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## Improving oncolytic viral therapy for glioma

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# CHAPTER 1

GENERAL INTRODUCTION



## GENERAL INTRODUCTION

Glioblastoma Multiforme (GBM) is a devastating disease for which no effective therapy exists. Sadly, the prognosis for patients suffering from this disease has improved only marginally during the last three decades. It is for this reason that GBM has become the target of many novel and experimental approaches, including gene therapy and oncolytic viral therapy.

Adenoviruses (Ads) are among the most widely used vectors in gene-therapeutic applications in both brain and various other organs. The use of adenoviruses can now be divided into two main categories: Gene therapy using replication deficient viruses, and oncolytic therapy, or virotherapy, which involves the use of (conditionally) replicating adenoviruses. Despite several promising preclinical results, neither of these approaches has seen significant success when employed in clinical trials against GBM. Several reasons for this lack of effect have been suggested including the high immunogenicity of adenoviruses causing their rapid elimination, the severe attenuation of first generation oncolytic viruses and especially the limited distribution of Ads following injection into the brain or brain tumor. In addition, there is a strong need to image the effect and distribution of Ads following gene therapy or oncolytic therapy, as a real threat of significant toxicity exists caused by either the virus or the ensuing immune response when Ad distribution cannot be monitored or predicted. Therefore, to improve the effect of adenoviruses, whether used in the context of gene therapy or virotherapy, this thesis discusses some of these key factors: imaging the distribution and effect of adenoviruses in the brain, improving the potency of conditionally replicating adenoviruses and improving the distribution of adenoviruses in the brain.

To appreciate the ensuing chapters in this thesis, this introduction will highlight GBM and adenovirus pathology and biology as well as those common pitfalls encountered when using adenoviruses to treat GBM.

### **Glioblastoma Multiforme.**

In 1926 in their seminal work *'A Classification of the Tumors of the Glioma Group on a Histogenetic Basis with a Correlated Study of Prognosis'* Bailey and Cushing when speaking about Glioblastoma Multiforme realized that: *"It is not only the largest single group in the series ... but at the same time is one of the most malignant ... In the five unoperated cases, the average duration of life from the onset of symptoms was only three months, which speaks well on the whole for the average survival period of twelve months for those surgically treated."*<sup>1</sup>. Now, more than 80 years later, little has changed in the prognosis of patients suffering from this disease.

### **Epidemiology**

In the United States, 14.8/100,000 persons are diagnosed each year with a primary brain tumor of which 20.3% are GBM (incidence rate, IR, 3.05)<sup>2</sup>. There

is a male predisposition (IR 3.86 vs. 2.39) and increasing incidence with age resulting in a maximum incidence of 13.45 in the 75-84 year age group. Despite this increase with age, GBM occurs in all age groups including the very young and malignant brain tumors are among the major causes of death in children. The two and five year survival rate for GBM patients according to these statistics are 8.7% and 3.3%, but the most current report studying the combination of Temozolomide and radiotherapy reported a two year survival rate up to 26.5%<sup>3</sup>. In this study, the overall median survival had increased to 14.6 months vs. 12.1 months for radiotherapy alone, the latter appearing remarkably similar to the numbers reported almost a century ago by Bailey and Cushing.

### Biology

Gliomas arise from glial cells or, as more recently proposed, from neural stem cells (NSC) and are classified according to their WHO grade I to IV, in which GBM are grade IV gliomas. GBMs are composed of poorly differentiated, often pleomorphic astrocytic cells with nuclear atypia and frequent mitotic activity. The presence of necrosis is needed for the diagnosis and is generally accompanied by microvascular proliferation. The GBM cell of origin has lately been the object of intense debate. A brain tumor cell could theoretically arise from a dedifferentiated glial cell after a series of oncogenic mutations, or from a NSC or precursor cell<sup>4,5</sup>. The latter explanation may appear especially plausible as these cells are inherently more permissive for transformation and possess an active self-renewal capacity<sup>6</sup>. Many markers have been found that are suggested to identify the tumor stem cell. However, CD133 positivity for instance has also been shown to arise in cells previously negative for this archetypical stem cell marker<sup>7</sup>, perhaps indicating dedifferentiation and thus complicating the stem cell hypothesis, demonstrating that further research is required before any hypothesis can be unequivocally accepted.

GBMs are generally subdivided into primary and secondary tumors, a distinction first made by Scherer in 1940<sup>8</sup>. Primary GBM presents without any evidence of malignant development of a lower grade precursor tumor and is also known as *de novo* glioblastoma. Secondary GBM requires progression from a less malignant astrocytoma and only 5% of all GBM can be termed as being secondary<sup>9</sup>. Primary GBM shows a poor survival when compared to secondary GBM, which is mainly attributed to age as primary GBM occurs in older patients. The other major prognostic factor is the Karnovsky Performance Scale (KPS) at diagnosis. Genetically, both primary and secondary GBM often show loss of heterozygosity (LOH) at 10q but differ in other genetic markers. PTEN mutation and EGFR amplification are found in primary tumors, while secondary gliomas frequently display TP53 mutations. The p16<sup>INK4a</sup>/RB1 pathway controls cell cycle progression from G1 to S-phase and appears to be important in both tumor types. Defects in this pathway occur in as much as 70% of all GBM<sup>10</sup>.

Numerous other genetic alterations have been described in glioblastoma. In addition to EGFR, growth factors such as PDGF, IGF, bFGF, FGF-2 and TGF- $\alpha$  are overexpressed, either by gene amplification or at the transcriptional level. Progression from a lower grade glioma usually involves an angiogenic switch, which appears to be mediated by VEGF, possibly in response to hypoxia induced increased transcription of the VEGF gene by the hypoxia-inducible factor (HIF) family of transcription factors <sup>11</sup>.

Mutations of the isocitrate dehydrogenase 1 (IDH1) gene have been demonstrated in a subset of tumors, notably lower grade gliomas and secondary glioblastomas, and are strongly associated with increased survival, possibly by inhibiting wildtype IDH1 induced HIF-1  $\alpha$  expression <sup>12-14</sup>.

The previous molecular distinctions have been important in the diagnosis and prognosis of brain tumors but have never been used to guide treatment of these patients. It has been shown that O(6)-methylguanine-DNA methyltransferase (MGMT) promoter methylation is a predictor of the clinical efficacy of concomitant Temozolomide and radiotherapy, providing a first molecular target to direct specific, perhaps even stratified, GBM treatment <sup>15, 16</sup>.

### **Infiltration**

Infiltration and migration are hallmarks of most glial tumors and the main cause of their resistance to therapy, even in the absence of distant metastasis. At the time of diagnosis, GBM cells will have spread well beyond the identifiable margins of the solid tumor. In many cases, these infiltrating cells will have reached as far as the other hemisphere <sup>17</sup>, leaving even a total hemispherectomy ineffective as treatment <sup>18</sup>.

Gliomas disseminate using white matter tracts, cerebrospinal fluid pathways, the perivascular space, subependymal space and by meningeal spreading <sup>19</sup>. These preferential pathways suggest the existence of an opportunistic factor in the infiltrating behavior, as less resistance to fluid or cellular movement exists in these areas. In addition, migration occurs mainly in white matter and much less in the densely packed grey matter <sup>20</sup>. However, to accommodate migration of tumor cells, the extracellular matrix will have to be actively modified, and migration requires active intracellular changes <sup>21</sup>. Cell adhesion to extracellular matrix components, cell locomotion, and widening of the extracellular space (ECS) are key factors promoting migration. This process is characterized by the degradation and turnover of ECM components by production of specific proteases and inhibitors, as well as specific proteins to create a microenvironment beneficial to migration (reviewed in <sup>19</sup>). Even though migration in any given case of GBM will be extensive, most migrated tumor cells will be in the zone directly adjacent to the solid tumor <sup>22</sup>. Therefore, tumor recurrence after surgery can primarily be expected in this area <sup>23</sup>.

The GBM can be considered to be a 2 component disease. Actively infiltrating cells do not divide and dividing cells do not infiltrate, although individual cells can

switch between these two states<sup>21, 24, 25</sup>. As these components behave differently to the most common treatment strategies, they should be treated differently. The current standard treatment has been generally aimed at the dividing tumor cell.

### Treatment

The current standard treatment of GBM consists of surgery and combined radio/chemotherapy. More than 98% resection, as identified on postoperative MRI or by the surgeon, is associated with an improved survival when compared to partial resection or biopsy for both GBM and anaplastic astrocytoma<sup>16, 26-28</sup>. Neuronavigation and intraoperative MRI have increased the percentages of resected tumor without increasing neurological complications<sup>29</sup>. A truly “complete” resection however is unachievable due to infiltration of the normal brain and therefore surgery should be aimed at maximizing the resection percentage while sparing eloquent brain and functions. Surgery relieves symptoms from tumor burden, rapidly removes tumor edema and creates a time span for adjuvant therapies.

Large studies have shown that radiotherapy combined with Temozolomide is more effective than either treatment alone<sup>3, 15, 30</sup>. As mentioned before, patients with a methylation of the O-6-methylguanine-DNA methyltransferase (MGMT) promoter respond better to this concomitant treatment<sup>15, 31</sup>, but although MGMT status should be determined to be able to provide an individualized prognosis<sup>16</sup>, stratification by this marker is not yet common practice. Therefore the current adjuvant protocol for all patients consists of radiotherapy (60Gy, 2gy at 5 days per week for 6 weeks) combined with Temozolomide (75mg/m<sup>2</sup>/day during the course of radiotherapy) followed by a 4-week break, and then up to six cycles of adjuvant temozolomide by a 5-day schedule every 28 days (150 mg/m<sup>2</sup>/day for the first cycle increasing to 200 mg/m<sup>2</sup> beginning with the second cycle). At recurrence, surgery can again be considered for a selected group of patients. Factors to consider include the mass effect, age, KPS, the expected extent of resection and especially the availability of another salvage therapy<sup>32</sup>. In addition, surgery may be indicated for relief of edema or to obtain histology<sup>33</sup>. Repeated irradiation should only be applied in a selected group of patients, mostly with focal recurrences, as whole brain radiotherapy (WBRT) cannot be re-administered without the risk of significant side effects. Individualized chemotherapy at recurrence is an option, but there exists debate over which agent would be most suitable<sup>34</sup>. Obviously, any chemotherapeutic or other peripherally administered agent will have to negotiate the blood brain barrier to be effective. For all treatment options at recurrence, the lack of randomized clinical trials makes an evidence based choice an utopia and most of these options will be in an experimental trial setting.

## EXPERIMENTAL TREATMENT

### Gene therapy – Adenoviruses

The grim prognosis but semi-localized (meaning no metastasis) character of GBM has made this disease a target for many experimental therapies. This introduction will focus on the use of gene therapy and especially oncolytic virotherapy.

Gene therapy relies on the delivery of DNA or RNA into cells. In cancer gene therapy, the gene of interest should either cure or kill the infected or surrounding cells. During the last two decades it has been realized that killing of infected cells is one of the core businesses of many viruses, which resulted in the use of viruses that were still capable of intracellular replication and lysis of the infected cell. This use of viruses, termed virotherapy or oncolytic virotherapy will be discussed later.

Ideally, any gene delivery method should protect the genetic material against degradation, bring the material across the cell membrane into the target cell and have an acceptable safety profile. This can be accomplished using either viral or nonviral vectors. Nonviral methods of gene delivery can be subdivided in physical and chemical approaches. Physical approaches include needle injection, electroporation, gene gun, ultrasound and hydrodynamic delivery. Chemical approaches use synthetic or organic compounds to deliver DNA by active cellular uptake via endocytosis. These compounds include cationic lipids, often formulated as liposomes, cationic polymers such as poly-L-lysine or combinations of these <sup>35-37</sup>. Although gene delivery by nonviral methods has the advantage to control toxicity, the efficacy of gene transfer is generally inferior to viral methods <sup>38</sup>. The main subject of this thesis concerns adenoviral delivery methods, therefore nonviral gene delivery methods will not be further discussed.

Viral vectors are genetically modified viruses that are able to transfer genetic material to the infected cell. This is an inescapable consequence of the lifecycle and definition of any virus, which involves infection of the target cell, using the cell machinery to replicate the viral genome and produce new virions that are released into the environment. Integrating vectors include retroviruses (RV, such as lentiviruses) and parvoviruses (such as adeno-associated viruses (AAV)) that integrate their single stranded RNA (RV) or DNA (AAV) as double strand DNA into the host genome providing sustained and non-pathogenic (in the case of AAV) gene expression.

The most commonly used non-integrating vectors are Herpes simplex viruses (HSV) and adenoviruses (Ad). As a vector for brain diseases, the neurotropic HSV has gained considerable interest. It contains a 152 kb linear double stranded DNA genome and can accommodate 40-50kb of foreign DNA which is significantly more than all other vectors.

Adenoviruses are the main subject of this thesis and therefore a more detailed description of this virus will be presented in the next paragraph.



## Adenoviruses

The family of Adenoviridae consists of non-enveloped icosahedral viruses, containing a linear double stranded DNA molecule of 26-45kb (Ad5: 36kb) in size that replicate in the nucleus of infected cells. More than 50 serotypes have been identified collected in 6 groups (A-F). In humans, adenoviruses (Ads) cause acute respiratory infections, pharyngitis, conjunctivitis, gastroenteritis and pneumonia (in young children) but human Ads do generally not produce proliferative infections in animals. The adenovirus capsid has a diameter of 70-110nm and is composed of 240 hexon capsomeres forming the 20 triangular faces of the icosahedrons and 12 penton capsomeres and associated fibers located at the vertices. Fiber length is 9 to 77.5nm. The fiber knob region mediates binding to the primary receptor CAR (Coxsackie and adenovirus receptor). This receptor is, at least in vitro, critical for infection by type 5 adenoviruses<sup>39</sup>. Following this initial attachment, a penton based RGD motif binds to cell surface integrin molecules ( $\alpha_v\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ) and mediates virus uptake in the cell by endocytosis. Recently it has been shown that viral infection *in vivo*, at least in the liver, is hexon and not fiber mediated, which could explain the discrepancy between some in vitro and in vivo results<sup>40</sup>. Upon cellular uptake the viral capsid is dismantled and the protein coated viral genome is efficiently delivered to the nucleus by microtubular transport.

The role of adenovirus proteins and genes is to prevent cellular attempts to halt adenoviral replication by cell cycle arrest or premature apoptosis and to create a cellular environment permissive to replication ultimately inducing cell death at the end of the viral replication process. This is a carefully balanced and elegant process involving a limited number of highly conserved adenoviral genes.

The adenovirus genome contains five important early (E) coding regions (E1a, E1b, E2, E3, E4) transcribed from 5 promoters, which are mainly concerned with viral gene expression and replication. E1 proteins are expressed within 1-2 hours after infection and are critically needed for replication. Deletion of this part of the viral genome is utilized to create the most commonly used replication defective vectors for gene therapy. The E1A gene is important in the development of conditionally replicating viruses and encodes protein products generated by alternative splicing. E1a proteins are needed to promote the viral replication cycle by inducing a cellular environment beneficial to viral replication. E1a binds to Rb thereby releasing bound transcription factors such as E2F. These factors will induce either p53 dependent (or independent) cell death or cell cycle activation and viral replication. A mutation in this region preventing binding of E1a to Rb inhibits viral replication in Rb wt cells<sup>41, 42</sup>. Early region 1b (E1b) proteins, and in particular E1b55kD, bind to p53 thereby promoting its degradation and interfering with programmed cell death and cell cycle arrest. The precise role of E1b55kD remains unclear as the increased p53 expression in p53 wt cells infected with E1b55kD deleted viruses does not appear to increase the death rate of these

cells<sup>43</sup>. Mutations in this gene could prevent binding to and degradation of p53 and prevent cell cycle arrest, which would limit replication to cycling (tumor) cells. E2 genes are completely involved in virus DNA synthesis. The E3 region of the adenovirus has two important roles, preventing elimination by the host immune response and causing cell lysis. E314.7kD and E310.4/14.5kD prevent TNF alpha induced apoptosis while E3gp19kD encodes a transmembrane protein that protects infected cells against adenovirus-specific cytotoxic T-cell immune responses. E311.6kD, or the adenovirus death protein (ADP), is required for efficient cell lysis at late stages of infection<sup>44</sup>. Because the E3 region is not needed for viral replication this region is often utilized to insert transgenes but viruses deficient in E3 have been shown to be less cytotoxic to human cells both *in vitro* and *in vivo*<sup>45</sup>. E4 gene products have been reported to regulate numerous actions including viral DNA synthesis, inhibition of cellular protein synthesis and regulation of mRNA shuttling and cell death<sup>44</sup>.

Adenovirus late genes L1 to L5 are expressed late during viral replication and are mainly concerned with adenoviral assembly. The normal adenovirus life cycle ends with lysis of the infected cell, which generally does not occur before 48 hours following infection depending on cellular replication rate. This cellular lysis occurs by a mechanism that may mimic apoptosis but more often has different characteristics, such as those found with autophagy or necrosis<sup>46, 47</sup>.

### Adenoviral vectors

The deletion of both the E1 and E3 region from the adenoviral backbone generates up to 8kb of space for the insertion of transgenes in such a replication deficient vector. The choice of transgenes is virtually unlimited and as an example this introduction will briefly outline those transgenes that have been clinically used in glioma therapy.

**HSV-tk**, herpes simplex virus thymidine kinase, converts ganciclovir to the toxic metabolite that kills the infected cell. This form of therapy is termed Gene-Directed Enzyme-Prodrug Therapy (GDEPT). Cell killing occurs not only in infected cells but also surrounding cells by a bystander effect which is needed because generally adenovirus will not infect all cells<sup>48</sup>. This GDEPT strategy has been assessed in multiple phase I/II trials and is currently being assessed in a phase III trial<sup>49-51</sup>.

Expression of **p53** in infected cells should cause these cells to undergo apoptotic cell death. The use of Ad-p53 has not proceeded beyond a single phase I study in glioma patients<sup>52</sup>, but this vector is produced commercially in China and the USA and already a registered medicine for HNSCC in China<sup>53</sup>.

**Interferon beta** (IFN-beta) is a cytokine with anti-tumor activity and Ad.hIFN-beta, an adenoviral vector expressing human IFN-beta has recently been injected into the tumors of patients harboring a GBM resulting in increased levels of apoptosis in infected cells<sup>54</sup>.

## Oncolytic adenoviruses

When (parts of) the E1 genes (and other genes needed for replication) are left intact, adenoviruses will be capable of replication in infected cells and lysis of these cells, releasing their progeny in the cellular environment to infect new cells.

As mentioned before, deletion of the E1b55kD gene prevents binding of this gene product to p53. Thus, in wt p53 cells, p53 will be expressed and induce either cell cycle arrest or apoptosis, both detrimental to adenovirus replication. In p53 mutant tumor cells, p53 will be unable to cause a cell cycle arrest, and viral replication can occur, the basis of the oncolytic virus ONYX-015<sup>55</sup>. This beautiful concept has been seriously challenged in the recent past<sup>43, 56</sup>. Both p53 functions and dysfunctions, as well as the functions of E1b55kD, have proven to be more complex and redundant than the single interaction between the two. Nevertheless, ONYX-015 has been extensively tested in clinical trials<sup>57-64</sup>, including a phase I trial for glioma<sup>65</sup>. In this trial, no significant toxicity or definition of a maximum tolerated dose (MTD) was noted after injections into the cavity wall following resection of relapsed high grade gliomas at a dose up to 10<sup>10</sup> Plaque Forming Units (PFU).

Another approach is the deletion of part of the E1a gene. This will render the virus unable to induce cell cycle progression in Rb wt (normal) cells. Several variations of this mutation have been published<sup>41, 42</sup> and a variant of this virus, Ad-delta24-RGD<sup>66</sup>, is currently being assessed in phase I/II trials against tumors including GBM.

## INCREASING EFFICACY

### Targeting

There have been numerous attempts to increase the selectivity or potency of adenoviral vectors or oncolytic viruses. Adenoviruses can be made safer by modifying the infection characteristics, thus sparing normal cells, and by changing the genes that are expressed in infected cells in such a way that therapeutic transgene expression or replication is limited to target cells. These methods are termed transductional and transcriptional targeting respectively. Clinically assessed examples of these strategies have been outlined above, but preclinically many more adenovirus variants have been published. Strategies aiming at improving selective infection include fiber modifications that direct infection to more specific cancer cell receptors including integrins<sup>67-70</sup>, EGFR<sup>71-76</sup>, and fibroblast growth factor-2<sup>77, 78</sup>. Although all of these approaches appear feasible *in vitro*, the translation to the clinic, or even to *in vivo* studies is not always straightforward as it seems that other factors such as the extracellular matrix and other receptors are important mediators of adenoviral infection, especially *in vivo*<sup>40, 79</sup>, complicating the intuitive approach of retargeting. This observation is corroborated by the lack of published clinical trials employing retargeted adenoviruses.

## Armed therapeutic viruses

Transcriptional targeting can be achieved by delivering a gene under the control of a suitable tissue or tumor specific promoter (TSP). Promoters of interest include surviving<sup>80</sup>, COX-2<sup>81, 82</sup>, GFAP<sup>83</sup>, midkine<sup>82</sup>, E2F<sup>84, 85</sup>, tTERT<sup>86-88</sup> and for prostate cancer, PSA<sup>89</sup>. All of these promoters are either active in cancer or specific tissue. Therapeutic transgenes such as p53 and HSV-TK can be put under control of the TSP, but also the adenoviral genes needed for viral replication (such as E1a, E1b), which yields TSP controlled conditionally replicating adenoviruses<sup>90-92</sup>.

## Combination therapy

Following the somewhat disappointing results of the first trials using adenoviral vectors or oncolytic viruses to treat human cancers, efforts were made to increase the activity of the first generation vectors, either by creating new vectors as described previously, or by combining existing adenoviral variants with more conventional therapies. One extra stimulus to pursue this strategy was the positive result observed when ONYX-015 was combined with cisplatin and 5-fluorouracil in patients with recurrent head and neck cancer, showing impressive tumor reductions<sup>58</sup>. In addition, combining gene therapy or oncolytic therapy with conventional treatments is rational because clinical trials using adenoviruses are generally performed in an adjuvant setting following, or combined with, these conventional treatments.

Replication deficient adenoviral vectors have been combined with other treatments for several reasons but most often because the transgene of interest was known to interact with the specific conventional therapy. The most prominent example is the combination of adenoviral expression of p53 and radiotherapy or chemotherapy<sup>93-98</sup>. Other transgenes have been assessed as well including pro apoptotic BAX and HSV-TK<sup>99-102</sup>.

Replicating adenoviruses have been a frequent subject of combination therapy<sup>103-113</sup>. Both conditionally replicating viruses as well as chemo- or radiotherapy are most effective against actively dividing cells. The mechanism behind the observed interaction has remained for the better part elusive despite many hypotheses that could at least be partially explanatory. *In vitro*, expression of the adenoviral E1a gene induces s-phase in quiescent cells, possibly sensitizing these cells to radio- or chemotherapy<sup>114-116</sup>. Otherwise, these conventional treatments may increase adenoviral replication or facilitate infection<sup>104, 117, 118</sup>. *In vivo*, other factors could participate. One of these factors is the immune suppression offered by radiotherapy and especially chemotherapy, decreasing the rate at which viruses are eliminated by the immune response following delivery<sup>119-121</sup>.

## Delivery

No matter how effective an adenoviral vector or oncolytic virus may be, either as single treatment or in combination with other therapies, it will cure no patient if it cannot reach the tumor. Delivery of adenoviruses to the brain and brain tumors

has been one of the most significant and insurmountable obstacles preventing the successful application of adenoviruses against this disease. Although this part of the introduction specifically refers to adenoviruses, the obstacle of efficient delivery is common for all gene therapeutic vectors or oncolytic viruses.

Prior to delivery it should be determined exactly where the virus needs to be delivered. Treatment can be directed to specific cells, specific locations in the brain or specific structures such as the white matter. When treating brain tumors, the target could be the (residual) enhancing tumor mass or migrating cells in the infiltrated periphery of the tumor. These different objectives ask for tailored delivery strategies.

### Methods of delivery

The brain can be accessed in three ways; through the skull, through the arterial blood supply or through the epidural or subdural space, intrathecally. Intrathecal delivery of therapeutics is relatively non-invasive but does not result in deep penetration of the agent to the brain parenchyma<sup>122, 123</sup>, and delivery of adenovirus by this route results in a predominant infection of the arachnoid and pia mater accompanied by an immune response-mediated toxicity<sup>124, 125</sup>. Although this route could hypothetically be utilized in the treatment of leptomeningeal metastasis or meningiomas, adenoviral toxicity seems a limiting factor for intrathecal delivery.

Intravascular delivery of adenoviruses offers the promise of highly selective tumor vascular bed targeting but is confronted by the challenge of passing the blood brain barrier (BBB), even when the BBB is disrupted in contrast enhancing tumors. Without manipulation of the BBB adenovirus is generally unable to reach the brain parenchyma<sup>126, 127</sup>. Following osmotic disruption with mannitol or disruption by bradykinin, both tumor and brain parenchyma can be targeted to varying extent<sup>126-128</sup>, but at the cost of significant toxicity and the risk of viral infection disseminating to other organs<sup>126</sup>. In addition, intravascular delivery will render the virus directly susceptible to the potent antiviral effect of the immune system.

With both previous methods not ideal for brain tumor treatment, direct interstitial delivery has been the most widely used route of administration. Most commonly used techniques are the injection of adenovirus into the tumor, or into the wound bed following resection of the tumor. Although invasive and with the risk of injection related complications, this method of delivery bypasses the BBB while this barrier itself limits peripheral toxicity of the treatment. Interstitial or intratumoral injections have been used in all clinical trials for GBM using adenoviruses up to date<sup>129</sup>. This method however did not result in widespread transgene activity in the tumor. When assessed, transgene expression could only be detected in a small area surrounding the needle or catheter tip<sup>52, 130</sup>. It is likely that freehand injections result in extensive backflow into the resection cavity that might significantly limit the viral dose available at the site of injection.

To be effective, viruses need to infect a considerable part of the tumor or be distributed widely in the parenchyma to infect migrated tumor cells. One method that could be used to achieve this goal could be convection-enhanced delivery (CED).

### Convection-enhanced delivery

Contrary to diffusion where particle movement and distribution are the results of random motion, advection consists of mass movement of particles present in a fluid. Convection is the sum of diffusion and advection and thus also implies mass movement, or bulk flow. Convection-enhanced delivery, CED, constitutes just that, delivery enhanced by convection. On first sight, CED is therefore a seemingly uncomplicated delivery method consisting of infusing fluids in the brain providing the possibility of eliminating simple diffusion and replacing it by convection. Several complicating factors should however be considered.

The interstitial space in the brain takes up approximately 20% of the total brain volume<sup>131</sup>. Any extracellular particle delivered by CED will initially distribute over this volume and therefore the total distribution volume could theoretically be 5 times larger than the volume infused, yielding a ratio of volume of distribution (Vd) to volume of infusion (Vi), Vd/Vi, of 5. The actually achieved Vd/Vi ratio is however dependent on many other factors, and not every agent is suitable for CED when the objective is to target an as large volume as possible. The Vd can be elegantly predicted by mathematical modeling of the infusion process<sup>132-138</sup>. Such simulations are important as they allow us to understand the observed effects of CED. A complete mathematical analysis would however be too complicated to include here and therefore some important aspects will be highlighted, some of which especially concern adenovirus delivery.

Convection needs to be accommodated in a medium, which in the brain is the extracellular space (ECS). The brain is considered to be a poroelastic medium and the normal movement of materials in the interstitial space of the gray matter is consistent with diffusion through a porous medium<sup>139</sup>, while in white matter evidence for a significant natural bulk flow of interstitial fluid exists<sup>140</sup>. In addition to this purely interstitial fluid movement, a paravascular fluid flow exists both in white and gray matter perivascular spaces<sup>141-143</sup>.

CED is dependent on the characteristics of the ECS. Important aspects for CED are the constituents and, especially for large particles, the width of the ECS. The width of the interstitial space, or pore size, in gray matter has been estimated to be 38-64 nm<sup>144</sup>, while the width of the perivascular spaces may exceed 500nm. With interstitial infusion, the interstitial space may be dilated which is especially apparent in white matter<sup>145-147</sup>.

In the ECS, the tissue binding and degradation rate of any agent significantly affects its distribution, with high rates decreasing the possible Vd. Because convection is determined not only by advection but also by diffusion, a low diffusion coefficient would similarly affect the Vd. In addition, as mass particle movement

and diffusion could both be influenced by tissue and particle characteristics, advection itself might well be correlated to the diffusion coefficient. Therefore the diffusion coefficient is an important parameter in CED.

Efflux of infusate from the targeted region is dependent on the interstitial pressure, infusion pressure, intravascular pressure and tissue mechanics. Interstitial pressure varies between tumor, necrotic areas and brain parenchyma, with high intratumoral pressures generally leading to rapid efflux out of the tumor into the surrounding brain parenchyma or necrotic areas <sup>148, 149</sup>.

Infusate backflow along the infusion catheter is an important factor and is dependent on catheter diameter, flow rate and the mechanical properties of the infused tissue <sup>133, 150</sup>. Backflow could result in severe toxicity when a toxic compound reaches the brain surface or ventricles <sup>151</sup>. In clinical trials, strict guidelines that describe the distance the catheter tip needs to be away from ependymal, brain or cavity surfaces, have been devised to prevent backflow <sup>149</sup>, but aside from the backflow occurring as a result of direct tissue disruption, it can never be completely prevented as it is inherent to the stresses and strains resulting from intraparenchymal infusions <sup>133</sup>.

### Imaging Adenoviruses

Imaging the distribution or activity of adenoviruses following gene therapy or virotherapy is technically difficult but the relevance is undeniable. In addition, imaging CED is of great importance as it should always be clear if the delivery method has been accurate to be able to comment on the specific activity or toxicity of any anti-cancer agent.

Imaging the distribution of Ads can be performed by imaging the particle itself, by imaging a surrogate marker showing a similar distribution to Ads or by imaging the effect of the Ad as a secondary marker of distribution.

Imaging the particle itself is complicated as the viral particle needs to be labeled with either a PET tracer or MRI contrast agent when applied in human studies while in animal studies bioluminescence or fluorescence reporters can be used. Of these two options, only the fluorescence reporters have been successfully used to image the viral particle. Examples include single labeling using Cy-3 and eGFP constitutively expressed in the viral capsid, which allows for repetitive imaging following viral replication <sup>152-156</sup>.

Using a surrogate marker has the advantage that the adenovirus does not have to be genetically or chemically modified. Using such surrogate tracers, both Adenovirus and Adeno-associated virus distribution has been imaged by MRI or iron staining using super paramagnetic iron oxide nanoparticles (SPIO) that have a diameter similar to the viral particles <sup>127, 157, 158</sup>. A disadvantage is that surrogate markers have to be chosen of which the distribution closely mimics that of the virus, preferably using the same cellular receptors.

Transgene expression can be used to determine which cells were infected following delivery of the virus. Examples include expression of PET markers such

as HSV-TK that can be imaged using PET tracers such as [18F]FHBG, [(3)H]FEAU or [14C]FIAU<sup>159-161</sup>, and the somatostatin receptor subtype 2 (SSTR) that can be visualized using the somatostatin analogues (99m)Tc-P2045 or 111In-DOTA-Tyr3-octreotate<sup>162, 163</sup>. Transgene expression is probably the most practical way of visualizing the distribution but it should be remembered that transgene expression does not primarily represent viral distribution, but rather the consequences and efficacy of infection.

Adenoviruses or oncolytic viruses lacking a transgene with imaging capabilities can be tracked only using indirect methods such as contrast enhanced MR imaging or Positron emission tomography of the effect of the virus on for instance a tumor. These methods are obviously rather crude and do not provide information about the actual distribution of the virus, but may be useful for therapy follow-up purposes.

## AIM AND OUTLINE OF THE THESIS

Oncolytic virotherapy and gene therapy provide an uncommon therapeutic option in the treatment of high grade gliomas with many pitfalls needing to be conquered. This thesis hopes to offer some improvements in oncolytic viral therapy for gliomas as discussed at the beginning of this introduction and repeated here:

- » Imaging the distribution and effect of adenoviruses in the brain
- » Improving the oncolytic potency of conditionally replicating adenoviruses
- » Improving the distribution of adenoviruses in the brain.

Chapter 2 investigates whether conventional <sup>18</sup>F-deoxyglucose positron emission tomography could be used to monitor the effect of oncolytic viruses *in vitro* using multicellular spheroids. This preliminary investigation could be important as oncolytic viruses could increase the metabolic activity of infected cells but subsequent lysis of tumor could equally decrease the overall metabolic activity. In addition, PET might provide an assessment of adenoviral activity in the postoperative period when MRI is not useful due to non-specific changes in contrast enhancement.

In chapters 3 and 4 we attempt to improve the activity of 2 oncolytic adenoviruses, one directed to integrins and one expressing p53, by combining these with radiotherapy in both a subcutaneous glioma model and an orthotopic model. In these chapters we discuss the improved efficacy of this combined treatment and the differential effects in subcutaneous and intracranial models.

Chapter 5 is devoted to the intracranial delivery of adenoviruses by CED, research that has been performed in the preparation of a clinical trial using CED and the oncolytic adenovirus Ad-delta24-RGD in glioma patients. This chapter tries to answer the important question if the size and characteristics of the adenoviral particle make it a feasible CED agent.



Chapter 6 contains a slight detour from the translational research covering the rest of the thesis and has been conceived from the problem that exists when trying to objectively compare several oncolytic viruses. In this chapter we attempt to find an objective assay for this comparison using mathematical modeling.

Chapter 7 contains a venture into the future of gene therapy where adenovirus is delivered using tumor targeting adipose tissue derived stem cells. Although clearly not clinically applicable anytime soon, studies like these further underscore the importance of adenoviral delivery and the methods that can be used to do so, now or in the future.

The most important findings in this thesis as well as an outlook into the future of oncolytic virotherapy and gene therapy are discussed in chapter 8 while chapter 9 contains a dutch summary.

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