15-LIPOXYGENASE-1 IN GENE THERAPY OF MALIGNANT GLIOMA

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Successful clinical trials for malignant glioma treatment have recently drawn attention to the potentials of gene therapy. 15-lipoxygenase-1 (15-LO-1) is known to inhibit tumor angiogenesis as well as enhance apoptosis, thus potentially acting destructively on tumor cells via two independent mechanisms. Therefore, it is a promising enzyme that could be used to treat malignant glioma. Nevertheless, because of the nature of malignant gliomas, it is believed that no single therapy is able to combat this fatal disease. Thus, this study investigated whether Ad15-LO-1 gene therapy alone, or in combination with herpes simplex thymidine kinase suicide gene therapy (AdHSV-tk) could benefit the survival of rat malignant glioma model.

In this study BD IX male rats were injected with 10^4 BT4C malignant glioma cells into the right corpus callosum and transduced 14 days after cell inoculation with multiple injections of Adh15-LO-1 (1.3×10^{12} vp/ml) or one of two combinations therapies of Adh15-LO-1 and AdHSV-tk (1.45×10^{12} vp/ml), which were then followed by 14 days of ganciclovir treatment (50mg/kg). Results in this study show that neither of the therapies improved inhibition of tumor growth nor benefited the survival. However, in a study group number two, where Ad15-LO-1 and AdHSV-tk/GCV were applied simultaneously a profound effect on the migratory properties of the tumor cells was observed. The treatment induced a shift in glioblastoma tumor phenotype towards enhanced migration and infiltration into healthy brain parenchyma, represented by formation of multiple satellite tumors. Moreover, co-option of the host vasculature became a compensatory mechanism to combination therapy, as the tumor cells ensheathed preexisting vessels and traveled along them to invade healthy tissue.

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Agnieszka Pacholska

ABBREVIATIONS

COX	Cyclooxygenase
CNS	Central nervous system
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
EMEA	European Medicines Agency
eNOS	Endothelial Nitric Oxide Synthase
FDA	Food and Drug Administration
EPC	Endothelial progenitor cells
FGF	Fibroblast growth factor
GBM	Glioblastoma multiforme
GCV	Gancyclovir
GM-CSF	Granulocyte macrophage colony stimulating factor
H&E	Hematoxylin and eosin stain
HETE	Hydroxyeicosatetraenoic acid
HIF	Hypoxia-inducible factor
HODE	Hydroxyoctadecadienoic acid
HSC	Hematopoietic stem cell
HSV	Herpes simplex virus
HSV-tk	Herpes simplex virus thymidine kinase
IFN	Interferon
IL	Interleukin
in vitro	In an artificial environment outside the living organism
in vivo	Within a living organism
i.p.	Intraperitoneal
LO	Lipoxygenase
LOH	Loss of heterozygosity
MGMT	O-6-methylguanine-DNA methyltransferase
MMP	Matrix metalloproteinase
NO	Nitric oxide
PAS	Periodic acid-Schiff stain
PEI	Polyethylenimine

PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PlGF	Placental growth factor
PPAR	Peroxisome proliferator-activated receptor
RNA	Ribonucleic acid
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vp	Virus particle
VPC	Viral packaging cell
WHO	World Health Organisation

CONTENTS

1 INTRODUCTION

Glioblastoma multiforme (GBM) is the most malignant of brain tumours and one of the most fatal and refractory cancers in humans. It is highly resistant to any conventionally applied therapies. At the moment there is no curative treatment for GBM, there is a clear need for the development of new treatments.

Anti-angiogenic treatment for cancer is a novel, promising therapeutic approach that brings new hopes in glioblastoma treatment. 15-lipoxygenase-1 (15-LO-1) is a multifunctional enzyme able to produce a vast number of metabolites with diverse bioactivities. 15-LO-1 is known to prevent VEGF-A induced neovascularisation in rabbit eyes and skeletal muscle, suggesting that this could be used as a potential new treatment strategy (Viita et al., 2009). 15-LO-1 metabolites are also linked to promoting tumour cell apoptosis (Viita et al., 2012). Taken together, this data suggests that 15-LO-1 may exhibit anti-angiogenic and anti-tumourigenic properties and therefore could be a potent enzyme to be used for gene therapy of GBM.

The most challenging factor for the treatment of GBM is its vast genotypic and phenotypic heterogeneity. It has been widely acknowledged that no single treatment is likely to eradicate highly malignant tumours and any therapeutic strategy should be further enhanced with other treatment modalities to improve its efficiency. One of the most successful gene therapy clinical trials for malignant is suicide gene therapy. Sitimagene ceradenovec is a herpes simplex virus thymidine kinase (HSV-tk)/gancyclovir gene therapy developed by Ark Therapeutics Group plc. Recent results from a phase III clinical study demonstrated significant improvement on time of re-intervention or death in comparison to standard care treatment (van Putten et al., 2010). The study also revealed that the treatment is well tolerated without significant safety issues and quality of life deterioration.

The future of cancer therapeutics may lay in combined therapies that target various aspects of cancer growth. Thus, the combination of 15-LO-1 anti-angiogenic therapy with HSV-tk suicide gene therapy, is a potential treatment approach.

2 REVIEW OF LITERATURE

2.1 Cancer

Cancer is a collective name for a group of diseases that features uncontrolled growth and spread of abnormal cells. It is the leading cause of death worldwide and undoubtedly a serious health problem. The World Health Organization (WHO) states that in 2008 alone there were 7.6 million of cancer deaths, accounting for roughly 13% of all mortalities. The same report predicts that the numbers will continue rising and in the year 2030 there will be 13 million cancer deaths worldwide (http://www.who.int/mediacentre/factsheets/fs297/en/). Cancer arises from a single cell and its transformation is caused by a mixture of environmental and genetic factors. Environmental factors involved in acquiring somatic mutations include radiation, chemical carcinogens and viruses. In most cases though, the carcinogenic element remains unknown (http://www.who.int/mediacentre/factsheets/fs297/en/).

Cancer as such is a very robust system, what means that it has the ability to maintain stable functioning despite wide range of external and internal stresses. It needs to sustain proliferation of tumour cells, which are under constant pressure from the surrounding microenvironment and human immune system. Cancers have the ability to maintain cellular heterogeneity. What this means, is that genetic heterogeneity is a major cause of acquired drug resistance and that the survival of only very small subpopulation of cancer cells may still give rise to recurrence of tumours or its metastasis. Thus, it is a main hurdle in designing successful cancer therapy.

In their milestone review, Hanahan and Weinberg identified six hallmarks of cancer (Hanahan & Weinberg, 2000). Those alterations in cell physiology include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Each of those changes in a cancer cell genotype is a step towards defeating natural human tumour protection mechanisms. In their most recent review, they have also characterized other two emerging hallmarks. One is the ability to evade the immune system and the other to reprogram energy metabolism. The acquisition of those features is made possible by two enabling

characteristics, that are the development of genetic instability and setting of tumour-promoting inflammation (Hanahan & Weinberg, 2011).

Cancers are heterogeneous and extremely complex diseases to cure. They are diverse and constantly adapting to the changes in their environment, what causes great difficulties in their management and urges the need for developing novel, potent therapy strategies. A great emphasis should be put not only on the development of therapeutics, but also on the prevention, detection and management of the disease to prolong patients' life and to improve its quality. Despite our extensive and still increasing knowledge in this field, we continue to fail in battling tumours. Nevertheless, thanks to the efforts scoping at understanding the biology of this multifaceted disease, some of the most common cancers have currently high cure rates. Unfortunately the prognosis for patients with gliomas, remains rather grim.

2.2 Glioma

Gliomas are the most frequent primary brain tumours in adults. They are invasive, highly aggressive and frequently infiltrate critical neurological areas within the brain. The aetiology of primary brain tumours is still unknown and the risk factors are unclear. The incidence rates of astrocytic and oligodendroglial gliomas are 5.27 and 5.17 new cases per 100 000 people per year respectively, while the incidence of glioblastoma is 3.55 per 100 000 persons per year (Oghaki & Kleihues, 2005).

Gliomas are classified according to their line of differentiation and subsequently graded on scale from I to IV, depending on the degree of malignancy they present. The most widely recognized is the WHO classification and grading system of tumours affecting the central nervous system (Louis, 2007). Gliomas are tumours of neuroepithelial tissue and are classified histologically as oligodendromas, astrocytomas or oligoastrocytomas, which are characterized by morphological features of the previous two cancer types. Astrocytomas, composed predominantly of neoplastic astrocytes, amount to 80% of all gliomas. More than 50% of them are the most malignant type of glioma that is glioblastoma multiforme.

Lesions, marked as grade I, have low proliferative potential and therefore can be cured by surgery alone. Benign grade II tumours are not curable by surgery only, as they tend to recur and may progress to higher grades of malignancy. Grade III neoplasms are malignant and usually lead to death in several years, despite radio- and chemotherapy applied. Finally, grade IV tumours are highly malignant, mitotically active and necrosis-prone neoplasms with rapid disease progression. Since they are usually resistant to chemotherapy and not sufficiently responsive towards other treatment options, they lead to rapid fatal outcome. Glioblastoma, most of embryonal neoplasms and many of sarcomas are examples of grade IV malignancies. Apart from the grade of the tumour there are also other factors, which are included when it comes to prognosis estimations. Survival is depending on the anatomic location of the tumour, extent of resection, proliferation indices, genetic alterations, age and health status of the patient. Those with grade II tumours, usually survive more than 5 years. The patients with grade III tumours are predicted to live 2-3 years, while the survival of patients with grade IV neoplasms depends mostly on the treatment applied (Louis, 2007).

2.2.1 Glioblastoma multiforme

Glioblastoma multiforme is the most malignant brain tumour and it is one of the most fatal and refractory cancers in humans. Glioblastomas are localized in the deep white matter of the cerebral hemispheres, manifesting itself in changes of personality followed commonly by headaches, nausea and epileptic seizures. It is important to mention that glioblastomas are poorly delineated. They are also characterised with central necrosis that may account for as much as 80% of total tumour mass. Last but not least, glioblastoma is particularly notorious for its rapid invasion of neighbouring brain structures, making it a difficult target for resection. Microscopically, GBM is a very poorly differentiated tumour of astrocytic origin. It is anaplastic with marked nuclear atypia and vigorous mitotic activity (Louis, 2007).

Several genetic mutations underlying formation of GBMs has been described. There are three main molecular alteration pathways associated with glioblastomas: loss of heterozygosity of chromosomes (LOH), loss of tumour suppressor genes and amplification of oncogenes. Loss of heterozygosity of specific chromosomal regions may predict patient outcome. For example LOH on 10q is the most frequent genetic alteration in glioblastomas and is associated with

poor survival rates (Oghaki & Kleihues 2005). When it comes to inactivation of tumour suppressor genes, the most common mutations occur in TP53 (31% of cases) and PTEN (24%) genes (Oghaki & Kleihues, 2005).

Primary GBMs are characterized as very aggressive, highly invasive tumours found mostly in older patients. They tend to expand very rapidly with a clinical history shorter than 3 months and without clinical or histopathological evidence of a pre-existing precursor lesion. Secondary GBMs are usually maladies of younger patients, namely 45 years of age in average, derived from low-grade astrocytomas 5 to 10 years after the primary diagnosis. They are not frequent, as they account for less than 10% of all GBMs, and they are associated with better survival prognosis.

GBMs are highly resistant to any conventionally applied therapy. At the moment there is no curative treatment for malignant glioma available. WHO pinpoints five main reasons for the therapeutic resistance to even the most aggressive treatments. Primarily, the most important reason is the vast genotypic and phenotypic heterogeneity of tumour cells. Another very important factor that contributes to cellular heterogeneity is the presence of cancer stem cells that harbour resistance mechanisms. Other reasons for poor treatment response are the blood-brain barrier, which inhibits delivery of the drugs, as well as highly invasive properties of glioblastoma cells that allow them to infiltrate healthy tissue. Last but not least, an abundant DNA repair system influences the effectiveness of chemo- and radiotherapy treatments of GBM (Louis, 2007).

2.2.2 Conventional treatments for glioma

Conventional therapy consists of surgical debulking that can be followed by radiotherapy and/or chemotherapy. Currently, surgery is the most common means for palliative treatment. It relieves typical symptoms of brain tumours such as headaches, vomiting and consciousness problems that are usually caused by raised intracranial pressure.

As the tumor edge is usually not well defined and vital anatomical structures are located nearby, surgery impact is often compromised. Patients that are chosen for more aggressive

surgeries are favored by age and low grade tumour located away from eloquent areas. Thus, many epidemiological studies have been compromised as they fail to control such variables as selection bias, histological criteria and tumour location. In the early nineties Quigley and Maroon have followed the data on 5691 patients who underwent tumour debulking and discovered that only four studies reported a significant difference in survival compared to untreated subjects (Quigley & Maroon, 1991). Today, even with the help of statistical modelling accounting for the variables, most of survival benefits are modest at best, with a maximum prolongation of life estimated at 4 months (Lacroix et al., 2001).

After surgical removal of the glioma, invasive cells will give rise to recurrent tumours, which commonly arises immediately adjacent to the resection margin or within a very short distance of the resection cavity. Therefore, it is beneficial to remove as much of the tumour and surrounding tissue as possible. Conventionally, the tumour is debulked from within, until visually normal brain is reached. As the cells tend to infiltrate the healthy tissue, it is obvious that such strategy is inefficient and unreliable. Therefore, some technologies were developed to maximize the resection margins. A guided intraoperative imaging with MRI was shown to enable more extensive resection in 28% of patients (Nimsky et al., 2003). Another approach developed at the Mayo Clinic is an awake craniotomy. Local electrical simulation is applied to check for brain activity and determine which areas are amenable for resection (Meyer et al., 2001). A very interesting and feasible approach is fluorescence-guided surgery. In this method 5-aminolevulinic acid is given preoperatively. This chemical, when taken up by glioma cells, is converted to fluorescent protoporphyrin IX (Stummer et al., 2006).

Generally, it is controversial if the overall effect of surgery in patients who do not have immediately life-threatening complications benefit their survival. Nevertheless, even partial resections may be advantageous, as it appears that the space obtained pushes tumour to enter the cell cycle, resulting in better responses to the radiation and chemotherapy treatments.

Radiotherapy is widely accepted as a treatment of choice for those affected with glioblastoma. The limitation to brain tumour radiotherapy is the radiation tolerance that may be below the threshold required to kill malignant glioma cells. Therefore, there are many studies focused on developing novel sensitizers to increase the radiation intensity in the tumour area.

Until recently, it was thought that chemotherapy was an inefficient treatment for patients with glioblastoma, mainly because of the blood-brain barrier being an obstacle in the delivery of therapeutics. Nowadays, the use of a particular chemotherapeutic agent becomes a standard in GBM treatment. Temozolomide is currently a chemotherapeutic drug of choice when it comes to glioblastoma treatment. It is well absorbed orally and easily crosses the blood-brain barrier. In general, it is very well tolerated, though there are reports of headaches, fatigue and nausea. Inactive upon administration, it is rapidly metabolized *in vivo* in systemic circulation at physiological pH to its active form. The standard chemotherapy starts at the first day of radiation and is maintained throughout its whole duration at 75mg/m2 (Stupp et al., 2002). The adjuvant treatment begins four weeks after the termination of radiotherapy and consists of 5 day cycles. In the first cycle the daily dose of Temozolomide is 150mg/m^2. The next cycle begins at day 28 with a dose of 200mg/m^2 and continuing for 6 more cycles. In the above mentioned study, patients responded in a significantly longer median survival (14.6 vs. 12.1 months). Moreover, 26% of patients were still alive after two years, while only 10% survived such a long time when treated with radiotherapy alone. Interestingly, it becomes clear that the tumour genotype has a profound effect on the treatment outcome. Tumour samples from Stupp's study were further analyzed and it was demonstrated that the strongest factor influencing treatment response was methylation of the DNA repair enzyme methylguanine methyltransferase (MGMT) (Heigi et al., 2005). The subgroup of patients who had methylated promoter of MGMT had much better survival rate when receiving combined treatment of Temozolomide and radiation than those who did not (46% vs. 14%).

Malignant glioma is refractory to most cancer cytotoxic agents. The response to treatment is usually short with rapid development of resistance. Interestingly, as mentioned above, certain patient groups achieve prolonged responses to chemotherapy, while others confined in the same study, do not. It is clear, that the emphasis should be put on developing novel molecular markers that will facilitate GBM patients division into subgroups responsive to given therapeutic approaches.

2.3 Angiogenesis in glioma

Tumourigenesis is a multistep, complex process that involves changes not only in tumour tissue itself, but also in the stromal microenvironment, including the vasculature. It is well known that one of the rate limiting steps for tumour progression is gaining access to the host vascular system and further generation of its own blood supply. Formation of new blood vessels may occur in three different ways. Primary, it happens via angiogenesis, that is the formation of new blood vessels by remodelling of the pre-existing ones. Another process is vasculogenesis, which involves *de novo* production of blood vessels from the circulating marrow-derived progenitor endothelial cells. Vasculogenesis occurs primarily in the embryonic development; however it has also proven to play a role in glioblastoma tumourigenesis (Kioi et al., 2010). Finally, a way of obtaining enlarged vasculature is by arteriogenesis, which refers to engorged arteriolar networks. However, arteriogenesis has not been shown to contribute significantly to tumour vasculature formation.

The most common way of acquiring vasculature for tumours is angiogenesis. An induction of tumour vasculature in order to gain access to the host vascular system is called 'angiogenic switch' (Hanahan & Weinberg, 2000). It occurs with tumour size progression as the growing mass requires constant supply of oxygen and nutrients, discarding at the same time waste products. Angiogenic switch is initiated when homeostasis is disturbed in favour of pro-angiogenic stimuli. Those can be secreted by both, cellular sources such as glia and endothelial cells as well, as by some environmental triggers like hypoxia or extracellular matrix (ECM) (Wang et al., 2005). In addition to activating pro-angiogenic pathways, hypoxic state downregulates natural anti-angiogenic pathways, and thus creates favourable conditions for new blood vessels growth. Tumour angiogenesis differs significantly from physiological angiogenesis. First of all, the structure of blood vessels is distinct from normal blood supply system. Tumour vessels are often structurally and functionally different from the normal ones and can be described as highly disorganised. Vessels apart from being dilated and irregularly shaped, demonstrate chaotic organisation, expressing features of venules, arterioles and capillaries parallely. Tumour angiogenesis is complex and thus is characterised by a number of steps, such as degradation of basement membrane, endothelial cell proliferation, invasion of the surrounding stroma and reorganisation into novel vascular network. This complexicity requires a plethora of regulatory factors such as growth factors, adhesion molecules and matrix-degrading enzymes that are described below.

2.3.1 Mediators of angiogenesis

Tumours express numerous pro-angiogenic factors that stimulate endothelial proliferation, migration and assembly into vascular networks. Neovascularisation in glioblastomas is thought to be driven mainly through vascular endothelial growth factor (VEGF) signalling (Plate et al., 1992). Members of the VEGF family are VEGF-A, -B, -C, -D, -E and placental growth factor (PlGF). Angiogenesis induced largely by VEGF-A leads to immature, dysfunctional vessels and impaired blood-brain barrier, with prominent vasogenic oedema. Its receptor, VEGFR2, is the main mediator of physiological effects of VEGF on endothelial cells such as survival, proliferation, migration and permeability (Dvorak, 2002). High levels of VEGF-A expression alone are known to initiate angiogenesis in quiescent vasculature (Pettersson et al., 2000). Histological grade of gliomas correlate with intra-tumoural levels of VEGF and its receptor (Schmidt et al., 1999) VEGF expression is independently regulated by such states as hypoxia and acidosis (Fukumura et al., 2001), but also by oncogenes or tumour suppressor genes such as Ras or p53 (Dvorak, 2002). It has been also reported that VEGF can be constitutively expressed as a consequence of genetic alternations (Rak & Kerbel, 2001). VEGF is among one of the most potent microvascular permeability factors that leads to leakiness and increase in extravasation of plasma proteins (Wang et al., 1996).

VEGF signalling is mediated mainly through VEGF receptor (VEGFR) 1 and 2. Generally, high grade gliomas produce more VEGF than low grade ones (Chaudhry et al., 2001). Interestingly, non-malignant astrocytes do not produce VEGF. This may indicate de-differentiation of glioma cells from normal cells, astrocytes at early postnatal development are able to synthesise this factor (Rosenstein et al., 1998). VEGFR-1 seems to have a more complex role in angiogenesis as it has been revealed to negatively regulate it (Fong et al., 1999), as well as to contribute to vascular sprouting and metastasis (Kearney et al., 2004; Hiratsuka et al., 2002). Thus, it is possible that the pathophysiological role of VEGFR-1 depends on the stage of angiogenesis. It is also believed that VEGF signalling in angiogenesis is mostly mediated by VEGFR-2 as it was shown that VEGFR-1-/- mice are able to develop normal vessels, while VEGFR-2-/- mice embryos die of failure of blood island formation and vasculogenesis (Hiratsuka et al., 1998; Shalabyet al., 1995). However, in addition to VEGF-A also other factors crucially contribute to angiogenesis in gliomas.

The members of the angiopoietin family are critical in the beginning stage of glioma angiogenesis. In glioma Ang-1 is expressed by stromal cells while Ang-2 by endothelial cells (Stratmann et al., 1998). It acts as an antagonising agent for vessel stabilising the Ang-1/Tie-2 pathway (Scharpfenecker et al., 2005). Ang-1 is the major activator of Tie-2, promoting blood vessel maturation and stability. Ang-2, however, is able to counteract by competitive inhibition of their binding. Upon glioma angiogenesis, initiation of both, Ang-2 and VEGFR-2, are parallelly induced in quiescent endothelial cells suggesting their critical role in this process (Vajkoczy et al., 2002). The beginning of their activity marks an increase in blood vessel permeability, microvascular dilation and sprout formation.

In contrast to the VEGF family and angiopoietins, there are number of factors that do not act primarily on endothelial cells, but influences other cell types. Gliomas have been demonstrated to manifest a significant level of fibroblast growth factor (FGF). It is expressed by tumour cells as well as by blood vessels themselves. Overexpression of FGF is known to increase angiogenic activity through chemotaxis induction and endothelial cell migration (Tanghetti et al., 2002). Interestingly, FGF and VEGF complement each other in protecting endothelial cells from apoptosis. FGF defends them from intrinsic stress-mediated apoptosis such as serum starvation, while VEGF protects from extrinsic apoptotic pathways (Alavi et al., 2003). Platelet-derived growth factor (PDGF) is a potent mitogenic activator for smooth muscle cells and pericytes. Its secretion by activated endothelial cells recruits these cells to the sites of newly formed vessels and contributes to the formation of basement membrane (Erber et al., 2004). Moreover, it has been demonstrated that its receptor is expressed on endothelial cells of astroglial tumours and it correlates with malignancy levels (Plate et al., 1992). PDGF overexpression is known to enhance angiogenesis by increasing VEGF-A expression in neovessels (Guo et al., 2003).

The final stage of angiogenic sprouting includes disruption of basal membrane and migration of endothelial cells. These processes are orchestrated mainly by a variety of matrix metalloproteinases and integrins. The proteolytic degradation of ECM plays a central role in vascular remodelling and the key enzymes are MMP-2 and MMP-9, which are secreted in an inactive form and become activated at the site of angiogenesis. Elevated levels of MMP-2 and MMP-9 are observed in brain tumours (Vince et al., 1999). The integrin family, in particular $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, become upregulated during tumour neo-angiogenesis and are expressed in small blood vessels of glioblastoma (Gladson, 1996).

2.3.2 New blood vessel formation

The formation of new blood vessels begins upon the breakdown of native cerebral vessels and is mediated by glial cells. As they accumulate around the vessels, they lift off astrocytic processes, what causes disruption of contact between endothelial cells (Zagzag et al., 2000). This leads to apoptosis and vessel involution, followed by necrosis and formation of hypoxic region. This step is known to be orchestrated by Ang-2 overexpression, which leads to regression of blood vessels (Stratmann et al., 1998). Subsequently, pseudopallisading cells around the regions affected by the lack of oxygen express hypoxia inducible factor-1 (HIF-1). This transcriptional regulator activates genes which are either responsible for metabolic adaptation to oxygen deprivation or more importantly, increase oxygen availability, mainly by activation of VEGF-A (Fukumura et al., 2001). As the native blood vessels regress, the basement membrane and surrounding ECM are degraded with the help of matrix metalloproteinases (MMPs) 2 and 9. Those molecules are proven to have not only a synergistic effect on basement membrane degradation, but also on promoting pro-angiogenic signalling by exposing endothelial cells to chemicals comprising tumour ECM. Importantly, high expression of those factors is associated with poor prognosis in glioma patients (Guo et al., 2005; Rao et al., 1996). The disruption in basement membrane allows endothelial cells to proliferate and migrate toward tumour cells which express pro-angiogenic factors. They upregulate cell surface adhesion molecules, such as integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, which help to increase adhesion, migration and cell survival (Gladson, 1996; Kim et al., 2000). Apart from the migration of endothelial cells, the role of pericytes in tumour vessel formation becomes more prominent. PDGF secretion recruits pericytes to the site of newly sprouting vessels to assist formation of the basement membrane around new vessels (Erber et al., 2004). As a result, a new vasculature emerges characterised with dilated, leaky vessels, disrupted basement membrane and increased ratio of small diameter vessels (Baluk et al., 2003).

Recently, it has been demonstrated that endothelial progenitor cells (EPC), hematopoietic stem cells (HSC) and myeloid precursors do take part in pathological angiogenesis. Usually, EPC are located in the bone marrow, however, a small population of those cells can be found also in the circulation. Interestingly, they may be mobilised from the bone marrow by hypoxic stimuli upon pathological neovascularisation (Tepper et al., 2005). Similarly, HSC and myelomonocytic population of cells are known to take part in tumour vascular remodelling (Lyden et al., 2001). The mobilisation and recruitment of EPCs is promoted by MMP-9

activation, which transforms membrane-bound Kit ligand to its soluble form. Consequently, c-kit positive progenitor cells detach from the bone marrow niche and are released into the circulation (Heissig et al., 2002). This process is further enhanced by such pro-angiogenic factors as PlGF and VEGF (Asahara et al., 1999; Hattori et al., 1999). Interestingly, EPCs not only incorporate into vascular endothelium, but are also able to secrete pro-angiogenic factors, such as inhibitors of DNA binding proteins (Lyden et al., 1999). The scale of their contribution is somewhat controversial. In the literature there are reports of EPCs being the leading cause of tumour angiogenesis, as well as having minimal contribution to its progression (Lyden et al., 2001; Machein et al., 2003).

2.3.3 Anti-angiogenic treatment for gliomas

Among solid tumours GBMs are characterised with highly angiogenic features and one of the highest degree of vascular proliferation and endothelial cell hyperplasia (Louis, 2007). Given the importance of angiogenesis for tumour growth and progression, it is assumed that its inhibition could be used to fight against malignant tumours. Abnormal blood flow, which is typical for tumour angiogenesis, is caused by heterogeneous leakiness of the tumour vasculature and it contributes to heterogeneous delivery of conventional therapeutics. Moreover, elevated interstitial pressure decreases the net transport of therapeutic compound to the tumour, as well as facilitates the access of tumour cells to the vasculature, increasing the risk of metastasis. Most current treatments target glioma cells directly, while anti-angiogenic strategies focus on endothelial cells. Tumour cells are very heterogeneous and therefore are a difficult target, while endothelial cells are a homogenous, non-neoplastic, stable cell population that at least in theory should be targeted easily. Another positive aspect is that the angiogenic vasculature is easily accessible for blood circulating drugs. Finally, it has been suggested that the abnormal vessels in gliomas may create vascular niches that house glioma stem cells (Gilbertson et al., 2007), therefore their destruction would have a positive effect on reduction of recurrences. Anti-angiogenic strategies are exciting new treatment modalities that bring new hopes for cancer treatment and control. In comparison to conventional chemotherapeutics anti-angiogenic drugs have less toxic side effects. However, despite promising preclinical results, it is still difficult to implement them to everyday use. One of the reasons are complex signalling cascades that control angiogenesis which most probably need

to be challenged with combined therapies, inhibiting pro-angiogenic growth factors and their cognate receptors.

2.3.3.1 VEGF and VEGF inhibitors

As it has been already mentioned, VEGF is a critical factor in angiogenesis in both normal and pathological conditions. Glioma is no exception. It has been shown that tumour cells are able to express both VEGF-A and its receptor VEGFR-2, suggesting a paracrine loop of activation (Plate et al., 1992). It is one of the most vascularized tumours with very high expression of VEGF and high microvessel density, what is associated with poor survival prognosis. Moreover, when VEGF-A expression was studied in malignant gliomas, it was produced 11 times more than in low-grade tumours (Schmidt et al., 1999). Therefore, many researchers focus on development of VEGF pathway inhibitors both against VEGF and VEGFRs.

Bevacizumab is a humanised monoclonal antibody that is directed against VEGF-A. It is approved by EMEA and FDA for metastatic colorectal cancer, advanced non-small cell lung cancer and metastatic breast cancer. Recently, it also received FDA approval for the treatment of recurrent GBM (Cohen et al., 2009). One of the biggest and most significant studies is a retrospective study, where 55 patients with recurrent GBMs were reviewed to determine Bevacizumab's efficacy, toxicity, and patterns of recurrence (Norden et al., 2008). Six-month progression-free survival was 42% for patients with glioblastoma and 32% for patients with anaplastic glioma, compared to a 21% rate for Temozolomide treated patients. Even more promissing results were achieved when combining Bevacizumab with Irinotecan. Twenty of the 35 patients had at least a partial response. The 6-month progression-free survival among all 35 patients was 46% and the 6-month overall survival was 77% (Vredenburgh et al., 2007). The results of effective actions of Bevacizumab and irinotectan were also confirmed by another study done on patients with recurrent glioblastoma (Desjardins et al., 2008). It was reported that 61% of patients had at least a partial response to treatment and that the 6-month progression-free survival was 55% and the 6-month overall survival was 79%. No haemorrhages were reported, and only one patient suffered from arterial ischemic stroke and a few developed venous thromboembolism. In general, Bevacizumab treatment is well tolerated. Currently a novel molecule that is able to bind to VEGF-A with several hundred

fold greater affinity than Bevacizumab is being developed. Aflibercept (also called VEGF Trap) is a soluble decoy VEGF receptor that is fused to the immunoglobulin constant region. Aflibercept has already been proven to have anti-glioma activity in combination with radiation therapy in subcutaneous GBM xenograft model (Wachsberger et al., 2007).

Apart from investigating VEGF ligands also small-molecule inhibitors of VEGFR2 are under development. One of them, Cediranib, which is a tyrosine kinase inhibitor targeting PDGFR and all the subtypes of VEGFR, showed a positive effect in recurrent glioblastoma patients (Batchelor et al., 2007). Of 31 subjects, there was a response rate of 56% and a 6-month progression free survival of 26%. Importantly, authors report that the blood vessels shrunk and then rebounded. This suggests that normalization of tumour vessels has a rapid onset, but it is reversible in quite short period of time, implying that there is a critical time window, which has to be considered when applying combination radiotherapy. Sunitinib, an inhibitor with similar properties that of Cediranib, has shown promising effects in animal studies (Zhou & Gallo, 2009). There are many other multi-targeted kinase inhibitors that undergo preclinical and clinical evaluation. Sorafenib targeting VEGFR-2, PDGFR and Raf kinase as well proved to be efficient in mouse orthotropic model of glioblastoma in combination with Temozolomide (Jones-Bolin et al., 2006).

2.3.3.2 Other anti-angiogenic strategies for malignant glioma

Apart from targeting VEGF and its receptor, there are also other strategies to inhibit angiogenesis. For example, another important growth factor for glial tumorigenesis is PDGF. There are several trials scoping to assess the efficacy of Imatinib a PDGF receptor inhibitor. Phase II clinical trials done both by European Organisation for Research and Treatment of Cancer Brain Tumour as well as by North American Brain Tumour Consortium did not show any efficacy when compared to standard patient care (Raymond et al., 2008). Despite discouraging results when using Imatinib as a single agent in GBM treatment, further studies went on to investigate its actions upon co-administration with other therapeutics. Much more encouraging data was shown by Reardon, who combined Imatinib with VEGFR tyrosine kinase inhibitor Vatalanib and hydroxyurea (Reardon et al., 2009). This study demonstrated a 9% response rate with progression free survival of 27% in a group of 33 patients with recurrent glioblastoma. Nevertheless, as the authors concluded themselves, the potential

efficacy could not be well estimated, because of the small number of patients. The most promising study so far is the one, where Imatinib was combined with Temozolomide (Reardon et al., 2008). The rationale for this combination therapy is the presumption that Imatinib can enhance chemotherapy delivery by decreasing tumour interstitial pressure, as well as diminishing tumour cell DNA repair. The 6-month progression free survival among patients enrolling was 36%, what compares favourably with that of treated with Temozolomide alone (21%).

FGF signalling stimulates endothelial cell proliferation, migration and tube formation. FGF receptor-1 is known to be overexpressed in glioblastoma multiforme.Thalidomide, that is suggested to inhibit basic FGF and VEGF, was one of the first agents used for anti-angiogenic treatment of glioma (D'Amato et al., 1994).

It is also possible to target angiogenesis through inflammation pathways. Studies have shown that interferon-1β is able to downregulate FGF expression as well as MGMT, making glioblastoma cells more sensitive to Temozolomide (Natsume et al., 2005). Cyclooxygenase-2 inhibitors are widely used in anti-cancer trials in various malignancies and several studies are investigating their possible use in GBM. High expression of cyclooxygenase-2 (COX-2) is known to be one of the markers for poor survival outcome in glioma patients (Shono et al., 2001). A phase II study that used a combination of Celecoxib and Irinotectan revealed a good response to the treatment, with a 25.1% progression free survival after 6 months (Reardon et al., 2005).

As discussed in above examples of anti-angiogenic treatment for GBM, it is becoming clear that there is a synergistic effect when it is administered together with chemotherapy. Hypothesis include the idea that there is a prevention of cancer cells repopulation after chemotherapy by anti-angiogenic factors, as well as, reversely, the one that chemotherapy may exert cytotoxic effect on circulating endothelial progenitor cells therefore reducing angiogenesis. Preclinical studies demonstrated anti-proliferative effect of chemotherapy on endothelial cells when administered according to the so called metronomic therapy, meaning in low doses over long periods of time (Klement et al., 2000).

Anti-angiogenic therapy may act in yet another manner in glioma, which involves radioresistance of cancer stem-like cells, eventually causing the recurrence of cancer.

Interestingly, it has been shown that anti-angiogenic therapy is able to reduce the tumour stem–like cell fraction in glioma xenograft tumours, giving new hope for sensitizing this cancer type to conventional therapies. Anti-angiogenic treatment is suggested to disrupt the vasculature and destroy the vascular niche, which is essential for stem-like cells survival. Unfortunately, it has also been shown that such disruption of vasculature and vessel normalisation may result in restoration of the blood brain barrier and therefore impede efficacy of chemotherapeutic agents (Claes et al, 2008).

Furthermore, tumours adapt quickly to new treatment strategies and respond with elaborate resistance mechanisms. In spite of encouraging results, patients improvement is only temporal and further disease progression leads to death. Resistance to long term anti-angiogenic treatment is probably related to upregulation of non-VEGF-mediated pathways of angiogenesis, recruitment of bone marrow-derived cells, increased phagocyte coverage and increased use of pre-existent vasculature (Bergers & Hanahan, 2008). Further improvement in understanding of molecular basis of angiogenic regulation is needed to improve development of novel anti-angiogenic modalities. More emphasis should be put in developing biomarkers that would select appriopriate treatment strategy for individual patients. It is also clear now, that anti-angiogenic therapies are not sufficient per se and need to be combined with other potent cytotoxic treatments to bring sufficient response.

2.4 Lipoxygenases

Lipoxygenases (LOs) are found in plants, fungi and animals. They are composed of a single polypeptide chain containing N-terminal β-barrel and a catalytic domain carrying a single atom of non-heme iron. Lipoxygenases belong to a heterogeneous family of enzymes that recognize the 1,4-pentadiene structure of polyunsaturated fatty acids and incorporate single oxygen molecules to their substrate fatty acids at specific carbon atoms. They are subdivided into 5, 8, 12 and 15 lipoxygenases, with respect to the carbon number which they predominantly oxygenate in arachidonic acid (Brash, 1999). Arachidonic acid is a secondary messenger and in response to various external stimuli is released from cell membrane and further metabolised either by cyclooxygenase or lipoxygenase pathways.

Products of lipoxygenases can act either as the final signalling molecules or as intermediates that are further transformed into secondary products such as leukotrienes. Biological effects of LO derived products can be mediated either through G protein-coupled cell surface receptors or in an intracrine manner by activating nuclear receptors like peroxisome proliferator activated receptors (PPARs). Lipoxygenases may as well modify membrane structures and provoke secondary oxygenations through peroxidation reactions (Fürstenberger et al., 2006). As oxidised metabolites are able to alter the redox balance within the cells, they mediate signal transduction and influence various gene expression pathways that regulate cell growth, cell survival, angiogenesis and immunomodulation just to name a few.

2.4.1 15-lipoxygenase-1

15-LO-1 is widely expressed in reticulocytes, macrophages, eosinophils and airway epithelial cells. It acts on different signal transduction pathways as well as has potential to produce reactive oxygen species together with lipid hydroperoxides. It plays role in various pathways like cell differentiation, maturation and inflammation (Nadel et al., 1991).

Unlike other members of its family, 15-LO-1 introduces molecular oxygen not only to the free form of polyunsaturated fatty acids but as well to those bound in more complex compounds like cholesterol estyrs, plasma lipoproteins and phospholipids. It transforms free arachidonic acid into 12-hydroperoxy-eicosatetraenoic acid and 15-hydroperoxyeicosatetraenoic acid, which are subsequently reduced to their hydroxyl derivatives (12-HETE and 15-HETE) (Kühn et al., 1993). 15-LO-1 additionally takes part in the transformation of linoleic acid to 13-hydroxyoctadecadienoic acid (13-HODE) (Haas et al., 1988). Complicating the matters further, 15-LO-1 has leukotriene and lipoxin synthase activities, which create numerous bioactive molecules able to interact in diverse cellular pathways (Bryant et al., 1985; Kühn et al., 1987).

Despite large efforts, 15-LO-1 remains a poorly understood molecule. It is a multifunctional enzyme able to produce a vast number of metabolites with diverse bioactivities. Moreover, there are substantial species-specific differences in the catalysis pathway and its regulation. Humans express only reticulocyte-type 15-LO-1 and mice only orthologous leukocyte-type 12-LO, rabbits express both (Kühn & Thiele, 1999). Given this, every animal has different

iso-enzymes and produces various amounts of metabolites what at least in part can explain controversial data on 15-LO-1 and its actions in diseases such as cancer or atherosclerosis.

2.4.2 15-lipoxygenase-1 in cancer

The levels of 15-LO-1 expression have been checked in a variety of normal and cancer tissues. Notably, it is expressed in healthy tissues and benign lesions, but its levels are significantly decreased for example in breast, bladder, colon, skin and lung carcinomas (Fürstenberger et al., 2006). Exceptionally, it was reported that 15-LO-1 is overexpressed in prostate carcinoma, correlating with the Gleason score (Kelavkar et al., 2000). The loss of expression during cancer progression may suggest that 15-LO-1 possess some anti-tumorigenic properties and therefore is suppressed in the process of carcinogenesis.

It is known for some cancer types that the overexpression of 15-LO-1 leads to growth arrest. For example, human osteosarcoma cells were shown to grow up to 50% slower when transfected with 15-LO-1 (Sigal et al., 1990). There are quite a few reports linking those findings to a potentially prominent role of 15-LO-1 in apoptosis. Interestingly, it was revealed that high doses of cyclooxygenase inhibitors promote the expression of 15-LO-1. It has been proposed to be essential for their anti-tumorigenic properties, as 15-LO-1 up-regulation showed to induce apoptosis and inhibit colon cancer cell growth (Shureiqi et al., 2000). Several publications relate this property to 15-LO-1 metabolites, particularly to 13-HODE. Sandstrom and co-workers proved that the addition of 15-HETE and 13-HODE induces apoptosis (Sandstrom et al., 1995). Further research on colon cancer revealed that 13-HODE may act through PPAR-γ to reverse the malignant changes (Sarraf et al., 1998). Similar findings were reported for esophageal cancer cells (Shureiqi et al., 2001). Activation of PPAR-γ acts anti-tumorigenic in yet another way. It is said to reduce pro-inflammatory cytokines such as tumour necrosis factor-α and interleukin-6, what leads to a decrease in activated macrophages (Ricote et al., 1998). Interleukin-4 has been proposed to orchestrate 15-LO-1 expression. Promising results were shown in oral cavity cancer cells that underwent 15-LO-1 mediated apoptosis induced by interleukin-4 (Kim et al., 2006). Additionally, another 15-LO-1 metabolite, 15-HETE, was reported to slow down human embryonic kidney cell proliferation (Yu et al., 2004). It may act similarly to 13-HODE, as Shappell and colleagues described 15-HETE mediated proliferation inhibition in prostate carcinoma cells

through activation of PPAR-γ (Shappell et al., 2001). Both, 15-HETE and 13-HODE are able to reduce expression of matrix metalloproteinase-9 by acting through PPAR-γ and PPAR-δ (Shu et al., 2000; Marx et al., 1998). It is of particular importance as matrix metalloproteinases are involved in tumour progression and metastasis.

Unfortunately, the role of 15-LO-1 in cell cycle and apoptosis is not that straightforward. There are a number of publications describing controversial actions of this multifunctional enzyme. Interestingly, the reports that highlight possible pro-tumorigenic role of 15-LO-1 were mostly conducted on various prostate cancer models. Kelavkar and colleagues found that overexpression of 15-LO-1 in human prostate cancer cells leads to increased rate of tumorigenesis (Kelavkar et al., 2001). Those findings were confirmed by two independent *in vivo* studies in mice, where adenoviral driven expression of 15-LO-1 induced neoplastic lesions (Kelavkar et al., 2006; Sen et al., 2006). The case of reverse expression levels of 15-LO-1 in prostate cancer as well as contradictory results in the studies of 15-LO-1 actions in tumour progression reminds us that lipoxygenases may act differently, depending on the tumour origin. It is therefore crucial to critically examine their role in cancer development for each particular type of tumour.

2.4.2.3 Anti-angiogenic properties of 15-LO-1

Angiogenesis is mediated by the interplay of a variety of factors secreted by tumour, endothelial and stoma cells. With the progress in understanding the biology of 15-LO-1, particularly its functions in atherosclerotic vascular remodelling, we become more aware of its possible roles in tumour neovascularization. Regardless of very few reports about its role in angiogenesis; there is strong evidence indicating anti-angiogenic properties.

In vivo studies on mammary gland and Lewis lung carcinoma models gave the first insight into the potential role of 15-LO-1 in tumour angiogenesis. Transgenic mice overexpressing 15-LO-1 were reported to inhibit angiogenesis and tumour growth, alongside with increased ratio of apoptosis (Harats et al, 2005). Also, a very recent report from Viita and coworkers presented that 15-LO-1 is able to prevent VEGF-A induced neovascularisation in rabbit eyes by reducing VEGF-A as well as VEGFR2 expression potentially being a new treatment strategy (Viita et al., 2009). Possible mechanisms of such anti-angiogenic actions of 15-LO-1

were enlightened in her previous work, where she reported very similar findings in rabbit skeletal muscles. Namely, the co-administration of 15-LO-1 together with pro-angiogenic molecules, such as VEGF-A and PlGF, significantly decreased their effects influencing on vasodilatation, vascular permeability and capillary perfusion (Viita et al., 2008).

Viita suggested three possible mechanisms to explain her findings. Primarily, 15-LO-1 suppresses the expression of VEGF-A and PlGF already at the mRNA level, probably by destabilisation of their transcripts by some yet unidentified mechanism. Another possible mechanism explaining anti-angiogenic properties of 15-LO-1 is the modulation of the bioavailability of nitric oxide (NO). Endothelium-derived NO (eNOS) is known to provoke branching and longitudinal extension of blood vessels, as well as influence vascular permeability and blood flow. It is in general linked to cancer, affecting angiogenesis, apoptosis, proliferation and invasion. Viita proved that 15-LO-1 blocks VEGF-induced angiogenesis and vascular permeability by reducing eNOS expression, resulting in decreased amount of bioactive free NO. Additionally, NO bioavailability may be depleted by another mechanism, as it has been shown that 15-LO-1 is able to catalytically consume it (O'Donnell et al., 1999). Interestingly, NO is promoting tumour progression not only by acting as a pro-angiogenic molecule, but it is also associated with inflammation and metastasis (Fukumura et al., 2006). Thus, in theory, decrease of NO levels by 15-LO-1 might promote anti-tumorigenic actions on various levels.

Finally, as it is known that 12/15-LOs generate endogenous ligands for PPAR-γ (Huang et al., 1999), Viita has proposed a connection between 15-LO-1 metabolites and PPAR-γ directed angiogenesis. It has been previously shown that PPAR-γ binding to the VEGFR2 promoter induces VEGFR2 expression, but when a ligand binds to PPAR-γ it causes inhibition of VEGFR2 expression (Xin et al., 1999). Therefore, the postulated mechanism is that 13-HODE acts as a ligand for PPAR-γ and prevents VEGF-induced VEGFR2 expression. In conclusion, Viita has shown strong proofs for anti-angiogenic properties of 15-LO-1. As they may be related to potential anti-tumorigenic effect, it is interesting to further investigate whether 15-LO-1 can be used for anti-angiogenic cancer therapy.

2.5 Cancer gene therapy

Gene therapy is a powerful technique of molecular medicine that allows treatment of a wide array of diseases. The aim of gene therapy is to introduce exogenous genetic sequences to correct abnormalities or provide them novel cellular functions. It has become a promising cancer treatment modality for highly malignant tumours including GBM. One of the basic issues in gene therapy is the choice of gene delivery method. Gene transfer vectors may be generally classified as non-viral and viral ones. Both vector types hold advantages and drawbacks which are discussed below.

2.5.1 Vectors in gene therapy

Non-viral vectors include DNA or RNA particles that are delivered into a cell without the use of viruses. The major is the ease of their use and production as well as reduced pathogenicity in comparison to viral vectors. However, poor efficiency of delivery has been holding back their applications. Major drawbacks include transient gene expression, manufacturing and stability problems, overcoming of extracellular and intracellular barriers and possible immune responses. Liposomes are commonly used non-viral gene delivery methods. As DNA is negatively charged, it is relatively easy to conjugate it with positively charged lipids forming a bilayer vesicular structure where negatively charged DNA is sandwiched between many liposomal particles (Sternberg et al., 1994). Another popular non-viral method of gene transfer is conjugation of DNA with certain polymers. A great advantage of this method is that those synthetic compounds may be modified in such characteristics as molecular weight and ligand attachment. DNA has been delivered in polylysine conjugates targeted with specific antibodies to specific cells *in vitro* and *in vivo* (Kim et al., 2004; Mousazadeh et al., 2007). Polyethylenimine (PEI) is another cationic polymer that can be successfully targeted to increase specificity. For example, in a study by Han, PEI was covalently conjugated with antibodies against matrix metalloproteinase-2 which is commonly expressed on the surface of cancer cells and is connected to angiogenesis and invasion (Han et al., 2008).

Viruses are very common gene transfer vectors. With the progress in molecular virology, we gained understanding of viral structures, mechanisms of pathogenesis, as well as their life cycles, enabling modifications of certain features. Each of the viruses holds both strengths and

weaknesses. Shortly, there is a great selection of viral vectors we can choose from according to one's needs.

Most commonly used viral vectors for glioma gene therapy include adenoviruses which are described in detail below, and retro- and lentiviruses. Retroviruses are small RNA viruses, which have been widely used in basic research, as well as in clinical trials for GBM gene therapy. They offer long-term expression with low toxicity and immunogenicity in brain tissue (Ram et al., 1993; Long et al., 1998). Another feature of retroviruses is their ability to transduce dividing cells, while leaving non-dividing cells unaffected (Miller et al., 1990). Some disadvantages of retroviruses include risk of insertional mutagenesis, low batch titers and instability of the viral particles (Roth et al., 1996; Tait et al., 1997). Finally, although retroviruses were eagerly used in initial gene therapy trials, their applications nowadays have been greatly reduced due to low transduction efficacy. Lentiviruses are based on the human immunodeficiency virus. An advantage of lentiviral vectors compared to retroviral based ones, is that they do infect also non-dividing cells, thus making even the quiescent tumour cells vulnerable to gene therapy (Sinn et al., 2005). Similarly to retroviral vectors, they offer the potential for stable single-copy gene insertion into a host cell chromosome, as well as low toxicity and large transgene capacity. There are also clinical and pre-clinical studies using some of the less common viruses such as adeno-associated virus (Maguire et al., 2010), herpes simplex virus (Manservigi et al., 2010), Semliki Forest virus (Roche et al., 2010) or baculovirus (Guo et al., 2010).

2.5.2 Adenoviral vectors

Adenoviruses have been studied for over half a century. Their biology and physiology is very well known (Bell et al., 1956). Adenoviruses have double-stranded DNA genome, nonenveloped, icosahedral particles with a protein shell encapsulating the nucleoprotein core. Infectious cycle starts with an early phase, lasting for about 6-8 hours, when the virus enters the host cell and its genome is transported to the nucleus for the transcription and translation of early genes to occur. The late phase consists of the virus assembly (Russel, 2000).

The first generation of adenoviral vectors have a deletion of E1 and E3 regions increasing the space for foreign DNA to 6.5kb. Deletion of both of those regions impairs the viral

replication. The defective E1 viruses require transcomplementation to be propagated. For this purpose producer cells such as human embryonic kidney-derived cell line (293 cell line), which provide the E1 gene products in trans are used (Graham et al., 1977). To further advance the safety, enlarge vector capacity and improve the length of transgene expression, the second and third generation vectors have been constructed. Apart from previous modifications they have E4 and E2 regions removed, making them less immunogenic (Almafitano et al., 1998). At the same time, there has been improvement made in the packaging cell lines. 911 and PER.C6 are said to carry smaller risk of homologous recombination, as they do not contain adenovirus sequences that overlap with those ones of the vector (Fallaux et al., 1996). The latest, fourth generation of adenoviral vectors, also called 'gutless' vectors, contain only IRT repeats and a packaging signal (Hardy et al., 1997). The great advantage of those vectors is that they can accommodate up to 37kb of foreign DNA and evoke minimal immune response to the viral backbone. The major hurdle though, is the contamination problem, as helper virus has to be used (Ng et al., 1999).

2.5.3 Adenoviruses in cancer gene therapy

Adenoviral vectors are one of the most commonly used vectors in clinical trials. Nowadays, almost 24% of all gene therapy clinical trials use adenoviruses for gene transfer purposes (http://www.wiley.co.uk/wileychi/genmed/clinical/). Adenoviruses bear many attractive features that give them advantage over other viral vectors. First of all, they have been extensively studied since 1950s, what gives us a relatively good understanding of their biology and a room for molecular modifications. Moreover, they do not cause life-threatening diseases and most of them are associated only with mild upper respiratory tract infections (Bell et al., 1956). Last but not least, an important advantage of adenoviral vectors is the ease of large-scale production, and the possibility of obtaining high titer stocks. However, there are several safety features related to the vector preparation process. It is known that the impurities may cause severe toxic reactions and immune responses (Hedman et al., 2003). Also, not without a concern is the threat of contamination with helper dependent viruses that brings the hazard of appearance of recombination competent viruses (Almafitano et al., 1998).

Adenoviruses have a limited duration of gene expression, what is a hurdle in therapy of hereditary diseases, but not so in the case of cancer gene therapy, as its goal is to kill target

cells. Importantly, infection is not dependent on cell cycle phase, allowing us to target both dormant and cycling cells and even those highly differentiated ones such as lung, brain, bladder and skeletal muscle cells (Zabner et al., 1997; Miller et al., 1998; Li et al., 1999; Bouri et al., 1999). Nevertheless, such a wide tropism to various cell types occurs to be problematic, particularly upon intravenous injection. One of the greatest problems of gene therapy is low tumour transduction rate in clinical trials. Binding of adenovirus to its receptor is a major rate-limiting step for gene transfer. In spite of CAR appearing ubiquitously on majority of epithelial cells, there are many reports suggesting that CAR expression in tumours is highly variable. Several investigations have shown that the more advanced and aggressive the tumour is, the less CAR is expressed, resulting in resistance to adenovirus infection (Kanerva et al., 2002; Dimitriev et al., 1998). Late stages of cancer are primary targets for gene therapy, thus differences in transduction rates caused by CAR down-regulation in advanced tumours, could partially explain low transduction rates in clinical trials.

Adenoviruses are strongly immunogenic, creating long-lasting humoral and cellular immune responses not only towards the vector but transgene and infected cells as well (Young & Mautner, 2001). Not surprisingly, this feature is often perceived as a positive side of adenoviruses as it could potentially activate the immune system to recognise tumour antigens, nevertheless one should never forget about potential hazards it brings along. In 1999 upon gene therapy treatment for partial ornithine transcarbamylase deficiency three out of eighteen patients became seriously ill and one of them has died (Marshall, 1999). Subsequently, one shall never exclude a possibility of a hypersensitive response to those vectors. Apart from the toxicity issues, potent immune response reduces the effectiveness of readministration of adenovirus, forcing the research to focus on removing neutralising antibodies (Rahman et al., 2001) or reducing immune responses. Another problem to be faced is that in the human population the neutralizing antibodies for adenoviruses are quite common and may result in decreased efficiency of these vectors (Belousova et al., 2010).

2.6 Gene therapy for glioblastoma

Gene therapy of cancer is one of the main applications for gene therapy and numerous strategies have been employed to combat glioma. The most common include suicide gene therapy, immunotherapy, anti-angiogenic gene therapy and oncolytic virotherapy. Despite numerous studies, only few trials have advanced from basic research to phase III clinical trials (Wirth et al., 2009; Maatta et al., 2009). Nevertheless, with advances in vector development and targeting methods, there is a hope that soon we will witness the translation of those innovative ideas into clinical success.

2.6.1 Suicide gene therapy

Suicide gene therapy relies on the principle of cancer cells modification to sensitize them to a chemotherapeutic agent that is otherwise non-toxic. Basically, a gene coding for an enzyme converting a non-toxic pro-drug into a lethal agent is inserted into recipient cells. Administration of a pro-drug results in the death of the target cells and usually the neighboring ones as well, through the so called bystander effect (Molten, 1986). Optimistically, several studies have shown that the application of suicide gene therapy may result in complete tumor regression despite transduction of only a small part of tumor cells (Freeman et al., 1993; Barba et al., 1993).

In comparison to conventional chemotherapy agents, pro-drugs do not have any intrinsic activity in unmodified cells and a high local tumour drug concentration can be obtained. The conversion of the pro-drug to the cytotoxic metabolite occurs only within the transduced tumour microenvironment, what allows for minimal systemic toxicity. The crucial property of suicide gene is to be absent or minimally expressed in other than the target organs and to be intrinsically non-toxic. Most popular suicide genes are bacterial, fungal or viral enzymes. An ideal pro-drug should have a high affinity as a substrate for the suicide gene, be able to penetrate into the solid tumor, have a long half-life to maximize bystander effect and exhibit no toxicity prior to activation.

2.6.2 Herpes simplex virus thymidine kinase/gancyclovir gene therapy

Herpes simplex tymidine kinase/gancyclovir (HSV-tk/GCV) was the first gene therapy strategy employing the so called suicide gene (Molten, 1986) and is the most commonly used one since. In this approach gene transfer of HSV-tk to the cancer cells is followed by exposure to gancyclovir. Once taken up by the cell, GCV is phosphorylated by HSV-tk to GCV-monophosphate and further by normal cellular kinases to GCV-diphosphate and triphosphate (GCV-TP). Further on, modified gancyclovir is incorporated into replicating strands of DNA, causing impairment of its synthesis, resulting in cell death (Cheng et al., 1983). GCV-TP is toxic only to dividing cells and since HSV-tk is nearly 1000 times more efficient at phosphorylating GCV than its cellular counterpart, it remains harmless to untransduced cells (Elion et al., 1973).

While GCV itself can passively diffuse into target cells, GCV-TP is highly charged making its further spread impossible, as it is insoluble in lipid membranes. Interestingly though, it was shown that not only the cells carrying tymidine kinase gene were affected by cytotoxicity, but surrounding cells as well. This phenomenon, of inducing cell death to neighbouring cells, is called the bystander effect (Molten, 1986). A wide range of sensitivity among different cell types was reported. For example, the murine neuroblastoma tumor line Neuro2a seems to be completely resistant to the bystander effect. The rat 9L gliosarcoma on the other hand, is highly sensitive, as only 10% of transduced cells exert the same inhibiting level as in the samples consisting of tk-9L modified cells only (Touraine et al., 1998). Mesnil proved that the bystander effect is primarily caused by the transfer of cytotoxic agents through gap junctional intercellular communication that is mediated by connexins (Mesnil et al., 1996). As different cell types have varying levels of gap junctions, transduction efficiency is crucial for successful HSV-tk/GCV therapy. To increase the efficacy of the treatment there is an ongoing research on gap junction inducers. The strategies are based either on pharmacological stimulation of connexin expression through application of such agents as lovastatin, apigenin and hydroxyurea (Touraine et al., 1998; Gentry et al., 2006) or on coexpression of connexins with HSV-tk (Nicholas et al., 2003; Carrio et al., 2001).

Apparently, the bystander effect does not rely solely on connexins, as it was shown that cells with low gap junction intercellular communication can still exhibit good bystander cytotoxity (Boucher et al., 2002). Clearly, there must be other factors that influence the bystander effect.

Some of them include the release of apoptotic vesicles from dying cells, containing GCV-TP that are further phagocytosed by non-HSV-tk-expressing cells (Freeman et al., 1993).

Undoubtedly, the host immune system has a great role in mediating the bystander effect. It has been proven that an intact immune system is required for exhibiting significant bystander effect *in vivo*. Nude and sublethaly irradiated mice did not show regression of subcutaneous tumours even when they were composed of 50% HSV-tk positive cells, while immunocompetent mice reacted with tumour rejection (Ramesh et al., 1996). Moreover, a number of studies reported a development of systemic antitumor immunity following the treatment by suicide gene therapy. One of the studies demonstrated that animals that underwent HSV-tk/GCV treatment were protected against rechallenge with melanoma (Vile et al., 1994). Moreover, there are proofs for existence of so called distant bystander effect. It has been reported that in some cases local suicide gene therapy may mediate the regression of distant metastasis by generation of systemic antitumor immune responses.

2.6.3 HSV-TK suicide gene therapy in glioma treatment

HSV-tk gene therapy is the most widely used suicide approach in clinical trials. The interest in the use of HSV-tk gene therapy method begun in the in the early 90's, when multiple animal studies proved that tumour regression as well as increased survival can be achieved by implanting cells producing HSV-tk (Culver et al., 1992; Rainov et al., 1996; Ram et al., 1993). It was soon concluded that viral packaging cells (VPCs) are not a good carrier of the suicide genes because they are inefficient in transducing tumour cells (Valery et al., 2002; Packer et al., 2000). One of the early studies showed superiority of adenoviral vectors over commonly used viral packaging cells (Puumalainen et al., 1998). In this study the transduction efficiency of the adenoviral vector was significantly higher (5.7-11.3%) than with the retroviral vector (0.01 - 4.0%). In the phase IIa trial with adenoviral gene transfer the mean survival of patients treated with AdHSV-tk was 15 months, while it reached only 8.3 months in the control group and 7.4 months in the VPC group (Sandmair et al., 2000). The phase IIb study on AdHSV-tk was the first randomized, controlled study that showed increase in the survival of patients. 17 patients were given gene therapy by local injections into the tumour bed. The mean survival of HSV-tk administered patients was significantly longer (70.6 +- 52.9 weeks compared to 39.0 weeks) (Immonen et al., 2004). The phase III study

showed in the preliminary results that patients treated together with temozolomide demonstrated a significant improvement in their survival when compared to standard care.

Suicide gene therapy acts has only direct cytotoxic effects. It has been shown that HSV-tk is also able to transduce and destroy dividing endothelial cells (Puumalainen et al., 1998), what means that the activation of suicide therapy can be also responsible for additional anti-angiogenic effect (Lawler et al., 2006). Last, but not least, it has been shown that HSV-tk/GCV therapy is effective as an adjuvant therapy, that is, it can sensitize gliomas to chemo- and radiotherapy (Nestler et al., 2004; Valerie et al., 2000; Rainov et al., 2001).

2.6.4 Other gene therapy modalities for glioma

Recently, a great interest is given to the potential of immune gene therapy. Herein, the idea is to boost our immune response towards GBMs. One possibility is to enhance cytokine expression e.g . IL-4 (Yu et al., 1993), IL-12 (Parker et al., 2000), IFN-β (Qin et al., 1998), IFN-γ (Gansbacher et al., 1990) and GM-CSF (Herrlinger et al., 1997), which are frequently used in cancer gene therapy. Another possibility is to vaccinate patients against autologous tumour antigens. Clinical trials using this approach proved the safety of such therapy for both intradermal and intratumoural vaccinations (Yamanaka et al., 2005). Finally DCs may also be fused with cultured autologous glioma cells and injected intradermally. Such approach demonstrated to be safe and resulted in partial tumour regression in some patients (Kikuchi et al., 2001).

Viral oncolysis involves the use of viruses to provoke host cell killing *via* viral multiplication and further spread to neighboring cells. Onyx-015 was the first oncolytic virus that was tested in clinical settings. It utilizes utilizes deletion of a E1B-55k protein which binds and inactivates p53 in order to prevent apoptosis. Once this region is deleted, adenoviruses are unable to replicate in the cells expressing p53. Yet, p53 mutation/depletion are very common in GBM, thus the replication results in cancer cell lysis (Jiang et al., 2009). ONYX-015, when administered to brain tumour patients, demonstrated that the oncolytic virus was safe. However, it did not show significant anti-tumor activity (Chiocca et al., 2004).

All of the above described gene therapy approaches for GBM have advantages and disadvantages. Gene therapy strategies have proven to be safe and well tolerated in GBM clinical trials. Unfortunately, their efficacy, in spite of successful preclinical studies, remains to be insufficient. This problem is mainly attributed to inefficient gene delivery and gene transfer. At present, the interest has shifted into multi-modality treatments, which combine various gene therapy approaches. It may be the solution to successfully target GBM as well as other malignant cancers.

3 OBJECTIVE OF THE STUDY

The objective of this study was to evaluate whether the 15-lipoxygenase-1 gene therapy is able to improve the survival of rat malignant glioma model.

The secondary objective of this study was to determine whether the combination of 15-lipoxygenase-1 and HSV-tk suicide gene therapy in various treatment modalities could influence the survival of rats bearing malignant glioma.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Adenovirus HSV-tk

Replication deficient, first generation E1-E3 deleted serotype 5 adenovirus vector carrying the HSV-tk gene driven by the cytomegalovirus (CMV) promoter. The virus was kindly provided by Ark Therapeutics Oy, Finland and its production is described elsewhere (Immonen et al., 2004). Titre of the AdHSV-tk virus used in this study was $1.45x10^{12}$vp/ml.

4.1.2 Adenovirus 15-LO-1

Replication deficient, first generation E1-E3 deleted serotype 5 adenovirus vector carrying human 15-LO-1 gene driven by CMV promoter. The virus was kindly provided by Helena Viita, PhD and the production is described elsewhere (Immonen et al., 2004). Titre of the Ad15-LO-1 virus used in this study was 1.3×10^{12} vp/ml.

4.1.3 BT4C rat glioma cells

BT4C cells, obtained from fetal BDIX rats exposed to N-ethylnitrosourea (Sandmair et al., 1999) were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Paisley, Scotland) and supplemented with 10 % fetal calf serum (GIBCO), 2 mM glutamine, 2 mM sodium pyruvate, and 50 μg/mL gentamicin at 37 °C in the presence of 5 % CO_2.

4.1.4 BDIX rats

Inbred, male BDIX rats 8 weeks of age were purchased from Charles Rivers Laboratories, France. Animals were housed at the National Laboratory Animal Centre, University of Kuopio, under standart conditions with twelve-hour period of light per day, constant temperature of 24°C and *ad libitum* food and water.

4.1.5 Ganciclovir (GCV)

GCV hydrochloride (Cymevene®) was purchased from, Roche, Espoo, Finland.

4.1.6 Antibodies

CD34 anti-rat goat polyclonal antibody was used at a dilution of 1:500 (R&D, USA, MN). The secondary antibody was a horse anti-goat antibody (1:200 dilution; Vector Laboratories, CA, USA). To identify matrix-associated vascular channels, after CD34 staining, tissues were stained with PAS (Sigma-Aldrich, UK)

4.2 Methods

4.2.1 Cell culture

BT4C cells were grown in 10 ml of Dulbecco's modified Eagle's medium and supplemented with 10 % fetal calf serum, 2 mM glutamine, 2 mM sodium pyruvate, and 50 μg/mL

gentamicin. After trypsinisation the cells were centrifuged for 3 minutes at 700 rpm and the supernatant was discarded. The cells were counted using the Burker-chamber and resuspended in DMEM medium to final concentration of $1x10^6$ BT4C cells per milliliter.

4.2.2 BT4C rat glioma model in BDIX rats

BDIX male rats (Charles River Laboratories, France) were anesthetized with IP injection of Ketamine (Ketalar®, 50mg/ml, Pfizer Oy, Espoo, Finland) and medetomidine hydrochloride (Domitor®, 1mg/ml, Orion Pharma Oy, Espoo Finland). The animals were stabilized in a stereotactic device (David Kopf Instruments, California, USA). A sagittal incision of roughly 2cm was made in the midline of the scalp. A burr hole was drilled 1mm caudal to the bregma and 2mm to the right of the sagittal suture (KaVo Elektrotechnisches Werk GmBH, Germany). 10µl injection containing 10 000 BT4C cells was done at a depth of 2.5-3.0 mm from bregma using a Hamilton-syringe (Hamilton, Bonaduz Ab, Switzerland; Figure 1). The incision was closed with 4-0 Proline sutures (Tyco HealthCare Ltd, UK). To reverse anesthesia 0.25µg of atipamazole hydrochloride (Antisedan®, 5mg/ml, Orion Pharma Oy, Espoo, Finland) was injected subcutaneously. The animals additionally received analgesia (Rimadyl®, Pfizer, USA; 5-10mg/kg) and the incision wound was treated with Bacitracin powder (Bacibact®, Orion Oyj, Finland) to prevent infection.

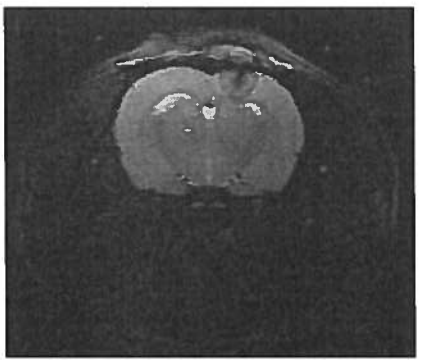

Figure 1. Magnetic resonance image of the coronal section of rat brain depicting a tumour developing above right corpus callosum.

4.2.3 Study groups timeline

This study consisted of four study groups (Table 1). Reference control group consisted of BD IX rats (n=10) which underwent tumor cell inoculation only and were sacrificed upon reaching humane endpoints (Figure 2a). The remaining three cohorts were the treatment groups. All of the animals received tumour cell inoculation on day 1 and multiple viral vector injections on days 14 and 15. Study group number one (n=10; Figure 2b) and three (n=20, Figure 2d) consisted of animals that received Ad15-LO-1 treatment on those days, while study group number two (n=18, Figure 2c) received both Ad15-LO-1 and AdHSV-tk gene transfers. Study group number three received additional AdHSV-tk gene transfer on days 21 and 22. For those animals that received AdHSV-tk, GCV treatment was started five days after the gene transfer at a total dose of 50mg/kg/day.

Table 1. Summary of the treatment group names and treatments applied.

Study group	Reference control	Number 1	Number 2	Number 3
Treatment	-	Ad15-LO-1	Ad15-LO-1 and AdHSV-tk applied simultaneously	Ad15-LO-1 injected before AdHSV-tk

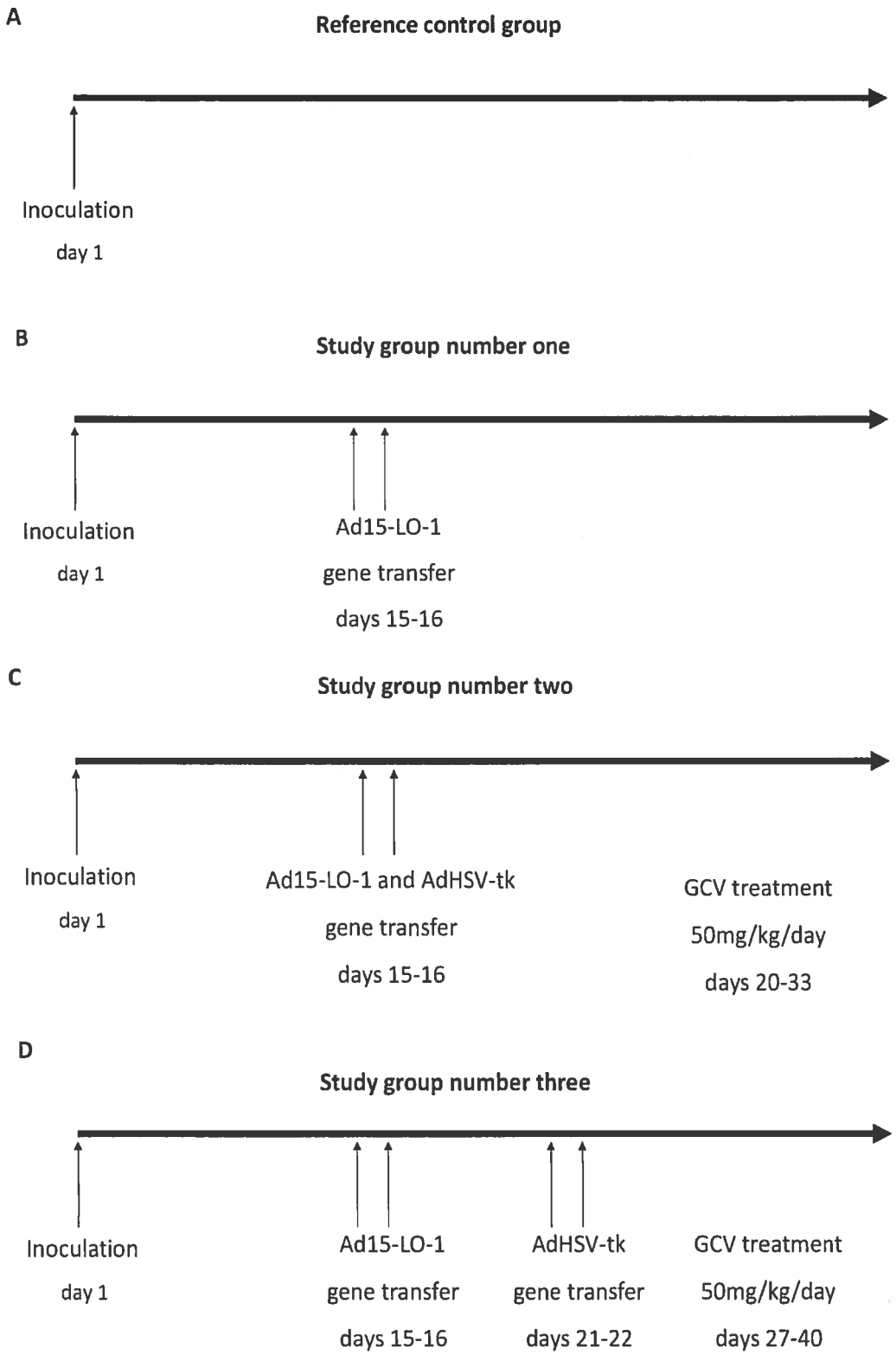

Figure 2. Study protocols for reference control group (A) as well as study groups one - receiving Ad15-LO-1 gene therapy (B), two – treated with simultaneous Ad15-LO-1 and AdHSV-tk gene therapy (C) and three, which received Ad15-LO-1 gene therapy followed with AdHSV-tk gene therapy (D).

4.2.4 Gene transfer

Those animals that were subject to gene therapy underwent gene transfer injections according to the schedule described in 4.2.3. The protocol for animal surgery and anaesthesia was followed as described in 4.2.2. The same burr hole made for cell inoculation was used for gene transfer. The animals scheduled to receive AdHSV-tk therapy were injected with 2.5 µl ($1.45x10^{12}$ vp/ml) of virus per site to the depth of 1.0mm, 1.5mm, 2.0mm and 2.5 mm from the bregma level. On the consecutive day two additional injections were applied at 2 mm depth at an angle, anteriorly and posteriorly, made by flexing the C-arm of the microinjection unit (Figure 2 and 3a). The animals scheduled to receive Adh15-LO-1 therapy were injected with 2.5 µl ($1.3x10^{12}$ vp/ml) of virus per site to the depth of 1.5mm, 2.0mm and 2.5 mm from the bregma level. On the consecutive day, two additional injections were applied at 2 mm depth at an angle, anteriorly and posteriorly, made by flexing the C-arm of the microinjection unit (Figure 2 and 3b).

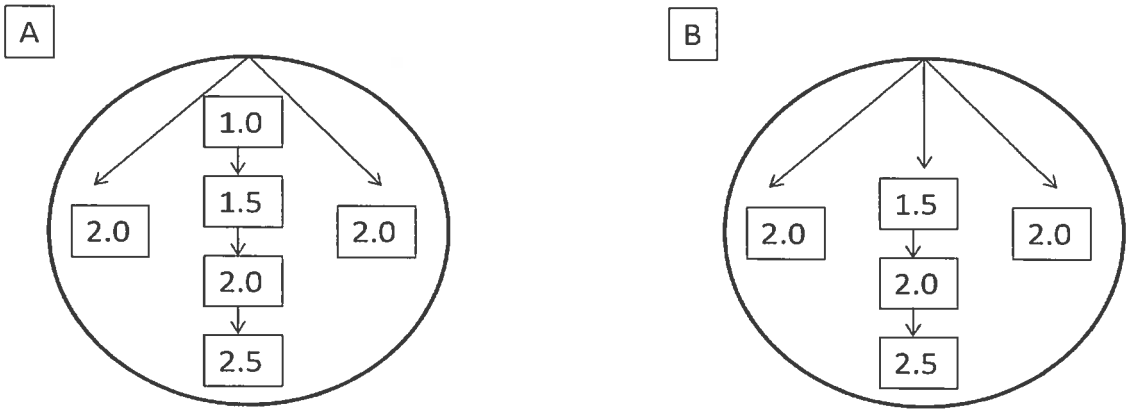

Figure 3. Multiple site injection technique for AdHSV-tk (A) and Ad15-LO-1 (B). Depth is in milimetres from the bregma level.

In the study group number 3, where the animals received simultaneous AdHSV-tk and Adh15-LO-1 therapy, the AdHSV-tk was injected to the depth of 1.0mm, 1.5mm, 2.0mm and 2.5 mm from the bregma level while the Adh15-LO-1 was applied in two injections 2 mm depth at an angle. On the consecutive day, the Adh15-LO-1 was injected to the depth of 1.5mm, 2.0mm and 2.5 mm from the bregma level while the AdHSV-tk was applied in two injections 2 mm depth at an angle.

The protocol for the closure of incision, reversal of anaesthesia and post-operative care was followed as described in 4.2.2.

4.2.5 GCV therapy

GCV hydrochloride (Cymevene®) treatment was started 5 days after the second AdHSV-TK gene transfer and terminated 14 days later. Each animal received a total dose of 50mg/kg/day that was administered IP in two doses of 25mg/kg (each 12 hours apart). During the course of the study the body weight of the rats was measured. The GCV treatment was discontinued and the animal was sacrificed if the body weight dropped 20% or more from the pre-treatment body weight.

4.2.6 Survival study

Animals were sacrificed with an overdose of CO_2 upon humane endpoints that were characterized with poor physical condition, neurological deterioration, paralysis or significant body loss. Survival was calculated in days from the day of BT4C cell inoculation.

4.2.7 Statistical analysis

Results were analysed in GraphPad Prism Version 5.01 (GraphPad Software Inc. USA). Survival analysis was done using Kaplan-Meier log rank test and the survival curves were compared using the Mantel-Cox Log-rank Test. $P<0.05$ was considered statistically significant.

4.2.8 Tissue processing

Brains harvested from the animals were fixed overnight with 4% paraformaldehyde solution and further paraffin embedded.

4.2.9 Immunohistochemistry

Four-micron sections from paraffin-embedded brain samples were used for immunohistochemistry. CD34 expression was detected from paraffin sections using the following procedure. After deparaffinization in xylene, rehydration in alcohol row and incubation in 0.25% Triton, sections were treated with 3% hydrogen peroxide for 30 minutes to block the endogenous peroxidase activity. To inhibit nonspecific antibody binding, the sections were preincubated in a solution containing 10% normal horse serum in PBS overnight. Subsequently, the sections were incubated with a primary antibody (polyclonal goat anti-rat CD34 (R&D, USA, MN), for 60 minutes at room temperature. 1:500 dilution was made by dissolving the antibodies in DAKO diluent (Dako Cytomation™, Glostrup, Denmark). The slides were rinsed for 15 minutes with PBS and after that incubated with biotinylated horse anti-goat secondary antibody (1:200 dilution) for 30 minutes at the room temperature, and rinsed with PBS again. The avidin-biotin-horseradish peroxidase system (Vector Laboratories, CA, USA) with DAB as a chromogen (Zymed, San Francisco, California) was used to visualize the immunoreactivity. To identify matrix-associated vascular channels, after CD34 staining, tissues were stained with PAS (Sigma-Aldrich, UK) and counterstained with Mayer's hematoxylin. The slides were further dehydrated in alcohol row, xylene and finally mounted.

5. RESULTS

5.1 Survival studies

This study evaluated the efficacy of Ad15-LO-1 therapy and two various modes of Ad15-LO-1 and AdHSV-tk/GCV combination therapies. BDIX rats were inoculated with BT4C glioma cells. Two weeks post-inoculation the animals were treated with the respective gene therapy module as described in Materials and Methods part. Survival was calculated in days from the day of tumour cell inoculation to the day of sacrifice. The animals were sacrificed when humane endpoints were reached, such as poor physical condition, significant weight loss or neurological deterioration. Kaplan Meier survival analysis was used to determine median survival and the Mantel-Cox Log-rank test was used to verify whether there is a survival benefit in comparison to reference control group. This study revealed that there was no statistically significant difference in survival between the reference control group and any of the treatment groups (Table 2). Detailed results of survival analysis for each of the study groups are described below.

Table 2. Statistical significance of various treatment modalities in comparison to reference control group.

	Ad15-LO-1	Ad15-LO-1 and AdHSV-tk/GCV	Ad15-LO-1 before AdHSV-tk/GCV
P value	0.11	0.76	0.13
Significance	No	No	No

5.1.1 Reference control

In this study group, a total of 10 BDIX rats were inoculated with BT4C cells. One of the rats died during the tumour cell inoculation due to anaesthetic complications and one did not develop a tumour. Kaplan Meier survival analysis showed a median group survival (n=8) of 37 days and most of the animals died within a short period of time (5 days; Figure 4).

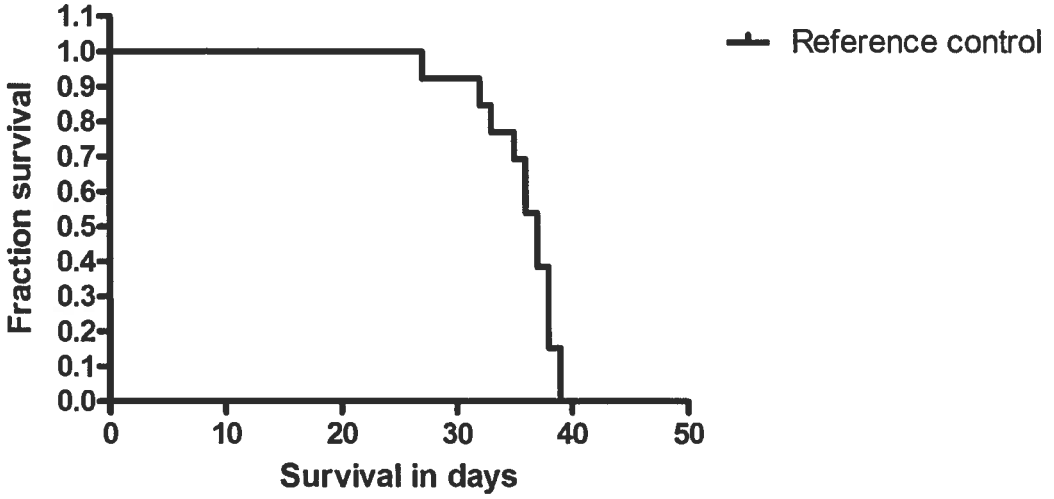

Figure 4. Kaplan Meier survival curve showing the survival in the reference control group (n=8).

5.1.2 Comparison of the reference control with the study group number 1

Out of 10 BDIX rats inoculated with tumour cells for the study group one (Ad15-LO-1). One animal died due to anesthetic complications during tumour cell inoculation and was not included in the further analysis. The remaining 9 rats were included in the survival study, but two of them died upon intervention and were censored from the survival study from that point onwards.

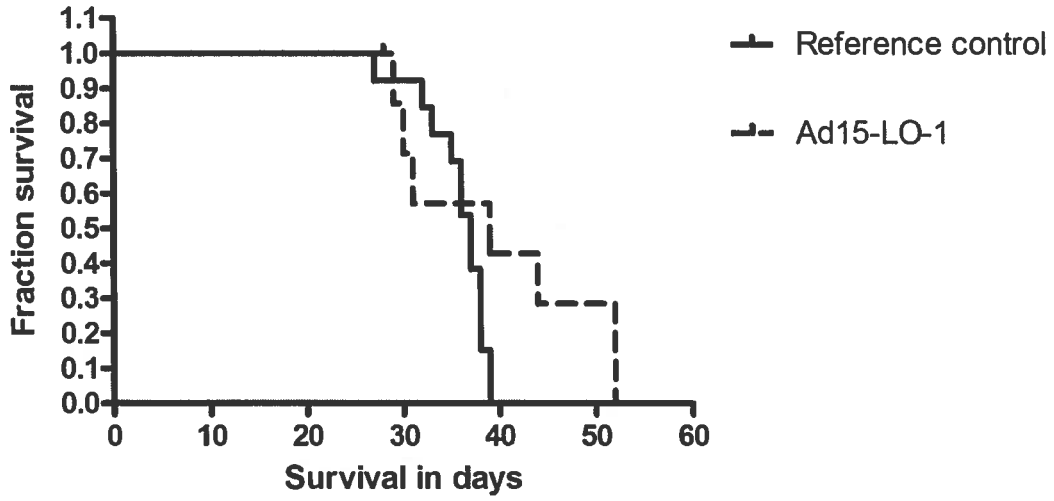

Figure 5. Kaplan Meier survival curve comparing the survival between the reference control group and Ad15-LO-1 treated group (n=9).

Ad15-LO-1 therapy did not improve the survival, when compared to the reference control group (P = 0.11). The median survival was 39.0 days (n=9). Hazard ratio was 2.43 (95% CI 0.82-7.19).

5.1.3 Comparison of the reference control with the study group number 2

Out of 18 BDIX rats inoculated with tumour cells for the study group two (Ad15-LO-1 and AdHSV-tk therapy applied simultaneously) 4 animals died due to anesthetic complications during gene transfer and 1 upon surgical intervention and were therefore not included in further analysis.

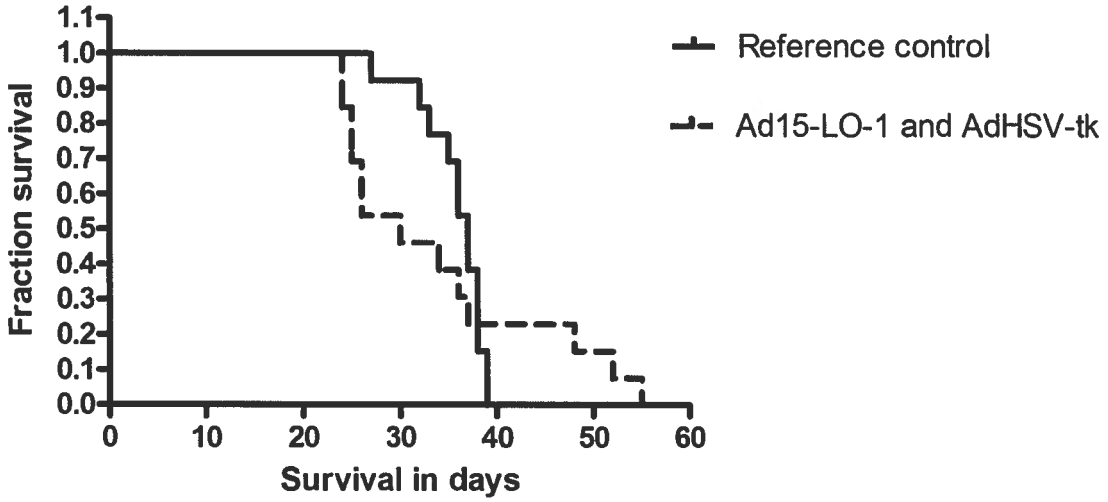

Figure 6. Kaplan Meier survival curve comparing the survival between the reference control group and Ad15-LO-1 and AdHSV-tk treated group (n=13).

Ad15-LO-1 applied simultaneously with AdHSV-tk therapy did not improve the survival, when compared to the reference control group (P = 0.76). The median survival was 30.0 days (n=13). Hazard ratio was 0.87 (95% CI 0.36-2.13).

5.1.4 Comparison of the reference control with the study group number 3

Out of 20 BDIX rats inoculated with tumour cells for the study group three (Ad15-LO-1 administered before AdHSV-tk) 2 animals died due to anesthetic complications during

tumour cell inoculation and were not included in the analysis. 10 rats died from anesthetic complications or had to be sacrificed because of poor physical condition upon the second gene transfer and they were not included in the analysis. One rat died upon intervention, and thus was included, but censored in the study. Interestingly, this study showed that multiple gene transfer applications put animals in severe health burden, particularly as they have to undergo anesthesia and surgery four times.

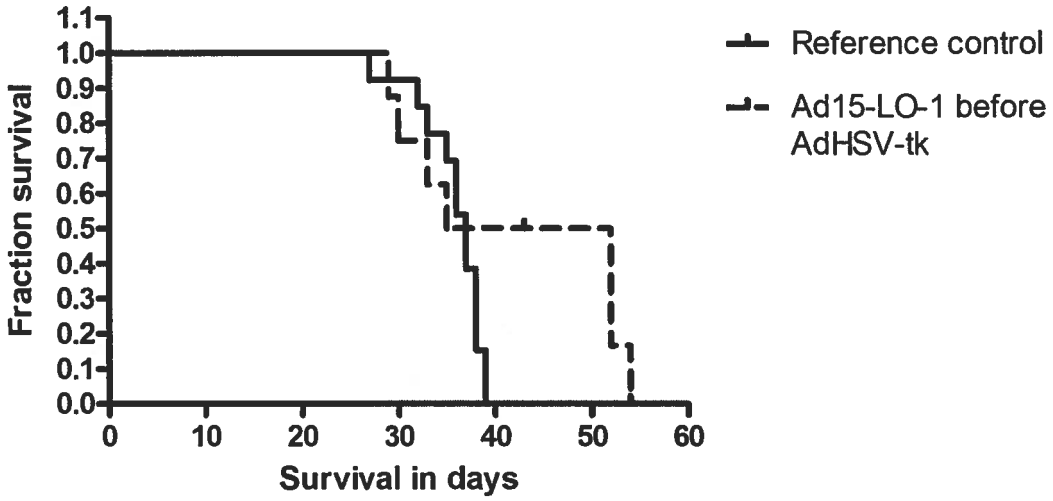

Figure 7. Kaplan Meier survival curve comparing the survival between the reference control group and Ad15-LO-1 applied before AdHSV-tk treated group (n=8).

Ad15-LO-1 therapy applied before subsequent administration of AdHSV-tk therapy did not improve the survival, when compared to the reference control group (P= 0.13). The median survival was 43.5 days (n=8). Hazard ratio was 0.85 (95% CI 0.45-1.25).

5.2. Increased invasiveness and satellite tumour formation

BT4C in BDIX model is known to represent the invasive properties of glial cells. On the edge of the tumours one can see that some migratory cells invade healthy brain parenchyma individually, by propulsion and elongation of leading pseudopods (Figure 8a), while others, invade as a group of cells by means of chain migration, what is a very effective penetration mechanism that confers high metastatic capacity (Figure 8b). Those mechanisms of tumor

migration are characteristic for BT4C in BDXI rats and were prominent in all animal cohorts. However, surprisingly the animals in the group number two, namely simultaneously administered Ad15-LO-1 and AdHSV-tk combination treatment group, were characterized by higher invasive activity of malignant cells, and the malignant invasion was seen even in the contralateral hemisphere (Figure 9a).

Microscopic investigation of H&E staining revealed that in the Ad15-LO-1 and AdHSV-tk combination group the treatment induced a shift in glioblastoma tumor phenotype towards enhanced migration and infiltration into the healthy brain tissue. In particular, a prominent increase in the number of small satellite tumours was seen (Figure 9b). Those invasive islets were not only surrounding the primary tumour, but intriguingly diverged far away from the original inoculation site. Characteristically, these satellites often contained discernible central vessel cores what indicates that in at least some cases those satellites might represent cross sections of vessels that were ensheathed by migrating tumour cells (Figure 10 a, b). Such invasion is similar to the growth pattern in human malignant gliomas as those tumours often invade brain along the host vasculature.

5.3. Vessel co-option as means for tumour invasion

The pattern of invasion appeared not to be random, but rather followed the blood vessels. To determine whether the cells migrated along pre-existing vasculature a CD34-PAS dual staining was made. It showed that the blood vessels were endothelium-dependent with a strong component of tumour cell lining. This confirmed the hypothesis that the malignant glioma cells co-opted preexisting vessels in particular those in the massive vascular plexus present in the brain parenchyma crevices (Figure 10 c, d).

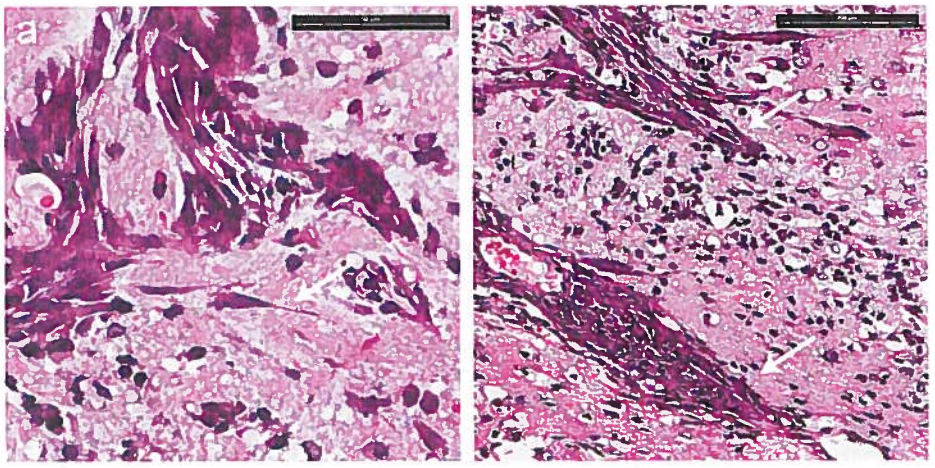

Figure 8. H&E staining of tumour edge representing two types of cell invasion. (a) Individual migratory cells invade by propulsion and elongation of leading pseudopods (arrow), magnification 40x. (b) Chain migration (arrows), magnification 20x.

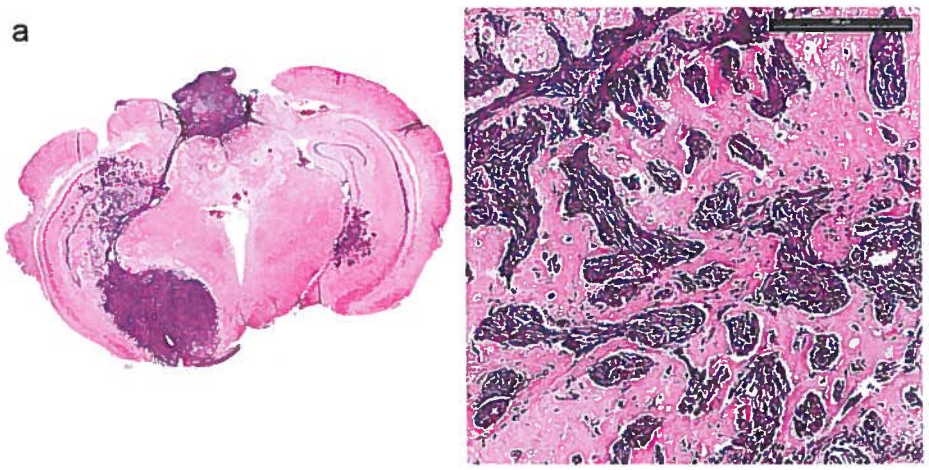

Figure 9. H&E staining revealing highly invasive phenotype after Ad15-LO-1 and AdHSV-tk combined gene therapy (a) Low magnification view of the brain (1,25x). (b) Multiple satellite tumours far from from the original tumour, magnification 20x.

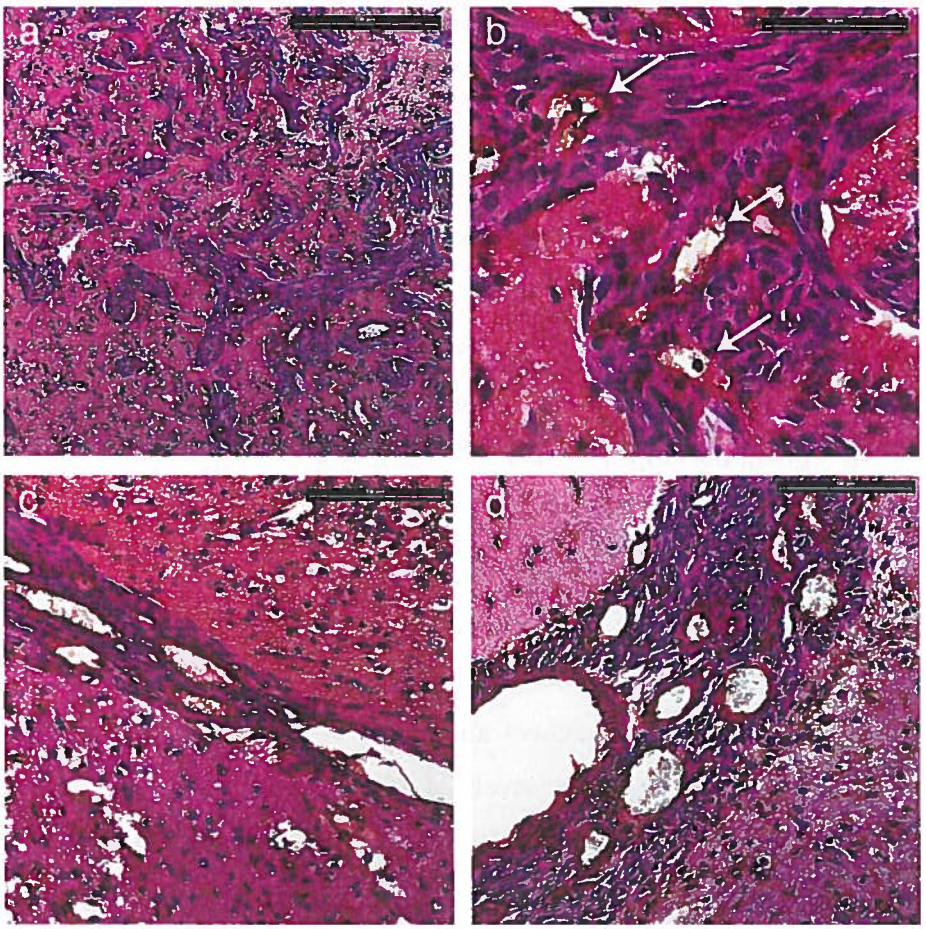

Figure 10. CD34-PAS dual staining. Satellites co-opting host vessels (arrows), magnification 20x, scale bar 200μm (a) magnification 40x, scale bar 100μm (b). Invasion to the contralateral hemisphere along massive vascular plexus (c, d) magnification 20x, scale bar 200μm.

6. DISCUSSION

6.1 15-LO-1 as a therapeutic molecule for gene therapy

Glioblastoma multiforme is the most malignant brain tumour and it is highly resistant to any conventionally applied therapy. At the moment, in spite of the advances in medicine, the final outcome of GBM patients remains grim with average survival of 14.2 months (Stupp et al., 2005). Since there is no curative treatment for GBM, there is an obvious need for further treatment development such as gene therapy.

15-LO-1 is a multifunctional enzyme able to produce a vast number of metabolites with diverse bioactivities, making it a promising molecule for combination gene therapy studies. Viita and coworkers showed that 15-LO-1 can prevent VEGF-A induced neovascularisation in several diverse manners and could be used as a potential new therapeutic (Viita et al., 2009). Interestingly, the levels of 15-LO-1 expression were measured in a variety of normal and cancer tissues and sound to be significantly decreased in numerous carcinomas (Furstenberger et al., 2006). The loss of expression during cancer progression may suggest that 15-LO-1 possess anti-tumorigenic properties and therefore is suppressed in the process of tumourigenesis. 15-LO-1 additionally produces metabolites that provoke cell apoptosis in colon, esophageal, gastric and oral cavity cancer cells (Kim et al., 2006; Sasaki et al., 2006; Shureiqi et al., 2001). To summarize, the studies presented that 15-LO-1 is a multifunctional enzyme that is able to inhibit angiogenesis process on several stages as well as produce metabolites that provoke cell apoptosis. Thus, it was concluded that 15-LO-1 would be a good candidate to be tested for gene therapy of GBM as well as in combination with other gene approaches.

6.2 Rationale for using anti-angiogenic gene therapy in GBM

Glioblastomas are characterised with highly angiogenic features and one of the highest degree of vascular proliferation and endothelial cell hyperplasia in solid tumours with increased secretion of VEGF (Louis, 2007). It is well known that the degree of tumour angiogenesis is directly correlating with tumour aggressiveness and inversely with the post operative survival of astrocytomas (Zagzag, 1995). An overexpression of promoting angiogenesis, VEGF in

tumour cells is believed to occur in the progression of low-grade astrocytomas to hight grade ones (Palte et al., 1994). Additionally, the overexpression of this protein results in up-regulation of matrix metalloproteinases 2 and 9 which are responsible against increased invasive potential (Munaut et al., 2003). Finally, VEGF is known to diminish the immune responce to growing tumours (Gabrilovich et al., 1998; Ohm & Carbone, 2001). It leads to a decrease in the dendritic cell population in cancer patients, as well as affecting T cell development (Ohm et al., 2003). Moreover, anti-angiogenic agents are known to normalise the tumour vessels and thus promote the delivery of simultaneously administered cytotoxic drugs. Therefore, anti-angiogenic strategies, particularly those affecting VEGFs, are exciting approaches that can brings new hopes for glioblastoma treatment, as they could affect multiple pathways in tumourigenesis.

Recently, the Food and Drug Adnimistration has granted accelerated approval to Bevacizumab therapy for recurrent GBM after standart therapy (Cohen et al., 2009). In particular the effectiveness of anti-angiogenic approach for glioblastoma patients was shown in dual and trimodal combinations with chemotherapy and radiotherapy where a synergistic activity was reported (Huber et al., 2005). The currently approved anti-angiogenic agents are monoclonal antibodies. There are some disadvantages in their applications, such as short half-life, requiring frequent injections, as well as high expenses and the risk of toxicity. Thus, novel anti-angiogenic therapies should be developed, such as those ones based on gene therapy.

6.3 Rationale for using combination gene therapy in GBM

Angiogenic treatments are cytostatic rather than cytotoxic in nature, what means that they promote stabilization of the disease rather than its regression. Therefore, it is understandable that they should be applied together with other therapeutics, particularly those that promote killing of the malignant cells.

The last decade has witnessed numerous clinical and pre-clinical studies in gene therapy which showed to be successful in diminishing tumour progression. The most common gene therapy approach is the HSV-tk suicide therapy, which proved not only to be safe, but also well tolerated and most importantly beneficial for the patients (Immonen et al., 2004, Wirth et

al., 2009). One of the most prominent examples is Sitimagene ceradenovec, which is a near market approval therapy developed by Ark Therapeutics Group plc. This adenoviral based HSV-tk gene therapy has significantly enhanced the survival of glioma patients from 37.7 to 62.4 weeks in a phase II randomised controlled clinical trial (Immonen et al., 2004). The phase III clinical study showed a significant improvement on time of reintervention or death in comparison to standard care (van Putten et al., 2010). Despite first gene treatment successes, it has been widely recognized that the suicide therapy approach could be further enhanced with other treatment modalities to improve its efficacy.

One of the most important characteristics of GBM tumours is their vast heterogeneity, which in effect makes a single therapy unlikely to eradicate the tumour. Thus, it is believed that combined therapies targeting different aspects of cancer growth could be more effective. Finally, anti-angiogenic therapy might act synergistically with suicide gene therapy, as it allows for vessel normalization what improves the spread of cytotoxic intermediates.

6.4 Survival studies

The results presented showed that there is no improvement in survival between the control reference group and any of the treatment groups tested. Control reference group had a median survival of 37.0 days with survival ranging from 27 to 39 days, while Ad15-LO-1 treatment group of 39.0 days with survival ranging from 28 to 52 days. Also, the combination of gene modalities did not show any significant difference in survival and study group number two (simultaneous Ad15-LO-1 and AdHSV-tk) had a median survival of 30.0 days with survival ranging from 24 to 55 days, while study group number three (Ad15-LO-1 before AdHSV-tk) had a median survival of 43,5 days with survival ranging from 29 to 54 days.

The lack of survival benefits in treatment groups could be attributed to the lack of effect of 15-LO-1 on rat malignant glioma. Nevertheless, there are also other possibilities. Some studies have shown that anti-angiogenic therapies are successful when they are present for a prolonged period of time (Drixler et al., 2000; Harding et al., 2006). Ad15-LO-1 gene therapy was given two weeks after initial tumour inoculation. Roughly additional five days have to be accounted for the time before the expression of the protein occurred. Taken that BDIX rat malignant glioma model has a short lifespan, there is very few time for the therapy to exert its

actions before the animals die. Another important factor is that once the treatment starts working, the tumours are already large. It is crucial, particularly for anti-angiogenic therapies to be applied early, when the tumour volumes are small and they only start their rapid expansion.

Apart from the reasons mentioned above, the lack of efficacy in simultaneously applied Ad15-LO-1 and AdHSV-tk treatment group may be in part caused by another factor. Both of the vectors were injected in approximately the same areas, what probably lead to transduction of neighboring or even the same cells by two various vectors. Upon GCV therapy initiation, the AdHSV-tk transduced as well as surrounding cells undergo apoptosis, killing some of the Ad15-LO-1 positive cells.

The rationale behind the study group number three design was the idea that if Ad15-LO-1 gene therapy is administered prior to AdHSV-tk therapy, then the anti-angiogenic properties of 15-LO-1 may normalize the vasculature and thus reduce the interstitial pressure what will facilitate the subsequent suicide gene transduction. However, no significant difference in animal survival was found, what in part may be the result of too late onset of anti-angiogenic therapy. Another reason could be the delay in application of suicide therapy, as the cytotoxic treatment was commenced when animals already suffered from large tumours that could not be eradicated by the therapy.

A peculiar phenomenon is the accumulation of early deaths in all of the treatment groups, in particular in study group number three, where in total 10 rats died either from anesthesia or needed to be sacrificed due to poor physical condition upon second gene transfer cycle. It is interesting to speculate, whether these early deaths were due to some toxic effects of the treatment. One of the possible adverse effects of the applied therapies was the fact that the animals underwent gene transfers on two consecutive days. Not only were they in stress due to surgical interventions, but most of all Ketamine that was used for anesthesia is known to increase the intracranial pressure, which is already elevated in rats bearing tumours. This could be an explanation for the poor performance of the rats after gene transfers.

When it comes to the discussion upon therapy toxicity, it is important to mention that GCV is known to cause some adverse effects in humans, such as fever, anaemia, poor liver performance, nausea and thrombocytopenia. Additionally, as in this study GCV was dissolved

in sterile water, the pH levels of the drug were very high, which could lead to some adverse effects when administered IP. To conclude, this could influence somewhat the performance of the animals in treatment groups and result in decrease in survival days upon the late phase of the study.

6.5 HSV-tk and 15-LO-1 combination gene therapy induces increased invasiveness

The results in this thesis showed that there is no statistic significance in survival between the control reference group and any of the treatment groups, whether 15-LO-1 was administered alone or in combination with HSV-tk gene therapy. Nevertheless, the combination treatment resulted in a change in tumor phenotype characterized with healthy tissue infiltration and intense migration. Interestingly, there were various ways malignant cells infiltrated healthy brain. Apart from single cell infiltration, whole clusters of migrating cells were seen particularly in vastly vascularised areas. Interestingly, multiple satellite tumours both surrounding the primary tumour mass as well as scattered far away in the healthy brain tissue were spotted. The areas with massive vascular plexus seemed to attract migrating cells, which ensheathed pre-existing vessels and traveled along them to invade healthy tissue. Such an intense co-option of the host vasculature could be a reflection of compensatory mechanism to therapy, suggesting that neoplastic cells were able to adapt to evade the treatment through escape from the original tumour site. The question remains whether the increase in invasion properties are due to combination gene therapy or one of the properties of 15-LO-1 itself.

6.6 Increased invasiveness as a response to anti-angiogenic properties of 15-LO-1

It is a well established fact that tumour growth, invasion and metastasis are angiogenesis-dependent. Data from recent studies show that anti-angiogenic therapy is successful in inhibiting tumour growth, however, it may influence local invasion as well as distant metastasis. Anti-angiogenic therapy might select for tumour cells that demonstrate better abilities to survive in hypoxic conditions. Therefore, it may select for those malignant cells that hold increased capacity to invade surrounding tissues and co-opt preexisting vessels. Tumours can grow only if the host provides them a vascular network sufficient to sustain their growth and the same time providing them a gateway to enter the circulation and metastasize.

However, when the blood supply is not obtained *via* angiogenesis then for instance, the tumour may obtain an efficient blood supply from pre-existing vascular bed in a process called vascular co-option (Wesseling et al., 1994). Another mechanism that helps the growing tumour to obtain necessary nutrients is vasculogenic mimicry, a process describing de novo generation of blood vessels without the participation of endothelial cells and independent of angiogenesis (Manitois et al., 1999). The study in this thesis showed that a driving mechanism for spread of malignant cells is vessel co-option.

The results in this study are alike to other reports, suggesting that some cancer therapeutics, in particular anti-angiogenic drugs, may provoke tumour adaptation and its further development into invasive phenotype. Increased infiltration and co-option of the host vasculature was observed by Rubenstein already over a decade ago when he used a systemic anti-VEGF inhibitionin a xenograft study of intracranial glioblastoma model (Rubenstein et al., 2000). Leenders and coworkers also reported that angiogenesis could be effectively blocked by anti-VEGF therapy, but that it may result in sustained tumour progression via co-option, rather than tumour dormancy (Leenders et al., 2004). Another study proved that using SU5416 inhibitor, affecting VEGF, results in a similar outcome and it can accelerate metastatic tumour growth and decrease overall survival of the animals (Ebos et al., 2009). In the clinics, it was shown that VEGF-targeted drugs are able to prolong progression-free survival of cancer patients, as well as cause increase in local invasion. Some of the studies associate pro-invasive adaptation in a subset of GBM patients, who develop multifocal recurrence of tumours upon anti-VEFG therapy with bevacizumab (Norden et al., 2008, Narayana et al., 2009). Those results suggest that treating GBM tumours with anti-angiogenic therapies may activate mechanisms that could promote invasion and metastasis. The rationale is based upon the idea that anti-angiogenic therapy might select for tumour cells that demonstrate a high degree of flexibility to survive the hypoxic conditions. Moreover, the anti-angiogenic therapy might select for tumour cells with increased capacity to invade the surrounding tissue and co-opt vessels that are less prone to anti-angiogenic treatments (Steeg, 2003). Nevertheless, anti-angiogenic therapeutics are perceived as a powerful strategy to halt tumour growth. It is believed that in the future their combination with tumour agents that impair invasion might be a highly beneficial strategy for tumours such as GBM.

6.7 Increased invasiveness as a response to other 15-LO-1 properties

It is appealing to consider whether increase in tumour invasiveness was a result of anti-angiogenic properties of 15-LO-1 or yet some other properties of this protein. As already mentioned, 15-LO-1 is a multifunctional enzyme able to produce numerous bioactive molecules interacting with multiple molecular pathways. As a result, studies analyzing the biological significance of 15-LO-1 in cancerogenesis are conflicting, as it is known to show various actions depending on the tissue investigated. For example, high levels of 15-LO-1 are detected in healthy tissues and benign lesions, but the amounts significantly decline in breast, bladder, skin, lung and colon carcinomas, what may indicate anti-tumourigenic properties (Furstenberger et al., 2006). Importantly, many groups reported that 15-LO-1 plays a prominent role in tumour cell apoptosis, which could be linked to properties of 15-LO-1 metabolites (Sandstrom et al., 1995; Shureiqi et al., 2001). However, it is obvious, that the actions of 15-LO-1 are not clear and that they are highly tissue dependant. Various publications reveal pro-tumourigenic properties of this enzyme, including increased tissue infiltration, in prostate cancer cells and models (Furstenberger et al., 2006). Furthermore, 15-LO-1 is known to mediate invasion of intrametastatic lymphatic vessels and to propagate lymph node metastasis of mammary carcinoma xenografts (Kerjaschki et al., 2011).

Regrettably, little research has been done on 15-LO-1 expression in GBM. A very recent publication proved that 15-LO-1 gene therapy was able to significantly prolong survival in a rat malignant glioma model (Viita et al., 2012). This property was linked to induction of lipid peroxidation followed by stimulation of caspase-3 mediated apoptosis. The authors concluded that 15-LO-1 has anti-tumourigenic properties in glioma. Prominently, no signs of increased invasiveness were seen in that study. A paper supporting previous results was published by Hsi and coworkers, who demonstrated that the inhibition of IL-13 decoy receptor could induce 15-LO-1 expression, which in turn cause 13-HODE driven activation of PPARγ and resulted in apoptosis (Hsi et al., 2011). Accordingly, despite insufficient research done in this area, there are hints that 15-LO-1 is not *per se* the reason for increased invasiveness. It seems more probable that together with its metabolites can act as tumour suppressor in the case of GBM rather than inducer. Consequently, the reasons for increase in invasiveness may be connected to the combination therapy rather than 15-LO-1 itself.

6.8 Other reasons for increased invasiveness

It was proposed that tumours that are treated with anti-angiogenic therapy may be prone to develop more invasive and malignant phenotype. However, also other cancer treatments are able to evoke glial tumour evolution into more malignant phenotype, for example radiation therapy (Wild-Bode et al., 2001). Glioblastoma is characterised by immense genotypic and phenotypic diversity that is known to be a major cause of acquired drug resistance. The stress from the therapy may promote the growth of malignant cell populations with high invasive properties that otherwise would remain dormant. Moreover, malignant cells are often de-differentiated and highly plastic and hence, they may adapt quickly to varying micro-environment conditions. Therefore, it is probable that any potent drug treatment or a combination of thereof that deteriorates the conditions for tumour growth may stimulate glioma cells to migrate and invade into healthy brain. Both anti-angiogenic and suicide therapies are able to significantly reduce tumour burden, and there is a possibility that the resulting increase in invasiveness became a compensatory mechanism to therapy stress.

Another possibility for change of tumour phenotype is a combined action of 15-LO-1 and HSV-tk treatments. It is known that suicide gene therapy has a strong influence on the tumour microenvironment through cell apoptosis. One of the potential mechanisms is through employment of the host immune cells. 15-LO-1 has an important role in immune responses and changes in the microenvironment caused by suicide gene therapy might result in changes in 15-LO-1 properties through yet unknown mechanisms.

In conclusion, the amplified invasiveness of malignant glioma cells might neither result from anti-angiogenic properties of 15-LO-1 *per se* nor from its possible carcinogenic potential, particularly as the invasive tumour phenotype was seen only in one of the combination therapy groups, but not in 15-LO-1 therapy group. Another possible reason for the increase in tumour migratory abilities could be a result of the changes in tumour microenvironment exerted by HSV-tk actions or finally simply an outcome of cell adaptation to potent anti-tumourigenic mechanisms exerted by the gene therapy treatment, which combined suicide, anti-angiogenic and pro-apoptotic approaches.

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