 2 mm lateral of the midline. Consequently $4x10^4$ 9L rat gliosarcoma cells in 1 µl of Hank's buffered saline were injected by a microliter syringe in the left forebrain, at a depth of 4 mm. The cells were injected over a period of 2 min. The needle was slowly retracted and the burr hole was closed with bonewax (Braun). The skin was closed with 9-mm clips. The same procedure and coordinates were subsequently used to inject recombinant virus; A volume of 10 µl was infused over 5 min. along the needletract (1 µl per 0.5 mm) starting 1 mm deeper than the injected tumor cells. Three days after tumor cell implantation, rats were randomised, different amounts of IG.Ad.MLP.TK or IG.Ad.CMV.TK or PBS as a control were injected into the tumor in different groups: 1.10^8 (n=15), 1.10^7 (n=10), 1.10^6 (n=10) IU IG.Ad.MLP.-TK, 1.10^8 (n=15), 1.10^7 (n=10), 1.10^6 (n=10) IU IG.Ad.CMV.TK and 10 µl PBS (n=20). Forty-eight hours after the injection of the virus, rats injected with either IG.Ad.MLP.TK, IG.Ad.CMV.TK or phosphate buffered saline (PBS) received twice a day intraperitoneal injections of GCV (15 mg/kg, Syntex, n=15) or PBS (n=30) for 10 days. Rats were observed daily and killed instantly when moribund or when neurological symptoms developed. Survival data of the rats were presented as Kaplan-Meier[16] plots. The log rank test was applied for statistical analysis of survival data.

Leptomeningeal metastases
A modified 9L gliosarcoma model of leptomeningeal neoplasia was used as described earlier by Vincent et al., (39). Briefly, male Fischer 344 rats (n=70) weighing 250-300 grams were anesthetized with ether, shaved suboccipitally and placed in a stereotactic frame with the skull in flexion. Consequently, $4x10^4$ 9L tumor cells in a volume of 30 µl using a 0.1 ml

syringe were injected directly into the CSF of the cisterna magna. After 3 days, rats were randomised and injected via the same route with; 2.10^9 IU IG.Ad.MLP.TK (n=25), 2.10^9 IU IG.Ad.CMV.TK (n=25) or phosphate buffered saline (PBS, n=20, control) in a volume of 30 µl. Rats injected with either IG.Ad.MLP.TK, IG.Ad.CMV.TK or phosphate buffered saline (PBS) received twice a day intraperitoneal injections of GCV (15 mg/kg, Syntex, n=15) or PBS (n=30) for 10 days. Rats were observed daily and killed instantly when moribund or when neurological symptoms such as paralysis of the legs, unsteady gait or incontinence developed.[40] The symptom free period of every rat was calculated starting with the injection of tumor cells on day 0. The symptom free latency of each group was presented in a Kaplan-Meier plot (16). The log rank test was applied for statistical analysis of symptom free latency data.

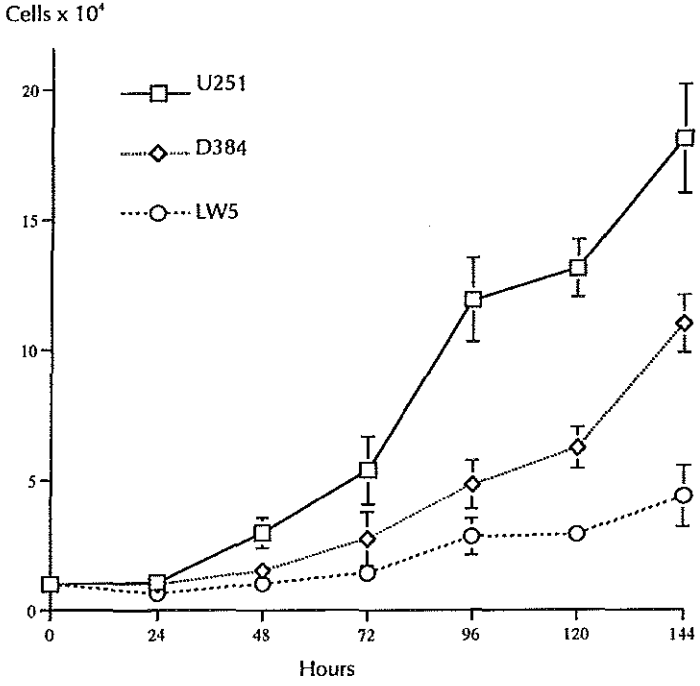
Results

In vitro experiments:

Tumor cell doubling time of human glioma cells
Population doubling time of the different tumor cells differed significantly between the glioblastoma tumor cell lines used. (U251 vs D384/ U251 vs LW5/ D384 vs LW5: day 1: ns/*/*, day 2: **/**/*, day 3: */**/*, day 4: ***/***/**, day 5: ***/***/**, day 6: **/***/***)(Student t- test;*= $p<0.05$, **=$p<0.01$, ***=$p<0.001$, ns=not significant) (Fig.2). The mitotically most active tumor cell was U251 with a mean doubling time of 34.4 hours. The calculated tumor cell mean doubling times in D384 and LW5 were 41.6 and 67.6 hours respectively(Fig.2 – appendix).

LacZ expression
Adenovirus mediated *Lac*Z gene transfer to tumor cells was determined by X-Gal staining of the cells. *Lac*Z activity could be microscopically detected in the nucleus (nuclear localisation signal)of all tumor cells infected with IG.Ad.MLP.*Lac*Z or IG.Ad.CMV.*Lac*Z (Fig.3 – appendix). Controls were negative for *Lac*Z activity (data not shown).

Figure 2



Tumor cell division activity and doubling time of human glioblastoma cells. U251, D384 and LW5 glioblastoma tumor cells were plated in 24 well culture dishes (Costar) at a density of 10^4 cells/well. Every 24 hours for 6 days, quadruplet wells were trypsinised and viable cells were counted by trypan blue exclusion method. Tumor cell doubling was determined as described in *Materials an Methods.* (see also Table 1.)

Killing efficiency

All cultured tumor cells infected with IG.Ad.MLP.TK or IG.Ad. CMV.TK were sensitive to a killing effect after treatment with GCV within 72 hours (Fig.4). Human tumor cells treated with TK/GCV were killed significantly at higher percentages than cells treated with TK/PBS (controls) (*MLP*-promoter: moi 10: U251**, D384*, LW5*, GLC-01**, A549*, 518 A2***, $9L^{ns}$, moi100: U251**, D384*, LW5*, GLC-01*, A549**, 518 A2**, 9L*, moi 1000: U251**, D384**, LW5**, GLC-01^{ns}, $A549^{ns}$, 518 A2**, 9L*; *CMV-promoter*: moi 10: U251**, D384**, LW5*, GLC-01*, A549***, 518 A2*, $9L^{ns}$, moi100: U251**, D384**, LW5*, GLC-01*, A549**, 518 A2***, 9L**, moi 1000: U251**, D384**, LW5*, GlC-01^{ns}, A549**, 518 A2**, 9L**)(Student t-

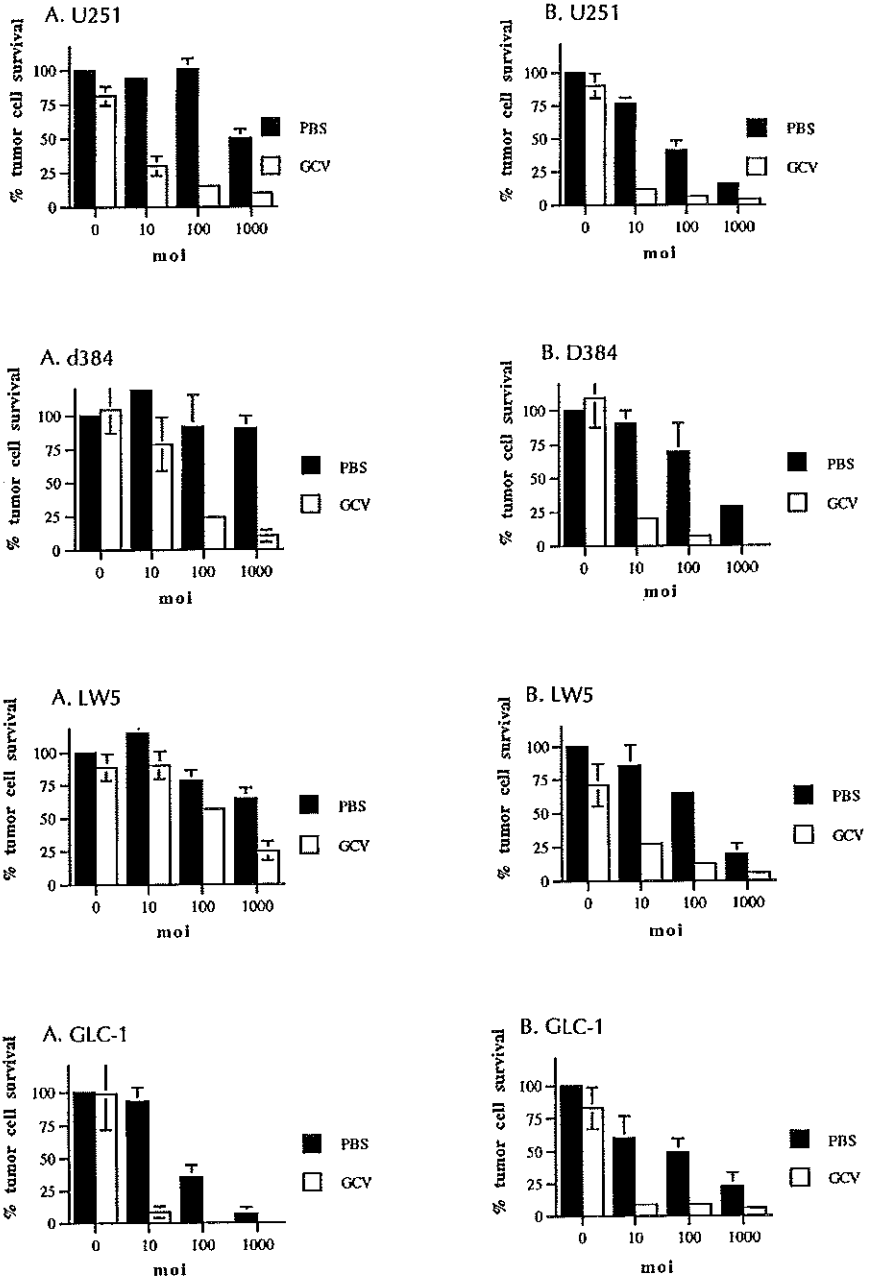
test;*=p<0.05, **=p<0.01, ***=p<0.001, ns=not significant). The tumor cell kill was also dependent on m.o.i. (Fig.4). However, human tumor cells infected at high m.o.i., also showed also cell death without GCV administration. Rapidly dividing tumor cells were killed at a significantly higher rate by TK/GCV treatment than slower dividing cells (U251 vs D384/ U251 vs D384/ D384 vs LW5: *MLP-promoter*: moi 10: */**/ns, moi 100: */***/**, moi 1000: ns/*/*; *CMV-promoter*: moi 10: **/*/ns, moi 100: ns/***/ns, moi 1000: **/ns/ns)(Student t-test;*=p<0.05, **=p<0.01, ***=p<0.001, ns=not significant), suggesting a correlation between division rates of human glioblastoma cells and tumor cell killing efficiency. (Fig.2,4).

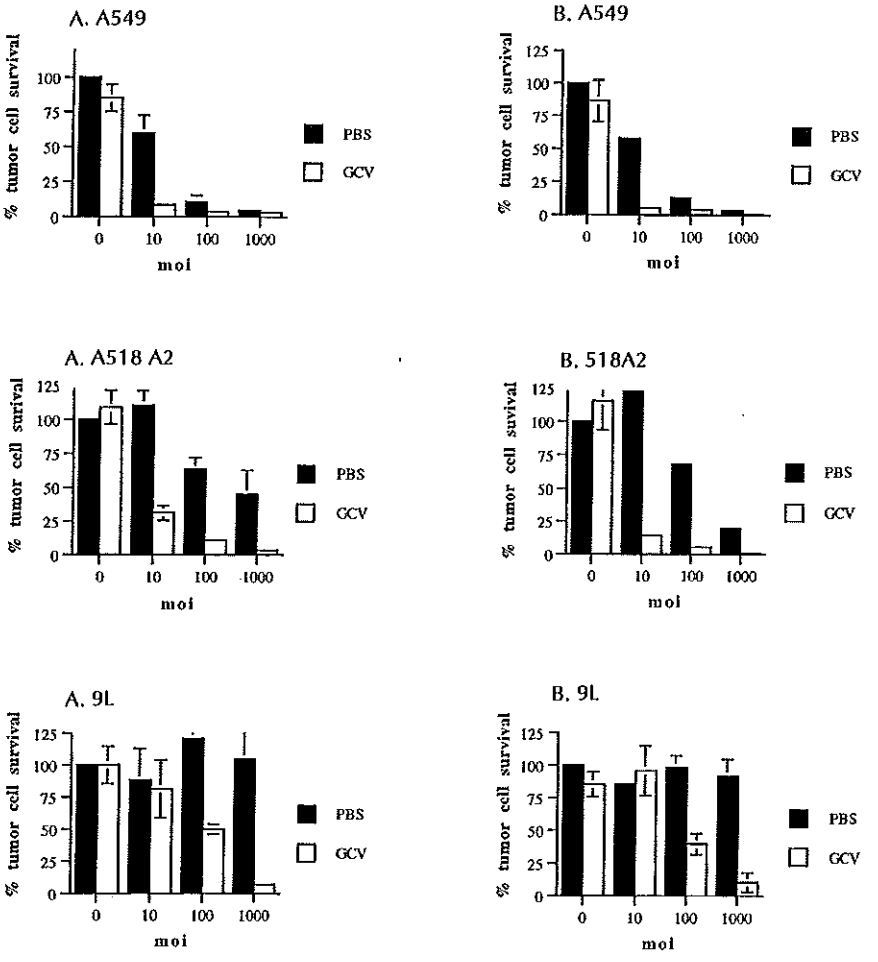
Mlp versus cmv promoter

Tumor cells infected with IG.Ad.CMV.*Lac*Z showed enlargement of cells and nucleus/plasma ratio was increased (Fig.3 – appendix) as compared to the IG.Ad.MLP.*Lac*Z, suggesting toxicity.

Most cell lines were killed more significantly after infection with adenovirus-TK containing the CMV-promoter as compared to the adenoviruses containing the MLP-promoter (Fig.4)(moi 10: U251*, D384*, LW5**, 518A2**, moi 100: U251*, D384*, LW5***, 518 A2*, moi 1000: U251**, D384*, LW5*, 518 A2**). No differences were found in the A549 and GLC-01 cells, although the GLC-01 cells* were killed more effectively at moi 100 with the MLP-vector. IG.Ad.CMV.TK was more toxic than IG.Ad.MLP.TK to U251, D384 and LW5 infected cells without GCV treatment (Fig.4).(moi 10: U251*, moi 100: U251**, moi 1000 U251**, D384**,LW5**)(Student t-test;*=p<0.05, **=p<0.01, ***= p<0.001, ns=not significant).

Figure 4


A. U251
B. U251
A. d384
B. D384
A. LW5
B. LW5
A. GLC-1
B. GLC-1
% tumor cell survival
moi
PBS
GCV
0
10
100
1000
25
50
75



Survival of tumor cells (U251 human glioma cells, D384 human glioma cells, LW5 human glioma cells, GLC-01 human small cell lung carcinoma cells, A549 human non-small cell lung carcinoma cells, 518 A2 human melanoma cells and 9L rat glioma cells) that were infected *in vitro* with IG.Ad.MLP.TK or IG.Ad.CMV.TK and subsequently treated with GCV or PBS. Tumor cells were plated at a density of $1x10^4$ cells/well and infected at m.o.i. 10, 100 and 1000. Twenty-four hours later the cells were treated with GCV at a concentration of 10 μg/ml or with PBS. After 72 hours the cells were trypsinised and surviving cells counted by trypan blue exclusion method. The figure depicts the percentage of surviving tumor cells as a function of m.o.i.. Open bars: treatment with GCV. Filled bars: treatment with PBS. 1. A. IG.Ad.MLP.TK B. IG.Ad.CMV.TK.

In vivo experiments:

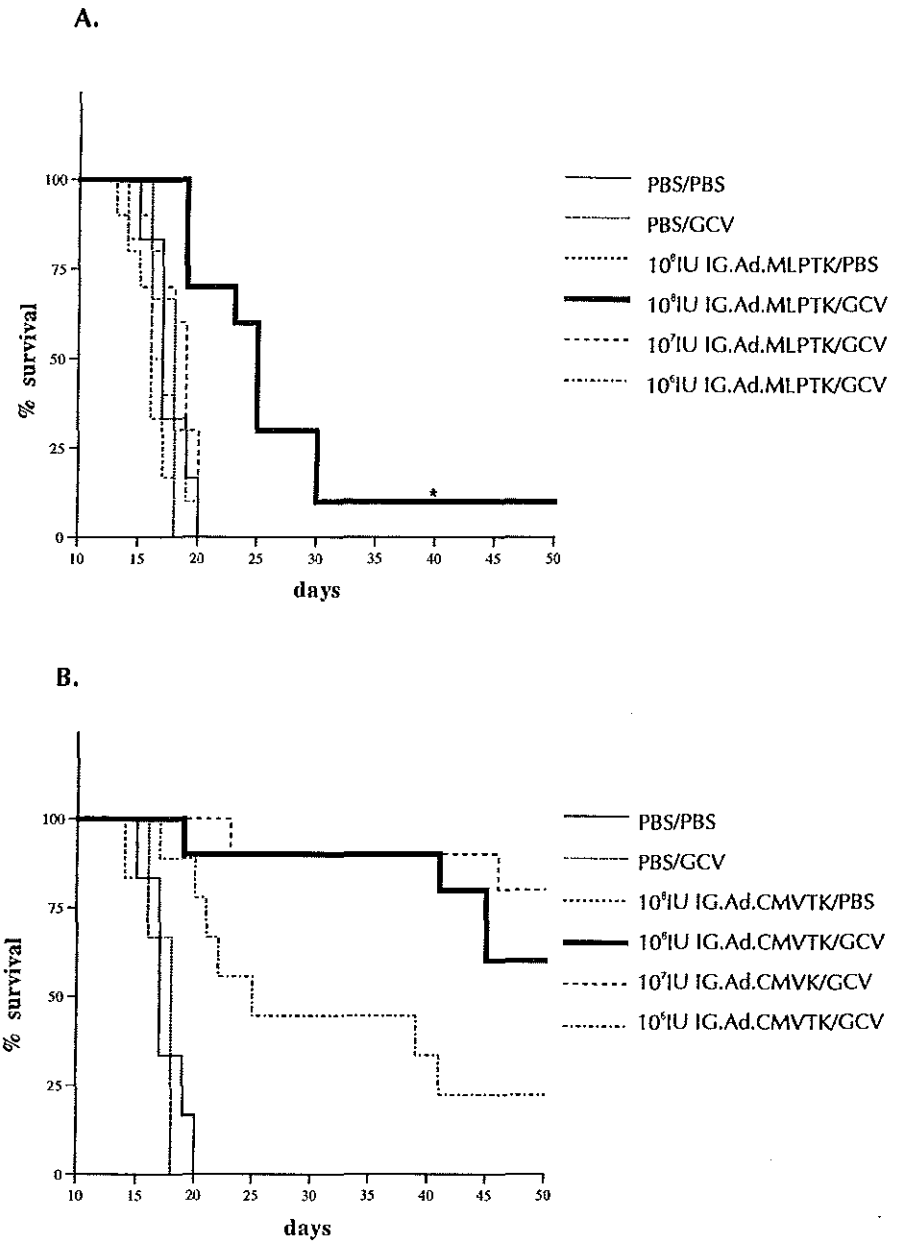
Rat brain tumor

Injection of brain tumor with IG.Ad.MLP.TK or IG.Ad.CMV.TK did not show any clinical or neurological signs related to vector administration. Treatment with both viruses significantly prolonged survival after GCV administration as compared to control rats given PBS instead of GCV (Fig.5). Control rats had a median survival (days) of 18.0±1.0 SD (PBS/PBS), 17.0±1.7 SD (PBS/GCV), 16.0±1.3 SD (IG.Ad.MLP.TK/PBS) and 17.0±1.5 SD (IG.Ad.CMV.TK/PBS) whereas IG.Ad.MLP.TK/GCV treated rats had median survival (days) of 25.0±9.2 SD (10^8 IU IG.Ad.MLP.TK/GCV), 19.0±1.7 SD (10^7 IU IG.Ad.MLP.TK/GCV), 16.5±0.7 SD (10^6 IU IG.Ad.MLP.TK/GCV). One animal treated with 10^8 IU IG.Ad.MLP.TK/GCV who had a survival time of 40 days, died of a superficial growing tumor that was caused by spill of tumor cells through the burr hole. No intracerebral brain tumor was present. IG.Ad.CMV.TK treated rats had a median survival (days) of 48.0±9.5 SD (10^8 IU IG.Ad.CMV.TK/GCV), 50.0±8.5 SD (10^7 IU IG.Ad.CMV.TK/GCV) and 25.0±13.3 SD (10^6 IU IG.Ad.CMV.TK/GCV). The data thus clearly show; 1) a dose dependent survival for both vectors, 2) a better therapeutic response after administration of IG.Ad.CMV.TK as compared to IG.Ad.MLP.TK, 3) that low doses of IG.Ad.MLP.TK (10^7 IU, 10^6 IU) are not effective anymore and 4) that administration of 107 and 108 IU of IG.Ad.CMV.TK had similar therapeutic effects, indicating that the maximal dose for single treatment was reached.

Rat leptomeningeal metastases

Similar to our findings in rats treated for brain tumors, animals treated by injecting the virus suboccipitally into the CSF showed no clinical or neurological signs related to vector administration. Administration of both vectors followed by GCV treatment resulted in a significant prolongation of the symptom free period as compared to control rats (Fig.6). Control rats had a median symptom free period (days) of 18±0.9 SD SD (PBS/PBS), 19.5±1.3 SD (PBS/GCV+), 18.0±1.4 SD (IG.Ad.MLP.TK/PBS) and 18.5±1.4 SD (IG.Ad.CMV.TK/PBS). Treated rats had median symptom free period (days) of 22.0±2.1 (IG.Ad.MLP.TK/GCV) and 29.0±5.4 (IG.Ad.CMV.TK/GCV). Treatment with IG.Ad.CMV.TK/GCV caused a longer symptom free period as compared to IG.Ad.MLP.TK/GCV.

Figure 5



Kaplan-Meier curves of rats with established brain tumors treated with recombinant adenovirus and subsequent GCV administration. Rats (n=90) were injected intracerebrally with $4x10^4$ 9L

tumor cells. After 3 days, rats were injected via the same route with 10^6, 10^7 and 10^8 IU of IG.Ad.MLP.TK or IG.Ad.CMV.TK. Controls were treated with PBS. Twenty-four hours after tumor cell injection 15 mg/kg GCV or 1 ml PBS was administered i.p. twice a day for ten days. Survival time of rats was assessed.

A. Rats treated with 10^8 IU IG.Ad.MLP.TK and subsequent GCV lived significantly longer ($p<0.001$, respectively, log rank test) than control groups (PBS/PBS, PBS/GCV and IG.Ad.MLP. TK/PBS). Control groups were not significantly different from each other ($p>0.01$). One animal treated with IG.Ad.MLP./GCV who had a survival time of 40 days, died of a superficial growing tumor which was caused by spill of tumor cells through the burr hole. No intracerebral brain tumor was present (*). (This rat is censored in the survival analysis).

B. Rats treated with 10^8, 10^7 and 10^6 IU of IG.Ad.CMV.TK and subsequent GCV lived significantly longer ($p<0.001$, $p<0.001$, $p<0.01$, respectively, log rank test) than all control groups (PBS/PBS, PBS/GCV and IG.Ad.CMV.TK/PBS). Control groups were not significantly different from each other ($p>0.01$).Additionally, rats treated with IG.Ad.CMV.TK/GCV lived significantly longer than rats treated with IG.Ad.MLP.TK/GCV.(10^8 IU ;$p<0.01$, 10^7 IU ;$p<0.001$, 10^6 IU ;$p<0.01$, respectively, log rank test).

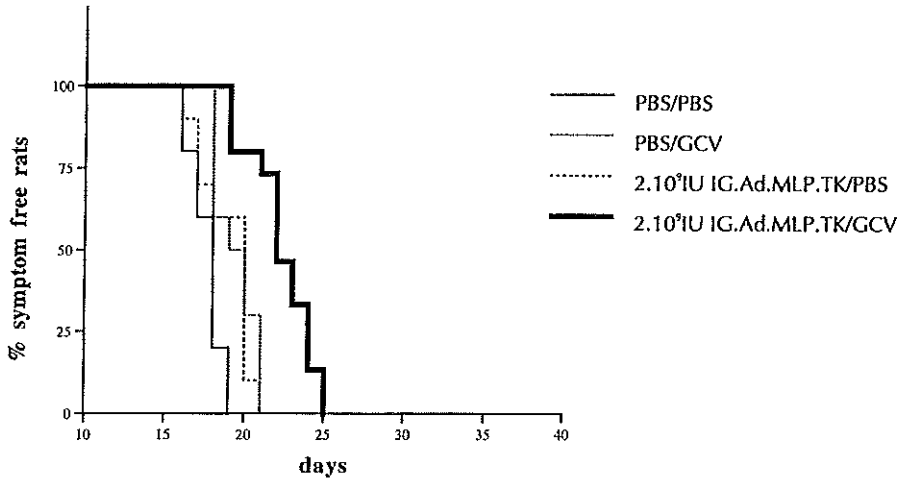
Figure 6

Kaplan-Meier curves of rats with established lepto-meningeal tumors treated with recombinant adenovirus and subsequent GCV administration. Rats (n=70) were injected intrathecally with $4x10^4$ 9L tumor cells. After 3 days, rats were injected via the same route with 2.10^9 IU IG.Ad.MLP.TK, IG.Ad.CMV.TK. Controls were treated with PBS. Twenty-four hours after tumor cell injection 15 mg/kg GCV or 1 ml PBS was administered i.p. twice a day for ten days. Rats were killed instantly when moribund or if neurological symptoms developed. Symptom free latency of every rat was assessed.

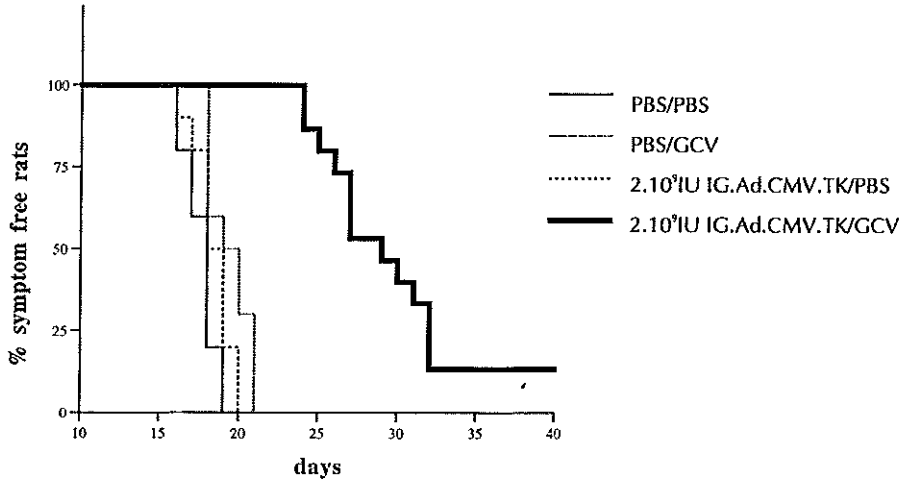
Rats treated with (A) IG.Ad.MLP.TK or (B) IG.Ad.CMV.TK and subsequent GCV had significantly longer ($p<0.001$, $p< 0.001$, respectively, log rank test) symptom free latency than all control groups (PBS/PBS, PBS/GCV and IG.Ad.MLP.TK/PBS or IG.Ad.CMV.TK). Control groups were not significantly different from each other ($p>0.01$).

Additionally, rats treated with IG.Ad.CMV.TK/GCV had significantly longer symptom free period than rats treated with IG.Ad.MLP.TK/GCV.($p<0.01$, log rank test).

A.


% symptom free rats
100
75
50
25
0
10
15
20
25
30
35
40
days
PBS/PBS
PBS/GCV
2.10⁹IU IG.Ad.MLP.TK/PBS
2.10⁹IU IG.Ad.MLP.TK/GCV

B.


% symptom free rats
100
75
50
25
0
10
15
20
25
30
35
40
days
PBS/PBS
PBS/GCV
2.10⁹IU IG.Ad.CMV.TK/PBS
2.10⁹IU IG.Ad.CMV.TK/GCV

Discussion

In this study we demonstrate a high efficiency of recombinant adenovirus mediated gene transfer to several human glioblastoma cell lines and cells derived from cancers known to metastasise to the CNS (Fig.3). The data also show that tumor cell kill after adeno-TK/GCV treatment was very efficient in all human tumor cell lines tested (Fig.4). A few investigators have reported a killing effect of adeno-tk/GCV treatment in several rodent brain tumor cell lines (10,27,40) *in vitro* and *in vivo*. We demonstrate that clinically important primary and secondary human CNS tumors can be efficiently transduced by adenoviral vectors and killed effectively by the TK/GCV treatment.

Tumor cell kill effectivity varied between the different human tumor cell lines (Fig.4). Unexplained variations of recombinant adenovirus transduction of different tumor cell lines have been described by others as well (4,13a). We have no satisfactory explanation for this phenomenon, but is most likely caused by differences in the numbers of receptors (37) and/or integrins (15,45,46) needed for attachment and internalisation of the adenovirus, respectively.

Additionally, our study suggests that killing efficiency in glioblastoma tumor cells correlate with cell doubling time (Fig.2, 4). The data show that mitotically active tumor cells (U251) are more effectively killed by the TK/GCV treatment than less actively dividing tumor cells (D384,LW5). This observation could be explained as follows; as the TK/GCV mechanism interferes with DNA synthesis, cell populations containing a relatively large fraction of 'S-phase' cells will be more sensitive than mitotocally less active cells. Our data suggest that tumor cell doubling time is an important parameters in the TK/GCV cell killing mechanism (10).

Adenoviral vectors harbouring different promoters have been developed by several investigators (2,5,10,27,40). Wills et al.(46) showed that the CMV promoter expressed higher levels of the p53 protein and demonstrated a significant stronger effect in vitro as compared to the MLP promoter. In our experiments, the majority of human tumor cells infected with the vector containing the CMV promoter showed significantly stronger anti-tumor effect in vitro and in vivo than the MLP promoter vectors. However, not all human cells showed higher cell kill with the CMV promoter (Fig.4). Higher expression and killing efficiency of the GLC-1 small cell lung cancer cells after adenovirus infection harbouring the MLP promoter was observed. We have no satisfactory explanation for this intercellular variation of promoter preference but it could be an im-

portant issue when clinical use of recombinant adenovirus is considered. Whether MLP or CMV constructs should be used in clinical studies depends on the balance between effectivity of tumor treatment and toxicity of the vectors to normal tissues.

Wills et al.(46) did not report on differences in toxicity of the vectors. In this study however, cytotoxicity in some human cells infected in vitro with IG.Ad.CMV.TK was significantly higher than with IG.Ad.MLP.TK (Fig.4). Cytopathic effects were also observed in IG.Ad.CMV.*Lac*Z infected cells (Fig.3) suggesting that toxicity is caused either by overexpression of the transgene or by the CMV promoter itself. This is in line with the observation that tumor cells infected at high m.o.i. with IG.Ad.MLP.TK without GCV treatment resulted also in toxicity (Fig.4). Another explanation for cytotoxicity in cells infected with recombinant virus containing the CMV promoter could be an excessive binding of transcription factors to the CMV promoter leading to cell death.

Effectivity of a single treatment with IG.Ad.MLP.TK or IG.AdCMV.TK and subsequent GCV administration in experimental brain tumors and leptomeningeal metastases is demonstrated in this study (Fig.5,6). The data show that the therapeutic effect is dose dependent, saturable, caused by the TK/GCV mechanism, and not directly related to a viral induced immune reaction (survival or symptom free period was not increased in rats treated with IG.Ad.TK/PBS). In line with our observations in vitro in the 9L cell line, the CMV promoter containing vectors were significantly more effective than the MLP constructs. As almost no toxicity of the CMV vectors to rat 9L cells was observed (Fig.4), these data can be explained only by the higher levels of gene expression by the CMV containing vectors as compared to MLP. Our data underscore the importance of adenovirus vectors for in vivo HSV-TK gene delivery.

None of the animals injected with the recombinant adenovirus in this study showed any neurological or other clinical signs related to the vector and/or GCV administration demonstrating the clinical feasibility of this approach. Since human tumor cells are more effectively killed than the 9L rat brain tumor cells (Fig.4), an even stronger therapeutic effect may be expected in a clinical situation. Additionally, larger volumes can be administered intratumorally or intrathecally as compared to the limited injection volume in rats. The data in this study also show that recombinant adenovirus can be transported within the CSF flow to kill leptomeningeal tumor cells effectively and is not immediately inactivated by humoral or cellular factors.

Several recombinant viruses have been described as vectors for HSV-tk gene delivery to tumor cells (6,8,10,10b,11,25,27,31). Retrovirus-TK

producing cells have shown to prolong survival of rats carrying experimental brain tumor or leptomeningeal neoplasia after GCV administration (11,25,31). However, only low virus titers can be achieved and the use of murine vector producing cells do have volume and immunological constraints in a clinical situation. Intrathecal injection of producer cells may also induce CSF pathway obstruction problems. Retroviral vectors will only integrate into the genome of dividing cells which can be a disadvantage, since the majority of cells in human malignant brain tumors remain in G0 phase (23,47). Clinical studies using murine fibroblasts producing HSV-tk retroviral vectors in patients with recurrent glioblastoma have been shown to induce some tumor regression but prolonged survival has not been reported yet (30). Transduction efficiency of tumor cells was very low in this study and seizures, severe headache, pancytopenia and meningeal inflammation have been noted during treatment. A phase 1 clinical trial for the treatment of leptomeningeal neoplasia using the same producer cells was closed temporarily after transient meningeal inflammation (32a). Immunogenic properties of the producer cells are most probably responsible for these side effects. Recombinant Herpes Simplex vectors which have the advantage to replicate in dividing cells but not in non-dividing cells, have also been proven to be effective in treating experimental brain tumors (7,9). However, the high immunogenic and toxic properties of the virus are major problems that need to be overcome before application. Adenovirus has the advantage that high titers can be obtained and that cell-free virus can be injected directly into the tumor. Both dividing and non- dividing cells are infected by the vector and insertional mutagenesis is unlikely because adenovirus does not integrate into the genome. Disadvantages however, could be the immunological and toxic constraints. Although immunological toxicity was not specifically addressed in this study, we previously demonstrated that no morphological changes or cytopathic effects were observed in the CNS of rats injected intratumorally or intrathecally with IG.Ad.MLP.TK (39,40). It is still a matter of debate whether significant immune responses occur in the relatively "immune privileged" CNS after single or multiple injections of recombinant adenovirus (7,10,27,37,40). Although immune responses have been described after instillation in several organs (5), our group (unpublished data) found no clinical toxicity in CNS of rodents after intracerebral or intrathecal injection of recombinant adenovirus. A possible explanation could be that in contrast to others, we retained the E3 region in our adenoviral constructs, which is responsible for evading host's immune response by down regulating MHC 1 expression in transduced cells (17). Based on the data described in this paper we consider clinical studies for

malignant glioma and leptomeningeal metastases aimed at assessing efficacy and toxicity of the TK/GCV treatment.

Conclusions

Gene expression in, and killing efficiency of human CNS tumors can be efficiently achieved with adenoviral vectors in vitro and in vivo. Our data also show the therapeutic effectivity and feasibility of treatment of experimental rat brain tumors and leptomeningeal metastases with recombinant adenovirus harbouring the HSV-tk gene and GCV.
Infection with recombinant adenovirus containing the CMV promoter showed higher GCV mediated killing efficiency in most human tumor cells tested than with adenovirus harbouring the MLP promoter. Infection by recombinant adenovirus harbouring the CMV promoter was however, also accompanied by cytotoxicity.

References

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CHAPTER 6

Safety and distribution of recombinant adenovirus HSV-tk after intracerebral administration in rats

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Abstract

HSV-tk gene transfer into tumor cells followed by Ganciclovir (GCV) administration is a promising approach for the treatment of central nervous system malignancies, since specifically mitotic cells are destroyed while surrounding brain tissue is spared. In this study, we investigated clinical signs or symptoms and histopathology of brain tissue after intracerebral injection of recombinant adenovirus containing the HSV-tk gene in rats followed by systemic GCV administration. Furthermore, systemic involvement and spread of the virus through the CNS was studied. Rats were injected in the left frontal lobe with 10^8 or 10^9 Infectious Units (IU) of IG.Ad.MLPI.TK, PBS (controls) or wild-type adenovirus followed by GCV or PBS for 16 days after which they were killed and histopathological examination of the brain was performed. No clinical adverse reaction was observed in any of the treated animals. After administration of 10^8 IU of IG.Ad.MLPI.TK histopathological examination revealed mild to moderate perivascular infiltration in the near vicinity of the injection site. Animals injected with 10^9 IU IG.Ad.MLPI.TK showed moderate to severe perivascular lymphoid infiltration, glial proliferation, edema and parenchymal loss extending to basal and baso-lateral area of the frontal lobe and also caudally, but generally not exceeding a distance of 2-3 mm from the injection canal. An additional effect of GCV administration was not observed. Rats injected with wild-type adenovirus showed more severe lesions compared to those treated with 10^9 IU IG.Ad.MLPI.TK, which could have been induced by proteins transcribed from the preserved E1 region. In this rat study, we demonstrate that intracerebrally injected recombinant adenovirus harbouring the HSV-tk gene causes dose dependent, but rather limited brain lesions not associated with short term major functional disturbances. In another experiment, rats were injected intracerebrally with IG.Ad.MLP.LacZ (containg the LacZ gene) or IG.Ad.MLP.luc (containing the luciferase gene) to study intracerebral and systemic spread of the virus, respectively. LacZ expression was observed at some distance and in the contralateral hemisphere of the injection site. However, no luciferase activity was measured outside the CNS.

Introduction

Most malignancies of the central nervous system are still devastating tumors with poor prognosis despite aggressive surgery, chemotherapy and radiotherapy (1-3). Transfer of the Herpes Simplex Virus thymidine kinase (HSV-tk) gene into brain tumor cells followed by Ganciclovir (GCV) administration is a newly developed experimental cancer therapy (4-9). Expression of the HSV-tk gene renders dividing cells sensitive to GCV(10). Since the HSV-tk/GCV mechanism specifically kills mitotic cells, central nervous system tumors which are embedded in non-dividing tissue are ideal candidates for this approach. Several investigators have shown the efficacy in vitro and in vivo and clinical trials are in progress. Recombinant retrovirus (4, 5, 11), Herpes Simplex virus (12, 13) and adenovirus (8, 14, 15) have been used as vectors to deliver the HSV-tk gene to the tumor cells in vivo. Recombinant adenoviral vectors have several advantages; they are stable, cell free virus can be administered directly in vivo and high titers can be obtained. They can infect a broad range of dividing and non-dividing cells very effectively and insertional mutagenesis is unlikely because the DNA is not integrated into the host cell genome. Recombinant adenoviral vectors used for most gene therapy studies, including the studies reported here, are derived from the subgroup C adenoviruses types 2 and 5 which are known to induce self limiting mild respiratory tract infections. The vector seems therefore an ideal candidate to be used for treating brain tumors. However, although the brain is regarded as an immune protected site, several sero-types of adenovirus have been shown to cause encephalitis in newborn infants, immune deficient humans and also occasionally in adults (16-31). Another limitation in the use of these vectors could be the pre-existing immunity to adenoviruses type 2 and 5 in the general population. Repeated injections could lead to direct complement mediated inactivation after two or more administrations (32).

Delivery of the adenoviral vectors to brain tumors has been achieved in experimental animals by either direct intra-tumoral injection or intra-arterial administration followed by osmotic disruption of the blood brain barrier (8, 14, 15, 33-35). In one clinical trial the vector is installed into the "tumor bed" after glioma resection to prevent regrowth of the tumor (36). Application of the vector in either way will also transduce healthy non-dividing brain tissue. Since the vector has also potency to be used in replacement therapy for neurodegenerative diseases and neural regeneration therapies, cytotoxicity of the vector on brain tissue is a very important issue to address. Previous studies showed that neurons, glial cells, choroid

plexus and ependymal cells can be transduced by recombinant adenoviral vectors without significant toxicity (37-42). Others, however, observed dose dependent intracerebral inflammatory responses after vector instillation in rats or primates (32, 36, 40, 43, 44).

We investigated the histopathology in the rat brain after intracerebral injection of adenoviral vectors harbouring the HSV-tk gene and subsequent GCV administration. Furthermore, the spread of the virus to other organs after intracerebral injection was investigated in rats.

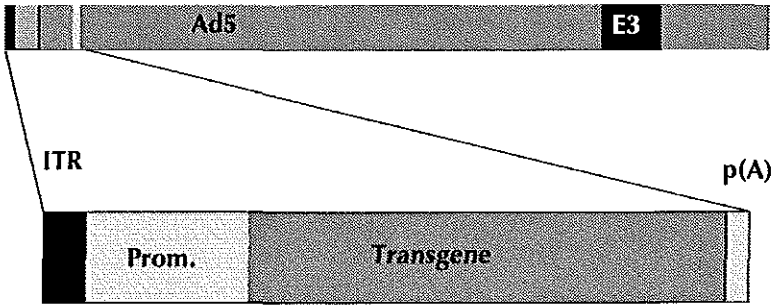
Methods en materials

Adenoviral Vectors

IG.Ad. MLPI.TK is derived from IG.Ad.MLP.TK as will be described elsewhere (44a). IG.Ad.MLPI.TK is similar to IG.Ad.MLP.TK (delta E1 459-3328) except that the E1 deletion in the vector is extended (delta 459-3510) and non-coding *LacZ* sequences present in IG.Ad.MLP.TK are deleted. The E3 region is contained in this construct. Similar adenoviral vector constructs containing the firefly luciferase (luc) or nls-*LacZ* genes (Escherichia coli *LacZ* gene preceeded by the SV40 large-T nuclear localisation signal) were made. The luc complementary (c)DNA was derived from pRSV.luc (45). Generation of these adenoviral vectors is described elsewhere (6).

Recombinant viruses were generated by co-transfecting 293 cells with Sal I linearized pMLP.TK or pMLP.luc and the large Cla I fragment of wild-type Ad5 DNA. Recombinant viruses were plaque purified twice, propagated, and titrated according to standard procedures (47). Virus titers determined by endpoint titration ranged from 10^{10} to10^{11} IU/ml. The respective resulting recombinant viruses were named IG.Ad.MLPI.TK and IG.Ad.MLP.luc. A map of the recombinant adenoviral vectors used is depicted in figure 1. The wild-type adenovirus subtype 5 was obtained from Prof.Dr.P.C. van der Vliet (Universtiy Hospital Utrecht).

Figure 1



Map of the constructed recombinant adenoviral vectors. Shown is the Human Ad 5 DNA containing the expression cassette. The cassette (enlarged) contains the Adenovirus type 2 major late promoter (MLPI). The promoter is preceded by an inverted terminal repeat, followed by either the nls*LacZ*, (Escherischia coli *LacZ* gene preceded by the nuclear localisation signal), Herpes Simplex derived Thymidine Kinase (TK) suicide gene or luciferase (luc.) gene. Transcription is terminated by SV40 poly-adenylation sequences.
Prom.: MLP promoter. **Transgene**: either TK, nls*LacZ* gene or luc. gene.
Ad 5: human adenovirus DNA. **ITR**: inverted terminal repeat. **p(A)**: SV40 poly-adenylation sequences.

Experimental procedures

Intracerebral injection of IG.Ad.MLPI.TK
Fischer rats weighing 150-200 grams were anaesthetized and placed into a stereotactic device. A burr hole was made 1mm in front of the bregma and 2mm off the midline. The rats were injected intracerebrally with 10^8 (1µl) or 10^9 (10µl) IU IG.Ad.MLPI.TK, 10^8 (1µl) IU WtAd5 (Wildtype adenovirus; subtype 5) or PBS via a microliter syringe into the left forebrain, at a depth of 4 mm. The needle was slowly retracted and the burr hole was closed with bone wax (Braun). The skin was closed with 9 mm clips. Twenty-four hours later the rats were treated twice daily for 14 days with either intraperitoneal injections of GCV (15mg/kg, Syntex) or PBS. The eight different groups were treated as follows: 1) 10^8 IU IG.Ad.MLPI.TK (1µl)/GCV (n=3); 2) 10^8 IU IG.Ad.MLPI.TK (1µl)/PBS (n=3); 3) PBS (1µl)/GCV (n=2); 4) PBS (1µl)/PBS (n=2); 5)10^8 WtAd5 (1µl)/PBS (n=2); 6) 10^9 IU IG.Ad.MLPI.TK (10µl)/GCV (n=4); 7) 10^9 IG.Ad. MLPI.TK (10µl)/PBS (n=3); 8) PBS (10µl)/GCV (n=2). The animals were observed daily for any clinical signs or symptoms and killed 16 days after intracerebral injection. The rats were perfused via the left cardiac

ventricle with ice-cold 4% paraformaldehyde. The brain was removed, and fixed in the same solution. The brains were cut transversely into 6 sections: 1) just caudally of the injection site, 2) in front of the optic chiasm opticum, 3) through the caudal part of the mammillary body, 4) through the anterior part of the pons and 5) through the middle of pons and 6) cerebellum. The sections, numbered 1-6 from rostral to caudal were embedded in paraffin and cut from the rostral side (except the frontal slice which was cut from the caudal side) into 4 μm slices. After staining with hematoxylin, phloxin and saffrane (HPS), the slices were examined microscopically.

A semiquantitative grading system for estimating severity and expansion of the lesion to areas at some distance of the injection site was applied. The severity (S) was scored in the most severely affected slice (generally slice nr. 1.) as 0, no lesions; 1, mild; 2, moderate; or 3, severe; depending on the presence and severity of perivascular lymphoid infiltration, gliosis, parenchymal loss and edema, and ventricular dilation. The extent (E) of the lesions was scored as: 1, injection canal only; 2, lesions within 0,5 mm from the margin of the injection canal; 3, lesions elsewhere in the frontal lobe but limited to the first slice only: 4, lesions also in the adjacent slice nr. 2; and 5, lesions also in slice nr. 2 and nr. 3. The total score for the reactive changes in each rat was determined by S x E.

Intracerebral, suboccipital and intracardial injection of IG.Ad.MLP.luc

Fischer rats (n=22) weighing 300 to 400 grams were anesthesized with ether. Rats were injected intracerebrally, suboccipitally or intracardially with either 10^8 IU IG.Ad.MLP.luc. or PBS (controls). The rats treated intracerebrally were treated by the same injection procedure as described above. A volume of 10μl containing 10^8 IU IG.Ad.MLP.luc. (n=3) or PBS was injected into the left forebrain. Rats treated suboccipitally were treated as described by Vincent at al (15). Briefly, rats were shaved suboccipitally and placed in a stereotactic frame with the skull in flexion. 10^8 IU IG.Ad.MLP.luc. or PBS (controls) in a volume of 30μl using a 0.1 ml syringe was then injected directly into the CSF of the cisterna magna. The proper position of the needle tip was confirmed by CSF aspiration prior to injection. For intracardial administration, rats were shaved on the chest and injected directly into the left ventricle of the hart. The proper position of the needle tip was confirmed by aspiration of pulsating blood prior to injection. A volume of 30 μl using a 0.1 ml syringe was used to inject 10^8 IU IG.Ad.MLP.luc. or PBS (controls) directly intracardially. The animals were observed daily for clinical signs or symptoms. Three or 14 days after injection, the rats were anesthetized with ether and perfused through the

left cardiac ventricle with ice cold PBS. The heart, lung, liver, spleen, gastrointestinal tract, kidney, gonads, brain and spinal cord were removed, snap-frozen and stored at -80°C. The frozen organs were placed in ice-cold 100mM potassium phosphate buffer pH 7.8. Subsequently the organs were disrupted with a tissue mincer and lysed by adding 0.1% Triton X-10C and 1mM DTT. After centrifugation, luciferase activity was determined in 20 µl of the supernatant by measuring the integrated light emission with a luminometer for 10 seconds in the presence of 25 mM adenosine triphosphate and 1 mM luciferin.

Intracerebral injection of IG.Ad.MLP.LacZ
Fischer rats weighing 150-200 grams (n=3) were anesthetized and injected with 10^8 IU IG.Ad.MLP.*LacZ* using the same procedure as described above. The same injection procedure was used as described above. The rats were injected intracerebrally with 10^8 IG.Ad.MLP.*LacZ*. The animals were observed daily for any clinical signs or symptoms and killed 3 days after injection. The rats were perfused under anesthesia via the left cardiac ventricle with ice-cold 4% paraformaldehyde, the brain was removed, and fixed in the same solution. The brain was cut into 5 transverse sections. Subsequently, the sections were cut with a vibratome into 100 µm sections, washed thoroughly with PBS and stained with X-Gal for 3 hours. The sections were then counterstained with hematoxylin/eosin and examined microscopically for reporter gene expression.

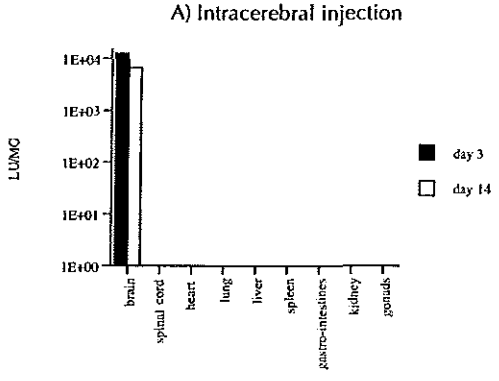
Results

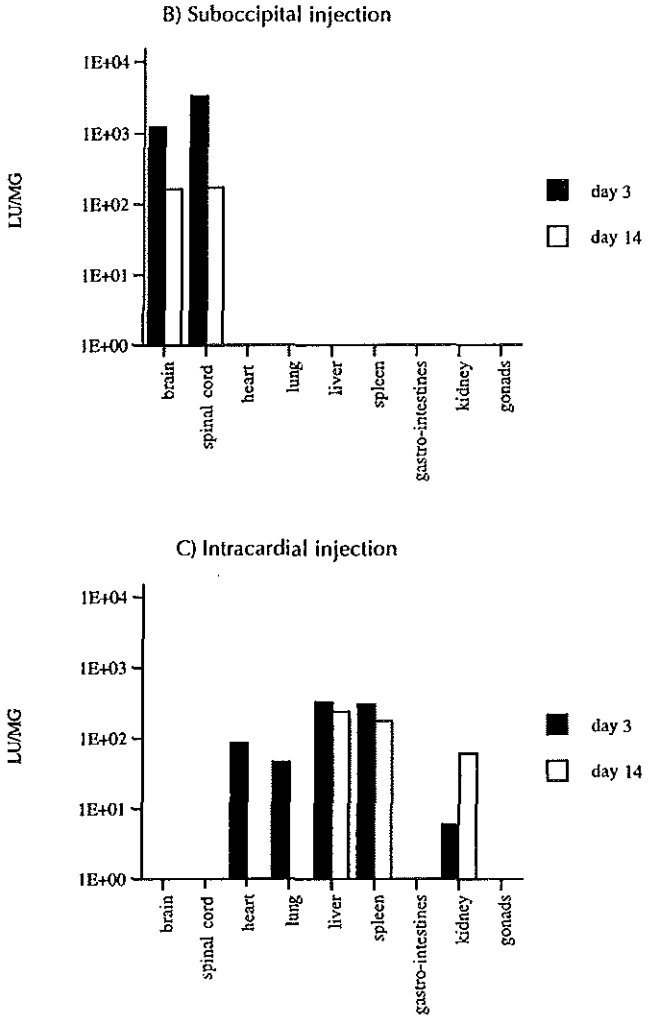
Intracerebral injection of IG.Ad.MLPI.TK
Intracerebrally injected animals with recombinant or wild-type virus did not cause any clinical signs or symptoms. After sacrifice, all brains showed histological changes due to the injection procedure itself. These changes included mild edema, mild mononuclear cells infiltration, especially with macrophages, mild glial cell proliferation, reactive vascular changes and multinucleated giant cells in the meninges. These reactions were restricted to the direct vicinity of the needle track (Fig. 2A – app.). All rats treated with IG.Ad.MLPI.TK showed mild (Fig. 2B – app.) to severe lesions (Fig. 2C - app.). The more severe lesions were characterized by multifocal perivascular lymphoid cell infiltration and glial cell proliferation, severe edema, parenchymal loss and punctuate mineralisation (signs of acute cell death however were not observed). The lesions extended mostly along the outer edge of the capsula externa to the basal and baso-lateral area of the

frontal lobe and in some cases also caudally. Expansion to the more caudally located structures (slides 2 and 3) generally was characterized by less severe lesions such as perivascular lymphoid infiltration and glial cell proliferation focally in the nucleus caudatus and thalamus. In sporadic cases, lesions were observed also in the contra-lateral hemisphere (Fig. 2D app.). Ventricular dilatation was not uncommon in the most severely affected rats. The results of the scores for severity, extension and total score of the lesions observed in the 8 groups injected intracerebrally are presented in table 1. It appears that 2 of the 6 rats treated with 10^8 IU IG.Ad.MLPI.TK. and 2 of the 2 rats treated with 10^8 IU WtAd5. showed mild lesions with total scores ranging from 1,5-4,5. In the high dose groups (10^9 IU) all IG.Ad.MLPI.TK treated rats showed moderate to severe lesions mostly limited to the first and second slice i.e. not outside 2-3 mm from the injection site, resulting in total scores ranging from 6-12,5. An effect of additional GCV treatment was not observed.

Intracerebral, suboccipital or intracardial injection of IG.Ad.MLP.luc
None of the injections caused clinical symptoms until 3 or 14 days after IG.Ad.MLP.luc injection. Luciferase activity was detected in the brains of rats treated intracerebrally, after 3 and 14 days, but not in any other organs. Suboccipitally injected rats showed luciferase activity in spinal cord and brain after 3 and 14 days but not in any other organ. Several organs of the intracardially injected rats (positive controls) showed luciferase expression after 3 and 14 days. Activity was detected in heart, lung, liver spleen and kidney. After 14 days luciferase could still be detected in liver, spleen and kidney (Fig. 3).

Figure 3





Mean luciferase activity after 3 or 14 days in brain, central cord, hart, lung, liver, pleen, gastro-intestines, kidney and gonads after intracerebral (A), suboccipital (B) or intracardial (C) (positive control) injection of 10^8 IU IG.Ad.MLP.luc. Rats were injected intracerebrally, suboccipitally or intracardially with either 10^8 IU IG.Ad.MLP.luc. or PBS (controls). Three or 14 days after injection, the rats were killed and hart, lung, liver spleen, gastro-intestines, kidney, gonads, brain and spinal cord were removed. The organs were lysed and luciferase activity was determined. Activity is presented as light units per milligram of soluble protein (LU/mg).

revealed reporter gene expression along the injection track and along the meninges near the injection site (Fig 4A – app.). Transduced cells were observed along the border of the corpus callosum with nucleus caudatus and white matter in the direct vicinity of the injection track. However, transduced cells were also observed along the corpus callosum directly contralaterally of the injection track (Fig. 4B – app.). No transduced cells were observed in frontal thalamus, nucleus caudatus or cerebellum (results not shown).

Discussion

Rats treated intracerebrally with 10^9 IU IG.Ad.MLPI.TK all showed moderate to severe brain lesions which were most pronounced in the vicinity of the needle track and along the outer edge of the capsula externa. Lesions were only sporadically observed at distances further than 2-3 mm from the needle track and if so they were only mild. Dose reduction to 10^8 IU caused a sharp decrease in severity and extend of the lesions with 4 or 6 rats, not different from the control rats. The dominant type of lesion is the perivascular lymphoid infiltration which suggests a T-cell mediated pathogenesis as described earlier for recombinant adenovirus lesions in lungs (48) and CNS (32,43).

The sporadic focal lesions consisting of perivascular lymphoid infiltration and proliferation observed in the contralateral hemisphere may be due to viral spread via the subarachnoidal space (by leakage from the injection site) or by transport via intracerebral fluid movements, due to retrograde transport of the virus via the normal commisural connections. Although clear-cut necrosis or apoptotic cell death was not observed, rats treated with high dose IG.Ad.MLPI.TK showed areas of loosely textured edematous brain tissue with decreased cellular density and multifocal punctuate mineralisation. These phenomena and the associated ventricular dilatation indicate acute parenchymal loss (cells and white matter). The increase in reactive changes with high dose IG.Ad.MLPI.TK suggest a recombinant adenovirus dose dependent inflammatory reaction. This could have some drawbacks in an eventual clinical setting. However, the relative dose applied to the human brain would be several times smaller, as the virus is injected into the tumor or tumor bed and over a larger area than was the case in the rat brain, leading to a considerable dilution. Furthermore, tissue damage observed in this experiment is relatively small when compared to the damage inflicted by neurosurgical tumor resection or by postoperative irradiation and might therefore be regarded as an acceptable side effect. In addition, it is reasonable to assume that the inflammatory response can partially be suppressed in humans by the use of

preoperative corticosteroids. Although investigators have suggested increased toxicity of high dose TK virus when combined with GCV in normal brain tissue (49), IG.Ad.MLPI.TK/GCV treated rats in our experiment did not show differences in histopathology as compared to IG.Ad.MLPI.TK/PBS treated rats (Table 1). Other GCV dosages and animal species used could be an explanation for the observed differences. Theoretically, GCV administration is not expected to aggravate toxicity after recombinant HSV-TK containing adenovirus, since in non-dividing tissue the TK/GCV suicide mechanism is not expected to be active.

Table 1 Severity (S), extend (E) and total score (S x E) of reactive changes in rat brains 16 days after intracerebral injection of adenoviral vector

Group	Rat Nr.	Treatment	Dose (IU)	Volume	Severity Score	Extension Score	Total Score
1	1	IG.Ad.MLPI.TK+GCV	1.10^8	1 µL	1–2	3	4,5
	2	IG.Ad.MLPI.TK+GCV	1.10^8	1 µL	0–1	1	0,5
	3	IG.Ad.MLPI.TK+GCV	1.10^8	1 µL	0–1	1	0,5
2	4	IG.Ad.MLPI.TK+PBS	1.10^8	1 µL	0–1	1	0,5
	5	IG.Ad.MLPI.TK+PBS	1.10^8	1 µL	0–1	1	0,5
	6	IG.Ad.MLPI.TK+PBS	1.10^8	1 µL	0–1	3	1,5
3	7	PBS+GCV	–	1 µL	0	0	0
	8	PBS+GCV	–	1 µL	0–1	1	0,5
4	9	PBS+PBS	–	1 µL	0–1	1	0,5
	10	PBS+PBS	–	1 µL	0–1	1	0,5
5	11	wtAd5+PBS	1.10^8	1 µL	1–2	3	4,5
	12	wtAd5+PBS	1.10^8	1 µL	1–2	3–4	5,2
6	13	IG.Ad.MLPI.TK+GCV	1.10^9	10 µL	2	3–4	7
	14	IG.Ad.MLPI.TK+GCV	1.10^9	10 µL	2–3	5	12,5
	15	IG.Ad.MLPI.TK+GCV	1.10^9	10 µL	2	3	6
	16	IG.Ad.MLPI.TK+GCV	1.10^9	10 µL	2	4	8
7	17	IG.Ad.MLPI.TK+PBS	1.10^9	10 µL	2–3	4	10
	18	IG.Ad.MLPI.TK+PBS	1.10^9	10 µL	2–3	5	12,5
	19	IG.Ad.MLPI.TK+PBS	1.10^9	10 µL	2–3	4	10
8	20	PBS+GCV	–	10 µL	0-1	1	0,5
	21	PBS+GCV	–	10 µL	0	0	0

Rats injected with wild-type adenovirus showed lesions indistinguishable from those due to IG.Ad.MLPI.TK at the same dose level, but slightly more severe and more extensive. We have no explanation for this obser-

vation. Fischer rats are not known to be permissive or semi-permissive for human adenoviral type 5 infection. Proteins transcribed from the E1A region in the replication competent Ad5 could be responsible for the slight increase in neurotoxicity as compared to the E1A-deleted recombinant adenovirus. Unpublished data of our group showed no pathological effects of intracerebrally injected IG.Ad.MLPI.TK or wild-type Ad5 virus in the brain of Cotton rats, that are known to be semipermissive for the wild type virus in the lung (50).

The luciferase experiment shows that intracerebral or suboccipital injection of recombinant virus does not reveal detectable expression of the transgene in organs outside the CNS (Fig. 3). Although intracardially injected animals (positive controls) did show expression in several organs, vascular spread of the recombinant virus with subsequent transgene expression outside the CNS after intracerebral or intrathecal injection was not detectable. These data contrast with the data from Goodman et al., (49) who demonstrated spread of recombinant adenovirus-tk (in primates) using PCR detection, outside the nervous system (marrow, liver, lung). Transgene expression in these organs however, was not addressed in their study. In our study spread of recombinant virus within the CNS is demonstrated (Fig. 3, 4 A,B – appendix). Rats injected suboccipitally with adenovirus containing the luciferase gene demonstrated activity within brain and spinal cord tissue. This can be explained by transport via the CSF flow all along the CNS. Intracerebral injection, however, did not reveal luciferase activity in the spinal cord, indicating that transport via the CSF contact is essential for spread throughout the CNS. We reported previously that recombinant adenovirus is transported very effectively along the CNS after intrathecal delivery (6). No clinical signs of viral meningitis were observed in our study. The *LacZ* experiments shows intracerebral spread of recombinant virus after injection into the brain. Although transgene expression was observed in the direct vicinity and at a distance of the injection site, some cells (neurons, glia cells) in the contralateral hemisphere were also transduced (Fig. 4A,B – appendix). Furthermore, spread of virus was restricted to anatomical borders within the brain (along corpus callosum). Whether recombinant virus migrates or is transported to the nucleus retrogradally into the contralateral hemisphere after infection of the axon in the vicinity of the injection site is still a matter of debate. It has been demonstrated that single neurons in mice can transport recombinant virus via a retrograde axonal route over a large distance within or to the brain (51-53), which demonstrates that intracerebrally injected virus particles has the potency to spread within the CNS.

Several investigators have studied recombinant adenoviral gene transfer into experimental rat brain tumors (6, 8, 14, 15, 33, 54, 55). Although several investigators have shown efficient CNS transduction by recombinant adenovirus (35-40, 42, 44, 56, 57), only few have investigated the pathogenic effects of the infectious virus on healthy brain tissue (40, 43, 44, 49). Byrnes et al., 'demonstrated that injection of recombinant adenovirus-*LacZ* into the rat brain leads to an inflammatory response that gradually decreases within 2 months, although expression of the transgene was still evident at that time(32). The inflammation was initiated by the virions rather than through the expression of new proteins from the vector. In another experiment Byrnes et al., 'showed that although adenoviral vectors can persist in the brain without causing chronic inflammation, they remain a potential target of a damaging cell-mediated immune response after a second exposure to the vector. We did not address this specific issue in our experiments but it should be investigated before considering human trials in which repeated injections over time are considered.

Toxicity studies in primates using recombinant adenoviral vectors have been conducted by only few investigators (40, 44, 49, 58). Toxicity studies in non-human primates showed only limited neurotoxicity. Goodman et al., '(49) however, reported severe toxicity in baboon's brain after recombinant adenovirus -tk (containing the RSV promoter) and subsequent GCV administration. Animals treated with high dose recombinant adenovirus-tk ($1.5x10^9$ IU) and GCV died or became moribund. Necropsies revealed necrosis at the injection sites. High dose adenovirus-TK without GCV administration and low dose virus ($7.5x10^7$ IU) administration resulted in minor lesions varying from mostly cystic lesions to microscopic foci of necrosis. The severe toxicity found by the latter investigators may result from differences in virus-batch solution, transgene containing vectors or the use of cortocosteroids.

Conclusion

We report in this study the relatively limited dose dependent neurotoxicity observed after adenoviral TK/GCV administration in a rat model. The toxicity observed was generally limited to the injection area. Systemic toxicity of the virus was absent. Previous studies have shown the therapeutic effectiveness of recombinant adenoviral vector harboring the HSV-tk gene in the treatment of brain tumors and leptomeningeal metastases. Clinical application of this approach is therefore considered acceptable.

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General discussion

Introduction

This thesis shows the effectiveness and feasibility of treating experimental CNS tumors in rats with recombinant adenovirus harbouring the HSV-tk gene and subsequent GCV. We concluded that based on these data, clinical application of this approach is considered acceptable. Phase 1 clinical trials using recombinant adenovirus-TK and subsequent GCV administration in humans with recurrent glioblastoma are starting at the moment or are already under investigation (1). A few aspects concerning the application of the adenovirus in human brain tumors and future prospects are discussed.

Adenoviral vectors

We used the recombinant adenoviral vector in our experiments because of the advantages over other viruses. High titers of the adenovirus can be obtained and the cell-free virus can be injected directly into the tumor or the CSF where an effective spread of the virus is established. Both dividing and non- dividing cells can be infected by the vector wich is an important advantage over retroviral vectors, since the majority of cells in human malignant brain tumors are in G0 phase. Furthermore, insertional mutagenesis by an adenoviral vector is unlikely because the DNA remains extrachromosomally. The immunological toxicity in rats found in our study (chapter 6) could be the only constraint when using the virus for clinical application. However, the inflammatory reaction observed after intracerebral injections of high titers of the vector is not comparable to the damage and inflammatory reaction inflicted by surgical resection or irradiation of brain tumors. In addition, the inflammatory response can partially be suppressed in humans by the use of standard peri-operative corticosteroids. Therefore, in our opinion, the intracerebral immunological toxicity can be regarded as an acceptable side-effect.

Experimental model versus human brain tumors

Rat brain tumors are experimentally induced tumors which can not be compared in their biological behaviour to human brain tumors. The 9L rat brain tumor cells are mitotically very active and show only subtle invasive growth into the brain parenchyma. The rat brain tumor model used in this thesis has therefore more or less "ideal" features for the treatment with recombinant adenovirus-TK and subsequent GCV administration:

The mitotic activity of these cells renders them very sensitive to the TK/GCV mechanism (this thesis) and the mild invasive growth makes the tumor very suitable for direct injection of the recombinant virus. Primary human brain tumors, for instance glioblastomas are aggressive infiltrating tumors that, although fast growing, are not comparable to the growth rate of the rat brain tumor *in vivo*. Secondly, the infiltration of glioblastoma cells into the brain parenchyma is very aggressive and extends sometimes to the other hemisphere (2,3). These infiltrating tumors are therefore not ideal for direct tumor injection of the recombinant virus since the infiltrating cells cannot be reached via this route. However, recurrent glioblastoma usually occurs at the resection site. Regrowth could therefore be delayed or prevented in the resection area by direct injection of the vector in the tumorbed. This could theoretically lead to a longer survival of patients with glioblastoma. Total eradication of the tumor will be difficult to achieve since infiltrative cells at a large distance of the injection site will not be transfected after direct tumor injection with recombinant virus. Secondary brain tumors (metastases) show only mild infiltration and are therefore perhaps better candidates for direct injection of the tumorbed after tumor resection. Intrathecal injection of recombinant adenovirus has only value in the treatment of leptomeningeal metastasis (chapter 4).

Minimal residual disease

Treatment of glioblastoma is nowadays usually confined to surgical resection followed by irradiation. Microsurgery and neuronavigation have improved surgical techniques but did not improve survival of patients with glioblastoma. Adjuvant experimental therapies as described in this thesis can in my opinion only improve survival when a "minimal residual disease" is left. This means that a maximum of cytoreductive surgery should be performed (followed by post-operative irradiation) after wich an experimental adjuvant therapy can achieve its optimum effect. Trials should therefore be confined to patients in good neurological condition who present with newly diagnosed primary brain tumors. In recurrent glioblastoma the disease is extended to a degree were it is unlikely to be controlled by experimental therapies.

Targeting and new routes of administration

Although gene therapy is still a promising new therapy, the delivery of the transgene to infiltrating brain tumor cells remains a major problem to overcome. New stratagies are being developed and are discussed shortly. Arterial (carotid) administration of virus (HSV, adenovirus) in combination with opening of the blood-brain barrier with bradykinin or mannitol

has been shown to transfect tumor cells selectively (4,5). This is probably due to fact that neovasculature in the tumor has a weaker blood-brain barrier as compared to the blood-brain barrier in normal brain. This route of administration could have some significant advantages over direct injection of the vector since tumor cells over large areas can be transfected. Another interesting approach is the vector targeting of neovascularisation of the tumor (6). In theory, destruction of the tumor vasculature will induce an anti-cancer effect. This approach has the advantage that also infiltrative growth of tumors can be arrested. Pressure perfusion of the interstitial space of brain parenchyma has been developed for the delivery of macromolecules to a large area surrounding the primary brain tumor (7,8). This method could be of importance for the delivery of vectors to infiltrating tumor cells surrounding the primary tumor. Combinations of the delivery methods mentioned could be applied to achieve maximum tumor cell kill. Further effectivity of gene therapy for cancer can be obtained by targeting the vector to tumor cell or tumor endothelium via receptor mediated augmented transfection (6). Replication competent recombinant adenoviruses have been demonstrated to induce an very effective tumor response (9). The virus could be of value in the treatment of infiltrating brain tumors although the toxicity of these viruses for normal tissue has to be assessed first.

Conclusion

Effective vector application to the tumor will be essential in the future for succesfull gene therapy for brain tumors. Methods to improve delivery should be investigated thoroughly. Vector targeting to tumorcells or tumor neovascularisation should also be studied further.

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Summary

The feasibility and use of recombinant adenovirus for the treatment of experimental brain tumors and leptomeningeal metastases in rats has been investigated in the different studies in this thesis. Chapters 2,3,4 and 5 contain the published (or submitted) articles of the different studies conducted by our study group.

In chapter 1 the different methods of gene transfer are outlined. Viral gene delivery methods have demonstrated to be very efficient as compared to non-viral gene delivery methods, although the latter have a higher safety profile. Construction, advantage and disadvantage of the most commonly used recombinant gene delivery viruses (retrovirus, adenovirus, herpes Simplex virus and adeno-associated virus) are discussed briefly.

Chapter 2: In experimental gene therapy for cancer genes encoding sensitizing enzymes are developed to mediate the formation of a cytotoxic drug from a non-toxic pro-drug in order to circumvent systemic toxicity. The most commonly used sensitizing transgene is the Herpes Simplex Virus thymidine kinase gene, also called "suicide gene". After transfection to the nucleus the gene is transcribed to the thymidine kinase enzyme. This enzyme is able to convert non-toxic GCV into a toxic nucleoside analogue, GCV- mono-phosphate. The nucleoside analogue prevents DNA elongation which results in cell death during replication. The system has the advantage that only dividing cells are killed after treatment with GCV. This has large potential in the treatment of CNS malignancies, since the majority of normal brain cells are non-dividing. Recombinant viral vectors (retrovirus, adenovirus, herpes simplex virus, adeno-associated virus) which have shown efficient brain tumor cell kill *in vitro* and *in vivo* using the TK/GCV system are discussed.

In chapter 3 the effectivity of recombinant adenoviral gene transduction and tumor cell kill after TK/GCV treatment is investigated *in vitro* and *in vivo*. First, rat (9L) and human brain tumor (U251) cells were infected *in vitro* with recombinant adenovirus containing the *LacZ* marker gene. Very efficient gene transduction was observed after staining with β–Gal in both cell lines, although higher activity was observed in human cells. The same virus was used to inject brain tumors in rats. After staining, microscopy of the brain slices showed very efficient transduction, mainly localized in

tumor regions adjacent to normal brain tissue. Microscopy revealed next to transduced tumor cells also transduced brain cells without cytopathic effects. Tumor cell kill *in vitro* was evaluated by infecting rat (9L) and human (U251) brain tumor cells with recombinant adenovirus containing the TK "suicide" gene followed by GCV administration. Very efficient cell kill was observed in both cell lines. Interestingly, human cells were killed more effectively as compared to rat cells. Finally, rats with brain tumors were treated with recombinant adenovirus containing the TK gene at different titers. Subsequently, the rats were injected with GCV for 10 days. Survival in rats treated with TK/GCV was prolonged significantly and directly related to the amounts of virus injected intra-tumorally. We also treated rats harbouring a brain tumor with recombinant retrovirus-TK producer cells. No difference in survival time was observed between recombinant adenovirus and recombinant retrovirus treated rats. Comparing between these recombinant viruses however is difficult. Recombinant adenovirus has the advantage that a cell free virus can be injected directly *in vivo* and that also non-dividing cells can be infected and killed when becoming mitotically active.

In chapter 4 the effectivity of treatment of leptomeningeal metastases in a rat model using recombinant adenovirus containing the TK gene is studied. First, a leptomeningeal metastases rat model was developed by injecting 9L brain tumor cells subocipitally directly into the CSF. Rats injected with these tumors developed tumor nodules all along the neuraxis and died within 20 days.
Four human tumor cell lines (A549; non-small cell lung carcinoma, 518-A2 melanoma, GLC-1; small cell lungcarcinoma, U251; human glioma) and 1 rat brain tumor cell line (9L) were infected *in vitro* with different titers of recombinant adenovirus containing a luciferase gene to quantify gene expression. All cell lines showed very effective gene expression after infection with the virus. The same cell lines were also infected with different amounts of recombinant adenovirus containing the TK "suicide" gene, followed by GCV administration to investigate tumor cell kill. All cell lines were very effectively killed by the virus. The study showed also that cell kill continued at least until 6 days after infection of the tumor cells and that human glioma cells (U251) were more effectively killed in time than rat brain tumor (9L) cells.
In the *in vivo* experiments described in this chapter, rats with leptomeningeal metastases were injected intrathecally (suboccipital) with recombinant adenovirus harbouring the *Lac*Z marker gene. The CNS was removed and stained with β–Gal to investigate gene transduction in lep-

tomeningeal tumors and healthy tissue *in vivo*. Microscopic examination revealed transduced tumor cells all along the neuraxis. Most transduced cells were observed in the tumor mass near the injection site. A few ependymal cells were also transduced, but no cytopathic effects were observed. Rats with leptomeningeal metastases were then injected intrathecally (suboccipital) with recombinant adenovirus containing the TK "suicide" gene. 24 hours after injection the animals were treated with GCV for 10 days. Rats treated with TK/GCV lived significantly longer as compared to control animals.

In chapter 5 adenoviral gene transfer and killing efficiency using the TK/GCV mechanism was evaluated in several human tumor cell lines occurring as central nervous system malignancies. The effectiveness was tested *in vitro* and in experimental rat brain tumors and leptomeningeal metastases models.
Recombinant viruses with the transcription of genes under the control of either the major late promotor (MLP) or the human cytomegalovirus (CMV) immediate early gene promotor were constructed and compared for gene transfer and killing efficiency *in vitro* and *in vivo*.
Human tumor cell lines occurring as brain metastases (A549; non-small cell lung carcinoma, 518-A2 melanoma, GLC-1; small cell lungcarcinoma), three human glioma cell lines (U251, D384, LW5) and 1 rat glioma cell line (9L) were infected with recombinant adenovirus containing the *LacZ* marker gene *in vitro*. All cell lines were effectively transduced with the *LacZ* marker gene after infection. The same cell lines were also infected *in vitro* with different titers of recombinant adenovirus containing the TK "suicide" gene followed by GCV administration. All cell lines were very effectively killed by the virus. We also demonstrate that tumor cell doubling time of glioma cells was directly correlated to the killing efficiency *in vitro* after TK/GCV treatment. The MLP and CMV promotor showed a few differences *in vitro* and *in vivo*. Tumor cells infected with recombinant adenovirus with the transcription under the control of the CMV promotor showed enlargement of cells as compared to the MLP promotor, suggesting toxicity. Most tumor cell lines were killed more efficiently after infection with adenovirus-TK containing the CMV promotor as compared to the adenovirus containing the MLP promotor. However, the CMV promotor also showed a higher cell kill of glioma cells without GCV treatment, suggesting toxicity. In the *in vivo* experiments, rats with either leptomeningeal metastases or brain tumor were treated with different titers of recombinant adenovirus-TK containing either the MLP or the CMV promoter and subsequent GCV treatment. Rats with

brain tumor showed a dose dependent survival for both vectors and a better therapeutic effect after treatment with the CMV promotor was observed as compared to the MLP promoter. Rats with leptomeningeal metastases showed also a significant longer symptom free period after treatment with both vectors. Treatment with the CMV promoter showed also a significantly longer symptom free period as compared to the MLP promoter in the rats with leptomeningeal metastases.

In chapter 6 intracerebral toxicity of recombinant adenovirus was examined histopathalogically. Also the systemic spread of virus after intracerebral injection was investigated.
Rats were injected intracerebrally in the right frontal lobe with 108 or 109 IU of recombinant adenovirus containing the TK gene, wild type adenovirus or PBS as controls. The rats were than treated for 16 days with GCV or PBS as a control. The brains of the treated rats were then microscopically examined. Rats injected with 108 IU of recombinant virus showed only mild to moderate perivascular infiltration in the near vicinity of the injection site. Brains of animals injected with 109 IU's revealed moderate to severe perivascular lymphoid infiltration, glial proliferation, edema and parenchymal loss extending to basal, baso-lateral and caudal area of the frontal lobe, but not exceeding a distance of 2-3 mm from the injection canal. An additional effect of GCV administration was not observed. Wild-type adenovirus injection revealed only slightly more severe lesions compared to those treated with 108 IU recombinant adenovirus. Systemic spread of the virus was studied after intracerebral injection of recombinant adenovirus containing the luciferase marker gene. The expression of the transgene was measured in brain, spinal cord, hart, lung liver, spleen, gastro-intestines, kidney and gonads, 3 and 14 days after intracerebral injection. Activity of the luciferase gene could only be detected in brain and spinal cord. In all other organs no detection of the transgene could be detected.

General conclusions

The studies in this thesis show an very efficient recombinant adenoviral gene transduction in various tumor cells *in vitro,* occurring as CNS malignancies. Efficient transgene expression was also demonstrated in rat brain tumors and leptomeningeal metastases *in vivo.* A high killing effectivity using the recombinant adenovirus containing the HSV-tk gene and subsequent GCV administration was obtained in human and rat tumor cells *in*

vitro. Significantly prolonged survival in rats with brain tumor or leptomeningeal metastases was obtained after treatment with recombinant adenovirus-tk/GCV. Transgene expression and killing efficiency was dose dependent *in vitro* and *in vivo*. We also demonstrated that the promoter driving the transgene in an adenoviral vector influences toxicity and efficiency of treatment. *In vivo* toxicity studies showed only limited damage after intracerebral injection of recombinant adenovirus. Systemic trangene expression was not measured after intracerbral or intrathecal injection. Rats did not show clinical deteriation during or after treatment with the recombinant virus.

The data show the effectiveness and feasibility of treating CNS tumors with recombinant adenovirus harbouring the HSV-tk gene and subsequent GCV. Based on these data, clinical application of this approach is therefore considered acceptable.

Samenvatting

In dit proefschrift wordt het gebruik van recombinant adenovirus voor de behandeling van experimentele hersen tumoren en leptomeningeale metastasen in ratten onderzocht in verschillende studies. Hoofdstuk 2,3,4 en 5 bevatten gepubliceerde (of nog te publiceren) artikelen van de verschillende studies.

In hoofdstuk 1 worden de verschillende methoden van genoverdracht besproken. Het is aangetoont dat virale genoverdracht zeer efficient is t.o.v. niet virale genoverdracht methoden, hoewel de laatste een hoger veiligheids profiel hebben. Constructie, voordelen en nadelen van de meest gebruikte recombinant genoverdracht methoden (retrovirus, adenovirus, herpes Simplex virus en adeno-associated virus) worden kort besproken.

Hoofdstuk 2: in de experimentele gentherapie worden genen ontwikkeld die coderen voor enzymen die een niet toxische stof kunnen omzetten in een toxische stof zodat systemische toxiciteit kan worden vermeden. Het meest gebruikte gen hiervoor is het Herpes Simplex virus thymidine kinase gen (HSV-tk), ook wel "zelfmoord gen" genoemd. Na tranfectie in de kern van een cel wordt het gen omgezet in het thymidine kinase enzym. Dit enzym kan niet toxisch Ganciclovir (GCV) omzetten in een toxisch nucleoside analoog, GCV-trifosfaat. Dit nucleoside analoog voorkomt DNA verlenging wat uitendelijk cel dood veroorzaakt als de cel in deling gaat. Het systeem heeft het voordeel dat alleen delende cellen worden gedood na behandeling met GCV. Dit kan een groot voordeel zijn bij de behandeling van maligniteiten van het centrale zenuwstelsel aangezien de meeste cellen in het centrale zenuwstelsel niet delen. Recombinant virale vectoren (retrovirus, adenovirus, herpes simplex virus, adeno-associated virus) die met het TK/GCV systeem reeds efficiente celdood van hersentumorcellen hebben kunnen bewerkstelligen *in vitro* en *in vivo* worden besproken in dit hoofdstuk.

In hoofdstuk 3 wordt de effectiviteit van recombinant adenovirale gen transductie en tumor celdoding na TK/GCV behandeling onderzocht *in vitro* en *in vivo*.

Allereerst werden ratten (9L) en humane hersentumorcellen (U251) geïnfecteerd *in vitro* met recombinant adenovirus die het *LacZ* marke-

ringsgen bevatten. Zeer efficiente gen transductie werd geobserveerd nadat de cellen werden gekleurd middels β-Gal, hoewel een hogere activiteit van het gen werd geobserveerd in humane tumorcellen. Hetzelfde virus werd gebruikt om hersentumoren in ratten te injecteren. Na kleuring van het virus demonstreerde de hersencoupes macroscopisch zeer efficiënte gentransductie die met name in de tumor is gelocaliseerd. Microscopisch onderzoek van de coupes demonstreerde behalve getransduceerde tumorcellen ook getransduceerde hersencellen zonder cytopathologisch effect. Tumor cel doding *in vitro* werd geevalueerd door ratten (9L) en humane (U251) hersentumorcellen te infecteren met recombinant adenovirus die het TK "zelfmoord gen" bevatten en vervolgens te behandelen net GCV. Zeer efficiente tumor cell doding werd geobserveerd in beide tumor cellijnen. Een bijzonderheid was dat humane tumorcellen effectiever werden gedood dan rattentumor cellen. Ten slotte werden ratten met hersentumoren geinjecteerd met verschillende hoeveelheden recombinant adenovirus die het TK gen bevatten. Vervolgens werden de ratten 10 dagen lang intraperitoneaal geinjecteerd met GCV. Overleving van ratten behandeld met TK/GCV was significant verlengd en direct gerelateerd aan de hoeveelheid virus die werd ingespoten. Ratten met hersentumoren werden ook behandeld met recombinant retrovirus-TK producercellen. Geen verschil in overleving werd geobserveerd tussen recombinant adenovirus en recombinant retrovirus-TK producercellen behandelde ratten. Het vergelijken van deze recombinant virussen in echter moeilijk. Recombinant adenovirus heeft het voordeel dat een celvrije oplossing direct *in vivo* kan worden geinjecteerd en dat ook niet delende tumor cellen kunnen worden geïnfecteerd en gedood als ze in deling gaan.

In hoofdstuk 4 wordt de effectiviteit van de behandeling van leptomeningeale metastasen met behulp van recombinant adenovirus welke het TK gen bevat bestudeerd in een rat model. Allereerst werd hiertoe een leptomeningeale metastasen rat model ontwikkeld door 9L ratten hersentumor cellen suboccipitaal direct in de liquor te injecteren. Ratten die geinjecteerd werden ontwikkelden tumoren door het hele CZS en overleden binnen 20 dagen.
Vier humane tumor cellen (A549; niet klein cellig long carcinoom, 518-A2; melanoom, GLC-1; klein cellig long carcinoom, U251; humaan glioom) en 1 ratten hersen tumor cellijn (9L) werd geinfecteerd *in vitro* met verschillende hoeveelheden (titers) recombinant adenovirus die het luciferase-gen bevatten om genexpressie te kwantificeren. Alle cellijnen demonstreerden een zeer effectieve genexpressie na infectie met het virus. Dezelfde cell lijnen werden ook geinfecteerd met verschillende titers re-

combinant adenovirus-TK, gevolgd door GCV toediening om tumorceldoding te kwantificeren. Alle cellijnen werden zeer effectief gedood door het virus. Het onderzoek toonde ook aan dat celdoding ten minste 6 dagen na infectie van de tumorcellen voortduurt en dat humane glioomcellen (U251) effectiever worden gedood per tijseenheid dan ratten hersentumor (9L) cellen.
In de *in vivo* experimenten die in dit hoofdstuk worden beschreven, worden ratten met leptomeningeale metastasen, intrathecaal (suboccipitaal) geinjecteerd met recombinant adenovirus die het *Lac*Z markergen bevatten. Het CZS werd verwijderd en gekleurd met β-GAL om gentransductie in leptomeningeale tumoren en gezond weefsel te onderzoeken. Microscopisch onderzoek toonde getransduceerde cellen langs de gehele neuraxis. De meeste getransduceerde cellen werden geobserveerd in de tumor massa dichtbij de injectie plaats. Hoewel enkele ependymcellen ook werden getransduceerd, werden geen cytopathologische effecten waargenomen. Ratten met leptomeningeale metastasen werden vervolgens inthrathecaal (suboccipitaal) geinjecteerd met recombinant adenovirus-TK. 24 na injectie werden de dieren 10 dagen behandeld met GCV. Ratten die behandeld werden met TK/GCV leefden significant langer dan controleratten.

In hoofstuk 5 wordt de adenovirale gen transfer en de TK/GCV dodingseffectiviteit geevalueerd in diverse humane tumor cellijnen die voorkomen als maligniteiten binnen het CZS. De effectiviteit van het virus werd getest *in vitro* en in experimentele ratten hersentumoren en leptomeningeale metastasen modellen *in vivo*.
Recombinant virussen met de transcriptie van transgenen door de "major late promotor" (MLP) of "human cytomegalovirus immediate early gene promotor" (CMV) werden eerst geconstrueerd en vervolgens vergeleken voor wat betreft gentransfer en dodingseffectiviteit *in vitro* en *in vivo*. Humane tumorcellijnen die voorkomen als hersenmetastasen (A549; niet kleincellig longcarcinoom, 518-A2; melanoom, GLC-1; kleincellig longcarcinoom), 3 humane glioom cellen (U251, D384, LW5) en 1 ratten gliomacellijn (9L) werden geinfecteerd *in vitro* met het recombinant adenovirus die *Lac*Z markergen bevat. Alle cellijnen werden na infectie effectief getransduceerd met het *Lac*Z gen. Dezelfde cellijnen werden *in vitro* ook geinfecteerd met verschillende titers recombinant adenovirus-TK gevolgd door GCV toediening. Alle cellijnen werden effectief gedood door het virus. Het onderzoek demonstreert ook dat tumorcel verdubbelingstijd van gliomacellen direct gecorreleerd is aan de dodingseffectiviteit *in vitro* na behandeling met TK/GCV. De MLP en CMV promotor lieten een

aantal verschillen zien *in vitro* en *in vivo*. Tumorcellen geïnfecteerd met recombinant adenovirus met de CMV promotor waren groter dan de cellen geinfecteerd met de MLP promotor wat zou kunnen duiden op toxiciteit. De meeste tumorcellen werden ook significant beter gedood na infectie met adenovirus-TK die de CMV promotor bevatten dan de virussen die de MLP promotor bevatten. Echter de CMV promotor demonstreerde ook een hogere cell doding na infectie maar zonder GCV behandeling, wat duid op toxiciteit. In de *in vivo* experimenten werden ratten met leptomeningeale metastasen of een hersentumor geinjecteerd met verschillende titers recombinant adenovirus-TK die of de MLP of de CMV-promotor bevatten, en vervolgens behandeld met GCV. Ratten met een hersentumor demonstreerden een dosis afhankelijke significant verlengde overleving na behandeling met beide vectoren, hoewel de CMV promotor een significant beter therapeutisch effect bewerkstelligde in vergelijking met de MLP promoter. Ratten met leptomeningeale metastasen hadden een significant langere symptoomvrije periode na behandeling met beide vectoren. Echter, ook hier bleek de behandeling met de CMV-promotor een significant langere symtoom vrije periode te bewerkstelligen in vergelijking met de MLP-promotor.

In hoofdstuk 6 werd de intracerebrale toxiciteit van recombinant adenovirus histo-pathalogisch bestudeerd. Hiernaast werd ook de mogelijke systemische verspreiding van het virus bestudeerd. Ratten werden intracerebraal geinjecteerd in de rechter frontaalkwab met 10^8 of 10^9 IU recombinant adenovirus die het TK gen bevatten, wild-type adenovirus of PBS als controle. De ratten werden vervolgens 16 dagen lang behandeld met GCV of PBS als controle. De hersenen van behandelde ratten werden vervolgens microscopisch bestudeerd. Bij ratten die geinjecteerd waren met 10^8 "Infective Units" (IU) recombinant adenovirus werd alleen milde tot matige perivasculaire lymphoide infiltratie in de directe omgeving van de plaats van injectie waargenomen. Bij hersenen van ratten geinjecteerd met 10^9 IU werd matige tot ernstige perivasculaire lymphoide infiltratie, gliale proliferatie, oedeem en parenchymverlies waargenomen die zich uibreidde naar basaal, baso-lateraal en de caudaal van de frontaal kwab, maar zich niet verder uitbreidde dan 2-3 mm vanaf het injectie kanaal. Een aanvullend effect van GCV werd niet waargenomen. Injectie van wild-type adenovirus demonstreerde iets meer uitgebreide leasies in vergelijking met de ratten behandeld met 10^8 IU recombinant adenovirus. Vervolgens werd de systemische verpreiding van het virus bestudeerd na intracerebrale injectie van recombinant adenovirus die het luciferase-gen bevat. De expressie van het transgen werd 3 en 14 dagen na intracerebrale injectie gemeten

in hersenweefsel, ruggemerg, hart, long, lever, milt, darmen, nier en testes. Luciferase activiteit was alleen meetbaar in hersenweefsel en ruggemerg. In alle andere organen werd geen trangene activiteit gemeten.

Algemene conclusies

De studies in dit proefschrift laten een zeer effectieve gentransductie zien m.b.v recombinant adenovirus in diverse tumorcellen *in vitro* die voorkomen als maligniteiten van het centrale zenuwstelsel. Effectieve genexpressie werd ook aangetoont in ratten hersentumoren en leptomeningeale metastasen *in vivo*. Hoge dodingseffectiviteit kon worden bewerkstelligd in humane en ratten tumor cellen *in vitro*. Een significant verlengde overleving in ratten met hersentumoren of leptomeningeale metastasen kon worden bereikt na behandeling met recombinant adenovirus-tk/GCV behandeling. Transgen-expressie en dodingseffectiviteit was dosis afhankelijk zowel *in vitro* als *in vivo*. Er werd tevens aangetoond dat de promotor die het transgen aanstuurt in een adenovirale vector de toxiciteit en effectiviteit van de behandeling beïnvloed. Slechts beperkte schade werd na intracerebrale injectie van reconbinant adenovirus in de *in vivo* toxiciteitsstudies gevonden. Systemische transgen expressie werd niet gemeten na intracerebrale of intrathecale injectie. Ratten gingen niet klinisch achteruit tijdens of na de behandeling met recombinant adenovirus.
Deze data laten de effectiviteit en geschiktheid om tumoren van het CZS te behandelen met recombinant adenovirus-tk en GCV. Gebaseerd op deze data is een klinische toepassing acceptabel.

Curriculum vitae

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Abbreviations

AAV	=	adeno associated virus
Ad 5	=	human adenovirus; serotype 5
β-gal	=	β-galactosidase
CNS	=	central nervous system
CMV	=	cytomegalovirus immediate early promoter
CSF	=	cerebral spinal fluid
DNA	=	deoxyribonucleic acid
GCV	=	ganciclovir
GCV-P	=	ganciclovir monophosphate
GCV-3P	=	ganciclovir triphosphate
HSV	=	herpes simplex virus
HSV-tk	=	thymidine kinase-gene derived from the herpes simplex virus
IG.Ad.MLP.TK	=	recombinant adenovirus containing the thymidine kinase transgene and mlp promoter; constructed and manufactured by Introgene
ITR	=	inverted terminal repeats
IU	=	infectious units
kb	=	kilobase
LTR's	=	long terminal repeats
luc	=	luciferase
MLP	=	major late promoter
MRI	=	magnetic resonance imaging
mRNA	=	messenger RNA
MuLV	=	murine leukemia virus
nls	=	nuclear location signal
PBS	=	phosphate buffered saline
PCR	=	polymerase chain reaction
pfu	=	plaque forming units
RCR	=	replicative competent retrovirus
RNA	=	ribonucleic acid
RSV	=	rous sarcoma virus
SD	=	standard deviation
TK	=	tymidine kinase
VPC	=	vector producing cell line

Dankwoord

Zonder de inspanning van anderen zou dit proefschrift niet tot stand zijn gekomen. Een aantal wil ik in het bijzonder bedanken;
Allereerst mijn promotor Prof. Dr. C.J.J. Avezaat, voor de kans die u me gegeven heeft dit onderzoek te verrichten. Hoewel de moleculaire biologie ver van de neurochirurgie en patientenzorg staat heeft u de vorderingen altijd met veel belangstelling gevolgd. Daarbij heb ik tijdens mijn opleiding ook alle ruimte gehad van u om dit proefschrift af te maken, waarvoor ik u zeer erkentelijk ben. Ik hoop nog in mijn komende opleidingsjaren nog tijd te vinden het neuro-oncologisch onderzoek met u verder uit te breiden.
Prof. Dr. D. Valerio voor de "mooie" en leerzame onderzoeksjaren op Introgene. Als clinicus was het aanvankelijk even wennen in het "wetenschappelijke wereldje" op Introgene. Het wetenschappelijke jargon heb ik me gelukkig snel eigen kunnen maken wat uiteindelijk tot een vruchtbare samenwerking heeft geleid. Bedankt voor kans die je mij hebt gegeven in jou lab te werken. Ik hoop dat we kunnen blijven samenwerken voor wat betreft neuro-oncologisch onderzoek
Mijn co-promoter Dr. P.M. Hoogerbrugge voor de kritische beschouwingen van experimenten maar met name van mijn artikelen en proefschrift. Het was leerzaam en prettig samenwerken met een clinicus die de vraagstelling van het wetenschappleijk experiment wist te transponeren naar de kliniek.
Dr B. Bout voor de "inwijding" in de moleculaire biologie. Het zal wel geen makkelijke klus zijn geweest om een clinicus de fijne kneepjes van het vak te leren. Ik wil je bedanken voor het geduld wat je hebt opgebracht en met name de manier waarmee je mij ten alle tijde positief wist te entousiasmeren, met name na mislukte proeven.
Zonder Juus Noteboom, Gerry van Someren en Carmen del Esandi was het proefschrift waarschijnlijk nooit tot stand gekomen. De vele hulp en toewijding tot het maken van de batches recombinant adenovirus heeft me de gelegenheid gegeven al deze experimenten te verrichten. Bedankt voor de grote hoeveelheid werk! Hierbij wil ik ook alle medewerkers van Introgene bedanken voor alle hulp en adviezen tijdens mijn onderzoek.
Dr Ch. Vecht wil ik bedanken voor het feit dat ik via hem op Introgene ben beland. Regelmatig was je op Introgene om je te informeren over de progressie van het onderzoek. Het zijn uiteindelijk twee zeer vruchtbare jaren geworden waarvan jij aan de basis hebt gestaan.

Drs. R. Bartstra wil ik bedanken voor de statistische berekeningen verricht in dit proefschrift en de vele wetenschappelijke discussie's die merkwaardigerwijs met name over vrouwen gingen.
Prof. D.W. van Bekkum wil ik bedanken voor de vele (regelmatig heftige) wetenschappelijke discussies die we hebben gevoerd tijdens en na werktijd, niet zelden tijdens een borrel. Het entousiasme, de daadkracht, en overgave waarmee u de wetenschap bedrijft is uniek en heeft mijn bewondering.
Maarten Driesse, Peter Sillevis Smit en Chris Zurcher wil ik bedanken voor de prettige samenwerking die hebben geleid tot diverse publicatie's.
Mijn collega's; stafleden en assistenten van de afdeling neurochirurgie wil ik danken voor de soepelheid waarmee mijn afwezigheid vanwege de wetenschap de afgelopen jaren werd opgevangen. Hierbij wil ik met name noemen: Marie-Lise van Veelen, Wimar van den Brink, Henk van Santbrink en natuurlijk niet te vergeten Rashid Janjua.
Mijn paranimfen Werner Overdijk en Rolf Bartstra wil ik danken voor de vrienschap en het feit dat jullie mij ter zijde willen staan bij de verdediging van dit proefschrift.

Mijn zusje Valerie heeft mijn respect afgedwongen door enkele maanden eerder te promoveren dan ik. Ik ben trots op je en erken de nederlaag. Veel geluk in Frankrijk! Mijn ouders bedank ik voor het feit dat ik besta en zij mij de mogelijkheid hebben gegeven dit leven te leiden.

Appendix: figures

Chapter 3, figure 2a, 2b

Microscopy of U251 and 9L tumor cells transduced with Ad.RSVβgal (x20). Human U251 and 9L tumor cells were cultured and infected at confluency with an m.o.i. of 100. Seventy-two hours after infection, the wells were stained with X-Gal, washed and fixed. Almost all U251 tumor cells are stained blue in the nucleus (a). 9L tumor cells 3 days after infection with Ad.RSVbgal (x10)(b).

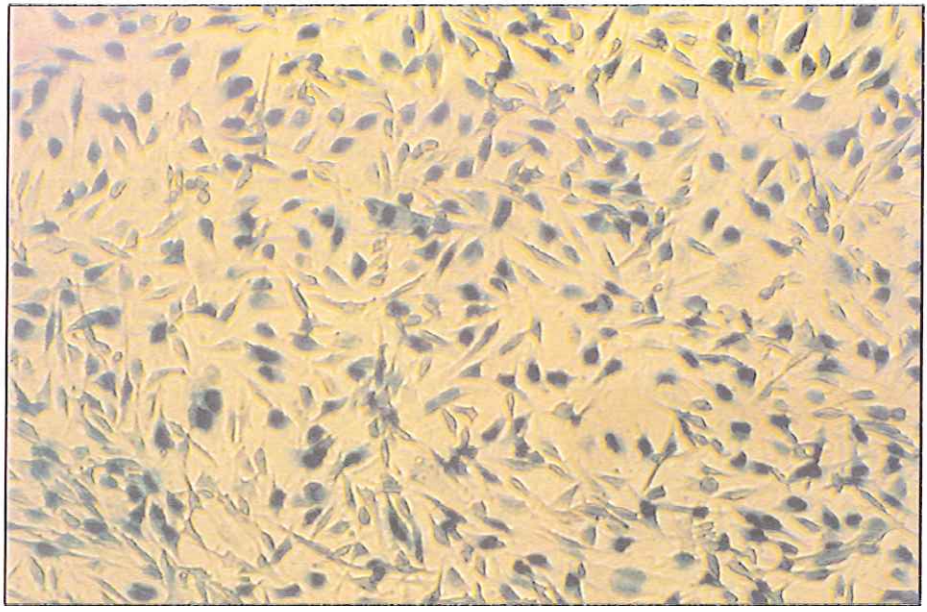


Figure 2a

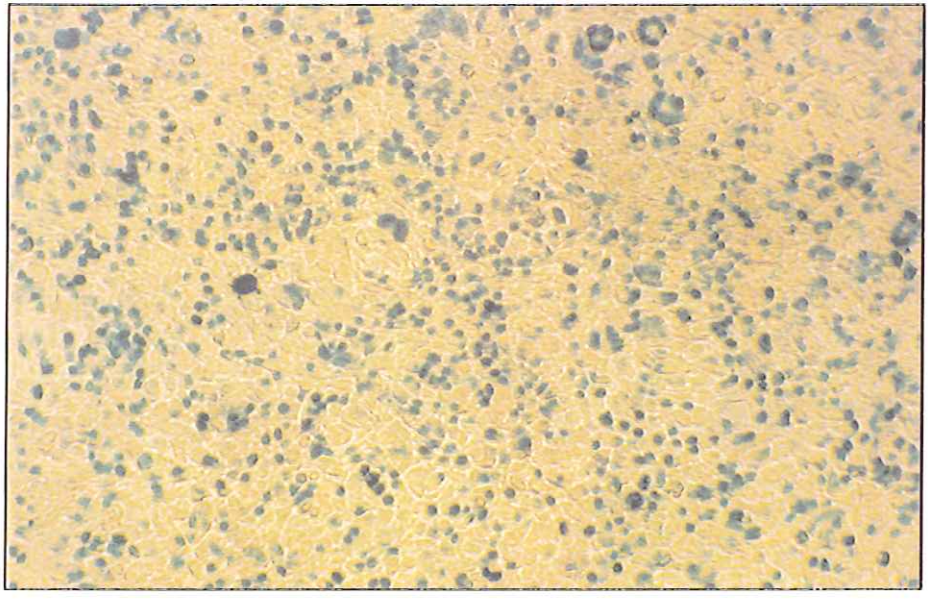


Figure 2b

Chapter 3, figure 3a

β–galactosidase expression in 2 slices of 9L brain tumor after injection of Ad.RSVβgal (a). Rats were injected stereotactically with $2.5x10^9$ pfu's in the tumor. Five days after injection the brains were fixed, cut in slices and stained with X-Gal. Microscopy (x20) of the part shown above (b). The slices were embedded in paraffin, cut in 2 mm sections and counterstained. The majority of the cells show β–galactosidase activity. A few *Lac*Z transduced normal brain cells are visible adjacent to the tumor. (x20) (c).

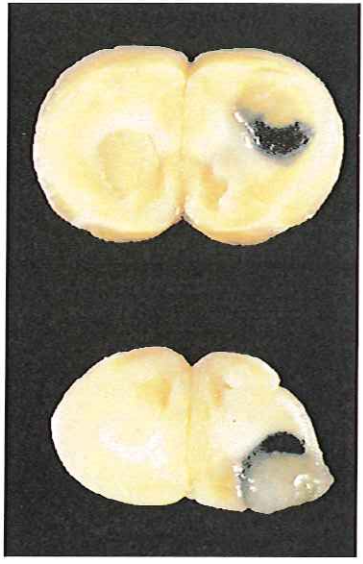


Figure 3a

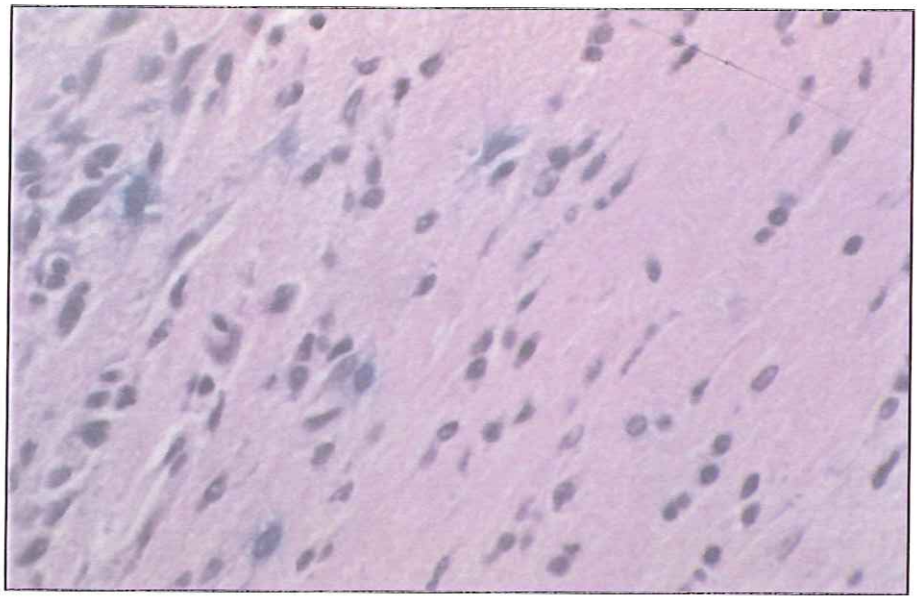


Figure 3b

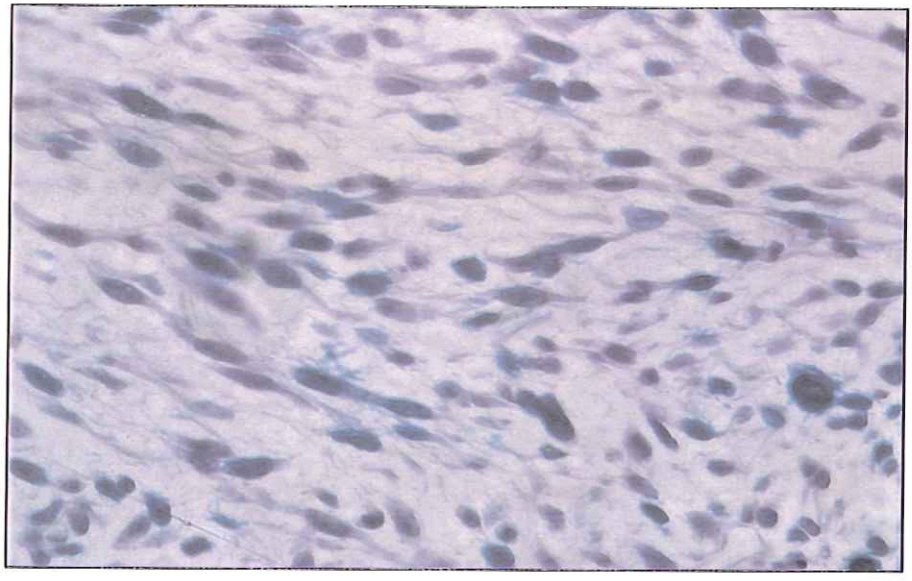


Figure 3c

Chapter 4, figure 4a, 4b, 4c, 4d

Microscopy of X-Gal stained brain and spinal cord containing leptomeningeal tumor cells

Figure 4a

Figure 4b

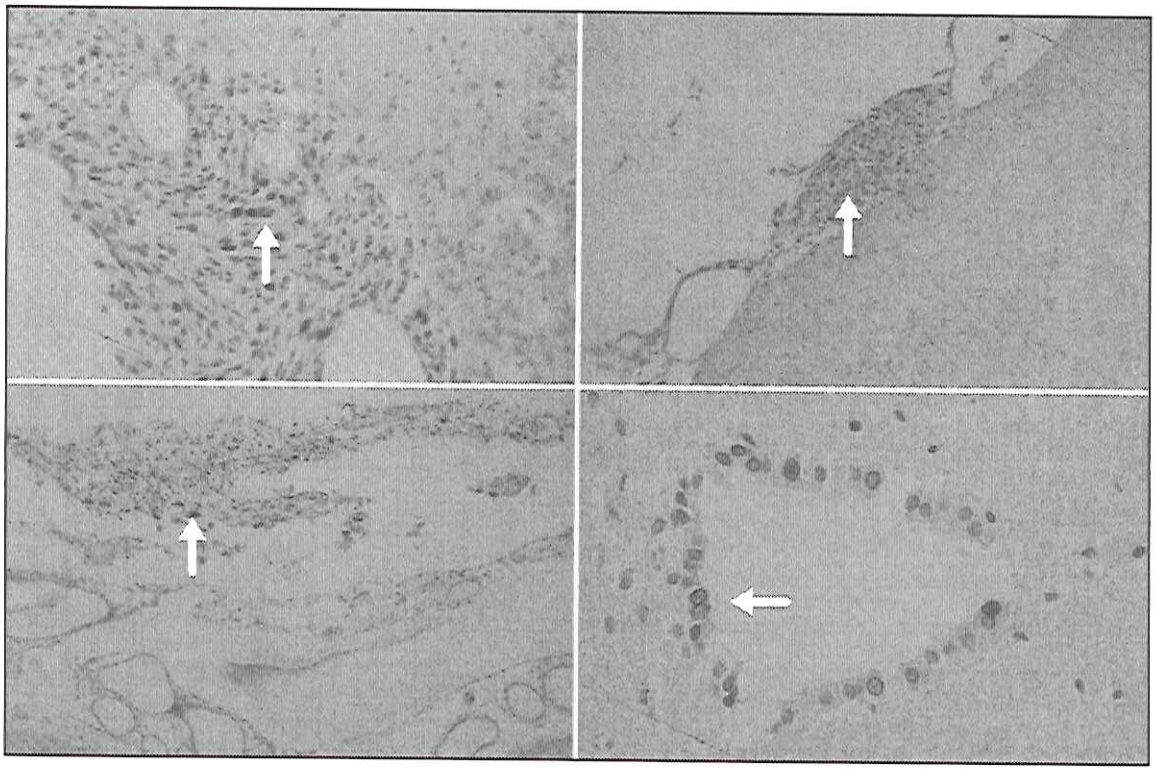


Figure 4c

Figure 4d

Chapter 5, figure 3a, 3b

Microscopy (x200) of human U251 tumor cells infected with IG.Ad.MLP.*Lac*Z (A) or IG.Ad.CMV.*Lac*Z (B) at m.o.i 100. Human tumor cells were plated in 24 well culture dishes (Costar) at a density of 104 cells/well. Cells in triplicate wells were infected at m.o.i. 0, 10, 100 or 1000. To asess β–galactosidase activity, the cells were stained with X-Gal 72 hours after infection and examined microscopically. All U251 tumor cells are transduced with the *Lac*Z gene. IG.Ad.CMV.*Lac*Z infected cells show enlargement of cells and nucleus/plasma ratio is enlarged as compared to the IG.Ad.MLP.*Lac*Z infected cells suggesting cytotoxicity.

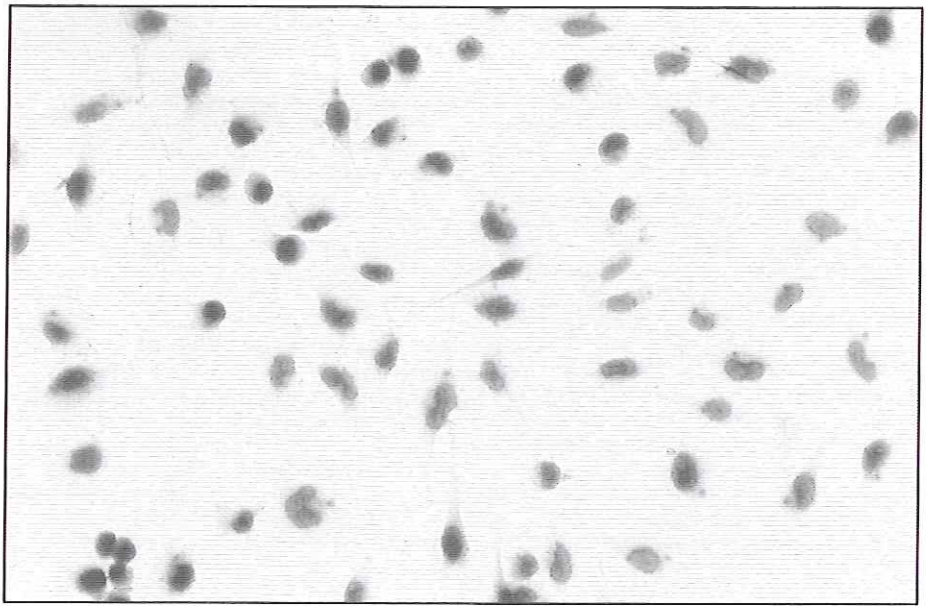


Figure 3a

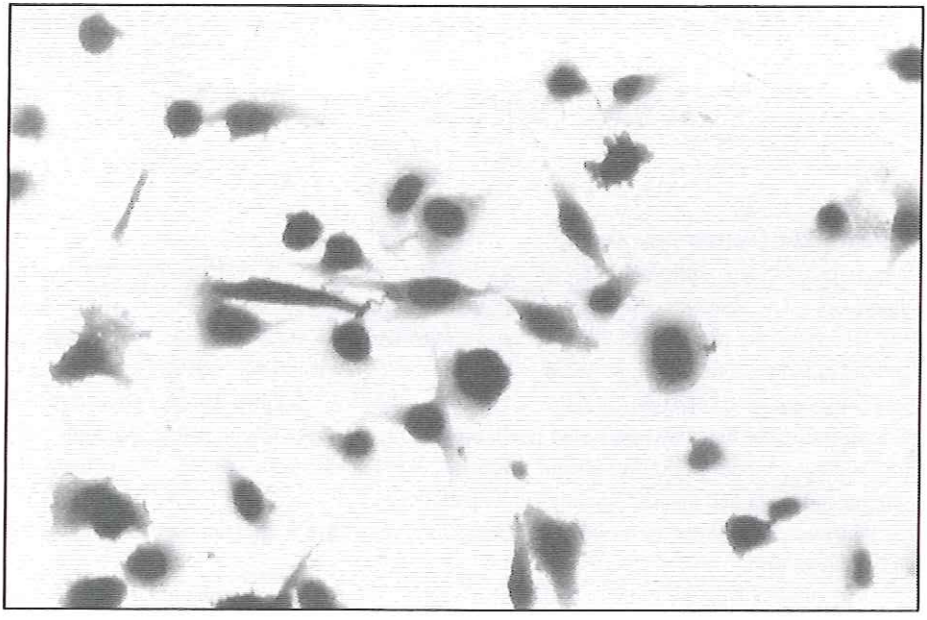


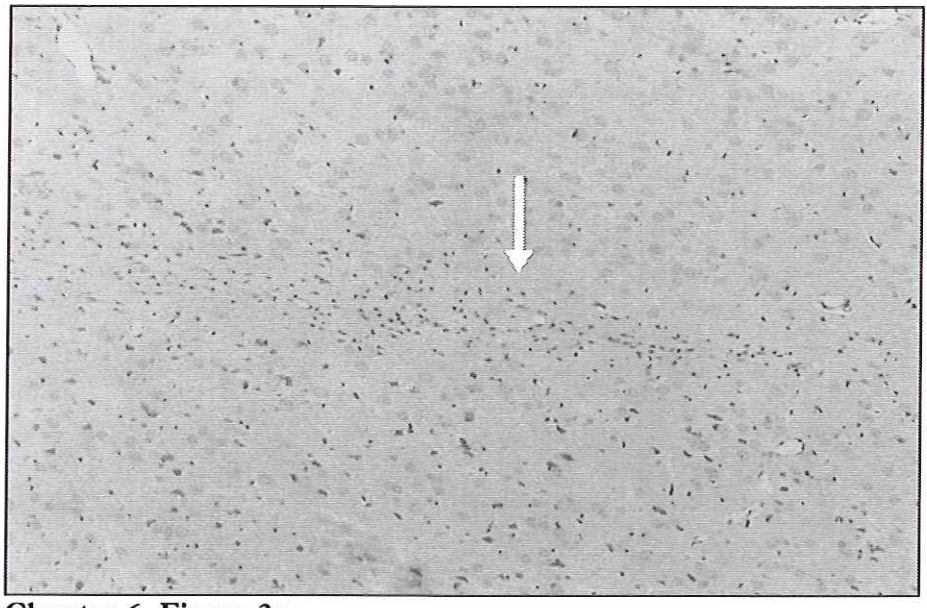
Figure 3b

Chapter 6, figure 2a, 2b

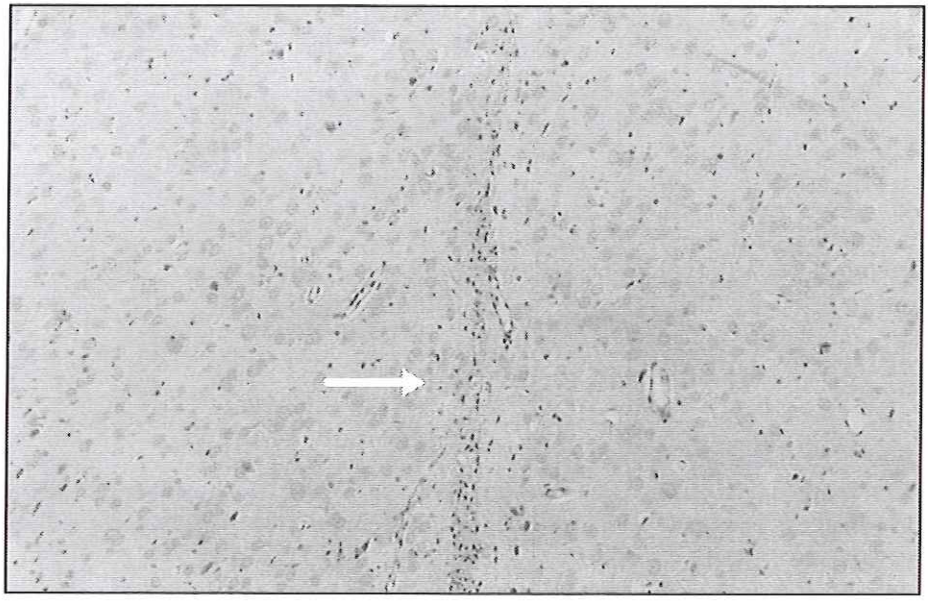
Photographs showing histopathology in rats injected intracerebrally with IG.Ad.MLPI.TK and subsequent GCV treatment. Rats were anaesthetized and injected stereotactically with 10^8 IU (1μl), 10^9 (10μl) IU IG.Ad.MLPI.TK, 10^8 (1μl) IU WtAd5 or PBS via a microliter syringe into the left forebrain, at a depth of 4mm. 24 hours later the rats were treated twice daily for 14 days with either intraperitoneal injections of GCV or PBS. The animals were killed 16 days after intracerebral injection. The brain was perfusion fixed, removed, cut transversely in 6 sections, embedded in paraffin, each section cut into 2 mm sections, counterstained with hematoxylin, phloxin and saffrane and examined microscopically by the pathologist.

2A: Reactive changes due to the injection procedure; mild edema, mononuclear infiltration and glia proliferation,

2B: Mild glia proliferation and mononuclear infiltration in the direct environment of the injection canal (10^8 IU IG.Ad.MLPI.TK+GCV).



Chapter 6, Figure 2a



Chapter 6, figure 2b

Chapter 6, figure 2c, 2d

2C: Moderate to severe glia proliferation, mononuclear proliferation, multifocal perivascular lymphoid cell infiltration, edema in the direct environment of the injection site (10^9 IU IG.Ad.MLPI.TK+GCV).

2D: glia proliferation, mononuclear infiltration , perivascular mononuclear infiltration and edema in the contralateral hemisphere (10^9 IU IG.MLPI.TK+GCV).

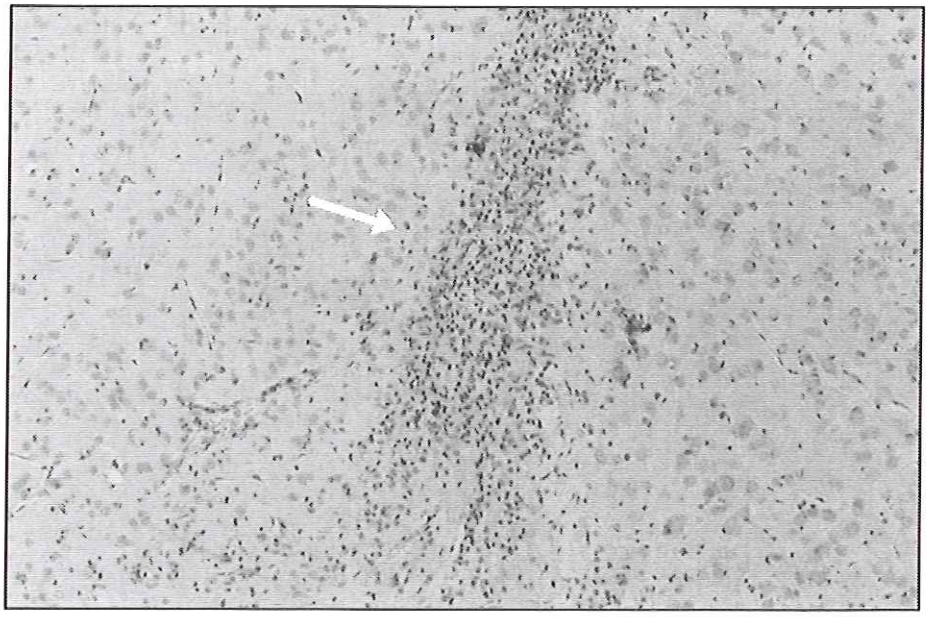


Figure 2c

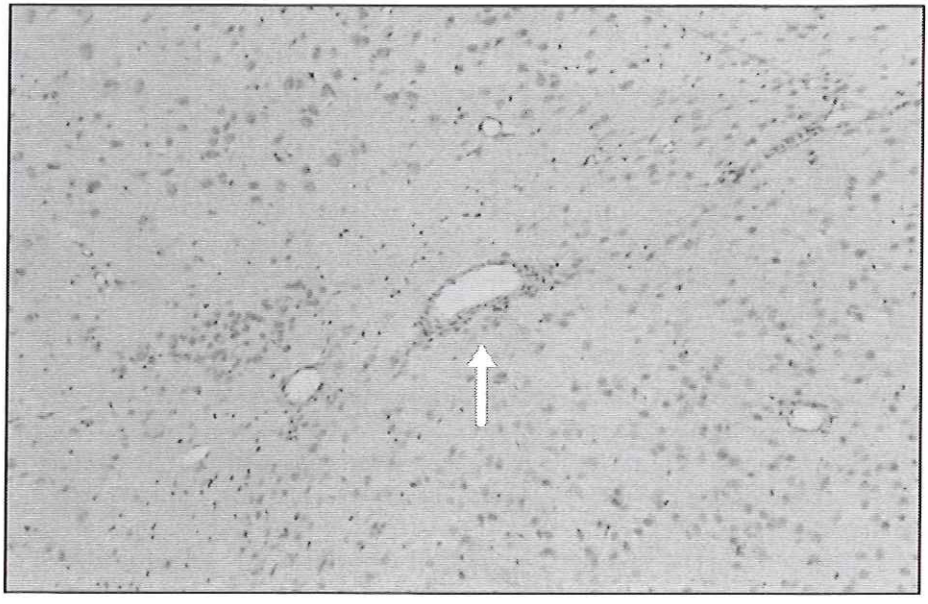


Figure 2d

Chapter 6, figure 4a, 4b

Photomicrographs showing X-Gal stained brain after injection of 10^8 IU IG.Ad.MLP.*Lac*Z.

Rats were injected intracerebrally with 10^8 IU IG.Ad.MLP.*Lac*Z and killed 3 days later. The brain was removed, cut with a vibratome into 100 μm sections and stained with X-Gal. The sections were then counterstained with hematoxylin/eosin and examined microscopically for reporter gene expression (blue cells).

4A: *Lac*Z reporter gene expression along the injection tract and along the meninges near the injection site.

4B: transduced cells along the corpus callosum in the contralateral hemisphere of the injection tract

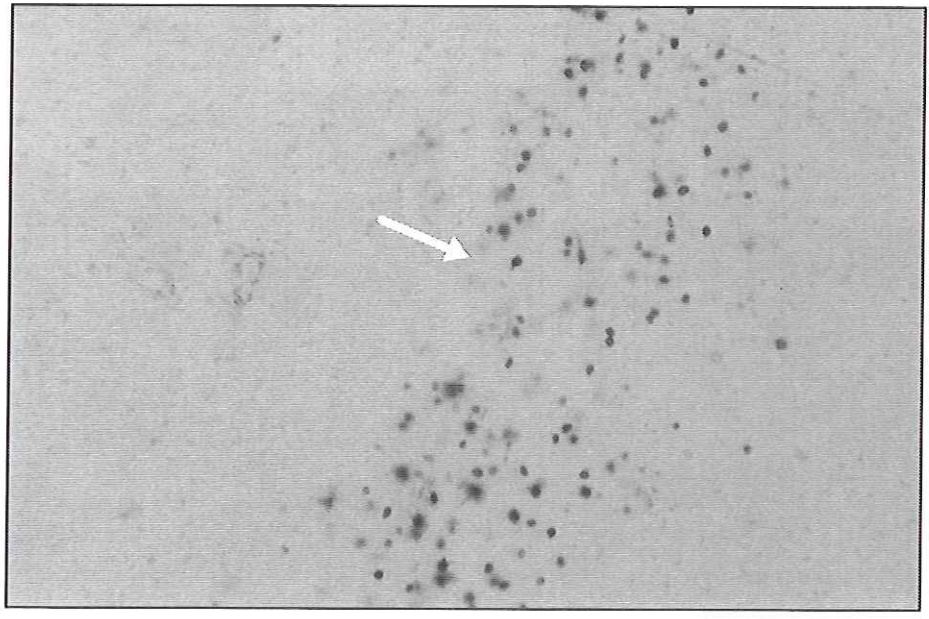


Figure 4a

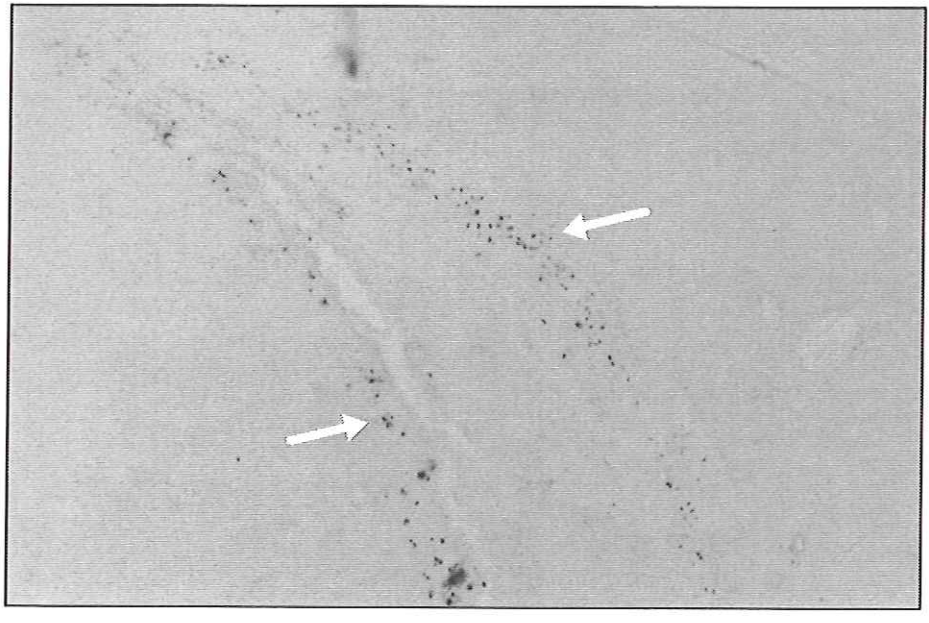


Figure 4b