Studies on viral gene transfer and angiogenesis inhibition in experimental glioma models

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1. LIST OF ARTICLES

I.

Read TA, Farhadi M, Bjerkvig R, Olsen BR, Rokstad AM, Huszthy PC, Vajkoczy P. Intravital microscopy reveals novel antivascular and antitumor effects of endostatin delivered locally by alginate-encapsulated cells. **Cancer Research.** 2001 Sep 15; 61(18):6830-7.

II.

Huszthy PC, Brekken C, Pedersen TB, Thorsen F, Sakariassen PØ, Skaftnesmo KO, Haraldseth O, Lønning PE, Bjerkvig R, Enger PØ. Antitumor efficacy improved by local delivery of species-specific endostatin. **Journal of Neurosurgery.** 2006 Jan; 104(1):118-28.

III.

Huszthy PC, Svendsen A, Wilson JM, Kotin RM, Lønning PE, Bjerkvig R, Hoover F. Widespread dispersion of adeno-associated virus serotype 1 and adeno-associated virus serotype 6 vectors in the rat central nervous system and in human glioblastoma multiforme xenografts. **Human Gene Therapy.** 2005 Mar; 16(3):381-92.

IV.

Thorsen F, Afione S, Huszthy PC, Tysnes BB, Svendsen A, Bjerkvig R, Kotin RM, Lønning PE, Hoover F. Adeno-associated virus (AAV) serotypes 2, 4 and 5 display similar transduction profiles and penetrate solid tumor tissue in models of human glioma. **Journal of Gene Medicine.** 2006 Sep; 8 (9): 1131-1140.

2. ABBREVIATIONS

AAV-Adeno-associated virus
Ad-Adenovirus
BCNU-1,3-bis(2-chloroethyl)-1-nitrosourea
CCNU- 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea
CDK-Cyclin-dependent kinase
CNS-Central nervous system
CSF-Cerebrospinal fluid
ECM-Extracellular matrix
EGF-Epidermal growth factor
EGFR- Epidermal growth factor receptor
E2F- E2 promoter binding factor
GBM-Glioblastoma multiforme
GFAP-Glial fibrillary acidic protein
HSV-Herpes simplex virus
IL-Interleukin
INK4a-Inhibitor of cyclin-dependent kinase 4 gene
LOH-Loss of heterozygosity
MMLV-Moloney murine leukaemia virus
PDGF- Platelet-derived growth factor
pRB-Retinoblastoma protein
***PTEN*-** Phosphatase and tensin homology gene
PTEN-Phosphatase and tensin homology protein
RV-Retrovirus
TGF-Transforming growth factor
***tk*-**Thymidine kinase gene
VEGF-Vascular endothelial growth factor
VEGFR- Vascular endothelial growth factor receptor
WHO-World Health Organisation

3. INTRODUCTION

3.1. Cancer, general considerations

A tumour may be defined as an abnormally growing mass of cells resulting from uncontrolled division, which serves no physiological function [1]. Benign tumours grow by local expansion and are confined to their tissue of origin. Such tumours are usually not life threatening and their surgical removal is in general curative. On the other hand, malignant tumour cells are characterised by their capacity to invade the extracellular matrix and to destroy adjacent tissues [2]. Eventually, cancer cells may enter the circulation or the lymphatic system and colonise other organs (metastasis). Such advanced cancer is a fatal disease, as it is not cured by surgical removal of the primary lesion. Accordingly, metastatic dissemination and the subsequent infiltration and destruction of vital secondary organs is the major cause of death in most cancer forms [3]. Malignant lesions found at the site of their origin are referred to as primary tumours, whereas those found in an organ distinct from where they originated are referred to as secondary tumours (Figure 1).

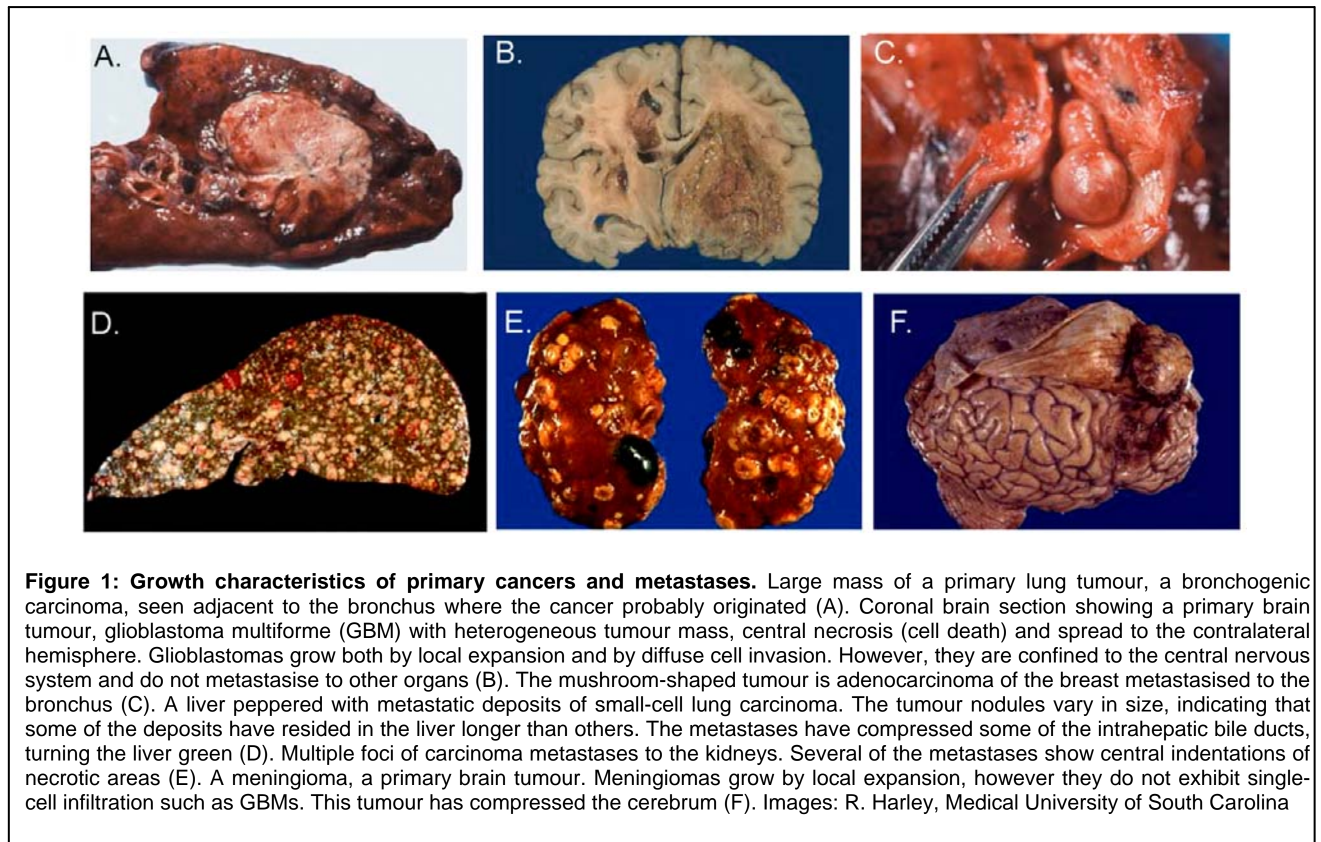


Figure 1: Growth characteristics of primary cancers and metastases. Large mass of a primary lung tumour, a bronchogenic carcinoma, seen adjacent to the bronchus where the cancer probably originated (A). Coronal brain section showing a primary brain tumour, glioblastoma multiforme (GBM) with heterogeneous tumour mass, central necrosis (cell death) and spread to the contralateral hemisphere. Glioblastomas grow both by local expansion and by diffuse cell invasion. However, they are confined to the central nervous system and do not metastasise to other organs (B). The mushroom-shaped tumour is adenocarcinoma of the breast metastasised to the bronchus (C). A liver peppered with metastatic deposits of small-cell lung carcinoma. The tumour nodules vary in size, indicating that some of the deposits have resided in the liver longer than others. The metastases have compressed some of the intrahepatic bile ducts, turning the liver green (D). Multiple foci of carcinoma metastases to the kidneys. Several of the metastases show central indentations of necrotic areas (E). A meningioma, a primary brain tumour. Meningiomas grow by local expansion, however they do not exhibit single-cell infiltration such as GBMs. This tumour has compressed the cerebrum (F). Images: R. Harley, Medical University of South Carolina

Today, more than 100 different cancer types have been acknowledged, with distinct phenotypic traits and growth characteristics. However, common to the diverse cancer types is their genesis. Early pre-malignant lesions arise after having acquired critical alterations within the genome of a cancer initiating progenitor cell, by a process known as malignant transformation [2]. It is assumed that a progenitor cell may either be a fully differentiated cell or an undifferentiated stem cell that has the potential to evolve into different tissue-specific cell types [4]. After transformation, newly acquired genetic traits of the cancer progenitor cell may enable it to proliferate in an autonomous fashion, no longer restrained by the physiological context of the tissue microenvironment.

Genes that contribute to the development of cancer are referred to as cancer predisposition genes. Based on their functions, such genes may be divided into three groups: tumour suppressor genes, stability genes and oncogenes [5]. Tumour suppressor genes encode key regulators of cell-cycle progression and survival. Their loss leads to the shortfall of normal mitogenic control or to the disruption of apoptotic pathways, that normally would arrest or eliminate such damaged cells [5]. Oncogenes are erroneously activated genes that encode proteins with essential roles in mitogenic signal transduction pathways, such as growth factors, cell surface receptors, intracellular messengers and transcription factors [6]. The activation of these pathways provides the "drive" for malignant proliferation. Stability genes encode proteins that maintain genomic integrity. They may be involved in nucleotide repair pathways or in more profound mechanisms such as those responsible for correct mitotic recombination or chromosomal segregation [5]. Defects in these genes contribute to genomic instability, allowing for further accumulation of mutations. The number of genetic alterations which have been found in cancer cells is immense [5,7]. Still, the vast array of cancer cell genotypes are manifested in a few essential changes in cellular physiology, which are collectively responsible for malignant growth [8]. They are: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angogenesis, and tissue invasion and metastasis (Figure 2). Each of these acquired capabilities represents a successful conquering by the early progenitor cell of an anti-cancer defence mechanism essential for normal tissue homeostasis.

It is becoming clear that cancer progression is not sustained by the tumour cells alone, but is also assisted by the host microenvironment [9]. Host fibroblasts are associated with tumour cells at all stages of cancer progression, and their structural and functional contributions to this process are beginning to emerge.

Growth factors, chemokines and extracellular matrix produced by fibroblasts facilitates the recruitment of endothelial cells and pericytes [10]. As cancer progresses, tumour-associated host cells may obtain phenotypes that are different from those normally seen in the tissue. For example, the molecular signature of tumour endothelial cells is distinct from that found in endothelial cells in normal tissue [11], providing evidence for a cross talk between the tumour and the host orchestrated by biological factors in the tumour-host microenvironment.

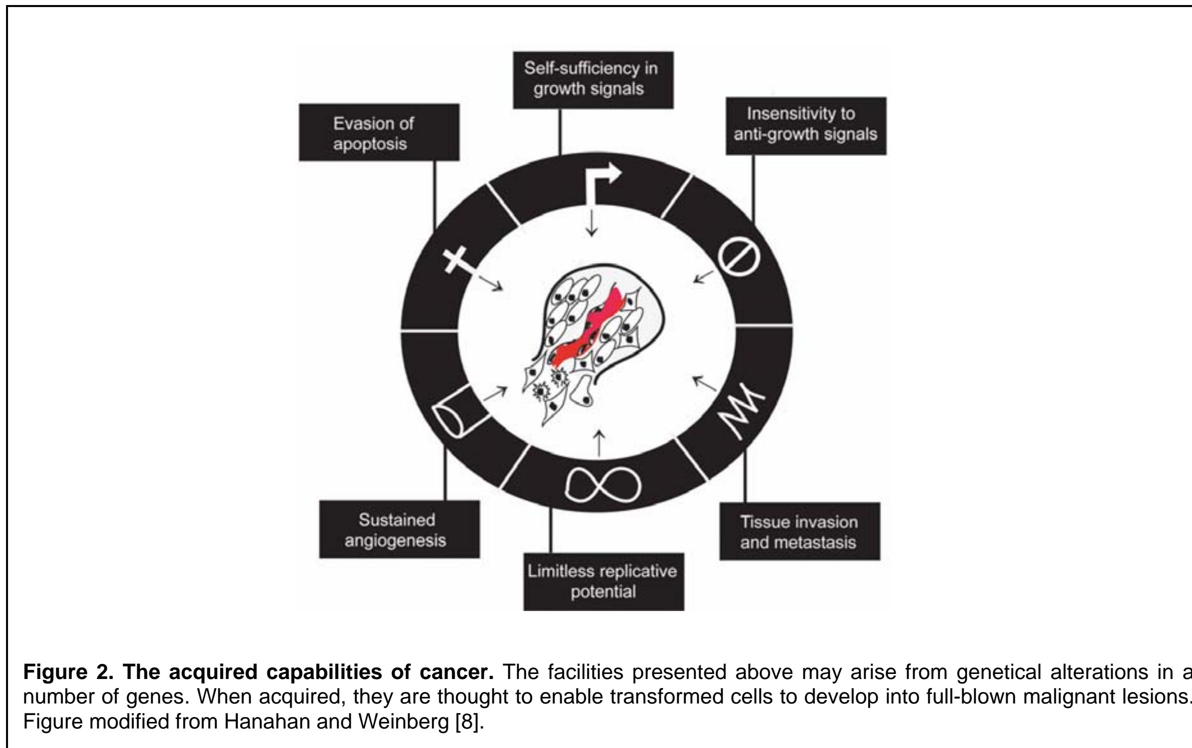


Figure 2. The acquired capabilities of cancer. The facilities presented above may arise from genetical alterations in a number of genes. When acquired, they are thought to enable transformed cells to develop into full-blown malignant lesions. Figure modified from Hanahan and Weinberg [8].

3.2. Astrocytomas, classification and malignant progression

Astrocytes, the most numerous glial cells in the brain, provide physical support to the neuronal network and have multiple functions in maintaining tissue homeostasis as well as in tissue repair [12]. Tumours that arise after malignant transformation and proliferation of astrocytes are termed astrocytomas. The World Health Organisation's classification of central nervous system tumours [13] categorises astrocytomas based on their grade of malignancy, determined by the presence of key histological features (table I). As a group, astrocytomas include tumours with a wide range of histological forms from benign to highly malignant. In the classification scheme, they are divided into grade I (juvenile pilocytic astrocytoma), grade

II (low-grade astrocytoma), grade III (anaplastic astrocytoma) and grade IV (glioblastoma multiforme; GBM) tumours. Grade II-IV tumours diffusely infiltrate into the surrounding brain tissue, hence they are also referred to as diffuse astrocytomas. In contrast, grade I astrocytomas display more circumscribed growth and have a different age distribution, intracranial location and biology; thus they comprise a group of tumours distinct from diffuse astrocytomas. Grade I astrocytomas retain their well-differentiated histological features as opposed to diffuse astrocytomas, which inevitably de-differentiate and progress to more malignant tumour phenotypes [13]. Diffusely infiltrating astrocytomas account for over 60 % of all primary brain tumours, with a world-wide incidence of about 5-7 new cases per 100.000 population per year [13].

The grade IV astrocytoma, glioblastoma multiforme, is the most de-differentiated primary tumour of the central nervous system. Two distinct types of GBMs are recognised, based on their clinical history. Secondary glioblastomas evolve from grade II and grade III astrocytomas in the course of several months or years (mean, 4-5 years). This glioblastoma form typically affects young adults (35-40 years) [14]. Primary glioblastomas; on the other hand, usually present with a clinical history of less than 6 months and they are more frequent in elderly people (mean, 55 years)[14]. Primary GBMs constitute around 95 % of all glioblastoma cases [15]. Although these glioblastoma forms are associated with different clinical presentations and genetic alterations, they can not be distinguished by routine histological evaluation [13]. The following sections will concentrate on diffuse astrocytomas, with special emphasis on glioblastoma multiforme.

WHO grade	WHO designation	Histological criteria
I.	Pilocytic astrocytoma	None of the below criteria
II.	Astrocytoma	Nuclear atypia
III.	Anaplastic astrocytoma	Nuclear atypia and mitotic activity
IV.	Glioblastoma multiforme	Nuclear atypia, mitoses, endothelial proliferation and/or necrosis

Table 1. The World Health Organisation's classification and grading system of astrocytomas.

3.3. Diffuse astrocytomas, aetiology and genetic susceptibility

Of the various environmental risk factors known to cause cancers, radiotherapy exposure has been clearly associated with the formation of diffuse astrocytomas [13]. Children that have

been treated by prophylactic cranial irradiation for acute lymphocytic leukaemia (ALL) have an increased risk of developing diffuse astrocytomas of all malignancy grades [16-19]. In a retrospective cohort of 9720 children receiving therapeutic irradiation for ALL, a 22-fold excess risk of subsequent tumour development in the CNS was documented, and the estimated cumulative proportion of affected children was 2.5%[20]. The gliomas appeared 7 to 9 years after radiation treatment [18]. Similarly, a 16-fold excess risk for the emergence of gliomas has been observed after irradiation of pituitary adenomas [21]. Neuroepithelial tumours have been observed to occur after cranial irradiation for several other tumour types as well [22-24].

Of the known hereditary defects known to be associated with oncogenesis, *TP53* germline mutations (Li-Fraumeni syndrome)[25], the Brain tumour polyposis syndrome (Turcot syndrome)[26] and *NF1* mutations (neurofibromatosis type I syndrome) [27] have all been implicated in the development of diffuse astrocytomas.

3.4. Diffuse astrocytomas, oncogenesis

The classical view presented in section 3.2., considers mature glia to be the founder cells of glioblastomas. However, the pathways of glioma oncogenesis have been extensively debated. It has been argued that neural stem cells (NSCs; self-renewing cells capable of differentiating into both neurons and glia) or glial progenitors (self-renewing precursors capable of differentiating into astrocytes or oligodendrocytes) may well be the targets of malignant transformation [28,29]. The notion that such primitive cells may give rise to gliomas offers some advantages compared to the classical hypothesis. First, it may be argued that the concept of de-differentiation of mature glia is a theoretical model that has not been adequately proven in experimental systems. Furthermore, this model does not explain the origin of mixed gliomas that contain both oligodendroglial- and astroglial components [29]. It is likely that NSCs and glial progenitors are more prone to proliferate than mature cells since they are already at a de-differentiated state. Therefore, these cell types would transform and develop more readily to multicellular lesions given a few key mutational events. Support for this thought has been gained from transgenic animal models, where the activation of two independent signalling pathways was sufficient to transform neural stem cells, but not mature astrocytes to give rise to glioblastoma-like lesions in mice [30].

In addition to diffuse infiltration, grade IV tumours are also associated with accelerated tumour cell proliferation and rapid local expansion. This is facilitated by the recruitment of

tumour blood vessels (angiogenesis), which supplies the rapidly dividing neoplastic cells with oxygen and nutrients. Since the brain is confined to the rigid case of the skull, the expansive growth of a neoplasm quickly meets physical barriers, resulting in elevated intracranial pressure and, in some cases, the compression of vital brain structures. In addition, the expansion of a tumour within- or adjacent to the cerebral ventricles may block the clearance of cerebrospinal fluid.

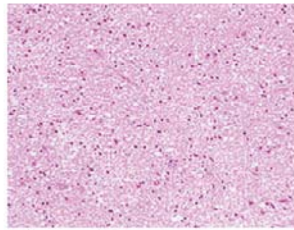
3.5. Diffuse astrocytomas, genetic alterations

As mentioned above, primary and secondary GBMs present with distinct clinical profiles. It has become clear that they are also associated with mutations affecting different genes [13]. Figure 3 reviews the most frequent aberrations that are associated with the two pathways of glioblastoma development. These genetic alterations will be briefly discussed below.

3.5.1. *TP53*

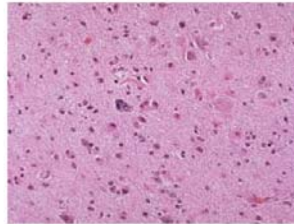
The *TP53* gene, found on chromosome 17p, encodes p53, a 53-kDa transcription factor.
p53 has a principal role in the cell in preventing the accumulation of mutations through the regulation of critical checkpoints in response to distinct exogenous stresses [31,32]. p53 is rapidly degraded in normal cells, however, it is stabilised in response to DNA damage, hypoxia, growth factors and activated oncogenes [31]. Important downstream effects of p53-mediated transcription include growth arrest, induction of apoptosis, senescence, differentiation and anti-angiogenesis [31,33]. The central hydrophobic core of the p53 molecule, encoded by exons 5 to 8, contains four highly conserved regions that are responsible for proper three-dimensional folding and DNA binding [34]. Inactivating mutations are most often found at 7 hotspots within these exons, which lead to loss-of function of the affected protein [35]. According to the International Agency for Research on Cancer database (IARC, http://www-p53.iarc.fr/Statistics.html), the distribution of somatic *TP53* mutations are found to vary between 5% and 45 % in human cancers, with around 27 % prevalence in various brain tumours. The introduction of wild-type *TP53* into deficent glioblastoma cell lines leads to growth arrest and morphological changes [36,37], suggesting that the loss of p53 is functionally involved in GBM oncogenesis.

Allelic losses of chromosome 17p and *TP53* mutations are present with similar frequency in low-grade astrocytomas, anaplastic astrocytomas and in glioblastomas [15,38]; thus *TP53* mutations are considered to be early events in astrocytoma progression.



Normal brain

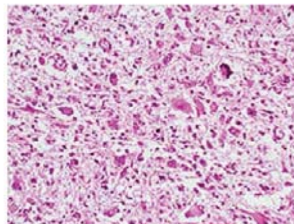
Astrocytes or precursor cells



WHO grade II

Low-grade astrocytoma

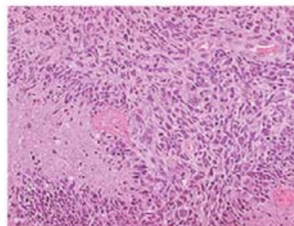
TP53 mutation (59%)



WHO grade III

Anaplastic astrocytoma

TP53 mutation (53%)



WHO grade IV

Secondary glioblastoma

LOH 10q (63 %)

EGFR amplification (8%)

$p16^{INK4a}$ deletion (19%)

TP53 mutation (65%)*

PTEN mutation (4%)

38 cases (5%)

Mean age: 45 years

Male/female: 0.65

Primary glioblastoma

LOH 10q (70%)

EGFR amplification (36%)*

$p16^{INK4a}$ deletion (31%)

TP53 mutation (28%)

PTEN mutation (25%)*

677 cases (95%)

Mean age: 62 years

Male/female: 1.33

Figure 3. Two pathways to glioblastoma development with associated genetic mutations. The numbers in parentheses indicate the percentage of mutations found in each tumour group taken from a recent population-based study [15]. Asterisks indicate the mutations that differ significantly in frequency between primary and secondary glioblastomas.

Since the transition from low-grade astrocytomas to anaplastic astrocytomas and to secondary GBM occurs in the course of several years, p53-related genomic instability is probably not the direct promoter of malignant progression. However, p53-deficiency may play a permissive role, by conferring selective growth advantage to *TP53*-mutated tumour cells. Supporting this notion, targeted germ-line inactivation of both *TP53* alleles did not give rise to gliomas in mice, even though all cells of the CNS proved to be p53-deficient [39]. Interestingly however, the majority of these animals spontaneously developed other cancer types, such as malignant lymphomas and sarcomas. Still, high-grade gliomas do develop when *TP53* knockouts are supplied with additional mutations in the tumour suppressor gene *NF1* (neurofibromatosis type I) [40].

3.5.2. *RB, CDK4, CDK6, INK4a*

The transition from low-grade astrocytoma to anaplastic astrocytoma has been associated with allelic losses on chromosome 9p and 13q and, less frequently, by 12q amplification. These chromosomal changes are mutually exclusive events [41,42], and it is now clear that they are all associated with the RB pathway.

The 107 kDa retinoblastoma protein (pRB) is a major regulator of cell-cycle progression [43]. About one-third of the high-grade astrocytomas have alterations at the *RB* locus [44], which maps to chromosome 13q14. Under normal, non-permissive conditions, the pRB protein is in a hypophosphorylated state bound to transcription factor E2F. This prevents E2F-mediated activation of several genes that are important for mitosis, consequently inhibiting cell cycle progression beyond the G1/S restriction point. Phosphorylation of pRB by cyclin-dependent kinases CDK4 and CDK6, in conjunction with cyclin D, and later by CDK2 releases E2F and leads to the transactivation of E2F-responsive genes, which take the cell into the S-phase [45]. Uncontrolled activity of the RB pathway may result from mutational inactivation of *RB*, amplification or over-expression of the cyclin-dependent kinases, or mutational defects or down-regulation of the cyclin-dependent kinase inhibitors.

p16 and p15, encoded by the genes *INK4a* and *INK4b*, respectively, are inhibitors of the cyclin-dependent kinases CDK4 and CDK6 [46]. Loss of chromosome 9q, leading to p16 inactivation occurs in about 50 % of high grade-astrocytomas [47] and is found in about two-third of the analysed glioma cell lines [48].

All these genetic events serve to activate the RB pathway, taking the cell beyond the G1/S restriction point. Histologically, this molecular transition is seen as a dramatic increase of mitotic figures, marking the progression from grade II astrocytoma to grade III anaplastic astrocytoma.

3.5.3. *PTEN*

While loss of heterozygosity on the long arm of chromosome 10 affects the majority of anaplastic astrocytomas and almost all glioblastomas [49-52], this abnormality is absent in low-grade diffuse astrocytomas [49] [51]. At least three tumour suppressor genes, shown to be dysfunctional or deleted in high-grade astrocytomas, are located here.

PTEN (phosphatase and tensin homology gene) maps to the 10q23 region. It has been found to be mutated in a wide range of sporadic advanced cancer types, such as melanomas and carcinomas of the prostate, breast, kidney and lung [53-55], as well as in haematological malignancies [56]. *PTEN* mutations have been found in 23-44% of the primary GBMs analysed in different series [15,57-59], however they are rare in secondary GBMs and are absent in astrocytomas [15,60]. The loss of PTEN function in tumour cells and in *PTEN*-deficient mouse cells leads to increased cellular levels of phosphatidylinositol (3,4,5) trisphosphate, activating AKT/PKB (Protein kinase B) [61-63]. Activated AKT/PKB promotes cell survival through several pathways, as for example by BAD phosphorylation, which suppresses apoptosis [64]. It has been shown that activated Ras can co-operate with AKT/PKB to give rise to gliomas in mice [30]. The introduction of wild-type *PTEN* into glioma cells that contain endogenous mutant alleles leads to growth suppression *in vitro* and *in vivo*, but it has no effect in cells which express wild-type PTEN [65,66]. Growth suppression after *PTEN* supplementation is caused by G1 cell-cycle block and by increased sensitivity to detachment-induced apoptosis [53,61,67].

Thus, *PTEN* is a principal tumour suppressor, whose absence seems to promote progression to the latest stage of this disease, histologically manifested as an increase in mitotic figures. However, it is clear from the rates of LOH 10q and that of *PTEN* mutations in GBMs that additional alterations at proximal loci contribute to the observed frequencies. Other tumour suppressor genes in this region include *MXI1* (Max-interacting protein 1) and *DMBT1* (deleted in malignant brain tumours) both of which have been shown to be deleted in the majority of glioblastomas [68,69].

3.5.4. *EGF-EGFR*

A major genetic aberration associated with high-grade diffuse astrocytomas is *EGFR* amplification, prevalent in about 40 % of the GBMs and in a few anaplastic astrocytomas [70-72]. According to a recent population-based study, *EGFR* amplifications are far more common in primary (36%) than in secondary (8%) glioblastomas [15].

EGFR is a 170 kDa transmembrane receptor, which conveys mitogenic signals from EGF and TGF-α (transforming growth factor-α). EGFR signalling has been implicated in the early embryonic development of glial cells as well as in the proliferation and survival of neural stem cells [73]. There is evidence that GBMs express EGF and TGF-α together with EGFR, establishing an autocrine stimulatory loop [74,75]. Approximately 31 % of the GBMs analysed in a recent series expressed a truncated receptor (EGFRvIII), which lacks the extracellular ligand-binding domain [76]. These mutant receptors are constitutively autophosphorylated; however, unlike wild-type EGFR, they are not down-regulated. Taken together, these traits lead to continuous mitogenic activation of the affected cells.

3.6. Diffuse astrocytomas, vascular induction

Low-grade diffuse astrocytomas do not initiate angiogenesis but migrate extensively throughout the brain parenchyma and co-opt with the physiological vasculature to meet their need for oxygen and nutrients [77]. The picture is dramatically altered during progression to glioblastoma, a lesion that exhibits prominent microvascular proliferations and regions of elevated vessel density [77,78]. The appearance of the angiogenic phenotype is clearly associated with poor prognosis [14].

Genetic alterations associated with the transition to GBM imply the erroneous activation of growth factor receptors, leading to strong mitogenic induction. As a consequence, the oxygen demand of rapidly dividing tumour cells may no longer be supported by the physiological vasculature, and hypoxic regions (regions with oxygen pressure below 30 mm Hg) develop [79]. Within these regions, tumour cells reach the lower limits of oxygen tension necessary for their survival. As a consequence, they undergo necrosis. Other tumour cells that are adjacent to the necrotic areas start to express the key transcription factor Hif-1α (hypoxia-inducible factor-1α), which together with Hif-1 β transactivate hypoxia-responsive target genes. Some of these are VEGF (vascular endothelial growth factor), nitric oxide synthase [80-82] and erythropoietin [83]. VEGF is likely the most potent endothelial cell-specific growth factor that is activated in this setting [84,85]. When angiogenesis is initiated, the proliferation and migration of endothelial cells forms a tumour vascular network, a process assisted by the angiopoietins [86]. In the following section, these two growth factors and their roles in GBM angiogenesis are discussed.

3.6.1. VEGF/VEGFR

Vascular endothelial growth factor, initially termed vascular permeability factor, is an endothelial cell-specific mitogen [87]. The VEGF family of proteins includes VEGF (i.e. VEGF-A), VEGF-B, C, D and E and placenta-growth factor, which all interact with one of the three tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR) and VEGFR-3 (Flt-4). During normal embryonic development, VEGF receptors are expressed by invading endothelial cells [88,89], whereas VEGF mRNA is found expressed by cells in the periventricular matrix zone [90], indicating that this family of growth factors participates in early angiogenesis. In GBM *in situ*, there is a marked up-regulation of VEGF expression in hypoxic areas close to necrotic regions [91,92] [93]. The concentrations of VEGF in the cyst fluid of GBMs have been shown to be 200 to 300-fold higher than those found in the serum of the patients [94], suggesting the importance of this growth factor in GBM angiogenesis.

Elevated VEGF levels have been shown to correlate with increased tumour vascularity and inversely with survival [94,95]. Knockdown of VEGF expression by antisense technology or with anti-VEGF antibodies transformed intracranial glioblastomas to less-vascularised low-grade glioma-like lesions in rodent tumour models [96,97], demonstrating the functional dependence of glioma vascularisation on VEGF. The VEGF receptors VEGFR-1 and VEGFR-2 are up-regulated on endothelial cells found in high-grade gliomas, but are not expressed on endothelial cells of the adult brain [85]. The interaction of VEGF with its receptors initiates a signalling cascade that promotes endothelial cell proliferation [98]. In addition, VEGF induces vascular permeability, allowing plasma proteins to extravasate, which creates a provisional matrix for endothelial cell invasion and subsequent blood vessel formation [99]. VEGF-induced plasma fluid extravasation also results in peritumoral vasogenic edema (an increase in the water content in the tissue, which leads to excessive enlargement of the extracellular space) [100]. In newly formed blood vessels, the survival of endothelial cells is also dependent on VEGF [101]. Hypoxia is a potent stimulator of VEGF expression, mediated by the Hif-1α/ Hif1-β heterodimer through a hypoxia responsive element (HRE) within the VEGF gene [102]. Interference with the Hif pathway prevents tumour growth *in vivo*, demonstrating the functional importance of the Hif pathway in neoplasia [80]. In addition to inducing transcriptional activation of VEGF, hypoxia also increases the stability of the VEGF-A mRNA [103].

Dominant oncogenes that are active in glioblastoma cells may contribute to elevated VEGF levels [104]. For example, activation of the PI3K/AKT pathway by mutant *ras* oncogenes induces VEGF expression [105-107]. EGFR over-expression is also demonstrated to up-regulate VEGF through the PI3K/AKT pathway, however it is not clear if it occurs by a Hif-dependent or Hif-independent mechanism [108,109]. In addition to VEGF, VEGF-B mRNA has also been found to be up-regulated in astrocytomas irrespective of histological grade, suggesting that this isoform may also contribute to tumour angiogenesis [110].

3.6.2. Angiopoietins/Tie-2

The angiopoietins Ang-1 and Ang-2 and their receptors Tie-1 and Tie-2 are involved in embryonic vasculogenesis and are co-expressed by most of the blood vessels and capillaries during early development [111,112]. Ang-1 recruits and sustains peri-endothelial support cells and is necessary for the maturation of newly formed blood vessels. Ang-2 seems to counteract these functions, by acting as an antagonist on the Tie-2 receptor [113,114]. Knockout models of Ang-1 display defective modelling of the primitive vascular plexus and lack of perivascular cells, an effect similar to that seen in Tie-2 knockouts [115]. On the other hand, Ang-2 over-expression can lead to endothelial cell apoptosis and to the regression of newly formed blood vessels in the absence of VEGF [113]. It is now clear that both angiopoietins bind to the Tie-2 receptor, Ang-1 as an agonist and Ang-2 as an antagonist.

The ligand for Tie-1 receptor is not known. In contrast to Tie-2 deficiency, which results in an unfinished capillary tree, the absence of Tie-1 results in a poor capillary integrity [114,116]. This demonstrates that the Tie receptors are responsible for distinct mechanisms during the process of blood vessel formation and maturation.

In transgenic mice, VEGF over-expression yields leaky blood vessels, whereas Ang-1 over-expression gives rise to tight blood vessels [117]. Thus; VEGF and Ang-1 may co-operate during embryonic blood vessel formation, VEGF by providing mitogenic stimuli for the endothelial cells, and Ang-1 by stabilising and enlarging the newly formed vessels [118]. Up-regulation of Ang-1 and concomitant down-regulation of VEGF has been implicated in the development of the blood-brain barrier [119]. Conversely; in the incidence of brain ischemia following stroke, disruption of the blood-brain barrier is associated with increased mRNA levels of VEGF and decreased levels Ang-1 mRNA [120].

Evidence suggests that the angiopoietins and Tie-1 are also involved in glioblastoma vascularisation [86,121,122]. Whereas Ang-1 mRNA is localised to tumour cells, Ang-2 mRNA is seen has been detected endothelial cells of hyperplastic and non-hyperplastic tumour vessels [121].

Ang-2 was found expressed in partially sclerotic vessels and in vascular channels surrounded by tumour cells in the brain tissue adjacent to the tumour. In human brain tumours, significantly elevated levels of Tie-1 mRNA is seen expressed both in the tumour endothelium and in the endothelium of the bordering brain tissue [123].

3.7. *In vivo* glioma models

There are two main reasons for modelling brain tumours in animals. The first is to identify the genetic events that contribute to oncogenesis within the central nervous system; thereby identifying possible targets for tumour therapy, as well as providing insight into brain tumour biology. The second reason is to obtain representative models to evaluate potential therapeutic strategies [124]. Three different *in vivo* models are discussed below, two are based on the grafting of foreign tumour material into animals, and one involves creating experimental tumours within the animal host by genetic manipulation. Foreign brain tumour material may be grafted intracranially or subcutaneously. The former is thought to be a more representative model, however the latter is widely employed for therapeutic purposes for its ease of evaluation by direct measurement with a calliper.

3.7.1. Cell line-based models

A number of glioma and glioblastoma cell lines generated from both rodent and human sources exist. The rodent cell lines are usually derived from tumours that have been induced in experimental animals by exposure to carcinogenic chemicals, for example to DNA alkylating agents such as ethyl-nitrosourea [125] [126,127]. Human glioma cell lines are derived from patient biopsy material by transferring the resected tumours to tissue culture flasks, which may subsequently be cultured for more than hundred passages [128]. Transplantation of the various cancer cell lines either to syngeneic- (rodent cell lines) or to immuno-compromised (human cell lines) animals leads to the development of tumours with typical growth characteristics.

The advantage of cell line-based tumour models is good reproducibility with respect to tumour take, growth characteristics and survival. In addition, immortalised cell lines are readily expanded *in vitro*, yielding almost unlimited amounts of tumour material for experimental use. A major disadvantage of cell-line based models when evaluating therapeutic modalities is the genetic- and phenotypic deviation of the obtained lesions from the patient situation.

The reasons for this deviation relate mostly to the genetic and epigenetic changes that occur during adaptation to the artificial situation of monolayer tissue cultures. During this process, tumour cell clones that are not able to adapt are lost. Therefore, the resulting cell lines tend to be genetically more homogenous than the parent tumours. Moreover, an additional genetic drift may occur during subsequent passaging in culture, which further increases the divergence from the original tumour. For example, an aberrant expression of ECM components was observed when GBM patient biopsies were transferred to culture and passaged as monolayers, suggesting that a certain (mesenchymal) differentiation may occur *in vitro* [129].

In general, transplantation of tumour cell lines to animal hosts do not recapitulate the diffuse infiltrative growth pattern of malignant brain tumours *in situ* [130]. These tumours grow as well-circumscribed lesions localised around the injection site [131,132]. However, since cell line-derived tumours often exhibit extensive angiogenesis, they are suited for studying anti-angiogenic therapies.

3.7.2. Biopsy xenografts

GBM models may also be generated by culturing the patient biopsy in the form of multicellular aggregates (spheroids) without previous passaging in monolayers [133]. Such ”organotypic” spheroids contain preserved vessels, connective tissue, and macrophages, displaying the cellular constituents of GBMs *in situ* [133]. In addition, the spheroids preserve other characteristics of the patient tumours, such as the same DNA ploidy and a similar percentage of proliferating cells [133,134]. Importantly, tumour cells maintained in this way display diffuse infiltrative growth similar to the parent neoplasm, when the spheroids are co-cultured with fetal brain tissue or implanted into the nude rat brain [131,132,135,136]. Standardised transplantation of equal amounts of biopsy spheroids derived from the same patient yields high reproducibility with respect to tumour take, growth rates and survival [131]. As this model better preserves the invasive characteristics of the original tumour, histological variations occur between tumours established from different patients. They are characterised by differences in cellularity, growth pattern and invasiveness [131]. When serially transplanted in nude rats, the xenografts display necrotic areas as well as endothelial proliferation, typical for GBM *in situ* [131]. Taken together, this model system seems to be more representative for human glioblastoma, making it a favourable choice for studying tumour biology and for evaluating therapeutic strategies.

This system has been employed both in intracranial and subcutaneous locations for the evaluation of gene therapeutic approaches ([137-140] and papers III-IV).

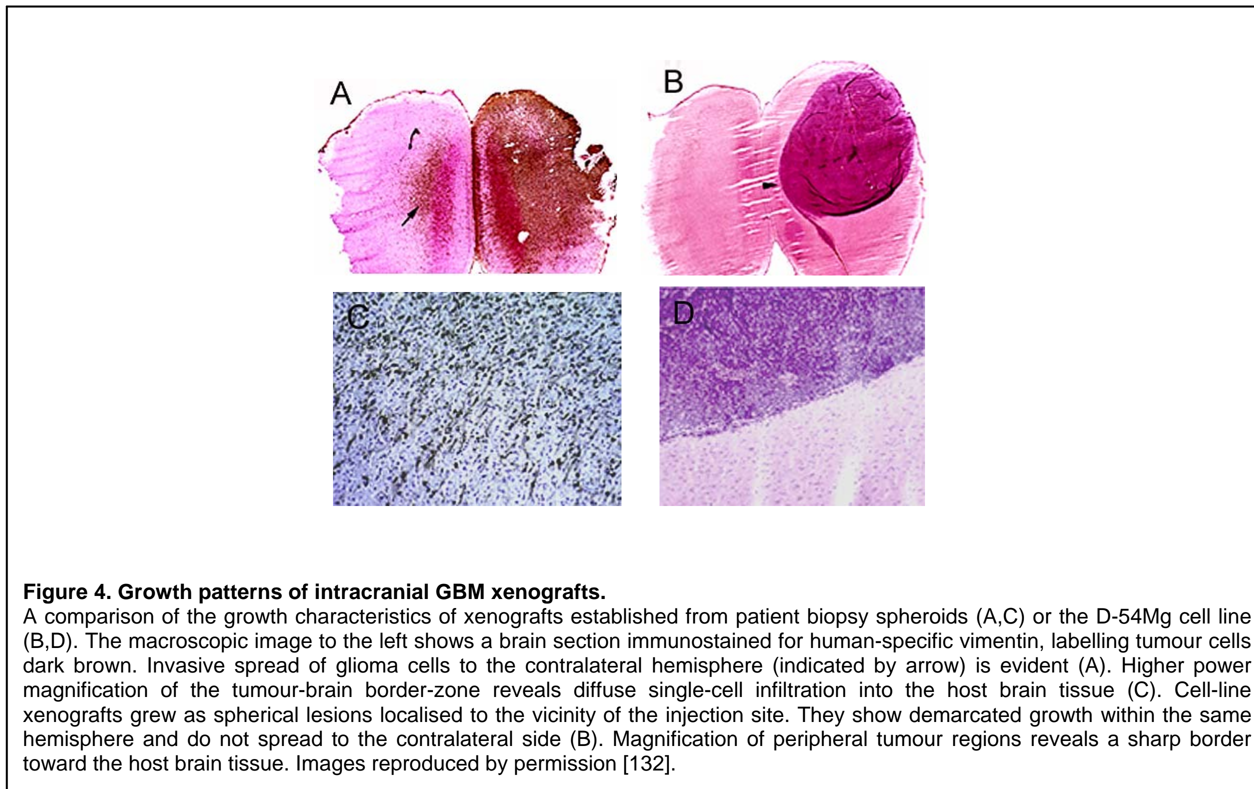


Figure 4. Growth patterns of intracranial GBM xenografts.
A comparison of the growth characteristics of xenografts established from patient biopsy spheroids (A,C) or the D-54Mg cell line (B,D). The macroscopic image to the left shows a brain section immunostained for human-specific vimentin, labelling tumour cells dark brown. Invasive spread of glioma cells to the contralateral hemisphere (indicated by arrow) is evident (A). Higher power magnification of the tumour-brain border-zone reveals diffuse single-cell infiltration into the host brain tissue (C). Cell-line xenografts grew as spherical lesions localised to the vicinity of the injection site. They show demarcated growth within the same hemisphere and do not spread to the contralateral side (B). Magnification of peripheral tumour regions reveals a sharp border toward the host brain tissue. Images reproduced by permission [132].

3.7.3. Transgenic models

Transgenic animals are generated by implementing defined genetic- or epigenetic changes in otherwise normal animal hosts [141,142]. These models have the advantage that the genetic alterations are precisely defined and are homologous throughout the organism. Therefore, they may be employed to dissect the minimum genetic alterations that are necessary for malignant transformation, or to define the interplay between the pathways involved in oncogenesis [124]. Rodent models with either gain-of-function (transgenic animals) or loss-of-function (targeted deletion) mutations have been developed. Tumour-bearing transgenic mice may be generated by directing the expression of an oncogene from a tissue-specific promoter [130,143]. In addition, tissue-specific enhancers may be added to elevate gene expression [144].

Targeted deletions were originally achieved by germ-line deletions, leading to gene knockout in every cell of the host animal. However, methods for conditional gene deletion have now been developed, where a tumour suppressor may be turned off in a tissue-or time specific

manner, making it possible to study specific deletions that would be fatal if they were constitutively lost in all tissues [142]. Another approach still is to generate tumours by somatic transfer of oncogenes with the use of viral vectors [30,145,146]. To recapitulate GBM development, Holland and co-workers infected mice with viral vectors which directed the expression of K-ras and AKT/PKB in infected cells either through the nestin- or the GFAP promoter (specific for neural-progenitors and astrocytes, respectively) [30]. This way, the expression of the oncogenes could be restricted to the desired cell types. While these models were originally developed to study oncogenesis and tumour biology, one might anticipate the use of such models to test therapeutic approaches directed against distinct genes or pathways.

3.8. Clinical treatment strategies

The current standard treatment for patients with high-grade gliomas consists of surgical resection followed by radiotherapy and chemotherapy [147,148]. In spite of improvements in these modalities, the median survival of patients diagnosed with diffuse astrocytic tumours remains disappointingly low, 2-3 years for anaplastic astrocytoma and 5-11 months for GBM [149]. Survival is primarily affected by the age of the patient as well as the histological grade of the tumour and its anatomic location [150,151].

3.8.1. Surgery

Surgery is performed to remove as much tumour tissue as possible [152]. Tumour resection improves the immediate neurological function of the patient by relieving the elevated intracranial pressure and by decompressing adjacent brain tissue [153]. Surgery allows for the insertion of single direction catheters, which drain excess accumulated fluid from the cerebral ventricles to other body cavities, such as the right ventricle of the heart or the abdominal cavity. This approach is especially useful for relieving chronic elevated intracranial pressure in cases where tumour growth blocks the normal clearing of cerebrospinal fluid.

The contribution of surgery to increasing patient survival has been a matter of debate. Retrospective studies indicate that radical surgery may result in significant survival benefit when compared to partial resection or simple biopsy procedures [150,153]. In a large study comprising 645 glioblastoma cases, patients undergoing total resection had a median survival of 11.3 months, versus 6.6 months for biopsy only [154]. Similarly, re-operation of recurrent gliomas may offer a reasonable extension of survival with good quality of life [153]. For both first-time operation and re-operation, there is a clear correlation between the pre-operative neurological status and the post-operative quality of life and duration of survival [153,155].

However, these trials have been subject to selection bias, since the extent of attempted resection is greatly influenced by the condition of the patient (age and performance status) and the size and site of the tumour.

Notably, no prospective randomised trials have compared surgery and radiotherapy-/chemotherapy with radiotherapy/chemotherapy alone for high-grade gliomas. Such a trial is probably not possible for ethical reasons, as current literature suggests that radical surgery should be attempted in all cases where the tumour is accessible and lies in non-eloquent areas [150,156].

3.8.2. Radiation treatment

Radiation treatment is routinely performed in the treatment of malignant gliomas as a supplement to surgery, as well as in cases where tumour resection is not feasible. All radiation treatment modalities are designed to achieve local tumour control. Post-operative radiotherapy of anaplastic astrocytomas and glioblastomas significantly improved survival when analysed in randomised controlled trials [157,158].

Ionising radiation delivered during radiotherapy may affect the target cells in a direct manner by random interactions between the photons and the biological molecules. The effects may also be indirect, mediated by reactive photon products, such as oxygen radicals. Ionising radiation mainly affects DNA, yielding both single- and double-stranded breaks, with double stranded breaks considered as the lethal effect. Radiation damage is most pronounced in the G_2 and the metaphase of the cell cycle [159]. Therefore, radiotherapy is most effective in cycling cells, such as those found in a growing neoplasm. Since the reactive intermediates that are produced have extremely short half-lives, they are only able to produce damage when they are generated within tens of ångstrøms from the DNA. However, the lifetime of the radicals, and therefore, their range and efficacy are enhanced in the presence of oxygen or other radiosensitisers. Unfortunately, glioblastomas tend to be more poorly oxygenated than the surrounding brain tissue, therefore maximal doses must be limited to avoid normal tissue damage [160].

Radiotherapy

For conventional radiotherapy of malignant gliomas, patients usually receive a total dose of 60 Gy divided into 30 fractions of 2 Gy per day, directed against the tumour [160]. Initial studies indicated that increasing the total radiation dose from 45 to 60 Gy led to a significant improvement of survival time [161,162].

Decreasing the size of each fraction may reduce damage caused by radiation to normal tissues. On the other hand, tumour cell death is more dependent on the total radiation dose. Therefore the total dose is often divided into a larger number of less intensive fractions (hyperfractionation therapy) [160].

Stereotactic radiosurgery

In stereotactic radiosurgery, high doses of precise electron beams are delivered using a linear accelerator or a "gamma knife". The gamma knife was originally designed for functional neurosurgery of inaccessible cerebral lesions, in particular small arteriovenous malformations and benign tumours such as acoustic neuroma and craniopharyngioma [163]. The beams from two hundred and one ^{60}Co sources placed within the gamma knife are directed towards a common focal point, creating spherical irradiation fields of 4-35 mm [164]. Tumours larger than 35 mm may be treated by employing multiple radiation fields. The advantage of this method is the possibility to deliver highly focused radiation to the tumour mass with a sharp fall of radiation dose toward the neighbouring brain tissue. Today, computer-guided dose calculation and volume planning is employed to deliver the radiation to the precise tumour shape. Promising results have been obtained using the Gamma knife for the treatment of low-grade gliomas, resulting in local tumour control and improvement of clinical status [165,166]. An analysis of the available clinical trials for high-grade gliomas suggests that any improvement reported is probably attributable to patient selection bias and does not support the continued application of strereotactic radiosurgery [167]. Other more recent studies have arrived at the same conclusion [168,169].

Brachytherapy

In cases where the tumour is localised and radiologically detectable, interstitial radiation therapy (or brachytherapy) may be used. This method employs radioactive pellets implanted into the tumour to kill the cancer cells, thereby minimising the exposure of normal brain tissue to radioactivity. Early clinical studies performed in the 1990s suggested improved survival of glioblastoma patients treated with brachytherapy combined with external radiation when compared to radiotherapy only [170,171]. Unfortunately, several of these studies were shown to be biased in patient selection and subsequent prospective randomised trials failed to reveal any survival benefit compared to standard treatment protocols [172,173].

Boron Neutron Capture Therapy

Boron Neutron Capture Therapy (BNCT) uses thermal or epithermal neutron beams to activate a boron drug concentrated within the tumour cells. Boron may be delivered to the tumours using appropriate pharmacological agents. When ^{10}B is exposed to radiation, it becomes unstable and disintegrates, releasing harmful radiation, which kills the cells. The penetration distance of the fission products $^{4}He^{2+}$ and $^{7}Li^{3+}$ is 9 and 5 µm, respectively; therefore it is mainly the cells that have incorporated the boron-containing substance that are destroyed [174]. The experimental application of BNCT has revealed significant survival benefit in rodent glioma models [175,176]. Recent clinical studies show that BNCT is relatively well tolerated by the patients [177-179] and early clinical data have suggested that BNCT treatment may yield occasional long time survivors [180]. However, analyses of the survival data for some of these patients (analysed following the stratification criteria according Curran et al. [181]) has not revealed significant survival benefit [182]. The following issues should be addressed to improve BNCT-treatment of brain tumours: the application of more selective and more effective compounds that would deliver sufficient amounts of boron to the tumour tissue, improvements in methods to evaluate boron content in the tumour, and the co-localisation of BNCT delivery facilities with medical centres to facilitate better treatment conditions for the patients [183]. Importantly, the potential of this treatment form would need to be re-evaluated in randomised prospective trials.

3.8.3. Chemotherapy

The use of chemotherapy is now well established for the treatment of several types of brain tumours. Chemotherapy of brain tumours is not curative, and is offered as adjuvant or neo-adjuvant treatment in addition to surgery and/or radiotherapy. The goal is to control tumour growth and to maintain good performance and quality of life for the patients [147].

Low-grade gliomas (astrocytomas and oligodendrogliomas) as well as CNS tumours of other origin (medulloblastomas, primitive neuroectodermal tumours, germ cell tumours and primary CNS lymphomas) have responded well to chemotherapy, resulting in significant survival benefit for the patients [151]. On the other hand, glioblastomas tend to become resistant and show only temporary responses [151]. For a chemotherapeutic treatment to be effective, the drug has to be distributed throughout the tumour at cytotoxic- or cytostatic concentrations for a sufficient time-period. Response to the drug depends on several factors, such as the mechanism of action, the kinetics of tumour cell growth, the chemosensitivity of the tumour cells and the mode of delivery [184].

Alkylating agents were introduced to cancer therapy nearly 60 years ago and are still in clinical use [158,185,186]. Of these drugs, alkylating nitrosourea compounds (BCNU, CCNU) are widely employed for glioma chemotherapy, either alone or in combination with other drugs [148,184]. Alkylating agents decompose to reactive chloroethyl-carbonium ions that deliver alkyl groups to DNA, and to organic isocyanates that inhibit nucleic acid replication and transcription [187]. Nitrosoureas are highly lipophilic; they have a relatively low molecular weight, and do not ionise readily at physiological pH. These properties cause efficient penetration of the blood-brain barrier when administered intravenously, resulting in good distribution within the CNS.

Another class of drugs is the vinca-alkaloids such as vincristine and vinblastine. These compounds inhibit the polymerisation of microtubuli, which mainly affects the mitotic spindle, blocking the cells in metaphase [188,189]. In addition, microtubuli arrest also affects tumour cell migration, invasion and intracellular transport mechanisms, which may yield secondary anti-neoplastic effects [184]. For adjuvant chemotherapy, the first line of treatment consists of BCNU or a three-drug regimen consisting of procarbazine, CCNU and vincristine, (PCV) [185]. The treatment of recurrent gliomas by chemotherapy may show limited effects, since the tumour cells often develop resistance toward nitrosoureas. This may be circumvented by the use of non-nitrosourea based agents. Temozolomide is an imidazotetrazine-derived prodrug that is converted into a DNA methylating agent *in vivo*. It is absorbed well upon oral administration with nearly 100 % bio-availability [190]. Temozolomide crosses the blood-brain barrier, resulting in widespread distribution throughout the CNS [191]. For recurrent GBMs, temozolomide has proven to be effective at first relapse with an acceptable safety profile [191-193].

A meta-analysis review which has processed the data from 16 clinical trials has detected an 8% increase in the 2-year survival for anaplastic astrocyoma patients and a 4 % increase in the 2-year survival in GBM patients when adjuvant chemotherapy was given in addition to radiotherapy [194]. More recently, the Glioma Meta-analysis Trialist Group (GMT) has performed a statistical review of individual patient data from 12 randomised trials, including a total of 3004 patients that have been treated with nitrososurea-based adjuvant chemotherapy for high-grade glioma [195]. Chemotherapy was found to provide a modest, but highly significant increase in the median survival time (of 2 months duration) compared to treatment with radiotherapy alone. A recent randomised phase III trial which enrolled 573 patients with GBM compared concomitant and adjuvant use of temozolomide versus radiotherapy alone [196].

The results were encouraging, with a 2.5 months increase in median survival for the combined chemo-and radiotherapy group compared to radiotherapy alone (14.6 vs. 12.1 months, respectively). The data were statistically significant and also clinically meaningful, with a 2-year survival rate of 26% versus 10% for radiotherapy alone. Thus, both nitorosoureas and temozolomide show similar clinical benefit for patients with high-grade glioma. However, the superior safety profile of temozolomide indicates that it may be the drug of choice for recurrent tumours [148].

Even though certain patients show marked and prolonged responses to chemotherapy, the overall contribution to survival is modest and needs to be re-evaluated in the light of a possible worsening of quality of life for some agents.

3.9. Experimental therapy

Based on the limited effects using conventional clinical therapies, there is a search for new and effective therapeutic modalities for malignant brain tumours. Some of the more novel molecular approaches are presented below.

3.9.1. Immunotherapy

Clinical studies conducted throughout the 1970s and 1980s have demonstrated that patients harbouring malignant gliomas present with impaired cell-mediated immunity [197]. The observed systemic immunosuppression appears primarily to result from cytokine dysregulation, mediated by soluble factors secreted by the glioma cells [197]. Transforming growth factor -β, and other related growth factors inhibit IL-2 production and the proliferation of T-cells [198-200]. Supporting this contention, the extent of immunosuppression shows significant correlation with tumour size [201], and surgical debulking has been associated with the partial restoration of systemic T-cell function [202]. In addition, gliomas may also have suppressed immunogenicity at the tumour site as a consequence of either down-regulation of major histocompatibility molecules [203] or Fas ligand over-expression, which induces apoptosis in infiltrating lymphocytes upon direct contact through Fas/APO-1 [204,205].

There is substantial evidence that boosting the compromised tumour-specific immune responses can improve survival time lengths in rodent glioma models [206-209]. However, the benefit of immunotherapy for the treatment of human brain tumours remains to be settled.

In the following paragraphs, passive serologic immunotherapy, cytokine therapy, adoptive T-cell transfer and vaccination strategies for *in vivo* T-cell activation will be discussed.

Passive serologic immunotherapy

In theory, monoclonal antibodies (mAbs) generated against tumour-specific molecules may be utilised to target and destroy neoplastic cells. Such therapeutic antibodies may be directed towards cell-surface receptors, thereby blocking signalling pathways that are necessary for neoplastic cell survival; or against extracellular matrix components that are known to be widely expressed by malignant gliomas [210]. For this application, the ECM molecules serve as "anchors" to guide the antibodies to the cells, which need to be armed with cytotoxic conjugates (such as a radioactive isotope or an immunotoxin) to exert their effects.

Passive serologic immunotherapy faces several challenges in order to be considered for the treatment of malignant gliomas. First, it is difficult to identify highly glioma-specific surface markers that are not expressed by normal brain cells. Although there are several antigens whose expression is up-regulated in malignant gliomas (such as the receptors for EGF [70], PDGF [211] and TGF-β [212]), none of these antigens are exclusive to tumour cells. Therefore, the application of antibodies directed against these molecules may induce normal tissue toxicity. Furthermore, the antigen of choice has to exhibit both stable- (lack of internalisation or down-regulation upon antibody binding) and robust expression throughout the glioma tissue to have any therapeutic effect [213]. The kinetics of transport within the tumour (affected by tumour vascularity, vascular permeability, extracellular fluid dynamics and interstitial pressure) of the antibody considered also needs to be favourable [210].

Early clinical evaluation of passive serologic immunotherapy for malignant gliomas has provided inconclusive results. Favourable outcomes have been reported from a phase II trial, which involved a ^{131}I-labelled mAb against tenascin (an ECM molecule which aids tumour cell invasion) in 33 patients with malignant gliomas applied by direct injection into the surgical resection cavity [214]. After antibody administration, the patients received conventional external beam radiotherapy followed by a year of chemotherapy. The median survival for all malignant glioma patients undergoing the combined treatment was 86.7 weeks; whereas for GBM patients it was 79.4 weeks. After accounting for established prognostic factors such as age and Karnofsky performance status, the authors concluded that the median survival times achieved by serologic immunotherapy exceeded that of historical controls.

Prolonged patient survival has also been observed in a phase II trial where 180 patients with malignant gliomas were treated with a ^{125}I-labeled monoclonal antibody directed against the EGF receptor [215].

Cytokine therapy

Cytokines are soluble effector molecules that are responsible for initiating, supporting or blunting specific immune pathways. The therapeutic over-expression of appropriate cytokines may potentiate immune surveillance and induce cellular anti-tumour immunity [216]. For glioma immunotherapy, the focus has been on cytokines that are involved in the promotion of tumoricidal T-cell activity, such as IL-2 [217,218], IL-4 [219,220], IL-12 [207,216,221] and the interferons (IFN-α, β and γ) [222,223]. Initial investigations have involved recombinant cytokine therapy, where high systemic doses of cytokines have been applied to stimulate strong anti-tumour responses. Although this approach has proved to be highly effective in rodent tumour models [224,225], it has not been met with success in the clinical setting. Major limitations have been the considerable tissue toxicity and the low half-life of the delivered molecules [226], preventing sustained therapeutic effect. Attempts to address these issues have resulted in new approaches using viral vectors [207,219] or producer cells [217,220,227] for site-directed delivery of the cytokines to the tumours. Both approaches have been highly successful in pre-clinical experiments [207,217,219,220,228].

Recently, the use of neural stem cells (NSCs) to deliver such therapeutic cytokines has been investigated [229]. Neural stem cells have been shown to track glioma cells and display tumour-tropism even after implantation in the contralateral brain hemisphere [227,230]. NSCs expressing IL-12 or TRAIL were highly efficient at inducing T-cell infiltration and tumour cell apoptosis, respectively, resulting in strong tumoricidal effects in rodent glioma models [227,228].

Adoptive T-cell transfer

Initial experiments of adoptive cellular therapy have been conducted using autologous, non-activated immune cells delivered through intratumoral- [231,232] or intrathecal [233,234] administration. Subsequently, the identification of IL-2 as a potent T-cell mitogen led to the application of lymphokine-activated killer (LAK) cells for glioma therapy. The clinical evaluation of LAK cells transplanted into the resection cavity has yielded confounding results [235,236]. In one study, neurological side effects associated with immunotherapy were seen in all of the treated patients [237].

Subsequent studies have focused on the adoptive transfer of specifically activated T-cell populations. The results have been inconclusive. In one clinical study including 12 patients with grade II-IV astrocytomas, anti-T-cell receptor (CD3) antibodies, bacterial superantigens and IL-2 were employed to stimulate T-cell activation before the cells were administered intravenously to the recipients [238]. Partial tumour regression was seen in four patients and only mild treatment discomforts could be related to therapy. No long-term adverse effects were observed.

Active immunotherapy (vaccination strategies for in vivo T-cell activation)

Substantial evidence has now accumulated to prove that the successful activation of cytotoxic, glioma-specific T-cells is of major importance to generate effective anti-tumour immune responses [239,240]. In general terms, the adoptive cell transfer of *in vitro*-expanded and activated T-cells does not generate true anti-tumour T-cell immunity, given the lack of specificity and the inability to generate protective memory [241]. Thus, identifying and successfully presenting immunologically relevant tumour antigens to T-cells *in vivo* is the main challenge for enhancing tumour-specific T-cell responses. Still, as discussed above, the difficulty of identifying glioma-specific, immunologically relevant tumour antigens remains a major hurdle.

Initial vaccination experiments have utilised either irradiated whole tumour cells or dendritic cells (DCs) pulsed with non-specific tumour-derived peptides or lysates in order to present a broad range of unknown, tumour-derived antigens to the T-cells *in vivo*. The subcutaneous implantation of irradiated, autologous tumour cells together with IL-2 secreting fibroblasts resulted in marked tumour necrosis and enhanced T-cell mediated anti-tumour immunity in a phase I study for glioblastoma [242]. Although there have been some encouraging results with such modalities, the main limitation of this approach is the poor antigen-presenting capability of the glioma cells. Thus, more recent strategies have focused on employing professional APCs, such as dendritic cells, rather than trying to augment the antigen-presenting capabilities of the glioma cells. In theory, the application of DCs should prove to be superior for T-cell priming, since DCs abundantly express many of the co-stimulatory molecules that are essential for the appropriate activation of naive T-cells. Recent evidence suggests that direct physical interaction between the DCs and the tumour cells may be essential to induce therapeutically effective immune responses [243] and that DCs are capable of processing apoptotic tumour cells to induce cytotoxic T-lymphocyte activity [244,245].

Thus, the most recent experimental approaches involve the intratumoral administration of DCs in the context of radiotherapy to induce tumour cell death. Therapeutic modalities based on this principle have been found to be effective in rodent models, resulting in robust T-cell infiltration, inhibition of glioma growth and prolonged survival [246,247].

3.9.2. Therapy targeted to the tumour vasculature

In 1971, Judah Folkman and colleagues proposed that solid tumour growth could be prevented by interfering with tumour vascular sprouting (angiogenesis)[248]. The angiogenic switch, which controls the onset or the shutdown of new vessel formation, is regulated by the balance between angiogenic growth factors (inducers) and inhibitors present in the tumour microenvironment [249]. Accordingly, local over-expression of angiogenesis inhibitors may be exploited for solid tumour therapy [250]. The main advantage of anti-angiogenic therapy is that it is directed toward the genetically more stable endothelial cells. In theory, this would circumvent the problem of acquired drug resistance that may develop in cases where treatment is aimed at the genetically volatile neoplastic cells [251]. To date, multiple angiogenesis inhibitors have been identified, and several of these have entered clinical trials [252]. Since high-grade gliomas are among the most vascularised lesions known, they are considered to be particularly attractive targets for anti-angiogenic therapy. The therapeutic over-expression of angiogenesis inhibitors, as well as the inhibition of key angiogenic factors have been attempted in pre-clinical experiments for malignant gliomas [253]. Most often, endogenous- or synthetic inhibitors of angiogenesis have been employed to control tumour growth. Some of these inhibitors are listed in table IV (appendix). The application of angiogenesis inhibitors has been attempted in the framework of different delivery systems. Early studies typically utilised the purified inhibitor in its protein form, often given as intravenous- or intratumoral injections. Disadvantages of this type of anti-angiogenic therapy are the necessity of applying repeated injections due to the short half-life of the inhibitors, as treatment needs to be continuous to maintain tumour dormancy. Obtaining sufficient amounts of clinical grade purified inhibitor is associated with high costs. For these reasons, subsequent research has concentrated on gene therapy by viral vectors or protein delivery by genetically engineered producer cells. Importantly, the anti-angiogenic strategies discussed above typically target immature vessels that participate in angiogenesis, which are sensitive to agents that block proliferation or induce regression of the tumour endothelium.

However, the relative number of proliferating endothelial cells in human malignant lesions is low, considerably smaller than those found in rodent tumour models [254], a notion that might explain the relatively poor efficacy of angiogenesis inhibitors in clinical trials. Recently, research efforts have focused on identifying molecular markers expressed by the entire tumour-associated endothelium to improve drug delivery to endothelial cells that are not participating in angiogenesis at a given time. Serial analysis of gene expression profiling and phage display technology have provided us with molecular signatures of the tumour vasculature [11,255,256]. Investigators have presented proof-of-principle for the efficient of re-targeting of a wide range of therapeutic molecules as well as viral vectors to endothelium-specific peptides [257,258]. Further unravelling of the cellular mechanisms involved in the specific inhibition of tumour vasculature will probably provide us with novel, specific markers associated with the tumour endothelium, which may be used in vascular targeting.

Endostatin

Endostatin is an endogenous angiogenesis inhibitor [259]. It is a 20-kD proteolytic fragment of collagen XVIII, an ECM component mainly found in blood vessel walls and in the basement membranes of the liver, kidney and the lung [260]. Endostatin may be cleaved from collagen XVIII by a range of proteolytic enzymes [261]. Since both capillary sprouting and tumour cell migration are associated with enzymatic degradation of the extracellular matrix, endostatin release is thought to be a direct consequence of these processes. Indeed, the serum levels of endostatin have been shown to be significantly elevated among patients with a variety of cancer types [262].

Research has revealed a variety of mechanisms by which endostatin signalling down-regulates endothelial cell survival, motility and invasion. Blockade of VEGF/VEGFR signalling [263,264], inhibition of metalloproteinases [265] and down-regulation of c-*myc* and cyclin-D [266,267] are all thought to be downstream effects of endostatin signalling. It has recently been shown that endostatin down-regulates the expression of several Hif-1 pathway activators leading to strong transcriptional inactivation of Hif-1α, thereby inhibiting its responsive pro-angiogenic gene targets [268]. Endostatin was shown to down-regulate Id1 and Id3, which play critical roles in angiogenesis, possibly by suppressing the expression levels of MMP-2 and $\alpha_v\beta_3$ integrin [269]. Importantly, Id1 transcriptionally represses thrombospondin-1, a potent angiogenesis inhibitor [270]. Other studies have shown that collagen XVIII expression is directly induced by p53, through its binding to the *COL18A1* promoter [33].

This evidence suggests that endostatin is part of the body's natural anti-angiogenic response cascade.

In the initial study by O'Reilly and co-workers, the administration of recombinant murine endostatin protein resulted in tumour growth arrest and the regression of several subcutaneous cancer types [259]. Although regression of pre-established tumours has not been reproduced, several studies have confirmed that endostatin is a potent inhibitor of a variety of experimental tumour types, such as melanomas, mammary- and lung carcinomas, hepatocellular carcinomas and gliomas [271-274]. The initial publication, which described complete tumour remission upon endostatin treatment, employed *E.coli*-produced recombinant protein [259]. Subsequent studies, where yeast-derived endostatin was used have also reported tumour growth inhibition [275,276]. Recent work has focused on endostatin gene delivery, such as the application of naked plasmid [277,278], liposome-complexed plasmid [279], adenovirus [263,272,273,280-287], retrovirus [266,274,288-297], adeno-associated virus [298-300] and lentivirus [301,302].

Endostatin protein delivery has also been attempted by *in vivo* transplantation of mammalian producer cells genetically engineered to secrete this inhibitor [303,304].

3.9.3. Suicide gene therapy

Suicide genes are exogenous transgenes which code for metabolic enzymes that convert non-toxic prodrugs into cytotoxic end-products [305]. Accordingly, the systemic administration of a prodrug only harms cells that express sufficient levels of the exogenous suicide gene. The application of suicide genes for glioblastoma therapy has been attempted in the framework of several vector systems, as reviewed recently by Pulkkanen and Herttuala [306]. Of the several suicide enzymes that may be exploited for these purposes [305], the herpes simplex virus thymidine kinase gene (HSV-*tk*) has been the most widely applied. Thymidine kinase converts the non-toxic nucleoside analogue gancyclovir (GCV) into its mono-phosphorylated form. Further modification by cellular enzymes yields toxic GCV-triphosphate, which inserts into DNA during synthesis, blocking replication and killing the target cell. A desirable feature of suicide gene therapy is the so-called bystander effect. The toxic product can escape from infected hosts to adjacent cells via gap junctions, leading to lateral spread of the cytotoxic effect. Thus, adjacent tumour cells not initially infected by the vector may also be efficiently killed.

Since most tumours are genetically heterogeneous, complementing- or knocking out a single gene product may not target the whole lesion efficiently.

The main advantage of suicide gene therapy is its broad target range, affecting every dividing cell that expresses the enzyme. However, the promiscuity of this approach renders specific targeting to the tumour cells important in order to protect normal tissue [307]. This may be accomplished by using gene delivery vectors that have enhanced tumour-specificity by virtue of their binding to cancer cell-related receptors. Another way to achieve specificity is to exploit tumour-specific promoters to direct transgene expression; such as those that promote hypoxia-, angiogenesis- or invasion-related genes.

3.10. Vehicles for the delivery of anti-neoplastic agents within the CNS

3.10.1. Microencapsulated producer cells

The application of genetically engineered producer cells to deliver cytokines or angiogenesis inhibitors has been investigated for the treatment of experimental brain tumours [308] [309]. The main advantage of this approach is the possibility to achieve sustained delivery of biologically active substances without the use of potentially hazardous gene therapy vectors. In general, the application of cell-based therapies employing non-autologous cells results in graft rejection. Encapsulation of the producer cells into microcapsules composed of immuno-isolating substances, such as alginate, offers a way to circumvent this problem [310]. Alginate forms a porous network with pore diameters of 5-200 nm, which allows the transmembrane exchange of soluble factors, meanwhile protecting the encapsulated cells from host immune cells. At least within the confines of the central nervous system, the immunological response is mainly cellular, which supports the application of this treatment modality for brain tumours as well as for other neurological disorders.

Several studies have characterised how the producer cells adapt to the microcapsules and examined the potential application of this delivery system within the CNS. Upon encapsulation into alginate, the producer cells established viable multicellular aggregates [311]. Importantly, 70 % of the cells within the capsules remained viable 4 months post-encapsulation, and only mild inflammatory reactions were observed when alginate beads were implanted into the rat brain [312].

The delivery of human growth hormone (hGH) to mammalian brain tissue by alginate-encapsulated murine myoblast producer cells has also been evaluated in an experimental study [313]. After intraventricular implantation in the mouse brain, hGH secretion from the bioreactors was detectable for 16 weeks, with peak levels observed after 8 weeks post-implantation.

Immunohistochemical studies revealed that the secreted hGH protein was distributed throughout a radius of 1.5-2 mm from the implantation site.

Similar experiments employing alginate-encapsulated hybridoma cells showed that antibody release increased up to day 12 *in vitro*, whereafter stable secretion was achieved at high levels [311]. Upon implantation into the rat brain, the secreted immunoglobulins were detected at least 1 mm from the implantation site, with a gradual decrease in concentration from the beads toward more peripheral areas [311]. These studies suggest that of alginate-encapsulated producer cells may be a safe and a reliable way to deliver therapeutic molecules to the CNS. Further experiments were conducted to evaluate the anti-vascular effects of this delivery system using 293-EBNA producer cells that secreted endostatin. In two studies, the encapsulated cells were implanted together with BT4C- or C6 glioma cells into rodent hosts at an intracranial or a subcutaneous location. The delivery of endostatin by this application exerted a potent anti-angiogenic effect, resulting in a significant inhibition of tumour growth [303,314].

In a clinical study aimed at correcting amyotrophic lateral sclerosis (ALS; a degenerative neurological disorder), microencapsulated producer cells secreting ciliary neurotrophic factor (CNTF) were surgically placed into the intrathecal lumbar space of patients [315]. By this approach, elevated levels of CNTF were obtained in the patients' cerebrospinal fluid (CSF) for at least 17 weeks post-transplantation. Importantly, the limiting side effects observed after systemic delivery of CNTF were not seen after local delivery. For malignant gliomas, no clinical trials have been performed employing microencapsulated producer cells. The efficacy and the potential toxicity of this approach needs to be evaluated in large animal models before clinical trials could commence [316].

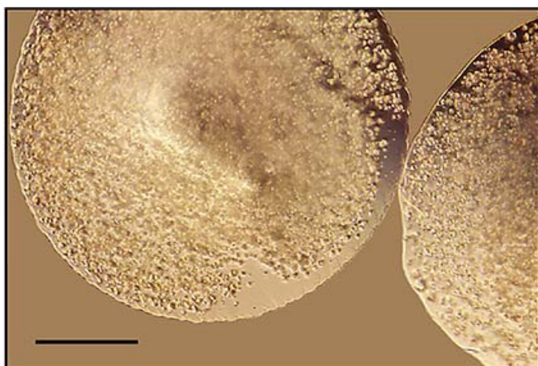


Figure 5. Light microscopic image of alginate bioreactors. Ultrapure alginate microcapsules containing viable 293 cells are non-immunogenic and can be engineered to release anti-angiogenic factors or other molecules that are thought to interfere with tumor growth within the CNS. The production of enzymes, growth factors applied to complement a range of CNS pathologies may also be envisioned using this system. Scalebar: 100μm. Image reproduced by permission.

3.10.2. Viral vectors

Retroviral (RV) vectors

Retroviruses are lipid-enveloped viruses, which contain a double-stranded RNA genome of 8-11 kB. Upon entry into the host, viral reverse transcriptase converts RNA to double-stranded DNA, which then integrates into the genome at random loci. Retroviral vectors based on Moloney murine leukaemia viruses (MMLV) can only insert transgenes into dividing cells. Therefore, when administered by site-directed delivery to the adult brain, these vectors would only infect neoplastic cells and endothelial cells participating in angiogenesis, conferring them an element of tumour-selectivity [317].

RV vector systems were among the first to be applied for brain tumour therapy [318,319], and they have been investigated in more than half of the clinical trials employing viral vectors for gliomas [320]. The most common strategy has been to use replication-incompetent MMLV vectors carrying the HSV-*tk* gene [321]. A major drawback of RV vectors for clinical use is the fact that human serum antibodies inactivate the viral particles that are released by mouse-derived packaging cells [322]. In addition, RVs are difficult to purify to high titres. Therefore, the most common application has been to transplant the vector producer cells (VPCs) into the resection cavity to ensure a continuos supply of active vector at the site where tumour recurrence is expected.

RV vectors have shown favourable safety profiles and some efficacy in early phase I and phase II trials for malignant gliomas has been reported [323-325]. However, a more recent randomised, prospective phase III trial which enrolled 248 patients with previously untreated GBMs failed to demonstrate significant survival benefit after RV-mediated suicide gene therapy [326]. The failure of the phase III protocol has mainly been attributed to the poor transduction rates, resulting in insufficient transgene delivery [326]. Since the producer cells were of a non-migratory fibroblast phenotype, they stayed in the vicinity of the injection site, limiting vector spread to more distant glioma microsatellites. The lack of penetration to more distal areas of the tumour mass by the viral particles has probably contributed to the poor transduction efficacy. Indeed, from experimental glioma models there is evidence that successful treatment of smaller tumours by RV vectors is feasible, but larger tumours seem to be refractory, underlining the contention that inadequate tissue penetration limits anti-tumour efficacy [319,323]. It is generally accepted that in the context of HSV-*tk* suicide gene therapy approach, a stable transduction of at least 10 % of the tumour mass is needed [327-330]. In contrast, the figures for gene transfer efficiency from clinical studies with RV vectors have been disappointingly low, in the range of 0.002-0.03 % [331,332].

Adenoviral (Ad) vectors

Adenoviruses contain a double-stranded linear DNA genome of 36 kB, which encode more than 50 proteins, generated through extensive splicing of the primary transcripts [333]. In humans, adenoviruses typically cause benign respiratory tract infections. Ads can infect a wide range of cell types with high efficacy without the risk of insertional mutagenesis, making them attractive tools for gene therapy.

Ads have evolved to exploit the cellular machinery for the production of viral progeny, as well as to evade the cell´s major regulatory pathways that would restrict their replication. During adenoviral infection, the Ad E1A protein binds the retinoblastoma tumour suppressor protein (pRB). The subsequent uncoupling of E2F from pRB initiates S-phase and to leads to the transcription of viral genes that are essential to replication [334]. The large E1B protein binds p53, thereby inhibiting p53-mediated anti-viral apoptotic response. These events ensure an active cell cycle, allowing the Ad to multiply its genome efficiently without facing host-cell apoptosis. Selectively replicating oncolytic vectors have been generated by deleting such essential early genes or by placing them under the control of tumour selective promoters. By deleting E1A or E1B from recombinant Ads, these vectors are restricted in normal cells that harbour functional pRB or p53, whereas they replicate in cancer cells where these proteins are defective or absent. Both E1A-deficient (Δ24) and E1B-deficient (ONYX-015) Ads have been constructed. Of these, the Δ24 vectors have shown superior therapeutic efficacy in experimental glioma models [335,336].

Since the native Ad receptor, CAR, was shown to be poorly expressed by some cancer types, limiting transduction efficacy in tumour tissues [337], a further modification was made by incorporating an integrin binding peptide into the Δ24 fiber knob. This novel vector, designated Δ24RGD, showed enhanced oncolytic effect, leading to tumour regression in 60% of the treated animals in an intracranial mouse glioma model [338]. Other manipulations of recombinant Ad vectors have included placing the E1A gene under control of the HIF-promoter [339] to create vectors that replicate in hypoxic tumour cells known to be particularly resistant to radio- and chemotherapy. The administration of this vector together with BCNU treatment to target normoxic cells has resulted in enhanced anti-tumour efficacy in two independent glioma models with 50% of the tumours showing complete long-term regression [340].

To date, 8 clinical trials focusing on adenoviral gene therapy of brain tumours have been published [306]. A recent phase I trial employing Ad-mediated delivery of *TP53* to recurrent GBMs confirmed tolerated doses up to $3x10^{12}$ viral particles with only minor vector-related

symptoms such as headache, fatigue or fever [341]. *TP53* transfer to the patient tissue was validated, and subsequent p21 activation and the occurrence of apoptoses were confirmed in the vicinity of the catheter in the resected tumour samples. Of note, the maximal spread of exogenous p53 expression was only 8 mm (mean: 4.9 mm) from the catheter, suggesting that these vectors may have an inherent inability to penetrate and transduce tissues for the distances required in the clinical setting. An assessment of survival revealed that one patient was alive for 3 years without recurrence, with 4 more patients surviving for more than 6 months [341]. Two phase II trials have been conducted in Finland, where 3.0 $x10^{10}$ plaque-forming units of Ad-HSV/tk was injected directly into the resection cavity of patients with primary- or recurrent malignant gliomas [342,343]. In the first study, comprising 21 patients divided into three groups, the application of Ad-HSV/*tk* resulted in a significantly increased survival of 15 months as compared to 7.4 months with RV- HSV/*tk* and 8.3 months (Ad-*lac*Z, control). A new phase II trial was conducted to corroborate these findings, where 36 patients were divided into two groups, receiving either Ad-HSV/*tk* or standard clinical treatment. This study confirmed the significant gain in survival obtained in the previous trial, with 70.9 weeks for the Ad-HSV/tk group vs. 39.0 weeks only in the standard care group. The treatment was well-tolerated.

Clinical trials have confirmed the safety of adenoviral vectors for local therapy in the human CNS, with mild treatment discomforts such as headache, low-grade fever and confusion reported in some of the studies [306]. Further randomised trials are necessary to evaluate the potential contribution to the various adenovirus-based gene therapy vectors to overall survival. Importantly, the evaluation of replicating Ads in phase II clinical trials awaits execution.

Herpes simplex virus (HSV) vectors

HSV is a common human pathogen that establishes life-long asymptomatic infections of the nervous system with occasional reactivation in epidermal cells, leading to cell lysis [344]. HSV type 1 (HSV-1) has a linear double stranded DNA genome of 152 kB, encoding more than 80 genes [345].

Much of the genome is redundant, allowing for the insertion of 50 kB foreign DNA without significantly affecting viral packaging [346]. The HSV envelope glycoproteins bind to ubiquitous cell-surface proteoglycans for entry, thus they have a naturally broad tissue host range. These traits make HSV vectors attractive gene delivery vehicles.

Intracerebral injection of wild-type HSV in rodent models resulted in lethal encephalitis [347]. Thus, in order to apply these vectors for therapeutic purposes within the CNS, it was important to abolish their neurotoxicity. Conditionally replicating HSV-based vectors have been generated where non-essential viral genes are deleted. The defective functions of the vectors can be compensated for by the up-regulation of complementary cellular enzymes in actively dividing host cells. Engineered mutants for the γ34.5 gene (e.g. HSV vector 1716) have reduced neurovirulence and do replicate; although at a lower rate, in dividing cells [348,349]. Double mutant vectors such as G207 [350] or MGH1 [351] which have an additional inactivation of the ribonucleotide reductase gene, have further reduced toxicity. An extra safety feature of these vectors is their sensitivity to gancyclovir and to temperatures above 39º C, which restricts proliferation in cases of possible encephalitis or fever [350].

In a phase I dose-escalation study for recurrent malignant glioma, the maximal given dose of $3x10^9$ PFU of G207 was tolerated well, without signs of acute toxicity, viral shedding or delayed reactivation of latent virus [352]. The vector was delivered by direct inoculation into the enhancing tumour region at five different sites. Eight of the 20 patients showed treatment response, noted as a decrease in tumour volume on MRI scans taken 1 month post-inoculation. Phase Ib/II trials are currently underway to establish the maximal tolerated dose as well as to assess the ability of G207 to replicate within the tumours.

Clinical studies have validated the safety of HSV1716 administered up to 10^5 PFU [353], as well as the ability of this vector to replicate in patient GBM tissue [354]. In a more recent trial enrolling 12 patients with high-grade gliomas, HSV1716 was injected at 8-10 sites in the cavity created after surgical resection, before the patients proceeded to radiotherapy or chemotherapy [355]. Three long time survivors were noted in this study, being alive 15, 18 and 22 months post-injection. Medical imaging has demonstrated tumour regression in one of the patients, despite no further clinical intervention.

In conclusion, HSV-based replicating oncolytic vectors show promise for the treatment of malignant gliomas. Their efficacy is currently being further evaluated in clinical ongoing trials.

Adeno-associated virus (AAV)

AAVs are small, non-enveloped viruses containing a single-stranded DNA genome of about 4.7 kB [356]. They were originally isolated as contaminants of human adenoviral stocks [357]. AAVs are naturally replication-defective, requiring helper functions from adenoviruses or HSV for productive replication and infection [358].

50-80 % of the adult population world-wide is seropositive for AAV serotype 2, with conversion occurring around 10 years of age [359-361]. AAVs usually spread with adenoviral infections [359]. In comparison to other viruses, AAVs are not associated with any form of disease in humans. In fact, some studies have even described anti-neoplastic features of AAVs and of other parvoviruses [359,362,363].

Wild-type AAVs contain two genes, coding for replicase (*rep*) and capsid structural proteins (*cap*). The Rep protein mediates site-specific integration of the AAV genome into the AAVS1 site of chromosome 19 [364,365]. Thus, AAV is unique in a sense that it integrates into a specific location of the chromosome, evading potential site-specific mutagenesis seen with other viral vectors.

To date, 8 AAV serotypes of human and simian origin have been isolated (AAV1-8)[366]. The serotypes maintain identical genomic organisation, but differ somewhat in their coding sequences. A major consequence is that they utilise different receptors for cell attachment and entry, thus each serotype has characteristic transduction patterns in different tissues [366]. The cell surface receptors responsible for binding and uptake of some of the AAV serotype vectors have been elucidated. Thus, AAV2 binds $\alpha_v\beta5$ integrin, fibroblast growth factor receptor-1 and heparan sulfate proteoglycan [367-369]; whereas AAV4 binds $\alpha2$-3 O-linked sialic acid [370] and AAV5 binds N-linked $\alpha2$-3 sialic acid and platelet-derived growth factor receptor [370-372]. Importantly, as they are bound by different cell-surface-receptors, the serotype vector of choice for each tissue may vary.

Superior safety profiles, high particle stability and broad host ranges render AAVs one of the most promising gene therapy vectors. A major drawback of AAV vectors however, is the small transgene capacity; 5 kB is considered to be the upper limit to be packed by a single virion [373]. Currently, 33 clinical trials are underway using AAV vectors (source: http://www.wiley.co.uk/genmed/clinical/). Many of these target neurological disorders such as Late Infantile Neuronal Ceroid Lipofuscinosis, Alzheimer’s Disease, Temporal Lobe Epilepsy, Parkinson Disease and Canavan Disease.

Initial studies have obtained proof of principle for AAV2-mediated HSV/*tk*-treatment of GBM xenografts [374,375]. In the study by Mizuno et al., U-251-SP gliomas transplanted into nude mice completely regressed after three AAV/*tk* injections followed by gancyclovir treatment. Six of seven mice became long time survivors at the end of the experiment (120 days), whereas all control animals died at 45 days post-implantation. However, a more recent report has shown limited efficacy of AAV2/*tk* on intracranial tumour growth, even when transduction efficiencies up to 39 % were initially observed [376]. The absence of therapeutic

benefit has mainly been attributed to the gradual loss of the *tk* transgene, which has been observed both *in vitro* and *in vivo*.

Recently, gene delivery by AAV serotypes other than AAV2 has been evaluated in more representative *in vitro*- and *in vivo* model systems based on human GBM biopsy material (paper III). In these studies, a proof of principle for the utility AAV serotypes 1 and -6 for gene transfer to glioblastoma tissue was shown. Importantly, AAV showed better penetration abilities in both model systems than Ad type 5 [139]. Among the different serotypes of AAVs examined, infection by AAV2 led to the most efficient transduction of glioma cell lines (paper III and paper IV). However, these studies and those of others [377-379] show that the intracerebral delivery of AAV1 results in more widespread transduction in the CNS than does AAV2 or AAV5.

In order to be considered for the treatment of human malignant gliomas, AAV vectors need to be further developed. Important modifications that have to be made relate to modification of the vectors to render them tumour-selective and to persist for prolonged periods in dividing tumour tissue, as well as the expansion of transgene packaging capability.

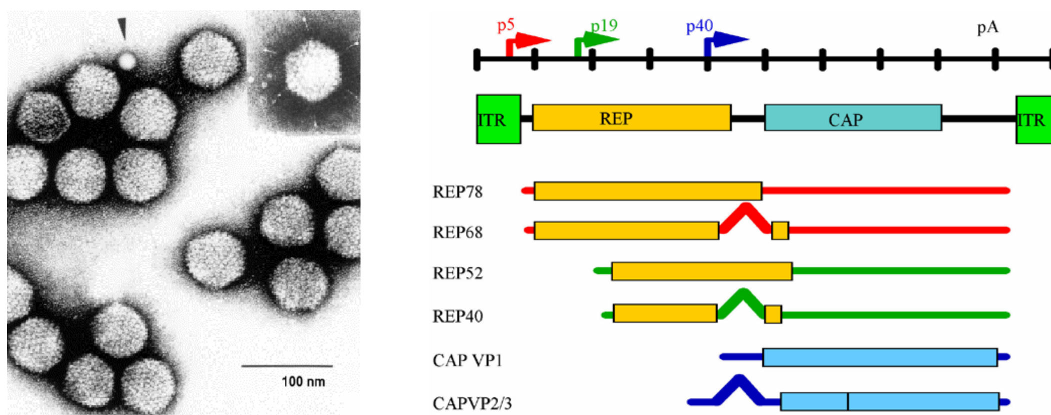


Figure 6. AAV. The figure to the left shows an AAV particle (arrowhead) in an adenoviral stock. The size difference between the two viruses may explain why AAV vectors better penetrate solid tissue and show a more widespread distribution in vivo than larger vectors such as Ads or HSV. Genomic- and transcriptional organisation of AAV (right). The ITRs encompass the rep and cap genes, which code for the replicase and capsid proteins, respectively. Four rep proteins and three capsid component proteins are generated after alternative splicing of the primary mRNA transcripts. Two of the replicase mRNAs are transcribed from the p5 promoter and two from the p19 promoter. All capsid-encoding transcipts are transcribed from the p40 promoter. The size of the AAV2 genome is about 4.7 kb.

4. AIMS OF THE CURRENT STUDY

Based on the introductory paragraphs describing the potentials as well as limitations of the viral gene transfer and angiogenesis inhibiton strategies, the following aims were outlined:

I.

To assess the effects of endostatin treatment on vascular physiology, tumour perfusion and tumour growth as well as to evaluate the potential utility of cell encapsulation therapy for malignant gliomas. Dorsal skin-fold chambers and cranial windows were prepared containing a glioma spheroid together with alginate-bioreactors secreting human endostatin. The morphology, density, functionality and the diameters of the developing blood vessels were studied by fluorescent intravital microscopy in the endostatin-treated animals and compared to control animals which received alginate beads without producer cells.

II.

To compare the anti-vascular effects and anti-tumour efficacy of human and murine endostatin in an intracranial rat glioma model. In this study, BT4C gliosarcoma cells were initially transduced by retroviral vectors encoding the human- or murine endostatin cDNA, selected to obtain a homogenous population and implanted intracerebrally into BDIX rats. Tumour volumes, blood-plasma volumes and vessel permeabilities were studied by functional magnetic resonance imaging. Vascular area fractions and microvessel densities were quantified after endothelium-specific immunostaining of the tumour sections. In addition, survival time lengths for the two treatment groups and the controls were compared.

III.

To compare and quantify the transduction efficacies of adeno-associated virus serotype 1 (AAV1) and AAV2 in a panel of human glioma cell lines *in vitro*. To assess the spread of AAV1 and AAV6 vectors in multicellular tumour spheroids prepared from patient biopsy material. To characterise the pattern of gene delivery by recombinant AAV1 and AAV6 vectors to intracranial glioblastoma xenografts and to assess the ability of these vectors to tranduce the host rodent brain.

IV.

To evaluate the transduction efficacies of recombinant AAV2, AAV4 and AAV5 vectors in a panel of human glioma cell lines. Furthermore, to investigate the abilities of these vectors to transduce GBM biopsy spheroids *in vitro* as well as intracranial GBM xenografts *in vivo*.

5. DISCUSSION

5.1. Experimental design

5.1.1. Tumour models

The main objective of papers I and II was to evaluate the effect of recombinant endostatin on angiogenesis and tumour growth in experimental gliomas. The conclusions of the studies are drawn from two different *in vivo* models and involve the use of basic molecular biology and cell culture techniques. In paper I, C6 glioma cells were implanted subcutaneously as well as intracranially, to allow for continuous observation of tumour-induced angiogenesis through dorsal skinfold-and cranial window preparations. Since the effect of endostatin has been observed to vary in tumours that grow at different anatomical locations, two different sites of tumour cell implantation were employed.

In paper II, BT4C gliosarcoma cells were delivered to the corpus callosum region, to establish intracerebral lesions at the site where many human gliomas manifest. Both cell lines display predictable tumour take and reproducible growth rates [380,381]. The BT4C cell line is syngeneic in BD-IX rats [382,383], whereas the C6 glioma cell line was induced in a strain of outbred Wistar rats, and is therefore only tumorigenic in immuno-compromised hosts [381]. Since both cell lines give rise to highly vascular lesions *in vivo* [384,385], these models are well-suited to evaluate therapeutic approaches based on angiogensis inhibition.

Disappointing results from clinical follow-ups of successful pre-clinical gene therapy trials are thought to be a consequence of inadequate transduction efficiency and the use of inappropriate pre-clinical model systems [130,326]. In many respects, cell-line based tumour grafts fail to represent the lesions encountered in the clinical setting [130]. However, there are model systems that preserve the biological heterogeneity of the cancer *in situ*. The GBM biopsy xenograft model [131], employed in papers III and IV is a favourable choice for evaluating gene transfer strategies for the treatment of glioblastomas. GBM spheroids that are generated directly from patient biopsies are not cultured as monolayers, and therefore retain the phenotypic features, as well as the same DNA ploidy and a similar percentage of proliferating cells as the original patient tumour [133]. The lesions that develop after intracerebral implantation of biopsy spheroids in nude rats display regions of necrosis as well

as microvascular proliferation (Figure 5, paper III); and importantly, diffuse single-cell infiltration into the host brain tissue is observed.

5.1.2. Viral vectors

MMLV vectors

In paper II, recombinant vectors based on Moloney murine leukaemia virus were employed to deliver the endostatin gene to BT4C glioma cells. The limitations of using murine retroviruses for *in vivo* gene transfer are the low particle numbers (titres) of the generated vector stocks and the observed inactivation of the viral particles in body fluids [322]. However, these limitations do not affect their application in tissue culture. For our purpose, retroviral transduction was chosen since it results in stable integration of the transgene sequences into the host genome. This ensures long-term expression of the transgene and continuos propagation of the expression cassette to daughter cells. Since implanted tumour cells undergo a vast number of cell divisions before they give rise to macroscopic lesions, chromosomal integration was important in order to maintain persistent and uniform endostatin levels in the tumours. After selecting for the neomycin resistance gene, nearly 100 % of the cell population was transgene positive, as observed for BT4C cells infected by EGFP-encoding retroviruses with identical vector backbones (paper II, figure 1C). Endostatin production in the BT4C ENDO cells was validated by western blot analysis of the conditioned media. Colonies were expanded from single-cell clones to ensure that the implanted cells had the retroviral cassette inserted into the same genomic location. This was essential in order to avoid possible variations in the levels of transgene expression between the tumour cells implanted into each animal. The different resistant clones secreted highly varying levels of endostatin. If not eliminated, such differences would be amplified after multiple cell divisions *in vivo*, giving rise to tumours-or tumour regions with varying endostatin expression. In this case, the therapeutic effect would not be uniform and would greatly enhance variation within the treatment groups. In addition, the cloning procedure facilitated the isolation of the tumour cell clone that secreted the highest amounts of endostatin, which was selected for the *in vivo* experiments.

AAV vectors

While a retroviral vector system was applied as an *in vitro* tool in paper II, in papers III and IV the objective was to gain a proof of principle for the utility of AAV vectors as gene delivery vehicles *in vivo*.

The failure of vector particles to infect sufficient number of cells in the target lesions is believed to be the main explanation for the disappointing results of gene therapeutic approaches in the clinical setting [321]. Three factors are thought to limit the spread of viral particles in the tumour tissue: the mobility of the viral particles in the tumour mass, the extent of host immune responses which may block virus dissemination, and the limited duration of lytic effect by replicating vectors [386]. Our current knowledge of vector biology suggests that AAVs may be the most suited gene delivery vehicles to address the problem of limited tissue penetration, since they are about one-fifth to one-tenth in diameter of other commonly employed viral vectors [387]. In addition, AAVs exhibit very low immunogenicity [388], and in the case of present neutralising complement in the patient, vectors of alternative serotypes may be used. The application of AAV vectors achieves persistent transgene expression in animal models of several diseases, such as haemophilia [389], retinal dysfunction [390,391] and several neurological disorders [392]. For example, vision was restored for the entire follow-up time of three years in a canine model of childhood blindness after subretinal administration of AAV vectors [391].

5.2. Experimental findings

5.2.1. The therapeutic effect of endostatin

Anti-tumour efficacy of endostatin

To, date almost 800 papers have been published concerning the structure and function of endostatin. The vast majority of the experimental studies confirm endostatin's inhibitory effect on tumour growth, in a variety of cancer types [271]. Endostatin has entered the clinical phase of development, and phase I trials suggest that endostatin administration is safe in humans [393-396]. To gain a proof-of-principle for the utility of this molecule for the treatment of brain tumours, we have applied endostatin in two different glioma models, a syngeneic BT4C-model and in the C6 model implanted in an orthotopic- or ectopic setting. We have observed clear anti-tumour effects with this inhibitor. However, the efficacy was discrepant, varying between the cell lines used as tumour models, the anatomical location of the lesions, the delivery system, as well as the species-specific form.

In paper I, recombinant human endostatin secreted by bioreactors implanted at the tumour site was shown to significantly inhibit C6 glioma growth in the subcutaneous setting. In contrast, in the cranial window model, the differences between the treatment group and the controls were not significant.

The discrepancy may be attributed to the fact that in the dorsal tumours, endostatin`s effect was most pronounced at day 14 and onward at which time point the tumours overgrew the available space in the cranial windows. Thus, the animals with cranial windows were sacrificed before endostatin could exert its effects. Another study underlines that tumour cells transplanted to the orthotopic location are more aggressive, reaching critical size before sufficient levels of the inhibitor are achieved [275]. In addition, tumour-host interactions, such as angiogenesis are organ-specific, varying between ectopic- and orthotopic locations [397], further contributing to the discrepancy observed.

In paper II, local delivery of human endostatin did not result in significant tumour size reduction and only a trend toward longer survival was seen. This is in contrast to the observations in paper I as well as in previous publications [303], where human endostatin delivered by alginate bioreactors resulted in significant inhibition of tumour growth. The differences in outcome may be attributed to the fact that the alginate bioreactors released higher amounts of endostatin than did the BT4C ENDO cells [303] (paper II and unpublished observations). Therefore, critical endostatin levels may not have been reached in the BT4C model in this setting. Still, some therapeutic effect by human endostatin was observed in the BT4C model in paper II, as large necrotic areas were present in the tumours treated with this endostatin form. Here, murine endostatin showed more potent anti-vascular and anti-tumour effects, which were comparable to that obtained with human endostatin in paper I and elsewhere [303]. In paper II, we show that at similar concentrations, murine endostatin has significant anti-tumour effect, whereas human endostatin treatment only shows a trend toward tumour inhibition. This might relate to the fact that the murine endostatin protein is more sequence-similar to its rat counterpart (difference: 8 amino acids) than is human endostatin (difference: 25 amino acids). Thus, more favourable interactions with the rat receptor and thus more potent signalling by murine endostatin is expected in our model.

Anti-vacular effects of endostatin

The anti-vascular effects of endostatin have been reported to relate to reduced vascular densities *in vivo*, increased apoptosis- and reduced endothelial cell proliferation *in vitro* [259,398-400]. In papers I and II, we studied endostatin-treated vasculature in experimental gliomas. This included analysis of vascular density, morphology and functionality. Whereas markedly dilated blood vessels were present in the control BT4C tumours (paper II), such vessels were not seen in the endostatin-treated lesions.

Immunostaining for blood vessels and subsequent assessment of microvessel density counts confirmed that endostatin-treated tumours were more scarcely vascualrised. It is worth noting, however, that reduced vessel densities alone do not necessarily predict a reduction in tumour perfusion, since this may be compensated for by an increase in vessel functionality (more vessels perfused) as well as an increase in vessel diameters. Therefore, we undertook studies involving fluorescent intravital microscopy (paper I) or functional MRI-analysis (paper II) to assess these parameters.

In paper I, tumour vascular density, microvessel diameters and vascular surface area fractions were significantly reduced after endostatin treatment. However, vessel permeability did not seem to be affected. In paper II, we show that murine endostatin significantly reduced plasma volume fractions, vascular area fractions and microvessel density counts. Both studies revealed that neoplastic blood vessels that have been treated by endostatin have smaller diameters and are more scarcely distributed throughout the tumour tissue than in non-treated controls. Importantly, tumour perfusion is impaired in the endostatin-treated tumours. As a consequence, necrotic areas develop as seen both on MR-images and on histological sections. Taken together, when sufficient levels of endostatin are reached in these experimental glioma models, vascular inhibition is achieved, offering the possibility of local tumour growth control.

5.2.2. Transduction of tumour cell lines by AAV

In papers III and IV, we sought to evaluate the abilities of recombinant AAV vectors of various serotypes to deliver reporter genes to human glioma cells and xenografts. We infected a panel of human glioma cell lines with AAV serotype vectors containing either the CMV promoter (AAV1 and AAV2, paper III) or the RSV promoter (AA2, AAV4 and AAV5, paper IV). In both studies, AAV2 proved to be the most efficient transducer of all the glioma cell lines. Transduction efficiency correlates with AAV serotype-specific uptake but also with intracellular processing mechanisms characteristic for the cell phenotype [401]. The molecular steps that characterise AAV infection have been intensively studied, and recently reviewed [402,403]. When added to target cells, AAV particles bind to primary attachment receptors and co-receptors before they are taken up by endocytosis in clathrin-coated pits [404,405]. Inside the cell, several successive steps involve AAV trafficking to the nucleus through the endosomal compartment [403]. Subsequently, viral uncoating and conversion of the single-stranded AAV genome to double-stranded replicative form takes place. In the absence of integration, AAV genomes reside as head-to-tail concatamers of over 12 kbp [406], which are responsible for long-term transgene expression.

The most significant factor affecting initial binding of the AAV particles is the distribution of serotype-specific cell surface receptors. Internalisation in clathrin-coated pits may be influenced by the abundance of serotype-specific co-receptors. Both primary attachment recpetors and co-receptors are required for AAV uptake, and the availability of these defines the host-range of a specific AAV serotype [403]. It has become increasingly clear that correct endosomal processing of AAV particles are important for transduction efficiency [403]. Entry of the AAV particles into the nucleus and subsequent conversion to transcriptionally active double-stranded form are both rate-limiting steps for transgene expression [403,407,408]. The rate of nuclear entry has been shown to diverge between permissive and non-permissive cell types [404,407,408].

Thus; from our observations, we suggest that the AAV2 vectors are taken up with greater efficiency through the plasma membranes and are processed more efficiently than AAV1 or AAV4 and AAV5 (paper IV) in glioma cell lines. For both AAV1 and AAV2 (paper III), transgene expression was enhanced after a ten-fold increase in multiplicity of infection. This may indicate that the levels of AAV-specific cell surface receptors was not the limiting factor for transduction at the lower vector concentrations. However, the more modest enhancement for AAV2-mediated transgene expression may relate to the fact that the receptors responsible for uptake of these particles were saturated at the highest amount of particles added. Our data show that the D37 and U373 cell lines were the most permissive for AAV serotype 2, whereas GaMg was poorly transduced. Analysis of αvβ5 integrin expression (co-receptor molecule for AAV2) in these cell lines correlates well with our transduction data. Several studies confirm that U373 has a high expression of this integrin [409-411]. In paper III and previous studies [139], A172 was poorly transduced by AAV2, whereas in paper IV it was transduced at high efficiency. Previous studies of integrin expression in this cell line suggest variable expression levels of this integrin form [409,410,412], possibly resulting in variations in its permissiveness for AAV2 between the experiments.

The receptors and the co-receptors for AAV2, AAV4 and AAV5 have been identified [367-372], whereas for AAV1, the host receptor remains to be found. Quantitative analysis of the known AAV receptors and co-receptors in glioma cell lines may provide better insight into the relationship between transduction efficiency and the expression of AAV-specific cell surface molecules.

It should be emphasised that the transduction figures may only be compared within the frame of the individual study, based on the different experimental set-ups. This includes the differences in the promoter- and enhancer sequences in the vector backbones, the viral particle numbers added, the time period of culturing before analysis and the passage number

of the cell lines. The vectors were purified using slightly different protocols, which means that they may vary in their infectivity, even when the number of vector genomes is the same.

As mentioned above, the expression of serotype-specific receptors is important, however not exclusive when trying to predict transduction effciency of a given cell type with a certain AAV vector. The rate of uptake and intracellular processing of AAV vectors in glioma cell lines will need further elucidation to explain the results obtained in the present study.

5.2.3. Tranduction of GBM xenografts by AAV

The dissemination of a viral vector stock after direct delivery to solid tumour tissue depends on the mobility of the viral particles within the tumour mass as well as the extent of anti-viral immune responses [386]. The cell density in the tumour mass is usually much higher than in normal tissues, due to high proliferation rates and to the loss of contact inhibition, making it less permeable to viral particles [386]. In addition, host-derived fibrous tissue barriers may effectively limit vector dissemination [413].

The distribution of AAV vectors upon injection into solid tumours has not been studied in great detail. However, previous work from our group has indicated that intratumoral injection of AAV vectors results in a more widespread transgene expression than what is achieved after injecting a non-replicating adenoviral vector in the same setting [139]. While reporter gene expression after injection of an adenoviral vector stock was limited to the tumour periphery, AAV-mediated transgene expression was found in central tumour regions. We suggest that the observed facts relate to the difference in the size of the AAV particles vs. the Ad particles (20 nm vs.100 nm), enabling AAVs to penetrate solid tumour tissues more effectively. Furthermore, the distribution of the endogenous Ad receptor, CAR, is generally low in glioma biopsy tissue [414], whereas the known AAV receptors are widely expressed [414-417].

In studies III and IV, we injected recombinant AAV vectors into glioblastoma tumours growing in the rat brain to study vector spread and possible differences in the host ranges between the AAV serotypes. As for AAV2, we observed that AAV1 particles transduce central regions of the tumour tissue (paper III and paper IV). However, in this case transgene expression was more scattered in the central tumour regions compared to the periphery, where more robust reporter gene expression was seen (figure 5, paper III). The observed expression pattern may relate to the fact that the interstitial pressure in experimental tumours is elevated in central regions, with the pressure gradient falling toward the periphery [418]. Thus, viral particles as well as other macromolecules are expected to diffuse toward the periphery after intratumoral injection.

This effect relates to particles that do not have their attachment molecules widely distributed in the targeted tissue, thus precluding immediate binding and uptake at the site of injection [419]. Conversely, the widespread distribution of AAV2 receptors and co-receptors in the mammalian CNS may explain the restricted tissue transduction in the vicinity of the needle track observed for these vectors [420](paper IV). Other studies confirm that AAV1 transduces larger tissue volumes in the CNS than does AAV2 [378,379,421], indicating that AAV1 particles are not bound and internalised at the vicinity of the injection site. To date, the cell-surface molecules responsible for AAV1 binding have not been identified, precluding the assessment of their receptors in the CNS and in GBM xenografts.

In our setting, the time of vector injection was three weeks post tumour spheroid implantation. At this time point, vascularised tumours had not yet developed, as no lesions could be discerned on magnetic resonance images. After AAV injection, the xenografts grew for two to four months (depending on the aggressive nature of the patient biopsy) before tumour-related symptoms appeared. In our experimental set-up, we anticipated that the AAV-infected tumour cells will pass on their transgene to daughter cells, and thus reporter gene expression should be achieved when the lesions have reached macroscopic sizes. In the majority of cases, however, transgene expression was disappointingly low, as only a few scattered cells were positive within most of the examined tumours. A brief discussion of the possible mechanisms for the low rates of gene transfer is provided below.

Episomal persistence of AAV genomes

In contrast to wild-type AAVs, recombinant AAV (rAAV) vectors do not display replicase (Rep)-mediated integration into the host cell chromosome [422], albeit non-site specific integration events have been encountered [423]. Prolonged expression of rAAV-delivered transgenes in quiescent tissues is mainly attributed to episomal persistence [406]. In dividing cells, chromosomally integrated vector genomes may be propagated to daughter cells, whereas episomal genomes are more likely to be lost. In a previous study, the rate of chromosomal integration by recombinant AAV vectors in dividing cells *in vivo* was studied after portal vein injection of AAV2 particles carrying the gene for human coagulation factor IX (F.IX) [424]. Upon hepatectomy and subsequent liver regeneration, the animals that carried the stably integrated transgene (delivered by a transposon) maintained factor IX expression at the same levels as before, whereas 85-95 % of the AAV-delivered transgene was lost. This number reflected the amount of cells containing episomal AAV genomes.

In this model, only a limited number of cell divisions occurred, leading the authors to argue that the 5-15 % of integrated vector genomes represents a maximum number. In growing tumours, however, numerous cell divisions occur before the lesions reach macroscopical sizes; thus, the transgene may be even further diluted. In a recent report [376], Hadaczek and colleagues studied the persistence of thymidine kinase expression from an AAV2-cassette in U87 glioma xenografts [376]. The authors observed loss of transgene expression both when the glioma cells were transduced and selected *in vitro* before transplantation as well as when the AAV vector was delivered *in vivo* to growing tumours. Gancyclovir administration yielded only a modest survival benefit in these animals, suggesting that loss of the *tk* gene resulted in the shortfall of suicide effect. In another study, where AAV2-GFP vectors containing the CMV or the EF-1 promoter were injected into experimental pancreatic carcinomas, only a few cells were transgene-positive [420]. The authors argue that vector dilution may cause the observed poor transduction efficacy. These studies point to the same conclusion: AAV genomes are lost during the multitude of cell division steps encountered in growing tumours. The correction of this trait by molecular strategies will be necessary if this vector system is to be considered for therapeutic application in humans. For example, developments in vector construction have made possible the inclusion of the *rep* gene into second-generation AAV-based gene therapy systems [425]. This would ensure site-specific integration of the AAV genome into the *AAVS1* in chromosome 19 and continuous propagation to daughter cells, increasing transduction efficiency [426].

Gene silencing

Gene silencing may have contributed to the loss of reporter gene-positive cells in the tumour grafts. In paper III, the vectors contained the cytomegalovirus immediate early promoter (pCMV), whereas in paper IV, the Rous sarcoma virus promoter (pRSV) was employed. Both sequences are able to direct constitutive gene expression in a number of cell types, with the CMV sequences displaying stronger transgene expression *in vitro* and *in vivo* [427,428]. However, the CMV promoter may be silenced by hypermethylation [429]. Silencing of the CMV promoter has previously been described in the context of AAV for gene delivery to human cancer cells *in vitro* as well as *in vivo* [430]. This problem can be circumvented by applying promoters that are not subjected to hypermethylation or to other silencing mechanisms, which substantially improves the persistence of transgene expression [431-433] [430].

Recent data suggests that the AAV vector genome in the cells is in the histone-associated chromatin form, which is capable of superior transcription [434]. However, histone

deacetylation efficiently silences transcription from AAV genomes [434]. Histone deacetylase inhibitors may improve tumour cell transduction by enhancing the acetylation of the histone-associated chromatin of the rAAV genome [434].

6. FUTURE PROSPECTS

Today, modern biotechniques provide us with tools to get a functional insight into living systems. More than before, we are enabled to understand the molecular complexity of the regulatory mechanisms and signalling pathways that drive tumour progression. Many specific as well as compensatory mechanisms exist within cancer cells and in the host environment that favour malignant growth. Therefore, inhibition of one particular pathway can often trigger compensatory changes, which counteract the desired therapeutic effects [435]. However, in a cell-signalling network there are key molecules that integrate several signal transduction pathways. Such integration sites may be critical for cellular function. A main objective should be to identify vital components within the cancer cell signalling networks that can be abolished without destroying normal tissue function. After discovery, these components may be targeted on the protein- or the mRNA level (i.e. gene silencing strategies), in the framework of cell-based or viral delivery systems.

In gene therapy, the lack of effect observed in clinical studies has been attributed to a low transduction efficacy, as well as lack of delivery to distant tumour sites [321,326]. Novel vectors have been developed with the ability to replicate within transformed cells, yielding excellent tumour-specificity. Malignant cells are destroyed by oncolysis, while nearby untransformed cells are spared [436]. Still, such conditionally replicating vectors have been based on larger size viruses such as adenoviruses and retroviruses that have limited penetration capacities in solid tumour tissues. AAV vectors, on the other hand, have been shown to spread and transduce host tissues more extensively [139], however their persistence is poor in rapidly dividing cancer cells. Therefore, one would need to capitalise on the beneficial traits of several viral vectors to develop new generation gene delivery systems. Better delivery to the tumour may also be achieved by additional treatments to induce penetrability (ex.g. by proteases) or by altering the physical relationships, such as by convection-enhanced delivery. Hopefully, further refinements will provide clinicians with more potent vector systems that have the capacity to penetrate the tumour tissue while only replicating in neoplastic cells.

Cell-based therapy with encapsulated producer cells makes possible the sustained release of therapeutic proteins for prolonged time periods, enabling us to maintain high local tissue concentrations of substances with a short biological half-life, such as endostatin.

Moreover, such producer cells can be genetically engineered to secrete other substances, such as differentiation factors, which could induce the alteration of primitive tumour cell phenotypes toward more benign, differentiated forms. Finally, based on extensive biological information obtained from patient biopsies, treatments may be individually tailored using genetically engineered cells that deliver active substances fitted to the biological profile of the tumour. The same pathways or molecules may be targeted using viral vectors, which may deliver the gene of interest. When these factors are brought into play with a concerted action, progress can be made.

7. REFERENCES

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Appendix

Table I. Classification of the tumors of neuroepithelial tissues[=]

Designation of the tumor group	Constituent neoplastic cells	WHO grades of tumor types*	Characteristics of the tumor
Astrocytic tumors	astrocytes	I: pilocytic astrocytoma II: astrocytoma III: anaplastic astrocytoma IV: glioblastoma multiforme	more diffuse infiltration and increasing cellularity with increasing grade, micro-vascular proliferation and necrosis in gr. IV
Oligodendroglial tumors	oligodendrocytes, occasionally mixed with astrocytes	II: oligodendroglioma, mixed oligoastrocytoma III: anaplastic oligodendroglioma	highly vascular, diffusely infiltrating lesions, pleomorphism is characteristic for the anaplastic type
Ependymal and choroid plexus tumors	ependymal cells and cells of the choroid plexus respectively	I: ependymoma II-III: anaplastic ependymoma	circumscribed lesions, rarely infiltrating, perivascular rosettes are characteristic
Pineal cell tumors	mature pineal parenchymal cells (pineocytoma) and pineal precursor cells (pineoblastoma)	II: pineocytoma III: pineoblastoma (rare)	moderately cellular with delicate connective tissue stroma (gr. II); increased cellularity, mitoses and necrosis in gr. III
Neuronal tumors	mature ganglion cells, occasionally with glial or neural elements	I: gangliocytoma II: ganglioglioma III: anaplastic ganglioglioma	low cellularity and highly differentiated cells exhibiting well-delineated growth; in gr. III, the glial components show features of anaplasia
Poorly differentiated and embryonal tumors	undifferentiated cells	IV: medulloblastoma, neuroblastoma, ependymoblastoma	rapid growth, often well-delineated, high cellularity

[=] a modified version of the classification presented in Neuroradiology (Zulch, 1980) * not all tumor types of each group are included

Table II. Genes often mutated during the progression of secondary glioblastomas

Gene product	Function of the gene product	Stage of malignancy*	References
TP 53 (mutated or lost)	-arrest of cell in G1 phase of the cell cycle -initiation of DNA repair -induction of apoptosis -promotion of cellular differentiation	gr. I-IV	Sidransky et al., 1992 Louis, 1994 Watanabe et al., 1996 Fulci et al., 1998 von Deimling et al., 2000
PDGF-A (overexpression)	mitogen for a variety of fibroblast-like cells	gr. I-IV	Hermanson et al., 1992 Westermark et al., 1995
PDGFR-α (overexpression)	receptor for PDGF	gr. I-IV	Hermanson et al., 1996 Galanis et al., 1998 Smits et al., 1998
RB1 (mutated or lost)	tumor suppressor gene	gr. II-IV	Henson et al., 1994
CDKN2A (mutated or lost)	tumor suppressor gene	gr. II-IV	Arap et al., 1997 Von Deimling et al., 2000
EGFR (amplified)	mitogen for a variety of cells	gr. IV	von Deimling et al., 1992 Galanis et al., 1998 Frederick et al., 2000

PDGF-A: Platelet Derived Growth Factor-A, PDGFR-α: Platelet Derived Growth Factor Receptor-α, RB 1: Retinoblastoma 1
CDKN2A: Cyclin-Dependent Kinase Inhibitor 2A, EGFR: Epidermal Growth Factor Receptor

* The stages of astrocytoma malignancy where mutation in the gene coding for this protein is usually found

Table III. Growth factors involved in the angiogenesis of gliomas

Growth factor	Mechanisms of angiogenic effect	References
VEGF	Mitogen to endothelial cells, induces plasminogen activator and plasminogen activator inhibitor.	Plate at al., 1992 Plate et al., 1994 Plate et al., 1997
aFGF and bFGF	Mitogen and chemotactic to endothelial cells.	Stefanik et al., 1991
PDGF	Stimulates the proliferation, cord formation and morphogenesis of endothelial cells.	Hermanson et al., 1988
EGF	Stimulates endothelial cell motility and proliferation. Stimulates the VEGF production in glioma cells	Goldman et al., 1993 Zagzag et al., 1995
TGF-α	Mitogen to endothelial cells, has a 40 % sequence identity with EGF. Competes with EGF for binding on endothelial cells.	Tang et al., 1997
TGF-β	Angiogenic *in vivo*, but inhibits the growth of endothelial cells *in vitro*.	Jennings et al., 1997 Jensen, 1998
Angiopoietin-1	Remodeling of vasculature. Possibly involved in regulation of endothelial-matrix interactions	Stratmann et al., 1998
Scatter factor (SF)	Mitogen to endothelial cells. Activates latent matrix metalloproteases	Moriyama et al., 1999 Lamszus et al., 1999

aFGF: acidic fibroblast growth factor, bFGF: basic fibroblast growth factor, VEGF: Vascular endothelial growth factor, PDGF: Platelet derived growth factor, EGF: Epidermal growth factor, TGF-α: Transforming growth factor α, TGF-β: Transforming growth factor β

Table IV. A selection of anti-angiogenic compounds tested in *in vivo* brain tumor models

Angiogenesis inhibitor (source)	Mechanism	Results	References
Polyclonal bFGF antibody	Binds bFGF, inhibits bFGF mediated angiogenesis	Significant reduction of tumor mass and vascularity	Stan et al., 1995
Angiostatin (endogenous)	Specific inhibitor of EC proliferation	Significant inhibition of tumor growth, reduction of vascularity, apoptosis	Kirsch et al., 1998
Endostatin (endogenous)	Inhibition of EC proliferation, leads to apoptosis	Reduction in intratumoral blood flow and tumor size, apoptosis	Read et al., 2001 Sørensen et al., 2002
Suramin (synthetic)	Inhibits the actions of several growth factors (bFGF, PDGF)	No growth retardation in cerebrally implanted glioma; growth inhibition of subcutanously implanted glioma	Takano et al., 1994 Olson et al., 1994 Bernsen et al., 1999
Soluble truncated VEGF receptor (synthetic)	Binds VEGF, inhibits VEGF induced angiogenesis	Significant increase of survival	Goldman et al., 1998
TNP-470 (synthetic)	Inhibits of EC proliferation by interfering with DNA synthesis	Reduced tumor growth and vessel density	Taki et al., 1994 Bernsen, 1998
$VEGF_{165}$-DT_{385} (synthetic)	Inhibition of EC proliferation	Significant inhibition of tumor growth	Olson et al., 1997
Monoclonal antibody A.4.6.1. specific for VEGF (synthetic)	Inhibits EC growth, abrogates the effect of secreted VEGF on EC	80 % decrease in tumor weight after 4 weeks	Kim et al., 1993

EC: Endothelial cells, TIMPs: Tissue inhibitors of metalloproteinases, bFGF: basic Fibroblast growth factor, VEGF: Vascular endothelial growth factor, $VEGF_{165}$-DT_{385}: VEGF bound to truncated Diphteria toxin, TNP-470: (3R,4S,5S,6R)-5- methoxy-4-[(2R,3R)-2-methyl-3-(3-methyl-2-butenyl)-oxiranyl]-1-oxaspiro[2,5]oct-6-yl(chloroacetyl) carbamate