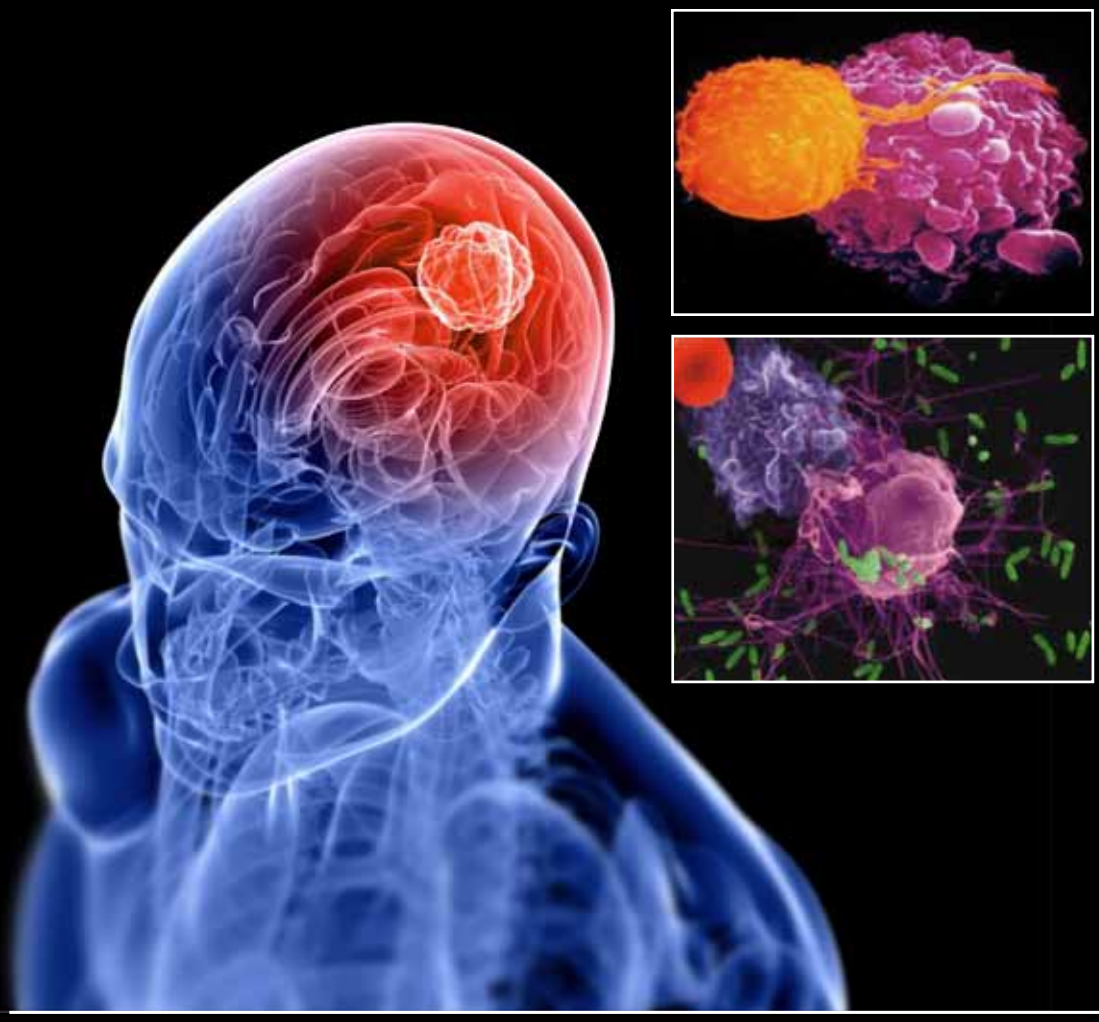
Therapeutic brain cancer targeting by gene therapy and immunomodulation

A translational study



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Contents

CHAPTER 1

General Introduction

General Introduction

1.1 Subject of this thesis

Malignant glioma is a disease with few therapeutic options. Malignant glioma has been widely considered as an example of a tumour in which the expression of tumour-specific antigens and the generation of antigen-specific immune responses are critically involved. Based on these notions we predict that efficient immunotherapy is a feasible treatment option for this type of tumour. In order to develop tumour-specific immunotherapy pertinent to this thesis, we will describe various strategies including stand-alone immune modulation as well as vaccination-induced immune responses in the tumour-bearing host, and monitor therapeutic efficacy of the resulting immunity, e.g. by tumour reduction.

We will first describe general aspects of the immune system in relation to tumour immunology (1.2). Subsequently, we will focus on malignant gliomas (1.3), conventional treatments (1.4), gene therapy (1.5), and newly developed immunotherapy (1.6). The latter is the main subject of this thesis.

Section A. The basics of tumour immunology for gliomas

1.2 The basics of tumour immunology

Historical data show that the immune system clearly plays a role in cancer progression. For example, immunosuppression is associated with cancer development. In fact, cancer is 100 times more likely to occur in people who take immunosuppressive medications (e.g., for organ transplant or rheumatic disease) than in people with normal immune function (e.g. Bouwes *et al.*, 1995; Ho and Murphy, 2008; DePry *et al.*, 2011). Lymphoma, skin, and cervical cancer are just a few types of cancer that have been associated with immunosuppression. For example, skin cancers are the most frequent malignancies in organ transplant recipients (Ulrich *et al.*, 2008). Calcineurin inhibition may result in a 200-fold increased skin cancer risk compared with the normal population (Kuschal *et al.*, 2012). Patients who have undergone renal transplantation have an estimated 3 to 5 times higher overall incidence of malignancy in long-term follow-up, than the general population, and this is explained in part to long-term immunosuppression. Conversely, heightened antitumour activity of the immune system has been suggested in the many reports of spontaneous cancer regression (Sato *et al.*, 2005; Galon *et al.*, 2006; Koebel *et al.*, 2007; Galon *et al.*, 2012).

Hence, numerous studies have now demonstrated that the immune system plays a significant role in the control of tumour development and progression (Vesely *et al.* 2011 and Schreiber *et al.* 2011). The components of both innate and adaptive immunity are involved in fighting tumour cells, however current data in general suggest that the pivotal role in natural antitumour immune response belongs to T-cell-mediated responses, with a prominent role for IFN-ɣ and perforin to prevent tumour

formation (Shankaran *et al.*, 2001) Patients with higher frequencies of intraepithelial CD8+ T cells, and a high CD8+/Treg ratio, exhibit improved survival compared with patients with lower frequencies (Sato *et al.*, 2005).

In addition, it has been demonstrated that the immune system of a naive host can restrain cancer growth for extended time periods (Koebel *et al.*, 2007).

Although the precise nature of the immune system's role in cancer occurrence and regression is actively being studied for each type of cancer, we do know today that tumours are immunogenic. Malignant cell growth is caused by a variety of genetic defects that occur in genes that encode for proteins involved in cell growth. Components of the immune system, including antibodies and T cells, do recognize and respond to the abnormal proteins that are overexpressed, mutated or selectively expressed on cancer cells relative to normal tissues (Hanahan and Weinberg, 2000).

Immune system-based cancer therapy

Immunotherapy against cancer is essentially the stimulation of the patient's immune system, using a variety of distinct strategies, to treat and eventually even prevent future malignancies. These may include active stimulation of immune system by either vaccines, stand-alone treatment with biological response modifiers or innate immunity activating substances, or by passive infusion of immune elements like T cells, antibodies or cytokines. These reagents may act through one of several mechanisms, which include 1) by stimulating the antitumour response, either by increasing the number of effector cells or by producing one or more soluble effector mediators such as cytokines; 2) by decreasing suppressor mechanisms; 3) by altering tumour cells to increase their immunogenicity and make them more susceptible to immunologic defenses; and 4) by improving tolerance to cytotoxic drugs or radiotherapy, such as stimulating bone marrow function by granulocyte (macrophage) colony-stimulating factor (G(M)-CSF).

Cancer-specific antibodies

Antibodies are important therapeutic agents for cancer. Recently, it has become clear that antibodies possess several clinically relevant mechanisms of action. Many clinically useful antibodies can manipulate tumour-related signalling involved in the maintenance of the malignant phenotype. However, antibodies can also initiate tumour-specific immune responses. Such antibodies exhibit various immunomodulatory properties and, by directly activating or inhibiting molecules of the immune system, antibodies can promote the induction of anti-tumour immune responses. These immunomodulatory properties can form the basis for new cancer treatment strategies.

Antibody-based therapy has become established over the past 15 years and is now one of the most successful and important treatment option for haematological and solid tumours. Tumour antigens that have been successfully targeted include epidermal growth factor receptor (EGFR) (by Cetuximab), ERBB2 (by Trastuzumab), vascular endothelial growth factor (by Bevacuzimab), cytotoxic T lymphocyte-associated factor (CTLA-4) (by Ipilimumab) and CD20 (by Rituximab).

Many mechanisms have been proposed to explain the clinical anti-tumour activity of passively administered unconjugated tumour antigen-specific monoclonal antibodies. The ability of some antibodies to disrupt signalling pathways involved in the maintenance of the malignant phenotype has received widespread attention. However,

the ability of antibodies to initiate tumour-specific immune responses has been less well recognized (Weiner *et al.*, 2012). Early research on the anti-tumour effects of therapeutic antibodies focused on the potential value of passive immunotherapy provided through antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) and by the induction of T cell immunity through cross-presentation.

Direct tumour cell killing can be elicited by receptor agonist activity, such as an antibody binding to a tumour cell surface receptor and activating it, leading to apoptosis. It can also be mediated by receptor antagonist activity, such as an antibody binding to a cell surface receptor and blocking dimerization, kinase activation and downstream signalling, leading to reduced proliferation and apoptosis. An antibody binding to an enzyme can lead to neutralization, signalling abrogation and cell death, and conjugated antibodies can be used to deliver a payload (such as a drug, toxin, small interfering RNA or radioisotope) to a tumour cell. Immune-mediated tumour cell killing can be carried out by the induction of phagocytosis; complement activation; ADCC; genetically modified T cells being targeted to the tumour by single-chain variable fragment (scFv); T cells being activated by antibody-mediated cross-presentation of antigen to dendritic cells; and inhibition of T cell inhibitory receptors, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). Vascular and stromal cell ablation can be induced by vasculature receptor antagonism or ligand trapping, stromal cell inhibition, delivery of a toxin to stromal cells, and delivery of a toxin to the vasculature (Scott et al, 2012). Since glioblastoma is a highly vascularized tumour and has high levels of vascular endothelial growth factor, there has been interest in the use of the vascular endothelial growth factor targeting, monoclonal antibody Bevacizumab, (also called Avastin), which is licensed for glioblastoma in the USA, at progression after standard therapy. However, this monoclonal antibody has not been adopted by the European Medicines Agency, because of (deemed) modest response rates and lack of direct comparisons with other agents (Morris, 2012).

Several studies have established the importance of Fc–FcγR interactions in antibody-dependent cellular cytotoxicity for the *in vivo* anti-tumour effects of certain monoclonal antibodies in murine models and clinical trials. Most clinically approved monoclonal antibodies that mediate ADCC also activate the complement system and thereby initiate CDC. Optimization of antibody-based complement activities can enhance anti-tumour activity. Agonistic antibodies are also being explored as immunomodulatory cancer therapies. Low doses of agonistic antibodies may provide a better risk–benefit profile compared with higher doses as they show bioactivity without associated risk. Antibody therapeutics might also have a role in the generation of de novo immune responses to the antigen targeted by the antibody through promoting antigen presentation to Fc receptor-bearing cells. Such responses may allow for the effects of therapeutic antibodies to persist after the dosing is completed.

However, there are multiple mechanisms by which antibody treatment of patients with malignant tumours may fail to achieve a therapeutic effect. These include the heterogeneity of target antigen expression in the tumour (which can be present initially or which can develop during therapy), physical properties and pharmacokinetics of antibodies that have an impact on uniform penetrance into a tumour and intra-tumour microenvironment (including, vascularity and interstitial pressure). Antibody dose and concentration in the tumour and possible receptor saturation kinetics can also

affect therapeutic impact as can signalling pathway promiscuity (which can lead to poor response to therapy and subsequent development of resistance), as well as immune escape through ineffective FcγR binding and immune suppression (Goodnow *et al*, 2010).

The future promise of antibody therapeutics in cancer is dependent on having a better understanding of the lessons learned from laboratory studies and clinical trials conducted so far.

Cytotoxic and helper T cells

T-cells represent a subset of lymphocytes that are crucial as effector cells and for the regulation of the immune response. The T-cell receptor (TCR) is a complex of membrane proteins that participates in the activation of T-cells in response to the presentation of antigen. Stimulation of TCR is triggered by MHC (Major Histocompatibility Complex) molecules on antigen presenting cells that present antigen peptides to TCR complexes and induce a series of intracellular signalling cascades. Engagement of the TCR initiates intracellular cascades that ultimately result in cellular proliferation, differentiation, cytokine production, and/or activation-induced cell death. These signalling cascades regulate T-cell development, homeostasis, activation, acquisition of effector's functions and apoptosis (Lin *et al.*, 2001). TCR activation is regulated by various costimulatory receptors. CD28 provides an essential co-stimulatory signal during T-cell activation, which augments the production of interleukin-2 (IL-2), increases T-cell proliferation and prevents the induction of anergy and cell death.

In the blood, 60-70% of T cells are CD4+ and 30-40% express CD8+. CD4+ T cells are generally designated helper cells (Th) and activate both humoral immune responses (B-cell help) and cellular responses (delayed-type hypersensitivity responses and others). CD8+ cells show a major cytotoxic activity (Tc) against cells infected with intracellular microbes, and against tumour cells. A portion of the circulating CD4+ cells play an important regulatory role that acts to down modulate immune responses. These regulatory T (Treg) cells consist of natural occurring Treg cells (CD4+CD25+ Treg) and adaptive or induced Treg cells (Tr1). Treg cells are able to inhibit the development of allergen-specific Th2 and Th1 cell responses and therefore play an important role in a healthy immune response (Ozdemir *et al.*, 2009).

Both CD4+ and CD8+ T cells differentiate into functionally distinct subsets after exposure to antigenic peptides processed and presented by antigen presenting cells (APCs), like dendritic cells, B cells and monocytes/macrophages. This is best described for the transition of CD4+ T cells from naïve to effector populations. Resting naïve CD4+ T cells (designated Th cells) release very low levels of cytokines. Soon after stimulation by antigen and APC, the Th cells begin to produce IL-2 and are designated Th0. As the Th cells continue to respond to the activating signal, they progress toward polar extremes of differentiation designated Th1, Th2, and Th17 cells, depending on the nature of the cytokines present at the site of activation. IL-12 produced by macrophages or NK cells induces differentiation toward Th1; IL-4 produced by NK1.1+ T cells, basophils, or mast cells induces differentiation toward Th2; and TGF-β produced by Foxp3+ regulatory T-cells, and IL-6 produced by yet to be defined innate immune cells or IL-21 produced by T cells and NKT cells induce differentiation toward Th17. Th1 cells are characterized by the production of IL-2, IFN-γ, and lymphotoxin, whereas Th2 cells produce IL-4, IL-5,

IL-9, IL-13, and granulocyte-macrophage colony stimulating factor (GM-CSF) and Th17 cells produce the cytokines IL-6 and IL-17 (Bonilla and Oettgen, 2010 ; Chaplin, 2010).

Every effective immune response involves T-cell activation; however, T-cells are especially important in cell-mediated effector immunity, which is the defense against tumour cells and pathogenic organisms inside body cells. They are also involved in rejection reactions. TCR stimulation may provoke different cell responses (proliferation, anergy to subsequent stimuli, cell death) in mature circulating T-cells, as in thymocytes. Deregulation of T-cell function, whether by defect or by excess, results in dire consequences for the organism i.e., immunodeficiency and autoimmunity respectively. It is also an extremely sensitive system, as very few peptide-MHC complexes on an antigen-presenting cell are needed to trigger a T-cell response. Since T-cells play various critical roles in orchestrating the immune responses, this knowledge should lead to an understanding of how breakdowns in immune regulation lead to autoimmune diseases and of how the immune system could be better manipulated to overcome afflictions such as cancer, infection and autoimmune diseases (Anderton, 2006).

Cancer and T-cells

A series of recent discoveries in molecular biology and immunology have resulted in new principles for the treatment of cancer, including gene transfer and immune therapy for activation of the host T cell system. Cytotoxic CD8+ T cells (CTLs) are key effector cells of the immune system and critical components of protective immunity against infectious diseases and cancer. After recognizing the MHC-peptide complex expressed on a cell, CD8+ T cell destroy the cell by perforating its membrane with enzymes or by triggering an apoptotic or self-destructive pathway. This CD8+ T cell will then move to another cell expressing the same MHC-peptide complex, and destroy it as well. In this manner cytotoxic T cells can kill many invasive cells. Ideally, CD8+ T cells could engender a very specific and robust response against tumour cells. CTLs have the potential to eradicate cancer cells with high specificity when present in high enough amounts. Therefore, induction of effective and long-term CTL immunity is a major goal of cancer vaccines. Efficient delivery systems and powerful adjuvants are needed to apply such vaccines in a clinical setting to overcome tumour-specific tolerance.

To activate such CTL *in vivo* a vaccination strategy is often used which combines vaccination with dendritic cells (DC) and adoptive T cell transfer. DC loaded with the material from the patients own tumour is injected to stimulate an anti-tumour immune response. To further amplify this response in absence of suppressive tumour-derived factors it is necessary to stimulate and expand patient lymphocytes *in vitro*, so that large numbers of trained and activated cells can be re-infused to fight the cancer.

Tumour immune evasion

Tumours employ a wide array of mechanisms to hide from the immune system, called tumour evasion. It is widely accepted that there are three stages of tumour immune evasion: lack of recognition, lack of susceptibility, and induction of immune suppression. These mechanisms are paramount to tumour survival and success. Once the tumour develops into the first stage, lack of recognition, the immune system can no longer respond properly to the tumour growth and cannot find the cells to eliminate. In the next phase resistance to the activity of the immune system

is gained by the tumour, by inactivating negative regulatory pathways and rendering cytotoxic molecules ineffective. And in the last stage the tumour takes control over from the immune system by recruiting immune suppressor cells and by excreting immune suppressive factors.

Lack of recognition

In the first stage of tumour immune escape, tumour antigen loss and down regulation of MHC class I molecules plays an important role. MHC class I molecules are used as recognition molecules by T cells and activate their response to certain peptides. Proteins produced in the cytosol of any cells are cleaved by proteasomes, the resulting peptides are loaded by the TAP transporter system onto MHC class I. These transmembrane proteins are transported to the cell membrane where interaction occurs with the immune system through cell-mediated responses. It was shown that only rarely a mutation in the MHC or beta-two microglobulin was the reason for low occurrence of MCH I molecules on the cell surface, but rather that the tumour has altered the process of peptide presentation to reduce recognition by T cells.

The tumour achieves this by reducing proteasome activity, simply reducing the amount of peptides being produced, which is also a helpful trait for survival against NK response. Also blocking the peptide processing by inactivating or down regulating the TAP system so that the peptides do not enter the endoplasmic reticulum. The tumour cells can also modify the peptide MHC I loading mechanism. All these methods reduce the MHC class I peptide presentation, leading to immune evasion (Seliger B. 2012).

Lack of susceptibility

In the second stage the tumour becomes non-responsive to cell mediated responses. One principle is based on upregulation of B7 ligands on its cell surface. These ligands are found on normal APCs to regulate T cell response. Once a T cell is activated it expresses increased levels of CTLA-4, PD-1 and other receptors that have a higher affinity to B7 than CD28 expressed by naïve T cells. These receptors decrease proliferation, cytokine production and cytolytic activity. Other methods of tumour resistance include upregulation of FasL and B7-H1 which induce T cell apoptosis. Increased expression of protease inhibitors inactivate perforin and granzyme responses from cell mediated immunity.

Immune suppression

The final stage of tumour evasion leads to cancer “taking over control” of the immune response. It does this by secreting immune suppressive cytokines and recruiting immune suppressor cells. There are two classes of cells most suspect in immune suppression, Treg cells and Myeloid derived suppressor cells. The function of these cells is to suppress the proliferation and function of T cells, which is achieved by the production of cytokines like IL-10 and TGF-beta (TGF-β). Also these cells absorb IL-2 which normally activates T effectors cells and NK cells. Treg cells also use CTLA-4 mechanisms to suppress activity of neighboring cells.

Tumours also use their modified metabolisms to change their surroundings. For example, by arginase 1 which decreases the amount of L-arginine in solution so that T cells can no longer proliferate. This tumour mechanism also

triggers iNOS which leads to increased reactive oxygen species (ROS), which damage surrounding T cells and decrease their effective response (Loos *et al.*, 2010). The HER2 oncogene is frequently over-expressed in human cancers and a promising target for immune therapy. Studies have shown that over-expression of mouse or rat HER2 leads to markedly reduced levels of major histocompatibility complex (MHC) class I and molecules of the antigen processing and presentation machinery (APM), thus resulting in a phenotype promoting tumour escape from the immune system. HER2 over-expressing tumours may be more prone to escape from HLA-A2 restricted CTLs suggesting that in such cases immunotherapy approaches inducing an integrated humoral, cellular and innate immune response would be most effective (Mimura *et al.*, 2011). In general, however, an immune response polarized towards a Thelper type-1 profile, favouring cell-mediated immune responses, is considered critical for immunotherapy of many types of cancer (Tuyaerts *et al.*, 2007)

1.3 Glioblastoma brain tumours

Glioblastoma multiforme (GBM), World Health Organization grade IV glioma, (see Figure 1.1 for molecular and pathobiological characteristics) is the most common and aggressive form of brain cancer in adults. In the United States alone over 18,000 primary brain tumours are estimated to occur each year. Of these 18,000, over 60% are diagnosed gliomas. The prognosis for this tumour is very poor. The median survival time of untreated tumours is only 3 months, with death most commonly due to cerebral edema or increased intracranial pressure. Even with the best available current therapy, which includes surgery, radiation, and chemotherapy, the median survival does not extend beyond 14 months, with 75% of GBM patients dying within 18 months of diagnosis (Deorah *et al.*, 2006), and with a 2-year median survival rate between 8% and 12% (Stupp *et al.*, 2005). Glioblastomas account for about 17% of all childhood cancers (McDonald *et al.*, 2011 ; Mahvash *et al.*, 2011). GBM tumours are inevitably recurrent either locally, usually within 2 cm of the original tumour, or at distant sites. Treatment of these recurrent lesions by a second surgery and further chemotherapy may increase the symptom free interval, but the 5-year survival remains 3-5 % (Deorah *et al.*, 2006). The poor prognosis of GBM is due to GBM cells infiltrating the brain and spinal cord thus preventing complete surgical resection. Moreover, these invasive tumour cells appear to be more resistant to cytotoxic drug therapy and have a higher proliferative potential (Furnari *et al.*, 2007 ; Huang *et al.*, 2007).

Additionally, molecular profiling of GBMs have indicated that there is great heterogeneity within these tumours (Mischel *et al.*, 2003), which may be due to the presence of a sparse population of cancer cells that exhibit stem-cell-like characteristics (Paugh *et al.*, 2010; Verhaak *et al.*, 2010).

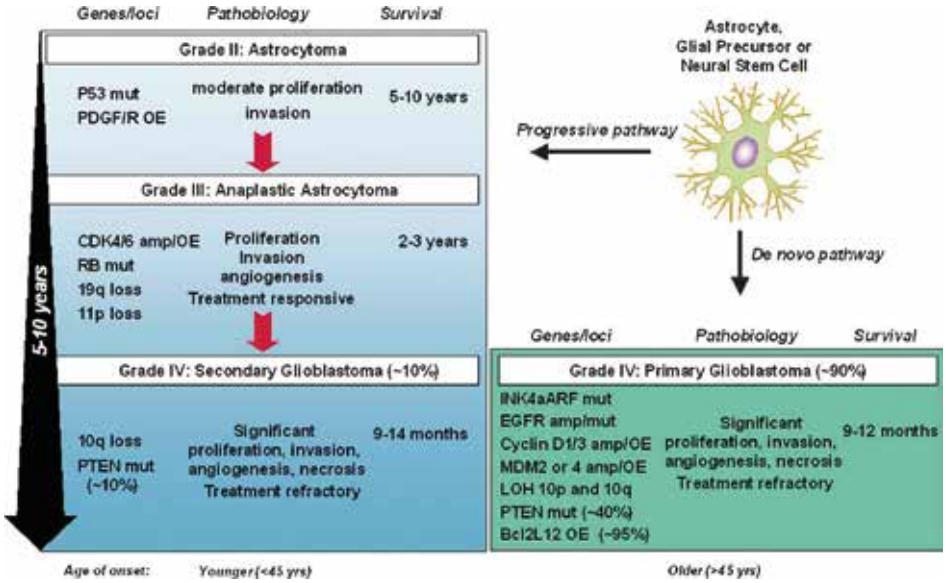

Figure 1.1. The relationships between molecular profile (left columns), pathobiology (middle column) and survival (right columns) that lead to the formation of primary (de novo) right hand pathway and secondary (progressive) glioblastomas left hand pathway. (OE) Overexpressed; (amp) amplified; (mut) mutated. (From : Furnari *et al.*, 2007)

Molecular basis of glioblastoma development

A number of structural genomic changes and dysregulation of signalling pathways in gliomas have been described. A normal developing brain requires signalling pathways involve Sonic Hedgehoc (Shh) and Patched (Ptch), which control mitosis and differentiation of cells in the internal and external granular layer in the brain. In approximately 25% of described human medulla blastoma there was a mutation in one of the many genes involved in the Wnt signalling pathway (Marino, 2005). Wnt is a family of paracrine/autocrine proteins that is expressed in the developing brain. Glioblastoma, the most common and most aggressive form, is characterized by marked cellular heterogeneity, high proliferative activity, aberrant microvascular proliferation, presence of necrosis and highly invasive growth Riemenschneider and Reifenberger (2009). Glioblastomas are characterized by complex genetic and epigenetic aberrations affect several pathways, in particular receptor tyrosine kinase/Ras, phosphoinositol 3-kinase, p53 and pRb signalling. Aberrant Wnt signalling is molecularly linked to many human cancers, and mutations in other Wnt pathway members, including b-catenin and Axin2, and aberrant production of Wnt ligands also have been associated with cellular transformation and tumour development. vi/Wntless/Sprinter/. GPR177, is a highly conserved transmembrane protein, being a component of the Wnt secretion machinery, which is required for glioma cell growth (Augustin *et al.*, 2012).

Cancer stem cells

Stem cell-like cancer cells are able to self-renew and retain the capacity to differentiate but lack proliferative control. These stem-like cancer cells are referred to as either tumour-initiating cells or cancer stem cells (CSCs), and have been identified

in a number of hematopoietic malignancies and solid tumours (Hope *et al.*, 2004; C. Li *et al.*, 2007; Ricci-Vitiani *et al.*, 2007; Singh *et al.*, 2004). CSCs have also been shown to be more resistant to conventional cancer drugs and have been implicated in the more aggressive nature of tumour drug resistance after remission (Bao *et al.*, 2006; Dean *et al.*, 2005). These studies suggest that targeting CSCs may effectively reduce tumour recurrence and significantly improve GBM treatment and the prognostic outcome of GBM patients (Dirks, 2008 ; Cheng *et al.*, 2010). Since CSCs are a biologically and molecularly distinct cell type within a tumour mass, CSCs might possess properties that, if more fully understood, would permit the rational design of novel therapeutic approaches for cancer. For example, Singh *et al.*, (2004) showed that human brain cancer cells express CD133.

Section B. The Long way to immunomodulation treatment against (brain) cancer

1.4 Conventional cancer therapy: Cutting, burning and poisoning of tumours

Surgery

The first report on cancer therapy dates from the Edwin Smith Papyrus, 1600 B.C., the oldest known historical text on surgery. It mentions primitive surgical excision of a tumour using a knife. But it has only been for the past two hundred years that surgical excision became established as the preferred option for treating solid tumours.

The first surgical excision was practiced in 1909 by Harvey Cushing, the American “Father of Neurosurgery”, as a young physician at the Johns Hopkins Hospital, when he operated a pediatric brainstem glioma (Dmetrichuk *et al.*, 2011; Pendleton *et al.*, 2011). He achieved remarkable increases of his patients expectancy of life – increased from 3 months expected survival to 6 to 12 months.

Surgery is the primary form of treatment for brain tumours in parts of the brain that can be removed without damaging critical neurological functions. Currently, the extent of surgical tumour resection (debulking) is the most important factor determining length of survival. Hence, the goal of surgery is to remove the entire tumour if possible, but a tumour is most likely to recur since in most cases not all the tumour cells are removed. Partial removal helps to relieve symptoms by reducing pressure on the brain and it reduces the residual amount of tumour to be treated by radiation therapy or chemotherapy. Any remaining tumour may then be treated with radiotherapy and possibly chemotherapy. The removal of 98 % of the tissue is considered to result in a significantly longer period of remission compared to lower degree of removal. Removal can be supported with a fluorescent dye known as 5-aminolevulinic acid to extend the chances of almost complete removal (Walker *et al.*, 1978). Glioblastoma on average consist of 10^{11} cells, a removal of 99 % implies a residual tumour mass of 10^{9} cells. Even with a dye it is hard to remove all tumour cells, especially given the infiltrative nature of the tumour.

Radiation therapy

Shortly after 1895, when Röntgen first reported the use of X-rays for diagnostic medical purposes, radiation therapy (RT) was introduced. Radiation remained a primary treatment option of certain tumours, especially solid tumours. Today radiation therapy can be tailored to the irregular shape of the tumour, allowing a reduction of intensity.

From 1987 onwards, after introduction of chemotherapy (BCNU, see below) irradiation could be applied to brain tumours (Walker *et al.*, 1978). Walker and coworkers demonstrated in a randomized trial that radiotherapy (5000 to 6000 rads to the whole brain) only improved median survival to 37.5 weeks, relative to BCNU only (25 weeks), and radiotherapy in combination with BCNU resulted in median survival of 40.5 weeks.

Today the radiotherapy (RT) (2 Gy per fraction once daily, per 5 days/wk, for 6 weeks) resulting in a total cumulative radiation dose of 60 Gy) has become part of the standard of care treatment for GBM (Fulton *et al.*, 1992). Radiation therapy uses high-energy x-rays or other types of ionizing radiation to stop cancer cells from dividing. It is often used in addition to surgery or when surgery is not possible.

Radiation therapy variants include:

Conventional radiation therapy is designed to deliver radiation to an entire region of the brain. Depending on the location and size of the tumour(s), the treatment can be either focused or whole brain radiation therapy (WBRT). For example, during stereotactic radiosurgery, a single, high dose of radiation is delivered to the tumour, minimizing damage to the surrounding brain tissue.

Fractionation includes giving the total dose of radiation over an extended period. This helps to protect healthy tissue in the area of the tumour.

Moreover, a 3-dimensional image of the tumour and brain region can be made in order to focus the radiation beams precisely to the tumour such that the surrounding healthy tissue is protected.

Finally, instead of X-rays, proton beam radiation therapy uses protons. Protons can pass through healthy tissue without damaging it. Interstitial radiation therapy (brachytherapy), is an internal form of radiation therapy, which involves surgically implanting radioactive material directly inside the tumour.

Notably, no significant difference was found in the time to progression or median survival time between three-dimensional conformal radiotherapy and whole brain radiotherapy (Showalter *et al.*, 2007).

Bao *et al.*, (2006) showed that cancer stem cells contribute to glioma radioresistance through preferential activation of the DNA damage checkpoint response and an increase in DNA repair capacity, making radiotherapy a palliative therapy.

Chemotherapy

Chemotherapy applies chemicals (drugs) that have a toxic effect on tumour cells as they divide. During World War I, exposure of soldiers to nitrogen mustard gases resulted in pancytopenia. This observation triggered interest for chemotherapeutic therapy, especially in patients with hematologic malignancies. Today chemotherapy is a well documented treatment option. At the end of seventies in 1977 we have the introduction of various chemotherapeutic agents such as

carmustine (BCNU or 1,3-bis(2-chloroethyl)-l-nitrosourea), procarbazine (PCB) or dacabazine (DTIC) (Eyre *et al.*,1986). However, we have controversial results. The efficacy of this treatment in conjunction with available adjuvant therapies could increase the quality and expectancy of life of GBM patients only for a few months, at best (Aoki *et al.*, 2007). Therefore, chemotherapy was non standard therapy for GBM, and only given in the context of clinical trials. However, Stupp and co-workers demonstrated in a publication in 2005 that surgical resection to the extent feasible, followed by adjuvant radiotherapy plus temozolomide (TMZ), improved survival in patients with histological diagnosis of GBM, better than surgery-radiotherapy without TMZ. They concluded that the addition of TMZ to radiotherapy for newly diagnosed glioblastoma resulted in a clinically meaningful and statistically significant survival benefit with minimal additional toxicity (Stupp *et al.*, 2005), and these results were confirmed by Athanassiou and co-workers (Athanassiou *et al.*, 2005). TMZ is an oral alkylating agent, with a relatively low toxicity profile (Yung *et al.*, 2000), which can easily cross the blood-brain barrier. After these publications in 2005 Temozolomide has been adopted as a standard therapy for the treatment of newly diagnosed GBM. But although for glioblastomas, the alkylating agent TMZ has become a well-appreciated option (Koukourakis *et al.*, 2009), it extends average survival time only for about 4 months. Importantly, although TMZ has an acceptable safety profile, adverse events may hamper its use in a considerable number of patients (Michalek, personal communication).

Chemotherapy is usually given in cycles: a treatment period is followed by a recovery period and so on. Apart from the oral, intravenous or intramuscular route, chemotherapy can be given via the intrathecal route into the cerebrospinal fluid or via wafers implanted directly in the brain. The wafers will dissolve and locally release the drug into the brain.

Upon chemotherapy, some cancer (stem) cells acquire drug resistance and survive. The surviving cell will divide and pass its drug resistance to progeny cells, thereby rendering the chemotherapeutic treatment of limited value. Hence, although chemotherapy kills most cells in a tumour, it is believed to leave tumour stem cells behind, which might be an important mechanism of resistance (Dean et al., 2005).

1.5 Gene therapy

The era of gene therapy started at the end of 80s and beginning of 90s of the last century (Anderson, 1992, Miller, 1992). It provided many interesting clinical trials about gene therapy application against brain cancer and specifically against GBM (Curtin *et al.*, 2005).

Cancer gene therapy is an experimental treatment that involves introduction of genetic material (RNA or DNA) into malignant cells to treat cancer. Gene therapy aims to selectively kill cancer cells while leaving healthy cells unaffected. This may be achieved by selective introduction of a gene, which mediates cytotoxic killing of tumour cells or genes, which encode immune system proteins instrumental in enhancing anti-tumour immunity (for example, Tai *et al.*, 2005). For example, the anti-tumour activity of a viral gene-delivering vector can be enhanced by arming the vector with genes that either activate chemotherapeutic drugs within the tumour tissue or that promote anti-tumour immunity (Grandi *et al.*, 2009). Gene transfer is currently

being investigated as a promising approach against brain cancer (reviewed in Fulci *et al.*, 2007).

We have seen really encouraging results for gene therapy-based approaches for GBM cancer treatment, but the largest limitation of gene therapy is the need to infect all tumour cells and/or evoke a bystander effect in the non-infected tumour cells. Examples include soluble cytokines, such as TNF or IL-1β, that generate an antitumour immune response that could involve the bystander effect of suicide-gene therapy.

This has been tried for example with recombinant replication-deficient adenoviral vectors able to transfer the HSV-TK suicide gene to human prostate cancer cells (Cheon *et al.*, 2000).

Other viruses have been used that could more easily replicate up to high viral titers in proliferating cells. However, one of the main problems that formed was that the increasing dose of viral particles introduced the risk for very strong immunoreactions against the viral particles and some clinical trials were stopped because of this (Marshall 1999).

1.6 Immunotherapy, the fourth pillar of cancer therapy

About one hundred years ago William B. Coley, an early 20th century New York City surgeon, observed that some of his patients with sarcoma would undergo spontaneous regression of their tumour, and that this regression was associated with antecedent bacterial infection, mostly erysipelas, or Streptococcus pyogenes, one of the leading causes of hospital-acquired post-operative bacterial infection and death [Coley, 1991; Starnes, 1992]. Since the function of the immune system was largely undiscovered at the time, he was the first to recognize the potential role of the immune system in cancer treatment. Hence, he intentionally injected cancer patients with a mixture of killed bacterial lysates, later called Coley's Toxins, to stimulate as we know today a bystander immune response triggered by a local inflammatory response. William Coley thus became the « godfather of cancer immunotherapy » [Hall, 1997]. Anecdotal regression of gliomas has been noted following intracranial infections (Bowles and Perkin, 1999).

The principle of activation of the patient's immune system, to better recognize the tumour and to eliminate invasive tumour cells that are not adequately removed by surgery, will be discussed in greater detail. This area in cancer therapy is based on knowledge accumulated in the field of vaccinology and immunology.

It has been demonstrated that the progression of certain cancers is associated with the expression of tumour-specific antigens and tumour antigen-specific immune responses (Jager *et al.*, 2001). Hence, theoretically, effective tumour rejection and immunity can be achieved by vaccination with tumour-associated antigen, the holy grail in tumour immunology.

However, active immunotherapy for cancer has shown limited clinical success. It has been clear that even with a fully functioning immune system, it is possible for tumours to evade recognition through the use of elusive escape strategies (Garcia *et al.*, 2003) . Although poorly understood, several mechanisms of tumour escape have been identified, as described in more detail in the section below on « Tumour immune

evasion ». For example, a change of or loss of MHC class I receptors is associated with the genesis of various tumours, while the presence of intact MHC class I molecules has been shown to participate in cancer resistance (Gu *et al.*, 2003). Other mechanisms include unresponsiveness to interferons (Garcia *et al.*, 2003), as well as tumour-induced immunosuppression as a result priming for and influx of inhibitory regulatory T cells (Zou, 2006) and associated induction of immuno-suppressive molecules including IL-10, CTLA-4 and related factors.

On the other hand, there is increasing evidence that the immune system can be engaged to combat cancer. This is supported by the observations that a deregulated immune system hampers rejection of cancer, while spontaneous rejection or inhibition of malignant tumours is associated with a well-functioning immune system (Palo *et al.*, 1977 ; von Mensdorff-Pouilly *et al.*, 1996). A recent study in colorectal tumour patients demonstrated that adaptive Th1 immune gene expression and high immune cell densities of CD3, CD8 and CD45RO cells in tumour regions correlates positively with patient survival (Galon *et al.*, 2006).

Interestingly, it has also been suggested that autoimmune diseases may contribute to a better prognosis in patients with malignant tumours (Palo *et al.*, 1977, Bystryn *et al.*, 1987). In these patients, the majority of the IgG specificities identified share considerable homology with both human and microbial peptides (Hansen *et al.*, 2011). This has lead to the hypothesis that molecular mimicry may initiate the observed anti-tumour autoimmunity. Studies related to this have shown long-term remission of malignant brain tumours after intracranial infection in four patients (Bowles and Perkins, 1999), and improved survival of cancer patients with microbial infection (Papachristou and Forstner, 1979 ; Pizzo *et al.*, 1984). This brings into question whether molecular mimicry-induced ‘‘autoimmunity’’ can be employed to treat tumours. Importantly, significant homology has been shown to exist between human proteins and proteins from other species (Sioud 2002). Moreover, use of artificial pathogen invasion signals, such as CpG motifs, or other innate immunity agonists, initiates and augments antigen-specific immune reactions (Schijns and Lavelle, 2011), and may break tolerance to self-tumour antigens, mimicking microbial infections during immunotherapy or vaccination (Coley, 1991, Takeda *et al.*, 2003). This suggests that self-tolerance to tumours may be broken by cross-reactivity against a homologous foreign antigen and improve median or overall survival.

The first active immunization attempts for glioma patients occurred in the 1960s (Bloom *et al.*, 1960 ; Trouillas and lapras, 1970). It was first hypothesized that brain tumours were hidden from the immune system because of location in the central nervous system an immunologically privileged site. This proved to be incorrect, since immune cells are readily able to cross the blood-brain barrier. In 1983 Mahaley and co-workers showed impressive results which have not shown follow-up in literature (Mahaley *et al.*, 1983, 1984)

At this time the era of immunology had already started and gradually became a very interesting and promising area of investigation for immunotherapy of brain cancer.

Section C. Aims and Outline of the present thesis

The hypothesis pertinent to this thesis is that glioma tumours can be therapeutically targeted by gene and/or immunotherapy in order to eliminate or delay further tumour development.

During the evolution of this thesis, it became apparent that instead of a single-agent-targeted immunotherapy based on just one or a few proteins or peptide antigens, produced synthetically or by recombinant heterologous expression, we preferred a multi-modular approach that is based on syngeneic lysates and cells, mixed with lysates and cells from an allogeneic tumour. This mixture of antigens is not defined, but expected to overlap to a large degree with the specific tumour antigens in the patient. Moreover, this strategy enables triggering of an immune response against a broad array of tumour antigens and also allo-immune reactivity, a classical allograft-directed immune response, typical for non-matching major histocompatibility between graft cells and the host.

Research in rat model systems would enable us to gain insight into critical aspects of therapeutic anti-tumour intervention strategies and to analyse basic mechanisms of action. These investigations were aimed to provide the pre-clinical basis for the development of a therapeutic post-surgical glioblastoma vaccine for patients diagnosed with GBM.

As mentioned above, gene therapy has been and is still considered a promising targeted approach for the elimination of glioblastoma. Gene therapy for cancer involves the use of DNA encoding a particular protein to treat malignant disease. The gene can be delivered to a cell using a carrier known as vector. The most common types of vector used in gene therapy are viruses, such as adeno-associated virus (AAV). In **chapter 2** we analyse if chronic expression of a fusion protein, composed of the mouse IsK potassium channel accessory molecule linked to enhanced green fluorescent protein (EGFP), leads to cell death of more than 50% of transfected U87-MG human astrocytoma cells as early as 2 days after transfection. Cell death is analysed by condensed chromatin measured within 2 days post-transfection and by the appearance of green fluorescent cellular debris within 5 days post transfection. However, the limited infection efficacy of tumour cells, as observed for the high multiplicity of infection (MOI) requirement *in vitro* for a variety of viral vectors (**Chapter 2**), and the expected low tumour cell targeting *in vivo*, forms a tremendous challenge for targeted gene therapy. We therefore switched from gene therapy towards immunotherapeutic treatments, which form a major theme of this thesis.

In **chapter 3** we give an overview of the rational, the experimental approach and clinical data of immunotherapeutic approaches of malignant gliomas, including the use of autologous and allogeneic tissue cells. We analyse if augmented immune reactivity is linked to improved tumour survival, which supports the concept to develop immune system activating treatments either as immune activating stand-alone immune activators, or as immune activating vaccines.

Enhancement of immune reactivity can be induced by administration of immunostimulatory ligands of innate immune cells receptors such the Toll-like receptors. In **chapter 4** we analyse the efficacy of immunotherapeutic Toll-like receptor (TLR)-7/8 activation by Resiquimod (R848) *in vivo* in the CNS-1 rat glioma model syngeneic

to Lewis rats. This innate immune system treatment is compared to the cytotoxic cyclophosphamide chemotherapy or to a combination of R848 plus cyclophosphamide immuno-chemotherapeutic treatment. If TLR7/8 activation by resiquimod alone is able to evoke partial or complete CNS-1 tumour regression, it is consequently not necessarily associated with a direct cytotoxic effect on tumour cells. We also determine if rats that may be cured from glioma tumours by innate immunostimulation can be shown to be fully immune to secondary glioma tumour re-challenge. Cyclophosphamide can also induced dramatic tumour regression but it remains to be determined if it can protect against secondary tumour challenge.

In **chapter 5** the beneficial anti-tumour efficacy evoked by immune activation is tested in a therapeutic vaccine setting in two other different rat glioma models. 9L is syngeneic to Fisher 344 rats and allogeneic to the SD rats, while C6 cells are syngeneic to SD rats and allogeneic to Fisher 344 rats. We test whether allo-immune reactivity is able to evoke anti-tumour immunity against a syngeneic tumour. We analyse if therapeutic immunization with a combination of allogeneic cells and syngeneic lysates induces rejection of malignant gliomas and offers a protective effect against challenge with syngeneic tumour cells.

In **chapter 6** we use the CNS-1 glioma model to explore the protective efficacy of various conditions in the vaccine preparation as well as in the dosing and timing schedule of a vaccine preparation, which is essentially based on allo- and syngeneic antigens in combination with distinct innate immune activating agents. We will analyse if particular costimulation agents are able to confer immunity against CNS-1 tumour development when combined with the allo- and syngeneic vaccine antigen preparation.

In **chapter 7** we continued with the same vaccine antigen preparation, which is evaluated as a therapeutic immunization when combined with the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF), following a low dose cyclophosphamide treatment. This prototype vaccine may form the basis for further evaluation in a clinical study.

In the last chapter of this thesis (**Chapter 8**), we discuss the importance of our pre-clinical findings in the different rat models and the perspective we have developed on the clinical application of our experience and know-how for immunotherapeutic immunization of GBM diagnosed patients.

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CHAPTER 2

Overexpression of mouse IsK protein fused to green fluorescent protein induces apoptosis of human astroglioma cells

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Summary

Intracellular K^+ plays an important role in controlling ion homeostasis for maintaining cell volume and inhibiting activity of pro-apoptotic enzymes. Cytoplasmic K+concentration is regulated by K^+ uptake via Na^+-K^+-ATPase and K^+ efflux through K^+ channels in the plasma membrane. The IsK (KCNE1) protein is known to co-assemble with KCNQ1 (KvLQT1) protein to form a K^+ channel underlying the slowly activating delayed rectifier Kz outward current which delays voltage activation. In order to further study the activity and cellular localization of IsK protein, we constructed a C-terminal fusion of IsK with EGFP (enhanced green fluorescent protein). Expression of the fusion protein appeared as clusters located in the plasma membrane and induced degeneration of both transiently or stably transfected cells. [Neurol Res 2007; 29: 628–631]

Keywords: Potassium channel; apoptosis; astrocytoma; fusion protein; green fluorescent protein; lipofection

Introduction

Expression of rodent IsK (also named KCNE1 or minK) was originally found to elicit a slowly activating voltagedependent K+ outward current in Xenopus laevis oocytes [18]. However, figure 2.2 attempts to express currents by transfection of some eukaryotic cell lines failed [8]. Also puzzling was the fact that IsK has a single putative transmembrane domain [17], whereas cation channels have two, four or six such domains. Furthermore, IsK shares no sequence homology with conventional K+ channels: in particular, it does not contain the P domain, a conserved motif ensuring specificity for K+ transport [22]. It was later on realized that IsK is an accessory protein which must co-assemble with the KCNQ1 (also named KvLQT1) K+ channel to display biologic activity [2,14]. The assembly with IsK increases the voltage dependence and current amplitude of KCNQ1 while slowing down the activation kinetics [4]. It has been suggested that KCNQ1 is the pore-forming subunit, whereas IsK is a regulatory protein affecting the permeation properties of the channel pore [1,16]. Multiple copies of IsK (at least 14) have been shown to be present in the complex [20]. The exact stoichiometry of functional K+ channels consisting in assembled KCNQ1/IsK subunits is not known. However, multiple stoichiometries resulting in channels with different pharmacologic properties have been described [21]. Consistently, overexpression of IsK in various cell types resulted in modification of endogenous K+ and Cl2 channels features [1,15] and antisense oligonucleotides directed against IsK inhibited Kz currents in cardiac myocytes [13]. Noteworthily, a human homolog to IsK harbouring similar properties as its rodent counterparts has been described12. In order to further study the activity of the IsK protein, we have constructed a C-terminal fusion of IsK with EGFP (enhanced green fluorescent protein) with the aim to easily analyse the future of cells overexpressing IsK.

Material and methods

The pTR-EGFP plasmid, an adeno-associated virus (AAV) vector with EGFP (Clonetech, Palo Alto, CA, USA) and neomycin resistance expression cassettes derived from pTR-UF [25] has been previously described [19]. A fragment containing the entire sequence of IsK9 cloned in pRc/cytomegalovirus (CMV) (Promega, Madison, WI, USA) was polymerase chain reaction (PCR)-amplified using the following primers: GCCAAGCTTGATATCCATCACACTGGCGG (5' primer; HindII site fused to pRc/CMV sequence 5' to IsK) and TTCCTGAACTGAAGCCATTGTCACCGGT (3' primer; 3' end of IsK sequence fused to AgeI site). The resulting PCR fragment was digested with HindIII and AgeI and inserted between the HindIII and the AgeI sites in the AAV vector plasmid pTR-EGFP19. This results in an in-phase fusion of the EGFP coding sequence at the 3' end of the IsK coding sequence (Figure 2.1).

U87-MG cell line was obtained from American type culture collection (Bethesda, MD, USA). Cells were cultured in Dulbecco's modified eagle's medium supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Life Technologies, Merelbeke, Belgium). Cells were transfected by lipofection using Vectamidine diC14-tBut (Biotech Tools, Brussels, Belgium) according to the manufacturer's protocol.

For isolation of stable clones, pTR-EGFP or pTREGFP:: mIsK plasmids were transfected into U87-MG cells. Twenty-four hours post-transfection, the cells were trypsinized and diluted (1 : 5) into complete medium containing geneticin (G418)

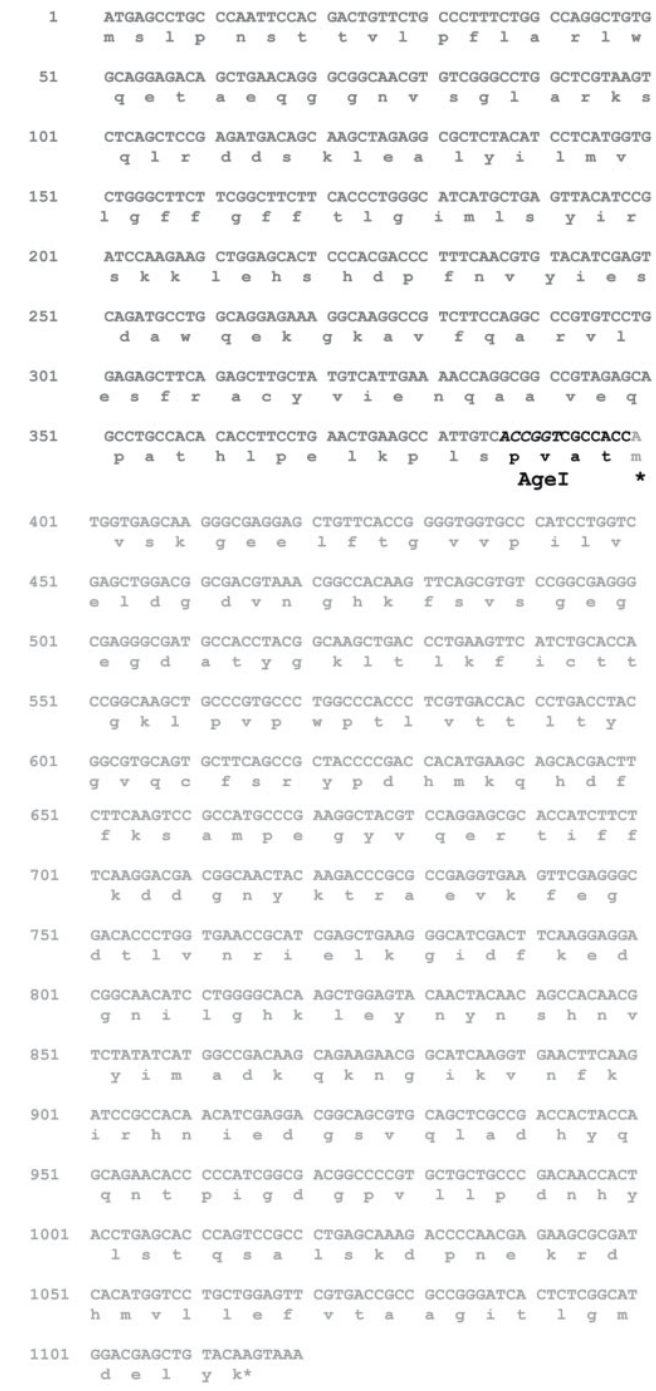

Figure 2.1: Nucleotide sequence of mIsK::EGFP fusion. Nucleotides are numbered from the first residue of the mIsK initiation codon. The AgeI site used to obtain an in-phase fusion of mIsK with the EGFP coding sequence is shown in italics. *initiation codon for EGFP; mouse IsK: nt 1–386; EGFP: nt 400–1116 (nt5nucleotide). Encoded amino acids are shown

(Life Technologies) at a concentration of 650 mg/ml. Neomycin-resistant clones were isolated after 2 weeks and further cultured in the presence of geneticin (G418).

For fluorescence-activated cell sorter (FACS) analysis, 10,000 cells were analysed in a FACStar analyser/sorter (Becton Dickinson, Mountain View, CA, USA) using the CellQuest program (Becton Dickinson). When appropriate, cells were stained with Hoechst 333425. Cultures were fixed with 4% paraformaldehyde in phosphate buffer for 20 minutes at 37°C. The cell monolayer was then incubated into a solution of Hoechst 333425 (1 mg/ml; Merck) for 15 minutes and rinsed twice with phosphate-buffered saline (PBS). After aspiration of the PBS, cells were coverslipped using FluorSave reagent (Calbiochem, La Jolla, CA, USA) and photographed using a Zeiss Axiophot-2 fluorescence microscope equipped with an Axiocam video camera (Carl Zeiss, Gottingen, Germany). Images obtained with a fluorescein isothiocyanate (FITC) filter (Chroma; excitation wavelength: 450–490 nm; emission wavelength: 515 nm) or a 4,6-diamidino-2-phenylindole (DAPI) filter (Chroma; excitation wavelength: 365 nm; emission wavelength: 420 nm) were acquired using the KS-300 software (Carl Zeiss) and analysed using Adobe Photoshop 6.0.

Statistical analysis

Data were analysed using chi-square test with Yates' correction or Mann-Whitney test using the GraphPad Instat software.

Results

The pTR-EGFP plasmid [19] encoding green fluorescent protein (GFP) under the control of the CMV promoter was used to produce a C-terminal fusion of the IsK protein with GFP (Figure 2.1). The resulting plasmid vector, pTR-mIsK::EGFP, also expressed the neomycin resistance gene in a separate transcription unit, allowing selecting cell clones expressing the fused coding sequence. Two days following transient transfection of the pTRmIsK:: EGFP plasmid into U87-MG glioma cells, the fusion protein appeared as a weak punctate fluorescence (Figure 2.2B) whereas cells containing pTR-EGFP harbored an homogenous cytoplasmic signal (Figure 2A). Interestingly, nuclear staining with Hoechst 333425 revealed that cells expressing mIsK harbored nuclear hallmarks of apoptosis such as chromatin marginalization/condensation whereas cells transfected with pTR-EGP displayed a normal nuclear aspect (Figure 2.2C,D). Cellular toxicity was further evident 5 days post-transfection because mIsK-expressing cells were shrunk (Figure 2.2F) with the presence of fluorescent ellular debris around the transfected cells (Figure 2G) supporting their continuing degeneration. Conversely, pTR-EGFP-transfected cells looked healthy (Figure 2.2E). Similar results were obtained with U373-MG and HEK293T cells overexpressing mIsK (data not shown). All these qualitative data thus suggest that IsK expression is deleterious and triggers cell degeneration. In line with these morphologic observations, quantitative studies demonstrated that the amount of GFPpositive U87-MG cells with nuclear signs of apoptosis was significantly increased 2 days following the transfection of pTR-mIsK::EGFP as compared with control cultures transfected with the pTR-EGFP vector (Table 2.1). This effect seemed directly related to mIsK expression because nuclear abnormalities were markedly higher in the GFP-positive cells than in the overall cell population (Table 2.1). Such nuclear alterations preceded cell death because the percentage of GFP-positive cells was

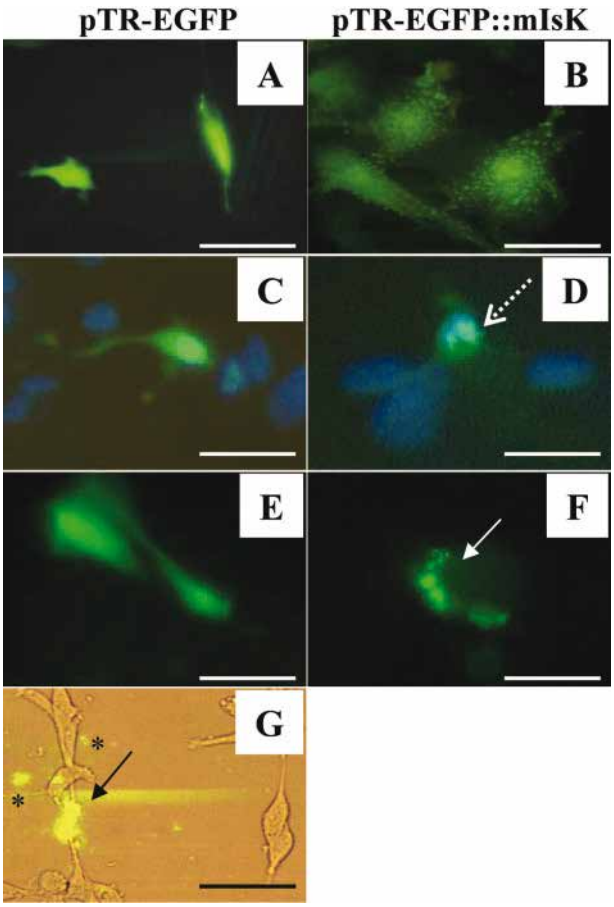

Figure 2.2: Transient transfection of pTR-EGFP and pTR-mIsK::EGFP into U87-MG cells. U87-MG cells were transfected with pTR-EGFP (A, C and E) or pTR-mIsK::EGFP (B, D, F and G) plasmids. Two days post-transfection, cells were fixed, incubated with Hoechst and examined under fluorescence microscopy using a FITC (A–G) or a DAPI filter (C and D). Images obtained using FITC and DAPI filters were fused (C and D). (G) Fluorescence in combination with bright-field microscopy. Dashed arrow: chromatin condensation triggered by mIsK::EGFP fusion protein expression; full arrow: pTRmIsK::EGFP-transfected cell; asterisk: cell debris; scale bar550 µm

significantly reduced 5 days following transfection of pTR-EGP::mIsK as compared with pTR-EGFP (Table 2.1). Noteworthily, these differences were not due to a difference in transfection efficiency between the two vectors used, because 2 days post-transfection, the proportion of GFP-positive cells was similar in cultures transfected by both pTR-EGFP::mIsK and pTR-EGFP vectors (Table 2.1). Similar data were obtained for U373- MG and HEK293T cells (data not shown). In agreement with these results, we found that the proportion of stable neomycin-resistant clones of U87-MG cells fully expressing EGFP was significantly lower after transfection of pTR-EGFP::mIsK as compared with transfection of pTR-EGFP control vector (Table 2.1).

Table 2.1: Transfection of human astroglioma cells with mISK::EGFP

	Transient					Stable
		2 days		5 days		
	n/N	GFP$^+$	Apoptotic cells	Apoptotic cells among GFP$^+$	% GFP$^+$	% GFP$^+$ clones
pTR-EGFP	5/164	7/164 (4.26%)	4/164 (2.43%)	0/7 (0%)	6.58 ± 4.31%	3/4 (75%)
pTR-EGFP::mIsK	18/814	29/814[a] (3.56%)	47/814[b] (5.77%)	18/29[c] (62.06%)	1.87 ± 0.62%[d]	0/12[e] (0%)

U87-MG cells were lipofected with pTR-EGFP and pTR-EGFP::mIsK plasmids. Two days post-transfection, cells were fixed and treated with Hoechst. Random fields from three separate transfections were examined under fluorescent microscopy. For each field, the percentage of GFP-positive cells (green fluorescence) and the percentage of apoptotic cells with fragmented chromatin (blue fluorescence) were counted. Five days post-transfection, the percentage of green fluorescent cells was evaluated by FACS analysis. Values are expressed as mean ¡ SD. Neomycin-resistant clones were isolated after 2 weeks and further cultured for 8 weeks in selective medium. The total number of clones and the number of fluorescent clones were counted under fluorescence microscopy using a FITC filter. n: number of fields; N: total number of cells examined; ND: not detected. Statistical analysis: chi-square test with Yates' correction: a, 0.83; b, 0.118; c, 0.0115; e, 0.0096; Mann-Whitney test: d, 0.057.

Discussion

The mouse IsK potassium channel accessory molecule was fused to EGFP. Transient expression of the fusion protein into human cell lines resulted in appearance of green fluorescent clusters on the cell membrane. Chronic expression of the fusion protein led to cell death of more than 50% of transfected U87-MG human astrocytoma cells as early as 2 days after transfection. Cell death was evidenced by condensed chromatin observed 2 days post-transfection and by the appearance of green fluorescent cellular debris in the transfected culture 5 days post-transfection. Furthermore, stable transfectants expressing GFP could not be obtained using the fusion construct.

Overexpression of IsK in various cell types resulted in modification of endogenous K^+ and Cl^- channels features [1,15]. Depending on the cellular background, overexpression of IsK is expected to either reveal endogenous previously silent channels or modify the activity of already active channels. It has been shown that a drop in intracellular K^+ creates a permissive state for apoptosis progression in various models [11,23]. Consistently, raising extracellular K^+ concentration as well as K^+ channel blockers have been shown to inhibit apoptosis [24]. Furthermore, apoptosis has been shown to be directly triggered by valinomycin, a selective K^+ ionophore, in thymocytes [5], vascular smooth muscle cells [7], tumour cells [6] and cortical neurons [24]. In the latter case, chromatin condensation and cell body shrinkage have been described. Exposure to the K^+ channel opener cromakalim also induced neuronal apoptosis [24]. In

accordance with these data, it has been suggested that cytoplasmic loss of Kz leads to caspase activation [3].

In conclusion, our results are consistent with activation of apoptotic pathways following IsK-mediated increase in K^+ efflux. Indeed, the observed cell shrinkage, appearance of cell debris and chromatin condensation are consistent with the hallmarks of apoptosis induced by low intracellular K^+concentration [24]. Alternatively, it could be that localized accumulation of GFP by clustering of multimerized K^+ channels causes toxicity. Indeed, it has been reported that overexpression of GFP can induce apoptosis [10].

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CHAPTER 3

Immunotherapy of Malignant Gliomas Using Autologous and Allogeneic Tissue Cells

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Summary

Immunotherapy of brain tumours is rapidly emerging as a potential clinical option [1-3]. The quality and magnitude of immune responses evoked by the new generation anti-tumour vaccines is in general highly dependent on the source or choice of peptide antigens, and as well, a suitable immunopotentiator. Poorly immunogenic antigens, such as those present in tumour cell lysates, may not reliably provide stimulation like recombinant or DNA-encoded protein antigens might be expected to. In addition, the efficacy of the vaccine may depend on inherent counteracting measures of the tumour which dampen immune surveillance and immune effector activity triggered by immunization [4]. Our body has many means of limiting an immune response to our own (self) proteins. In particular, patients with gliomas exhibit a broad suppression of cell-mediated immunity [5-8]. Unfortunately, for most tumour vaccines the induction of local or systemic immune effector cells does not necessarily translate into objective clinical responses or increased survival [9]. Here we review immunotherapeutic approaches against gliomas and recent pre-clinical and clinical initiatives based on cellular or active immunization of the patient's immune system using autologous and allogeneic tissues or cultured cells. Available evidence shows that single modality cancer therapies likely remain suboptimal. Combination regimens targeting the immune system at multiple coordinated levels must be developed, and possibly combined with strategies to inhibit immune suppressive factors if significant clinical benefit is to be achieved.

Keywords: Astrocytoma, allogeneic, biotherapy, brain tumour, CTL, glioma, immunotherapy, immunization.

Introduction

Glioblastoma multiforme (GBM) is the most common and malignant of all gliomas, with 75% of patients dying within 18 months of diagnosis. Brain tumours are graded according to their likely rate of growth, from grade I (benign) to grade IV (most malignant), with grades III and IV considered high-grade gliomas. Grade IV glioma is also known as glioblastoma multiforme (GBM). The prognosis for patients with this tumour is very poor. Glioblastoma overall survival rates are less than 3.3% at 5 years [10]. In the United States alone over 18,000 primary brain tumours are estimated to occur each year. Of these 18,000, over 60% are diagnosed gliomas. The median survival time of untreated GBM tumours is 3 months, with death most commonly due to cerebral edema or increased intracranial pressure. Even with the best available current therapy, which includes surgery, radiation, and chemotherapy, the median survival time is 14.6 months [11]. Due to their highly infiltrative nature complete surgical resection is difficult. These tumours are, therefore, inevitably recurrent either locally, usually within 2 cm of the original tumour, or at distant sites. Treatment of these recurrent lesions by a second surgery and further chemotherapy may increase the symptom free interval, but the 5- year survival remains 10%. The present review discusses various typical immunotherapeutic strategies in a comprehensive way, with a focus on active therapeutic immune intervention. All these therapies will serve as additions to, rather than a replacement of current medical practice. Due to many examples it is impossible to cover all preclinical approaches and past or ongoing clinical trials. We will, therefore, address only the most promising or remarkable strategies, but omission from or inclusion into this review should not be interpreted as rejection or support of a particular approach; they are examples. We propose that immune adjuvant therapies need to be explored to improve median or overall survival.

The long road to current glioma therapeutic Vaccine approaches

The first report on cancer therapy dates from the Edwin Smith Papyrus, 1600 B.C., the oldest known historical text on surgery. It mentions primitive surgical excision of a tumour using a knife. It has only been for the past two hundred years that surgical excision became established as the preferred option for treating solid tumours. Radiation therapy was introduced shortly after 1895, when Roentgen first reported the use of x-rays for diagnostic medical purposes, and remained a primary treatment option of certain tumours, especially solid tumours. Today radiation therapy can be tailored to the irregular shape of the tumour, allowing a reduction of intensity. During World War I, exposure of soldiers to nitrogen mustard gases resulted in pancytopenia. This observation triggered interest for chemotherapeutic therapy, especially in patients with hematologic malignancies. Today chemotherapy is a well documented treatment option. For glioblastomas, the alkylating agent temozolomide has become a well-appreciated option [12].

About one hundred years ago William B. Coley, an early 20th century New York City surgeon, observed that some of his patients with sarcoma would undergo spontaneous regression of their tumour and that this regression was associated with antecedent bacterial infection (erysipelas, or streptococcus pyogenes in the first

patient) which eliminated malignant cells [13, 14]. He intentionally injected cancer patients with a mixture of killed bacterial lysates, later called Coley's Toxins, to stimulate a cytokine storm. William Coley thus became the godfather of cancer immunotherapy [15]. He was the first to recognize the potential role of the immune system in cancer treatment. Activation of the patient's immune system to better recognize the tumour and to eliminate invasive tumour cells that are not adequately removed by surgery will be discussed in greater detail. This area in cancer therapy is based on knowledge accumulated in the vaccine and immunology fields.

Immunization attempts for glioma patients date back to the early 1960s [16]. Two investigative teams hypothesized that brain tumours were hidden from the immune system because of location in the central nervous system. To ensure that immune cells located systemically could "see" the tumour, they placed autologous glioma cells into the thighs of patients. The vaccines given into the thighs actually started growing there and metastasized to regional nodes [16, 17]. Ten years later, Bloom and colleagues administered irradiated, whole autologous tumour cell vaccines. Again, there was no effect, but the results may well have been different had they used an adjuvant, such as that used by Trouillas and Lapras [18] who immunized 20 patients with autologous tumour and Freund's complete adjuvant. These early attempts at active immunotherapy had obstacles consisting of potentially radiation resistant cells, and the absences of, or inappropriate, immune adjuvant. Degrees of sophistication in vaccine development have occurred in the past 50 years; and at last the benefits of active immunotherapy are finally documented.

Key considerations for development of immunotherapy Approaches

Tumour Directed Immune Responses

Distinct from classical preventive vaccines, which mostly require the generation of antibodies, the goal of most therapeutic GBM vaccines is to stimulate tumour-specific cytotoxic T cells (CTL) to function at the tumour site. Over the past 20 years we have only begun to learn how to accomplish this. A key factor for the success of a vaccine concerns the requirement of expression of unique tumour antigens, preferably in the context of class I Major Histocompatibility Complex (MHC), on suitable target tumour cells. Upon stimulation of the immune response, the host can specifically target these antigens, by producing antibodies, or by activating immune effector cells which recognize specific antigens in context of MHC Class I antigens. Studies have shown that human glioma cells do express variable levels of class I MHC antigens, and that IFN-□ upregulates these class I antigens [19]. In addition, Fas is expressed on glioma cells [20], providing a possible mechanism for tumour cell death [20]. Therefore under the appropriate conditions, glioma cells can be a target of CTL.

Remarkably, recent animal experiments in various tumour models showed that apart from CD8-positive cytotoxic T cells, which are considered the current "gold standard" effector cell in tumour immunotherapy, CD4 T cells can eliminate tumours that are resistant to CD8-mediated rejection [21]. Indeed, CD4 T cells collaborated with natural killer (NK) cells to achieve this antitumour effect. These findings suggest that adaptive CD4 T cells can also be successful effector cells, and in some cases, even

outperform CD8 positive T cells as mediators of cell death. This observation of efficient tumour elimination by CD4 T cells, using indirect mechanisms that do not require MHC expression by the tumour cell, suggests that it might be possible to attack even poorly MHC expressing tumours by designing strategies to elicit CD4 T cell responses against the tumourigenic proteins [21].

Adoptive Transfer of Effector T Cells

Adoptive transfer of lymphocytes into patients is classified as a variant of passive immunotherapy. Adoptive transfer of lymphocytes into brain tumour patients is not a new concept. Indeed, thinking that the blood brain barrier inhibited the passage of immune cells into the brain, one of the first clinical trials involved the adoptive transfer of autologous, unstimulated lymphocytes intrathecally into glioma patients, accompanied with or without interferon [22]. Since the advent of recombinant growth factors, adoptive immunotherapy now in principle, is based on the concept of infusing *ex vivo* expanded effector cells after their *in vitro* activation against tumour antigens and/or culture in growth factor containing media, back into the patient. The whole gamut of effector cells have been tested including autologous or allogeneic cells that are nonspecifically or specifically activated [23, 24]. In newly diagnosed malignant gliomas, this approach revealed several objective clinical responses without adverse effects [25]. Expansion of autologous tumourspecific T lymphocytes *in vitro* with IL-1, IL-2, and/or IL-4, followed by injecting directly into the tumour site gave response rates that were not consistently encouraging [26]. A further variant of this approach included genetic manipulation of such *ex vivo* isolated cells by the introduction of immunostimulatory transgenes [27].

Tumour Associated Antigens (TAAs)

One important step in producing a therapeutic vaccine is the identification of appropriate glioma-associated antigens. Limited knowledge of molecularly defined antigens explains why initial approaches used tumour lysates derived from autologous irradiated glioma cells as the source of tumour antigens. This approach warrants specific activation of the immune response against a broad set of potential tumour associated antigens, which may enhance immune therapy but may also result in immune reactions against unwanted antigens [28]. Glioma associated, rather than glioma specific, appears to be the more correct terminology since the antigens appear to be present at very low levels on normal brain cells, but overexpressed on glioma cells. Importantly, for the majority of studies no evidence of adverse autoimmunity was noted after such immunizations. The premise was that by providing the entire tumour cell, all the appropriate antigens would be made available to the antigen presenting cells of the immune system. Unfortunately, these procedures did not lead to successful therapies [26]. The advent of molecular biology enabled approaches aimed to identify the specific glioma-associated antigens (GAA) at the molecular level [3, 20, 29, 30]. Jadus and colleagues analysed adult and pediatric brain tumour cell lines and some primary tissues for tumour-associated antigens [30, 31]. The glioma cell lines were characterized for 20 tumourassociated antigens by quantitative reverse-transcriptase real time polymerase chain reactions (qRT-PCR), and where antibodies were available, the protein expressions were confirmed microscopically using flourescently-tagged antibodies or by intracellular flow cytometry. In general, the glioma cell lines had high mRNA expression

for the antigens and also made the proteins. Thus, primary malignant brain tumours and the cell lines express many tumour associated antigens; a truncated list of them is given in Table 3.1. From these data we conclude that either primary tumour specimens or cell lines could serve as suitable sources for antigens for vaccine development. In general, the surgical specimens from the adult glioblastomas had a more robust antigenic profile than those from the pediatric tumour specimens. Since many of the tumour associated antigens display HLA-restriction, we also conclude that given the known HLA type of the tumour patient, one might predict which antigens might likely be associated with his tumour [30]. Certainly in conjunction with cytogenetic information, especially genomic imbalances, one might predict overexpression of certain antigens.

Interestingly, recent genomic studies have revealed the genes of human glioblastomas and provided insight into associated molecular pathways [32-34]. The genetic subtypes are associated with prognosis. These analyses may provide further refinement on potential glioma-associated target antigens. The Cancer Genome Atlas (TCGA) Research Network Comprehensive genomic characterization defines human glioma genes and core pathways [35].

The tumour antigens most suitable to activating the host specific T cell response are still under investigation. Prominent tumourassociated antigens that others have considered suitable for vaccine development include tenascin (glioma-specific extracellular matrix), gp240 (chondroitin sulfate-associated antigen found in glioma), MAGE 1 and MAGE 3 [36, 37]. For development of their vaccines, Okada et al. focused on epitopes that specifically bound to the class I HLA-A2 such as EphA2, IL-13Ra2, YKL-40 and GP- 100 [29]. More specifically, the IL-13 Ra2 and EphA2-derived epitopes were shown to stimulate immune activity. The successful use of these antigens may also largely depend on the immunopotentiator system used for switching on and maintaining the specific immune reaction directed towards the antigen of interest [38]. This will be addressed later.

Activation of Antigen Presenting Cells

The activation of the cytotoxic T cells generally requires the stimulatory interaction with T helper cells, which recognize antigen in context of Class II expression. Therefore cells that express high levels of class II antigens are most efficient antigen presenting cells (APC) for triggering helper T cells. Although macrophages and microglia

Table 3.1. Tumour-Associated Antigens Overexpressed in Primary Malignant Brain Tumours

Aim-2	Ezh2	HNRPL	Prame	Sox11	Ube2V
Art-1	Fosl1	IL-13Ra2	PTH-rP	SSX-2	Whsc2
Art-4	Gage-1	Mage-1	Sart-1	Survivin	Wt-1
B-cyclin	Galt-3	Mart-1	Sart-2	Tert	YKL-40
CD133	GnT-V	MELK	Sart-3	TRP-1	
EGFRvIII	Gp100	MRP-3	Sox 10	TRP-2	
Epha2	Her2	NY-Eso-1	Sox 2	Tyrosinase	

express Class II, dendritic cells (DC) are the most efficient antigen presenting cells [39]. Dendritic cells are also called "nature's adjuvant", and represent the key APCs for induction of primary immune responses. As sentinels they sample peripheral tissue for potential antigens and bring them to draining peripheral lymph nodes to present the processed antigens to potential antigenspecific T lymphocytes.

Therefore events that trigger the maturation and activation of these cells under natural conditions at the tumour site, or in the cervical lymph nodes are considered valuable for natural tumour surveillance. By contrast, maturation of APCs at the site of the vaccine inoculums or in the local draining lymph node are likely important for producing an effective vaccine [38]. There is substantial evidence that DCs exposed to tumour antigens under the right circumstances are potent stimulators of cytotoxic T cells.

Dendritic Cell Vaccines

In recent years there have been numerous attempts to use dendritic cells in therapeutic "vaccines" [3, 40-42]. The isolation of dendritic cells by elutriation enabled the handling and *in vitro* loading with antigens. Unfortunately, there is no consensus on the best protocol to isolate and use DCs; different DC subtypes may exert distinct functions and efficacy. Recently, several dendritic cell vaccines have been tested in glioblastoma immunotherapy. These were based on either crude tumour lysates or acid-eluted peptides from cell cultures derived from surgically removed glioblastoma multiforme [9, 43-45]. In some patients, a peripheral CTL response was detected. However, only sporadic objective responses and modest increases in the patient's survival were observed, with no long-term survivors, from these approaches [9, 43-45]. Importantly, no evidence of adverse autoimmunity was noted after these immunizations.

Siesjo et al. showed that co-administration of autologous tumour cells with autologous DC decreased tumour growth [46]. Liau et al. demonstrated that autologous DC incubated with autologous tumour protein increased survival, and increased tumour-specific cytotoxic T cells within the tumour [9]. These initial studies that were performed in only a few patients led to clinical trials. Different methods to activate these DC were explored. Expanding the immune response was first approached by using non-specific immunomodulators, such as BCG, IL-2, interferon (IFN)-α, which had been registered as approved immunostimulants.

Immunopotentiators Co-Administered with Tumour Antigens

As mentioned earlier the success of glioma immunotherapy will depend on better understanding of glioma biology but also from lessons learned from the vaccine and immunology fields [47]. It is beyond the scope of this review to mention the plethora of distinct immunopotentiators used in various pre-clinical and clinical settings, in most cases with different types of antigen formats.

Here we briefly mention a few types which employ in most cases immunostimulants that were approved for human application. In a rat model, IL-4 was shown to have the most potent therapeutic results, mediating local endothelial cell activation, recruiting immune T cells, and stimulating antibody production [45]. IFN-α was also a possible candidate because this cytokine stimulated endothelial cells to produce CXCL-10, a chemokine shown to induce homing of cytotoxic T cells (Tc1) to

the tumour site [48]. Granulocyte macrophage-colony stimulating factor (GM-CSF) has also been used as an effective adjuvant to attract large numbers of antigen presenting cells to the vaccination site [25]. The heat shock protein (HSP) 70, in particular, has been shown to directly activate NK cells and indirectly stimulate the dendritic cell population; in addition, this extracellular secreted product induces the release of IFN-γ from peripheral blood leucocytes of tumour patients thereby further stimulating the immune response [49]. Other studies showed that poly I:C, the ligand for the toll-like receptor (TLR)3, was effective in triggering the maturation and functional activity of DC [50]. Further studies used lysine and carboxymethyl cellulose stabilized poly I:C (poly ICLC) to activate cells, however no significant advantage to survival in humans was reported [29]. Yet another approach used CpG-oligonucleotide (ODN), which binds to TLR9, and thereby stimulates immune responsiveness [7]. Although no registered effective treatments are currently available, sporadic positive clinical responses were observed following immunization with vaccines prepared from the patients' autologous irradiated glioma tumour cells mixed with GM-CSF, irradiated GM-CSF secreting K562 cells, or IL-4-secreting fibroblasts (www.clinicaltrials. gov NCT00694330) [25, 45, 51]. In general, increases in patient survival were observed, however these approaches yielded no longterm survivors [9, 43, 45]. Importantly, no evidence of adverse autoimmunity was noted after these immunizations.

Collectively, these studies emphasize the importance of both the correct activation of the cell population presenting the antigen, as well as the accurate delivery of the targeted antigen, as part of the vaccine approach. It should be realized that a potent new antigen is not enough to make a vaccine. The antigen should be presented to the immune system in a formulation which activates the desired immune pathway required for tumour eradication. Vaccine immunopotentiators comprise a diverse group of molecules or formulations, which have been used routinely as critical components of inactivated antimicrobial vaccines for almost a century. Advances in vaccine research are expected to emerge from better knowledge of immune pathways and antigen delivery [52]. Apart from the historic examples of immunopotentiator technologies a variety of other options have not been explored as yet because of restrictions for use in human subjects [47].

Immunotherapeutic Concepts

The human body has evolved a complex immune system to eliminate pathogens and abnormal cells with minimal damage to healthy tissues. The immune system has multiple levels of regulation to guarantee the appropriate balance between immune activation and immune suppression. Over the last two decades the fundamentals of this regulation have become clearer. This knowledge now provides new opportunities for rational intervention based on a tailored response of the immune system against tumours that may lead to clinical benefit.

Since the observations by William Coley there has been a long held belief that the immune system can prevent the emergence and growth of cancer. Indeed, Galon and coworkers elegantly demonstrated that an adaptive immune response influences the behavior of human colon cancer tumours by in situ analysis of tumour-infiltrating immune cells [53, 54]. Also, in ovarian cancer the presence of intraepithelial tumour infiltrating lymphocytes is associated with significantly longer clinical remission after chemotherapy and with improved overall survival [55]. Similar observations have

been made for other tumours, including renal carcinoma [56], prostate cancer [57] and breast carcinoma [58]. The observation that improved survival is linked to the capacity to mount natural immune responses supports the concept to stimulate the patient's immune effector responses by active immune programming.

As for most traditional vaccines the quality and magnitude of immune responses evoked by new generation immunotherapeutic anti-tumour approaches is in general highly dependent on the choice of vaccine antigen and the provision of a suitable immunopotentiator or concurrent immune activation strategy; especially for poorly immunogenic antigens, such as tumour cell lysates, recombinant or DNA-encoded protein antigens [38, 47].

Despite decades of failed attempts to develop effective cancer vaccines, several candidates are now progressing towards commercial approval. Several distinct technological concepts have been pursued to achieve immunotherapeutic cancer treatment, which are summarized in Table 3.2.

i) Classical vaccines consisting of tumour antigens, isolated either from tumour cell lines or *ex vivo* tumour tissue obtained by surgical removal, and formulated or co-administered together with an effective immunopotentiator into a vaccine formulation. This vaccine is injected parenterally and able to unlock the required immune response [1, 29]

ii) The source of tumour associated antigens (TAA) may also be generated by recombinant biotechnology procedures, such as laboratory- based expression from mammalian, bacterial, yeast, insect or plant cells, or as synthetic protein peptides.

iii) The loading of TAAs, either contained in surgically removed tumour cells or isolated as tumour-derived peptides, or as synthetic or recombinant molecular mimics, on the patient's own dendritic cells, which are isolated from the patient by leukapheresis, and after a culture period in the laboratory, transfused back into the patient [9, 40]. These autologous dendritic cells are activated in the culture dish by a mixture of recombinant cytokines or immunostimulatory ligands.

iv) Instead of protein antigens, investigators have used the genes encoding these antigens, either in RNA or DNA format or inserted in replicating viral or bacterial vectors which produce the TAAs in the patient after parenteral injection. Such genetic vaccines contain immunostimulatory sequences which directly activate immune cells after recognition by specific receptors. In addition, the provision of gene-encoded antigens allows for intracellular expression and correct processing of candidate antigens by the patients antigen presenting immune cells.

v) As a variation of the above strategy, the same genes can be delivered directly into isolated dendritic cells by transfection in culture dishes before infusion. This approach assures the correct targeting of the gene-encoded TAAs into the patients dendritic cells in the laboratory before infusion.

vi) Instead of providing TAAs in the context of a favorable immune system activating conditions, clinical trials have been designed with patient-derived tumour-specific T cells. These cells have been developed in the patient and are isolated and expanded in the laboratory in specialized culture media supplemented with T cell growth promoting molecules before reinfusion back into the patient.

vii) Another strategy involves the administration of immunostimulatory agents which strengthen the endogenous immune response of the patient in order to better attack the tumour. For example, this approach comprises intravenous

Table 3.2. Survey of Immunotherapy Approaches

	Tumour Associated Antigens (TAA)	Immunopotentiation	Category
1	From lysates extracted from cell lines or tumour tissue	Classical vaccine adjuvants, or cytokines such as interleukin (IL)-12, GM-CSF, TNF, haptens, BCG, etc	Classical vaccine
2	Recombinant proteins or peptides	Classical vaccine adjuvants, or cytokines such as interleukin (IL)-12, GM-CSF, TNF, haptens, BCG, etc	Modern vaccine
3	Antigens from 1 or 2 loaded on patient-derived autologous dendritic cells	Activating cytokine (or cocktail)	Dendritic cell vaccine
4	RNA, DNA, or viral/ microbial vector encoding the TAA	Direct parenteral injection, without or with immunopotentiators	Genetic vaccine
5	RNA, DNA, or viral vector encoding the TAA	Transfection into dendritic cells	Dendritic cell vaccine
6	Endogenous TAAs expressed/released by the tumour	Patient-derived or allogeneic T lymphocytes cultured in medium facilitating T cell survival and expansion, sometimes gene modified T cells expressing transgenic T cell receptors specific for defined TAA	Adoptive T cell transfer
7	Endogenous TAAs expressed (released) by the tumour	Cytokine or immunostimulant administration (IFN, IL-2, IL-12, TLR agonists etc.)	Non-specific stand-alone immunostimulation
8	Endogenous TAAs released from the tumour after local destruction of tumour tissue by ablation	Local inflammatory responses resulting from apoptosis or necrosis of tumour tissue, optionally combined with immunostimulants	Tumour necrosis therapy (TNT) by non-specific local immune activation
9	One of the categories of exogenous or endogenous TAAs described above	Blockade of endogenous immunosuppressive cells or molecules (PD-1, CTLA-4, TGF-β, IL-10, regulatory T cells, etc.)	Inhibition of local or systemic immune suppression
10	Two or more combinations of the above approaches		Multi-modal

administration of recombinant cytokines (IL-2, IL-12) or microbe-derived immunostimulants, including toll-like receptor agonists, like synthetic CpG motifs [59], stabilized synthetic RNA oligonucleotides (ODN) [60] or resiquimod® [61].

viii) The above approach activates the systemic immune activity and may enforce the critical local anti-tumour effector responses to a limited extent. Therefore, alternative strategies involve local immune manipulation by local administration of immune activating agents in combination with local destruction of tumour tissue, and associated liberation of TAAs following surgical ablation or tumour targeting by "magic bullets" such as TAA-specific or tumour cell DNA-targeting antibodies . Examples include the radiotherapeutic strategy, called Cotara®, that employs the isotope 131Iodine conjugated to an antibody that binds the necrotic core found in all solid tumours [62], or antibody-targeted delivery of chemokines by chemokine/antibody fusion proteins, which results in high local tumour-associated concentrations of chemokines that attract monocytes, neutrophils and lymphocytes [63]. Yet another variant of this concept includes locoregional or intratumour application of oncolytic viruses, e.g. NDV [64] or HSV [65], which cause a proinflammatory immune response in the vicinity of the tumour tissue.

ix) All above-mentioned approaches are based on expanding tumour–specific immune effector elements. In addition, it is known that during immune surveillance reactions, local or systemic immunosuppressive regulation contributes to the escape of solid brain tumours and that such processes may also contribute to the inhibition of suboptimal, vaccination-triggered immunotherapy [5-7, 66]. Hence, inhibition of immunosuppressive molecules, such as CTLA- 4 [67] PD-1 [68-71], TGF-β [72], IL-10, or immunosuppressive regulatory T cell [50, 73, 74], may also contribute to anti-tumour immunity. Abrogation of immunosuppression can be achieved by a stand–alone approach, or as a push-and-pull tactic in combination with one of the active immunotherapeutic approaches mentioned earlier [75].

x) Combinations of two or more of the above-mentioned approaches.

Tumour Mediated Immune Suppression

Despite stimulatory strategies, the production of active immunotherapy in cancer patients at an advanced stage of disease may be hampered by the suppression of systemic immune responsiveness in these patients [4]. Radiation and chemotherapy can induce generalized immune suppression because these treatments reduce the production of immune competent cells. Such therapies can provide the natural selection process resulting in the development of resistant tumours [4, 76, 77]. Furthermore, the administration of highly immunosuppressive glucocorticoids to control brain edema would tend to point toward vaccine administration early after diagnosis rather than later, after patients develop systemic immune suppression. Evidence collected long ago showed that glucocorticoids such as dexamethasone transcriptionally inhibit IL-2 synthesis in T lymphocytes. They interfere with nuclear factor activating protein-1 binding to the IL-2 promoter and also with calcineurin dependent pathways for T cell activation [78]. Since endogenous immune cell activation and proliferation must be engendered upon successful immunization and is reliant upon mRNA and protein synthesis [79], vaccination would be optimal if given to patients who are not steroid-dependent.

In addition to drugs, the tumour itself can regulate immune reactivity. It

has been recognized for some time that patients with malignant gliomas demonstrate a profound immune suppression when compared to normal persons, suggesting that gliomas produce an immune inhibitory environment [20, 80]. Such immune suppression is mediated by soluble cytokines and growth factors. For example, transforming growth factor (TGF)-β, and interleukin (IL)-10 have been reported to be secreted by glioma cells [81, 82]. These cytokines functionally impair T cell activity and are responsible for the development of immunotolerizing T regulatory (T reg) cells [83]. Gliomas also produce prostaglandin E2 (PGE2), and IL-6, both potential immune suppressive agents, as well as macrophage chemoattractive protein (MCP-1) [29, 84, 85]. In addition, local synthesis of Vascular Endothelial Growth Factor (VEGF) inhibits DC maturation on the one hand, and induces tumour-promoting angiogenesis on the other hand [86]. Apart from these soluble factors, gliomas may express membrane-bound Fas ligand (FasL), which induces apoptotic cell death of infiltrating immune cells when interacting with Fas, and/or PD-1, an inhibitory co-receptor, a B-7 family member, which attenuates T cell receptor signalling of infiltrating lymphocytes [29]. These mechanisms can shut down anti-tumour immunity and can be viewed as a counterattack by the tumour.

CD8- and CD4-positive T cells become inhibited after expression of CTLA-4 and subsequent interaction with its ligands, CD80 or CD86. Hence, the use of the anti-CTLA-4 antibody has resulted in better and more sustained anti-tumour responses [87]. Another recent approach to reversing this immune suppression is the use of small molecule inhibitory drugs to block the common signalling pathway to these suppressive activities, including the Signal Transducers and Activators of Transcription 3 (STAT3) activation pathway [80]. Thus it has to be appreciated that multiple factors must be taken into account when considering an immune therapy approach. Regardless of the stimulatory concepts attention should be given to factors which promote immune escape.

In addition, immunosuppressive enzymes may provide another way for tumour cells to evade immune responses. Ninety percent of human glioblastomas are positive for indoleamine 2,3, dioxgenase (Ido-1) [4, 88]. Ido expression is accompanied by a lack of accumulation of specific T cells at the tumour site.

Recent Encouraging Pre-Clinical Results

As mentioned earlier, initial tumour vaccine approaches used tumour lysates derived from irradiated glioma cells as the source of tumour antigens or whole irradiated tumour cells themselves. However, while the tumour cells contain a plethora of tumour associated antigens, they may be present at relatively low levels. Additionally, tumour specimens can contain normal, nonmalignant cells as well as tumour cells. Nevertheless, in a rat model where systematic subcutaneous administration of either allogeneic or xenogeneic tumour cells, or that combined with syngeneic cell lysates, proved safe and protective in early and advanced malignant glioma growth [89]. These results suggest that injections of allogeneic cells and/or lysates, or xenogeneic cell lines, can activate the immune system and can break anti-self/tumour. Also, these cells likely contained critical antigenic determinants shared with the implanted tumour, leading to a reduction in tumour growth. These data therefore, support the potential viability of this cancer vaccine strategy as an adjuvant treatment to prevent tumour relapse in cancer patients after standard surgical removal of the tumour. The impact of such data may be far reaching when translation of this strategy to patients proves possible. Indeed, alloresponsive effects may prove to be powerful.

Immunotherapy Approaches Utilizing Allogeneic Cells for Cancer Treatment

Table 3.3 provides examples of open or pending clinical trials utilizing allogeneic cells for immunotherapy of cancer. There are cellular therapy trials using allogeneic effector T cells a) sensitized to tumour associated antigens or patient human leukocyte antigen, b) genetically modified T cells with targeting elements for brain tumour antigenic receptors as well as to T cell receptor (TCR) signalling, or T cells sensitized to highly antigenic viral proteins. For the latter, cytomegalovirus-specific T cells are used because of subclinical reactivation in CMV-exposed brain tumour-bearing individuals [90- 92]. Since the allogeneic effector cells are administered directly into the brain they are protected, at least for a short while, from destruction by the host's immune cells. The use of allogeneic cells also obviates the use of immune cells from immunosuppressed cancer patients. The peripheral blood mononuclear cells of glioma patients has been documented to contain higher numbers of T regulatory cells [8]; as well, the CD4 to CD8 ratios of T cells are about 1:1 instead of 2:1 as it is in normal individuals [93].

Other immunotherapy approaches listed in Table 3.3 involving allogeneic cells employ whole tumour cell vaccines using allogeneic tumour cells with TGF-β

Table 3.3. Immune Therapies Using Allogeneic Cells or Tissue

Site/Investigator	Description	Disease	Study Phase-Enroolment	References
City of Hope, Duarte, CA/ B Badie	Allogeneic T Cells modified with chimeric IL-13α2 -TCR ζ	Brain tumour	I - 10	[97]
Penn State Univ, Hershey, PA/ K Lucas	Allogeneic, CMV specific CTL	Brain tumour I/ II - 10	[98]	
UCLA, Los Angeles, CA/L Liau & C Kruse	Alloreactive CTL and IL-2	Brain tumour	I - 15	[93, 99]
NovaRx, San Diego, CA/H Fakhrai	Allogeneic Tumour Cell Vaccine with TGF2 knockdown	Non-small cell lung carcinoma and brain tumour	II -75	[100]
Baylor, Houston, TX/ J Fay	Autologous DC pulsed with Allogeneic Melanoma Tumour	Melanoma	I/II - 33	Clinicaltrials.gov, NCT00313235
IDM Res Lab, Sanofi-Aventis, Paris, France/ M Salcedo	Autologous DC pulsed with Allogeneic Melanoma Tumour	Melanoma	I/II - 15	[101]
Univ Pittsburgh, Pittsburgh, PA/ G Chatta	Autologous DC pulsed with Allogeneic Prostate Tumour	Prostate cancer	I - 12	Clinicaltrials.gov, NCT00970203

knockdown, or the use of autologous dendritic cells that are pulsed with antigens derived from allogeneic tumour cells. While the majority of clinical trials still remain in the Phase I or I/II arena, no phase III clinical trials have been completed at this time. The results from a handful of immunotherapy trials are finally being reported. A prominent example is DCVax®- Brain, an immunostimulant cancer vaccine, based on experimental autologous cellular therapy, produced by the American pharmaceutical company Northwest Biotherapeutics, Inc., which exhibits promising efficacy (www.clinicaltrials.gov NCT00045968) [9]. DCVax-Brain vaccine is manufactured using a patient's dendritic cells loaded with a tumour cell lysate prepared from surgically resected tumour tissue. The clinical data from a cohort of 141 newlydiagnosed glioblastoma patients treated in a Phase II study is still being collected, however, assessments from their Phase I trials suggest overall safety, with delayed time to disease recurrence and increased survival, especially in glioblastoma patients with stable disease at entry [see http://www.nwbio.com/clinical_dcvax_ brain.php]. Indeed, for those patients treated in the Phase I trials, the company is reporting that the median survival is 33.8 months, with 9 of 19 patients still alive at 8-82 months from initial surgery. A multiinstitutional Phase II trial where 82 patients were treated was supported by Pfizer Pharmaceutical company, Celldex Therapeutics [see http://www.celldextherapeutics.com/]. The trial is described at (www.clinicaltrials.gov NCT00458601) and they report interim positive results from the Phase 2b study. This involves a non-cell based vaccine using an EGFRviii peptide conjugate, CDX-110, given in conjunction with temozolomide [94, 95]. Newly diagnosed WHO Grade IV glioma patients were treated. The trial is still active for follow-up, but not currently recruiting patients. The company ImmunoCellular Therapeutics, Ltd (http://www.imuc.com/) has supported a Phase I study using ICT- 107 for glioblastoma. This is a dendritic cell-based vaccine that targets multiple glioma associated antigens [44, 96]. In a June, 2010 company report they say that the median overall survival had not yet been reached at the 26.4 months analysis point, with 12 out of 16 treated newly diagnosed patients alive (http://www.imuc.com /pdf/Brain-Cancer-Vaccine-Looks-Promising-in-Small-Trial-1.pdf# zoom=100). Other clinical trials involving immunotherapy for brain gliomas can be found at www.clinicaltrials.gov by refining search terms using key words at that site.

Conclusion

To conclude, the results of the most recent clinical trials suggest that systemic immunotherapy using dendritic cells or peptide vaccines are capable of inducing an immune response in malignant glioma; increased patient survival has been reported, though no phase III clinical trials are completed to this time. Apart from better targeted radiotherapy and more fine tuned surgery, we will experience a gradual continuing increase of immunological insight that will enable novel intervention strategies. Successful vaccine approaches will likely result from the "golden" combination of antigen(s) and immunopotentiators [97-101].

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CHAPTER 4

Development of immune memory to glial brain tumours after tumour regression induced by immunotherapeutic Toll-like receptor 7/8 activation

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Summary

The efficacy of immunotherapeutic TLR7/8 activation by resiquimod (R848) was evaluated *in vivo*, in the CNS-1 rat glioma model syngeneic to Lewis rats. The immune treatment was compared with cytotoxic cyclophosphamide chemotherapy, and as well, was compared with the combination of cytotoxic and immunotherapeutic treatments. We found that parenteral treatment with the TLR7/8 agonist, resiquimod, eventually induced complete tumour regression of CNS-1 glioblastoma tumours in Lewis rats. Cyclophosphamide (CY) treatment also resulted in dramatic CNS-1 remission, while the combined treatment showed similar antitumour effects. The resiquimod efficacy appeared not to be associated with direct injury to CNS-1 growth, while CY proved to exert tumouricidal cytotoxicity to the tumour cells. Rats that were cured by treatment with the innate immune response modifier resiquimod proved to be fully immune to secondary CNS-1 tumour rechallenge. They all remained tumour-free and survived. In contrast, rats that controlled CNS-1 tumour growth as a result of CY treatment did not develop immune memory, as demonstrated by their failure to reject a secondary CNS-1 tumour challenge; they showed a concomittant outgrowth of the primary tumour upon secondary tumour exposure. Rechallenge of rats that initially contained tumour growth by combination chemo-immunotherapy also failed to reject secondary tumour challenge, indicating that the cytotoxic effect of the CY likely extended to the endogenous memory immune cells as well as to the tumour. These data demonstrate strong therapeutic antitumour efficacy for the immune response modifier resiquimod leading to immunological memory, and suggest that CY treatment, although effective as chemotherapeutic agent, may be deleterious to maintenance of long-term antitumour immune memory. These data also highlight the importance of the sequence in which a multi-modal therapy is administered.

Keywords: TLR agonist, brain tumour, glioma, immunotherapy.

Introduction

Glioblastoma multiforme (GBM) is the most common malignant brain tumour in adult patients. Due to its highly infiltrative nature GBM is notoriously difficult to treat and complete surgical resection is difficult. GBM tumours are inevitably recurrent either locally, close to the original tumour, or at distant sites. Moreover, chemotherapy and/or radiotherapy have shown only limited success. As a result the overall prognosis for this tumour has changed little over the last two decades. Today the average survival time for a newly diagnosed patient is between 12 and 15 months and new forms of therapy are desperately needed to change the clinical course of this highly malignant tumour. GBM therefore requires additional forms of therapy to prolong the lifespan and quality of life of patients. Immunotherapy is now emerging as a novel fourth option for clinicians.

Immunotherapy stimulates and teaches the patient's immune system to recognize and eradicate malignant tumour cells. If successful it has the added advantage of generating a memory response to prevent tumour reoccurrences after cessation of treatment. Our immune system has evolved to protect our body by eliminating pathogens and abnormal cells with minimal damage to healthy tissues. It is complex and has multiple levels of regulation to guarantee the appropriate balance between immune activation and immune suppression. Over the last two decades the fundamentals of this regulation have become more clear.

It has been amply shown that the immune system can prevent the emergence and growth of cancer. For example, an adaptive immune response influences the behavior of human colon cancer tumours as evidenced by in situ analysis of tumour-infiltrating immune cells. [1,2] Also, in ovarian cancer the presence of intraepithelial tumour infiltrating lymphocytes is associated with prolonged clinical remission and improved survival. [3] Similar observations have been made for other tumours, such as renal carcinoma, [4] prostate cancer [5] and breast carcinoma. [6] These observations in patients are further supported by the observations in experimental systems that an impaired immune system is less able to protect the host against the development of spontaneous and chemically-induced tumours. [7-9] In addition, individuals with cancer sometimes develop spontaneous reactivity against the antigens of the tumour. [10] Hence, it can be concluded that tumours can be recognized and eliminated as a result of natural tumour-specific immune responses that develop in the host.

The observations that the capacity to mount natural immune responses is linked to improved survival supports the concept to develop immune activating tumour vaccines, stand alone immune activators, or biological response modifiers. Presently, such strategies are tested in clinical trials for the treatment of different types of solid tumours, including glioblastoma, as recently reviewed by Hofman and coworkers. [11] To date, these approaches have provided only modest clinical results. Nevertheless, they have shown promise by successfully generating antigen specific effector T cells capable of reacting with the tumour, and significant survival advantage or improved quality of life in subgroups. Remarkably, effector immune cells may fail to produce tumour regression because newly triggered and successfully expanded tumour-specific lymphocytes are actively inhibited within the draining lymph nodes or upon entrance into the tumour. In recent years it has become well-established that many tumours, including GBM, use various mechanisms of immune suppression or evasion, including immunediting, and

the generation of T regulatory (Treg) and myeloid derived suppressor cells (MDSC). [12] These cells act to inhibit the beneficial effects of immune activation [13] by direct cell contact mechanisms or by secretion of inhibitory molecules, such as IL-10, and TGF-b. [14,15] As a result, the suppression of immunity in tumours may present a major challenge to clinicians interested in using tumour vaccines or other methods of immune activation to treat tumours at the time of diagnosis. Immunotherapy may be further complicated in situations where the immune system promotes tumour development by selecting for tumour escape variants with reduced immunogenicity. [16]

Hence, successful immunotherapy for the treatment of solid tumours may require two entirely different steps: (1) the use of potent immune activators such as single immunostimulants or tumour vaccines comprising suitable adjuvants; and (2) reagents that can reverse immune suppression induced by the tumour. In the last two decades there has been a strong interest in using toll-like receptor (TLR) agonists as immunostimulants and adjuvants for therapeutic vaccines because of their stimulatory effects on innate immune responses which precedes the shaping of adaptive immune effector and memory cells. [17,18] TLRs are socalled pattern recognition receptors that are found on a variey of innate immune cells [17] and able to recognize pathogen-specific molecular patterns (PAMPS). They discriminate these PAMPS from invading pathogens as non-self molecules, which represent a signal for the receptor-expressing immune cells to become activated and produce pro-inflammatory cytokines and costimulatory molecules resulting in recruitment and activation of immune cells.

The imidazoquinoline-based small molecules imiquimod and resiquimod are synthetic ligands that have been shown to activate human TLR 7 and 8, and TLR7 in mice and rats. TLR8 is not functional in mice. These TLRs also recognize viral and synthetic single-stranded RNAs. The TLR7/8 agonist R837 (imiquimod) has been licensed as a key ingredient in Aldara cream for the topical treatment of genital warts, basal cell carcinoma and bladder cancer. [19,20] In mouse studies, imidazoquinolines were able to act as adjuvants promoting adaptive immune response to co-administered prophylactic antigens. [22,23] This observation is in line with the notion that single-stranded RNA induces an antigen-specific immunity characterized by a potent cytotoxic T cell response. [24]

However, very little is known about how systemically administered TLR7/8 agonists affect immune responses in general and anti-tumour immunity to glial brain tumours. Interestingly, Xiong and Ohlfest recently showed that topical imiquimod (Aldara) applied on the skin has therapeutic and immunomodulatory effects against intracranial tumours in a mouse model. [25] Weekly application increased survival of mice against implanted syngeneic GL261 glioma tumours. Resiquimod is related to imiquimod, as they are both synthetic small molecules that activate Toll-like receptor (TLR) 7. [26]

In the present study, we investigated the anti-tumour immune effects of parenterally injected resiquimod (R848) in a CNS-1 glioma model in immunocompetent Lewis rats. This model represents a valuable *in vivo* system for preclinical studies because of histopathological and pathological features which highly resemble human GBM. [27] Our results show that TLR7/8 agonist resiquimod (R848) affects immune responses leading to growth arrest of large established glioma tumours, and that R848 treatment, at the concentrations used, does not inhibit CNS-1 tumour growth directly.

Remarkably TLR7/8 activation by R848, as a therapeutic stand-alone therapy, is able to reject smaller established CNS-1 tumours, leading to solid, immunological memory against tumour rechallenge. Hence, this TLR7/8 activation approach provides a new opportunity for rational therapeutic immune interventions based on strengthened anti-tumour immune responses that may translate into successful clinical outcome in patients affected by glioblastoma.

Results

Therapeutic adminstration of the TLR7 immunostimulant resiquimod arrests growth of large (35-day old) CNS-1 tumours. We sought to determine immunotherapeutic strategies for controlling the malignant growth of syngeneic CNS-1 glioma tumour cells in Lewis rats using the newly described small molecule TLR7/8 agonist resiquimod, also referred to as R848.

To examine whether immunocompetent Lewis rats, which had developed large syngeneic CNS-1 tumours, would benefit from R848 treatment (100 µg/kg, 30µg/dose), we started to treat established, large five week-old, 10- 20,000 mm^3, CNS-1 tumours, at 38 days after tumour implantation. Indeed, resiquimod treatment was able to arrest or slow tumour growth in 2/3 animals, as shown in Figure 4.1, however, complete regression of these large tumours was not noted within the observation period.

We next decided to investigate the antitumour activity of resiquimod against lower tumour burden in rats with less advanced CNS-1 tumours. In a subsequent pilot dose-finding experiment we noticed a dose-dependent inhibition of CNS-1 tumour growth, when treatment was started earlier at day 10 after tumour implantation. Injection of a low dose of immunostimulatory resiquimod (3.3 µg/kg = 1 mg/dose) did not inhibit CNS-1 tumour growth, while a higher dose of either 10 or 50 µg/dose, representing 33.3 and 166.6 µg/kg respectively, clearly evoked reduction in tumour growth (data not shown). We therefore decided to test robustness of this protective

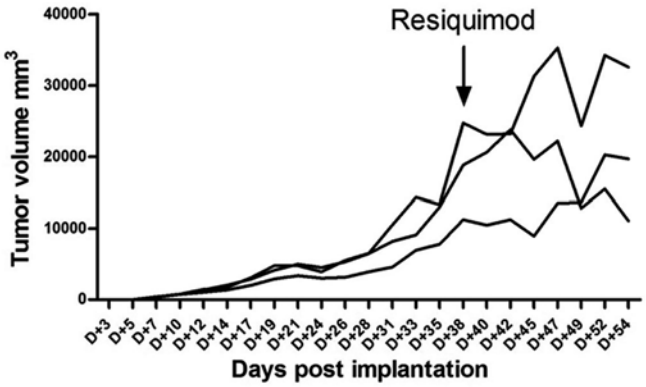

Figure 4.1. Indication of inhibition of established large CNS-1 tumours in Lewis rats (*n* = 3) treated with resiquimod starting at day 38. Individual growth as a function of time after implanation is shown for each individual animal. The arrow indicates the start of resiquimod treatment.

treatment in a larger experiment. Figure 4.2A shows that administration of R848, at a dose of 100 mg/kg (this is about 30 µg/ dose), when given three times per week (Monday, Wednesday and Friday) profoundly reduces tumour growth, relative to a control group receiving no treatment ($p < 0.001$) . We stopped the therapeutic weekly treatment regimen after day 42 (week 6) and further continued to monitor tumour

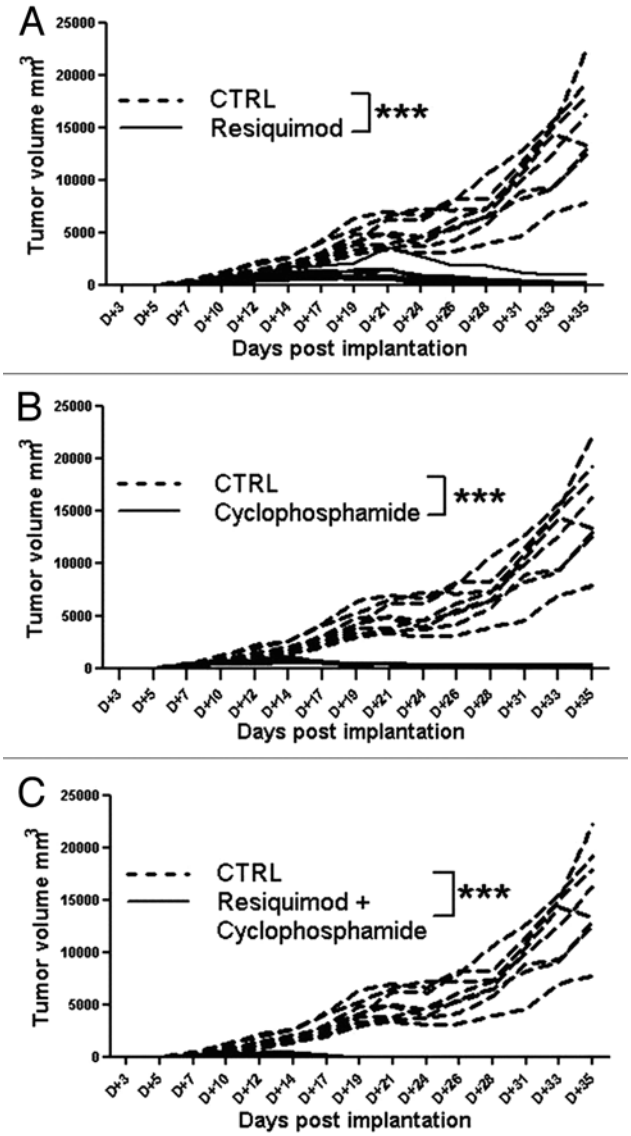

Figure 4.2. Therapeutic administration of TLR7/8 agonist resiquimod strongly inhibits tumour growth. CNS-1 tumour development after implantation in groups of rats (n = 8) treated with either resiquimod (A), Cyclophosphamide (B) or a combination of resiquimod and cyclophosphamide (C). Individual growth as a function of time is shown. The stippled lines show individual tumour growth of untreated controls.

growth.

Therapeutic administration of high dose cyclophosphamide alone or in combination chemo-immunotherapy of CY with resiquimod protects against CNS-1 tumour growth. We next examined whether resiquimod immunotherapy combined with chemotherapy further improves anti-tumour immunity. Hence, in a parallel arm of the same animal experiment we investigated the therapeutic efficacy of the cytotoxic alkylating agent cyclophosphamide (CY), given once every two weeks, which is a well-know direct cytostatic antitumour agent, but has also been shown to mediate immune suppression or even tumour regression by abrogation of immunosuppressive

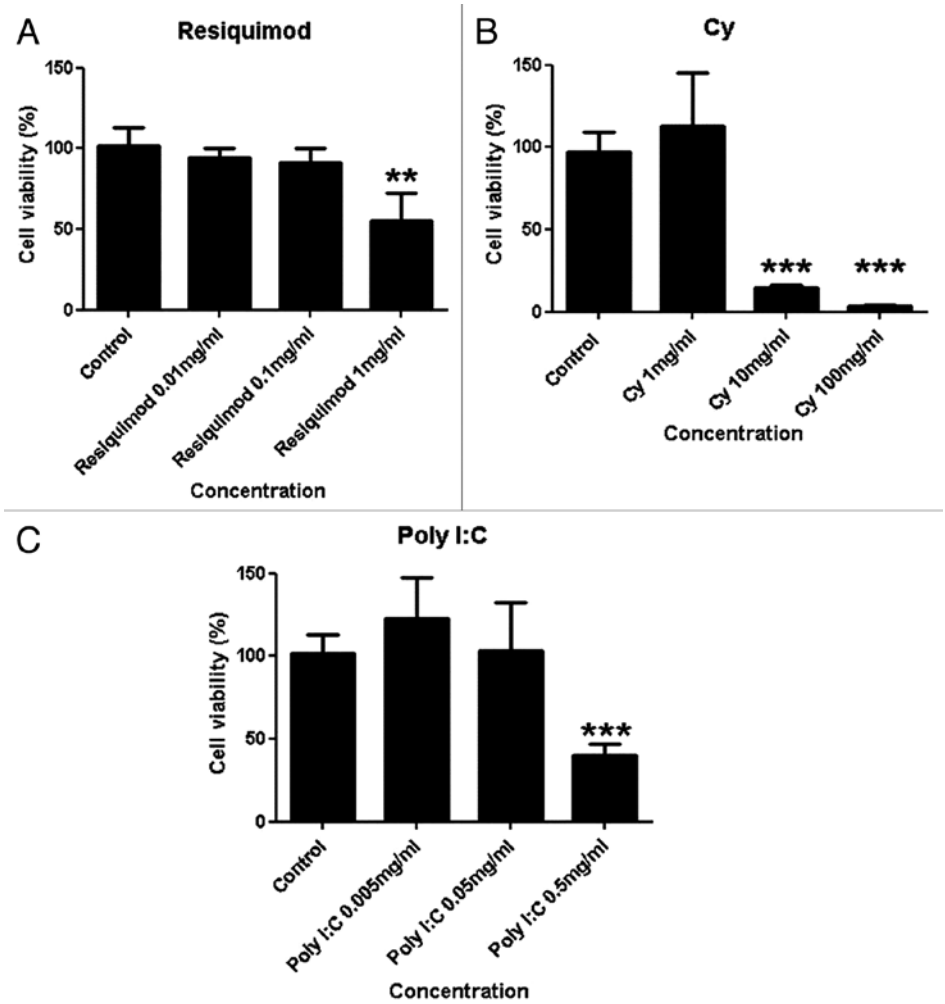

Figure 4.3. CNS-1 cells (200,000 cells per well) were exposed for 24 hours to increasing concentrations per well of either resiquimod (A), or CY (B-. Viability was measured (in triplicate) in a standard (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay, and was expressed as a percentage of viability measured for cells cultured in medium only (control). Representative data of two experiments are shown.

T regulatory cell function. [28] In addition, we tested the combined treatment of CY plus R848 to investigate beneficial synergy of CY with active immunotherapy, as a chemo-immunotherapy variant. [29,30] Figure 4.2 shows that inhibition of CNS-1 glioma tumour growth was observed for a group which received CY alone (Fig. 2B) or resiquimod in conjunction with CY at a 100 mg/kg dose (Fig. 2C; $p < 0.001$). The combined administration of CY and R848 seemed to further inhibit tumour development as noted by earlier tumour regression. Poly I:C injected in a similar regimen, at a dose of 30 or 50 µg, did not inhibit CNS-1 growth (data not shown). A lower dose of 30 mg/kg of CY alone also failed to inhibit tumour growth.

TLR7 agonist does not directly inhibit CNS-1 tumour growth. To examine whether parenterally injected resiquimod may have been able to directly affect CNS-1 tumour growth we tested the cytotoxicity of resiquimod *in vitro*, in parallel to cyclophosphamide and poly I:C, a prototype TLR3 agonist. Figure 4.3 shows a direct cytotoxicicty of cyclophosphamide when used at high dose, and no apparent direct growth inhibition by resiquimod, at concentrations reflecting the *in vivo* dose. The effective concentration of resiquimod of 30 µg/dose used *in vivo* is not directly cytotoxic *in vitro* and is therefore very unlikely to evoke direct contralateral tumour killing, while the *in vivo* concentration of 100 mg/kg (30 mg/dose) is clearly cytotoxic for CNS-1 cells cultured *in vitro* (200,000 cells /well).

CNS-1 glioma cells do not express TLR 7/8. We checked expression of TLR7 and TLR8 by CNS-1 cells using RT-PCR, but where unable to detect receptor expression, while expression of both receptors could be detected in rat spleen tissue (Fig. 4).

Glioma tumours cured by resiquimod therapy alone evoke immunological memory when rechallenged with syngeneic CNS-1 tumour. To examine whether immunocompetent Lewis rats, which had rejected CNS-1 cells as a result of resiquimod treatment, had developed immunological memory against CNS-1 tumour cells, they were re-challenged at day 49 after the first tumour implantation (one week after treatment arrest and wash out of R848) using a tumour dose of CNS-1 which evoked tumour growth in naive age-matched control rats.

As shown in Figure 4.5 all recipients of R848 therapy which had eliminated the first CNS-1 tumours, completely rejected the secondary CNS-1 challenge, with no evidence of measurable secondary tumour growth (Fig. 5B, $p < 0.01$), while the same dose of CNS-1 tumour cells induced reproducible tumour development in all untreated

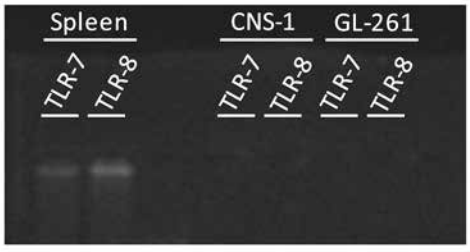

Figure 4.4. Lack of detection of TLR 7 and TLR 8 expression in both rat glioma CNS-1 cells and murine glioma GL261 cells. However, in rat spleen tissue, used as positive control, expression of both TLR7 and TLR8 can be detected.

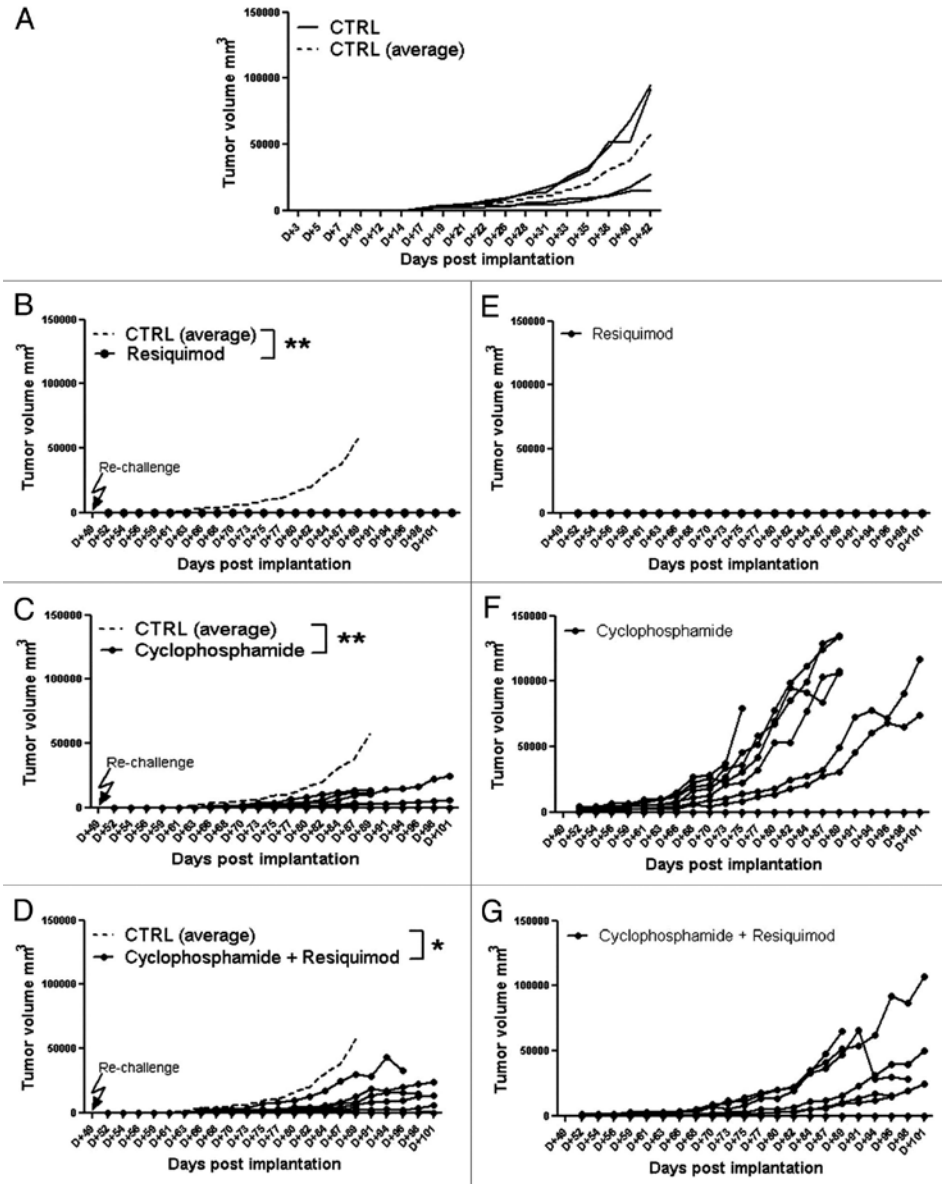

Figure 4.5. Individual CNS-1 tumour development as a function of time after secondary tumour implantation (A-D) in groups of rats (*n* = 8) treated either with Resiquimod alone (B), Cyclophosphamide alone (C) or a combination of Resiquimod and cyclophosphamide (D). The CNS-1 implanted, untreated age-matched group is shown in A (*n* = 4). Mean tumour growth of group A is shown as a stippled line for the untreated control group in B, C and D. Panels E, F and G show the growth of the recurrent individual primary CNS-1 tumours at the original inoculation site, for the resiquimod treated (E), Cyclophosphamide treated (F) and combination group (G) at the days post primary tumour challenge.

age-matched Lewis rats (Fig. 5A). This observation was confirmed in a subsequent experiment as evidenced by a complete rejection of a secondary tumour challenge, after cessation of treatment for a period of three months, in a group of four R848 treated Lewis rats, which had rejected the first CNS-1 tumour. Again this additional tumour challenge evoked progressive tumour growth in naive age-matched controls.

Glioma tumours controlled by treatment with CY alone or by CY-resiquimod combination therapy fail to evoke immunological memory against rechallenge with syngeneic CNS-1 tumour.

Lewis rats that had initially rejected CNS-1 cells as a result of CY treatment alone, or after CY-R848 combination treatment, were tested for immunological memory against CNS-1 tumour cells, by a re-challenge at day 49 after the first tumour implantation. Upon secondary tumour challenge both, the CY only treated, as well as the CY-R848 combination treated hosts, did displayed an initial inhibition of secondary tumour development (Fig. 5C and D, $p < 0.01$ and < 0.05, respectively), but eventually progressive CNS-1 growth was noted in most animals, This indicated a deleterious effect of the CY on the endogenous immune cell component that was initially engendered, sensitized to and keeping the CNS-1 tumour cell growth suppressed. Further substantiating this conclusion, the CY-treated rats, alone as well as in combination therapy, also showed a recurrence of their primary tumour, which apparently was not completely resolved (Fig. 5F and G, respectively). In both groups, seven out of eight animals showed progressive tumour growth.

Safety. In our studies we did not observe any signs of toxicity in rats treated with the systemic resiquimod.

Discussion

In the present study we demonstrate that immunotherapy based on the innate immune cell activator resiquimod, is effective as a treatment modality for eradication of established CNS-1 glioma tumours.

Our CNS-1 glioma cell implants are syngeneic, haplotype RT-1^{l}, for Lewis rats and represent an excellent *in vivo* glioma model, because of its glial phenotype, reproducible *in vivo* growth rates and histological features that closely resemble human glioma. [31] It has been demonstrated that CNS-1 tumour cells are immunoreactive for glial fibrillary acidic protein (GFAP), S100 and vimentin, as well as neuronal adhesion molecule, retinoic acid receptor α, intracellular adhesion molecule and neuron specific enolase. [31] This model therefore provides an excellent *in vivo* model in which to investigate immunotherapeutic intervention strategies against glioblastoma multiforme in immunocompetent hosts.

Natural immune responses against glioma tumours are often elicited as demonstrated by histological evidence of local inflammation and tumour-specific lymphocytes, likely directed against tumour specific antigens. However, the GBM tumour microenvironment is characterized by the presence of a variety of immunosuppressive cells and their inhibitory products, which may eventually result in the escape of the tumour from immune surveillance. [32-35] However, when an effective therapeutic dose of resiquimod was injected three times per week, we observed a dramatic reduction in tumour volume. While most untreated or control tumour-bearing animals had to be sacrificied, either due to massive tumour volumes or due to ulceration of

the tumour, the groups receiving a dose of more than 10 mg resiquimod per injection eventually showed complete regression of the tumour volumes. When therapeutic treatment was arrested, at day 49 after implantation, the tumour had shrunk to minute or nonmeasurable sizes. *In vitro* studies revealed that resiquimod (0.01 or 0.1 mg/ml), in contrast to CY, did not directly inhibit CNS-1 tumour cell growth.

These results may seem contradictory with other data showing that when tumour cells express TLR7/8, activation of this TLR type leads to cell survival and chemo resistance. [36] We have therefore checked expression of TLR7/8 by CNS-1 cells by RTPCR, but where unable to detect receptor expression by PCR. However, even if TLR7/8 activation by resiquimod would have stimulated tumour growth the net effect *in vivo* would apparently still be tumour regression.

Interestingly, all rats proved immune to re-challenge with CNS-1 glioma cells (Fig. 5) as evidenced by complete inhibition of tumour development. Immune memory against rechallenge was confirmed for rats which received the additional tumour inoculation even after three months of treatment arrest, while naive rats developed tumours. In view of the short half-life of the imiquimod family members of only few hours it is very unlikely that resiquimod had some remnant activity after a three month resting period before administration of a tumour rechallenge. The complete inhibition of secondary tumour growth suggest that immunotherapeutic treatment during the first tumour growth, using resiquimod, a known innate immune response agonist activating TLR7/8, results in tumour regression that results in the development of T cells with immune memory. Hence this innate immune triggering acts as an in situ therapeutic vaccine, alerting the adaptive immune system to recognize and eliminate the syngeneic secondary CNS-1 brain tumour. In future studies we will set out to decipher the exact mechanism underlying this intriguing observation of in situ immune memory priming.

In addition, we evaluated the effects of CY on CNS-1 tumour development. Cyclophosphamide (CY), although primarily used as cytotoxic therapy and expected to suppress the immune system, has been shown to abrogate immunosuppressive T reg function, and beneficially synergize with active immunotherapy when used at an appropriate dose and timed correctly. [29,37,38]

CNS-1 tumours regressed, as a result of CY treatment, and even faster after combined CY-R848 chemo-immunotherapeutic treatment. Importantly, the administration of CY, after the animals had developed immunity to CNS-1, was deleterious (Fig. 4). The explanation for why, after tumour rechallenge, the animals that were treated with CY only or by the CY-R848 combination were not able to inhibit secondary tumour development relates to the CY also causing damage to the CTL that had developed in situ at the beginning of the treatment. Additionally, it provides an explanation for why the CY-treated rats also exhibited recurrence of the primary tumour. These data highlight the need to carefully arrange the administration of combined therapeutics involving cytotoxic chemotherapeutic agents with immunotherapeutic agents so one agent does not interfere with the effects derived from the other. However, the delay in tumour growth after rechallenge of the cyclophosphamide group, suggests that there is an immune effect, which is most likely dependent on T cells, although a memory response by B cells cannot be excluded formally. In both senarios T cells are necessary for T help and likely also for T cell effector function. The effect of T cell depletion will be subject of follow-up studies addressing the biological mechanism of action responsible

for rechallenge immunity.

These results provoke two intriguing questions. How does R848 eradicate CNS-1 tumours, and how does immune memory develop during this treatment? In addition, it is of interest to know how CY hampers antitumour immunity. The exact mode of action and associated immune pathway responsible for the observed resiquimod-mediated anti-tumour immunity needs to be defined in detailed follow-up studies. Most likely resiquimodbased immunotherapy is able to activate a spontaneous, natural, innate anti-tumour immune response, that under normal circumstances is unable to control tumour growth, likely as a result of delayed or actively suppressed immune control. Non-specific immune attack of the tumour evoked by TLR7/8 activating resiquimod, but not by poly I:C treatment activating TLR3 (data not shown), may release tumour antigens into the surrounding tumour environment which are sampled by locally attracted antigen presenting cells and which allow presentation to and priming of adaptive immune lymphocytes, in the draining lymph nodes. Alternatively, or in parallel, an in situ "vaccination" occurs as a result of R848 therapy. TLR7 activation by the related imiquimod causes human and rodent dendritic cells to become tumouricidal. [39] Eventually, a sufficient number of tumour-specific naive adaptive immune cells, such as cytolytic T cells, are triggered and expanded in draining lymph nodes as a result of parenteral R848 immunotherapy and enabled by activated antigen-presenting cells. These presumed cytolytic T cells selectively recognize and eliminate the tumour and provide immunological memory, as illustrated by the rejection of secondary tumour cell implants. However, dedicated follow-up studies need to address to involvement of anti-tumour killer macrophages or NK cells, or IFNs for the resiquimod-induced glioma growth regression and immune memory.

In conclusion, our data show that injection of the innate immune cell receptor agonist resiquimod as a therapeutic TLR7/8 activating stand-alone therapy, is able to cure established CNS-1 tumour growth in Lewis rats. They suggest that immunotherapeutic parenteral treatment of established glioma tumours by resiquimod, as defined in the protocol, significantly improves anti-brain tumour immunity in a way that leads to immune memory, which is superior to CY treatment alone. Our studies have thereby identified a promising novel antitumour immunotherapy which may lead to clinical benefit.

Materials and Methods

Tumour Model. Rat CNS-1 cells (2 x 10^5 cells/200 ul) were implanted subcutaneously (SC) using a 21 gauge needle into the right flank of 8–12 week-old (300 g body weight) male Lewis rats. For each treatment group and control, 4–8 rats/group were used. The same tumour implantation procedure was performed during re-challenge experiments, on the contralateral side, for rats which had controlled the tumour growth after first exposure. All animal studies were approved by an independent ethical committee.

Monitoring Tumour Growth. The sizes of the CNS-1 tumour volumes were measured using a caliper three times per week on Mondays, Wednesdays and Fridays to monitor the effects of each treatment group.

Completion of Experiment. Tumour implanted rats were sacrificed if

they showed unfavorable signs of discomfort, as defined by the ethical committee. For example if they appeared moribund due to weight loss, lethargy, ruffled fur, or when tumours showed ulceration. A mixture of Rompun and ketamine was used for anesthesia, followed by a dose of sodium pentobarbital for euthansia.

Chemicals and reagents.

Immunomodulators and potentiators. Rats were subcutaneously (SC) injected in the flank, contralateral to the tumour-implanted side, with resiquimod (R848) (purchased from Invivogen, catalog number tlrl-r848), a Toll-like receptor 7/8 agonist, in a range of 3.3–166.6 µg/kg, corresponding to 1–50 µg/dose, three times per week on Mondays, Wednesdays and Fridays. Resiquimod(R-848, S-28463) was shown to be more soluble and more potent in inducing cytokine expression than its family member imiquimod which has a half-life of 2–3 h in humans [40].

In a parallel arm of the experiment we evaluated the effect of cyclophosphamide administration on CNS-1 glioma development. Cyclophosphamide (CalBiochem, cat. no. 239785) was given at 30–100mg/kg. CY was injected once every two weeks on Fridays.

Cytotoxicity Assay. The direct cytotoxicity of resiquimod, CY and Poly I:C (Invivogen, cat. no. Tlrl-pic, tlrl-pic-5), which was included as a reference TLR-3 agonist, was determined by exposing CNS-1 cells at a concentration of 10,000 cells per well in a 96-well plate in DMEM culture medium (cat. no. 30–2002, ATCC), supplemented with 10% fetal bovine serum (FBS; Lonza, cat. no. DE14–801E), for 24 h. The viability of CNS-1 cells, measured in triplicate, was measured in a standard (3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide assay, absorbance was read at 590 nm, and was expressed as a percentage of viability measured for cells cultured in medium only.

TLR 7 and TLR 8 detection by RT-PCR.

Samples collection. Normal spleen tissue was obtained by surgical resection from a male non-treated Lewis rat and cut in pieces of 1 mm^3 with a sterile surgical blade. CNS-1 and GL-261 cell lines were cultured as described above and a pellet of 1 x 10^6 cells was used. Cells or tissue sample were put in lysis buffer using the SV Total RNA Isolation System (Promega Corp., Leiden, The Netherlands).

RNA extraction and reverse transcription. After extraction of total RNA it was reverse-transcribed by using the Thermoscript RTPCR System (Life Technologies, Inc., Paisley, UK) as previously described [41].

Oligonucleotide primers used for PCR amplification: Primers for the PCR amplification were obtained by Real Time Primers LLC, PA, USA, according to successful approach for TLR-7, [42] or as customized primers for TLR-8 obtained from Real Time Primers LLC, PA, USA.

PCR. PCR was performed according to the manufacturer's recommendations, with Platinum® PCR SuperMix (Life Technologies, Inc., Paisley, UK). Aliquots of the RT products were subjected to PCR in a total volume of 50 µl, with 100 nM adequate paired primers. PCR products were visualized on a 2% agarose gel with GelRed™ Nucleic Acid Gel Prestaining Kit (Biotium, CA, USA), visualized on an UV transilluminator and photographed using a Canon Powershot G10 photograph, equipped with a conversion lens 032 LA-DC58K.

Statistical analysis. ANOVA followed by the students t-test was used to compare groups, with a p value of < 0.05 (*), $p < 0.01$ (**), $p < 0.001$ (***), considered statistically significant.

Disclosure of Potential Conflicts of Interest

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CHAPTER 5

Therapeutic vaccination against malignant gliomas based on allorecognition and syngeneic tumour antigens: Proof of principle in two strains of rat

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Summary

In the present study we investigated whether allogeneic glioma cells can be utilized to evoke prophylactic or therapeutic immune-mediated elimination of syngeneic glioma in two rat strains. Fisher 344 and Sprague—Dawley (SD) rats were injected with two syngeneic glioma cell lines, 9L and C6, respectively, resulting in progressive tumour growth. 9L is syngeneic to the Fisher 344 and allogeneic to the SD rats, while C6 cells are syngeneic to SD rats and allogeneic to Fisher 344 rats. Both rat strains were subcutaneously injected with their respective allogeneic tumour cells, which proved unable to grow progressively. The allogeneic cells were either rejected immediately in SD rats or within 25 days in Fisher rats, after limited tumour outgrowth. Both rat strains were subsequently challenged with their respective syngeneic glioma tumour cells and once more 10 days later with a fivefold higher dose. SD rats, even after reinjection with five times the original dosage of C6 cells, remained tumour free for at least 360 days. Similarly, Fisher rats, after initially rejecting allogeneic tumours, failed to develop syngeneic tumours.

To determine anti-tumour immunity against established glioma tumours under more demanding therapeutic conditions, rats were first injected subcutaneously with their respective syngeneic tumour and vaccinated once or repeatedly (at 5-day intervals) with a mixture of the allogeneic or xenogeneic cells, with or without a lysate from the same syngeneic tumour, which served as a therapeutic vaccine preparations. The control group received either no treatment or syngeneic instead of allogeneic cells. In both strains of rats, we demonstrated that the therapeutically vaccinated groups were able to significantly reduce tumour growth, while complete rejection of tumours was noted in the SD rats. Immunization with syngeneic tumour cells alone failed to evoke anti-tumour immunity.

We conclude that therapeutic immunization with a combination of allogeneic cells and syngeneic lysates induces rejection of malignant gliomas and offers a protective effect against challenge with syngeneic tumour cells. This immunization approach may prove useful as a post-surgery adjuvant therapy in future cancer treatment protocols, or even as a stand-alone therapeutic tumour vaccination.

Keywords: Immunomodulation; GBM; Rejection; Therapeutic vaccine; Tumour.

Introduction

In the United States alone over 18,000 primary brain tumours are estimated to occur each year. Of these 18,000, over 60% are diagnosed gliomas. Glioblastoma multiforme (GBM) is the most common and malignant of all gliomas, with 75% of patients dying within 18 months of diagnosis [1]. The prognosis for this tumour is very poor. The median survival time of untreated tumours is 3 months, with death most commonly due to cerebral edema or increased intracranial pressure. Even with the best available current therapy, which includes radiation, chemotherapy and surgery, the median survival does not extend beyond 14 months. These tumours are inevitably recurrent either locally, usually within 2 cm of the original tumour, or at distant sites. Treatment of these recurrent lesions by a second surgery and further chemotherapy may increase the symptom free interval, but the 5-year survival remains 10% [1—3].

It has been shown that the progression of certain cancers is associated with the expression of tumour-specific antigens and tumour antigen-specific immune responses [4]. Hence, theoretically, effective tumour rejection and immunity can be achieved by vaccination with tumour-associated antigen, the holy grail in tumour immunology. However, active immunotherapy for cancer has shown minimal clinical success. It has been clear that even with a fully functioning immune system, it is possible for tumours to evade recognition through the use of elusive escape strategies [5]. Although poorly understood, several mechanisms of tumour escape have been identified. For example, a change of or loss of MHC class I receptors is associated with the genesis of various tumours, while the presence of intact MHC class I molecules has been shown to participate in cancer resistance [6]. Other mechanisms include unresponsiveness to interferons [5], as well as tumour-induced immunosuppression as a result priming for and influx of inhibitory regulatory T cells [7] and associated induction of immunosuppressive molecules including IL-10, CTLA-4 and related factors.

On the other hand, there is increasing evidence that the immune system can be engaged to combat cancer. This is supported by the observations that a deregulated immune system hampers rejection of cancer, while spontaneous rejection or inhibition of malignant tumours is associated with a well-functioning immune system [8,9]. A recent study in colorectal tumour patients demonstrated that adaptive Th- 1 immune gene expression and high immune cell densities of CD3, CD8 and CD45RO cells in tumour regions correlates positively with patient survival [10]. Interestingly, it has also been suggested that autoimmune diseases may contribute to a better prognosis in patients with malignant tumours [11,8]. In these patients, the majority of the IgG specificities identified share considerable homology with both human and microbial peptides [12]. This has lead to the hypothesis that molecular mimicry may initiate the observed anti-tumour autoimmunity. Studies related to this have shown long-term remission of malignant brain tumours after intracranial infection in four patients [13], and improved survival of cancer patients with microbial infection [14,15]. This brings into question whether molecular mimicry-induced ‘‘autoimmunity’’ can be employed to treat tumours. Importantly, significant homology has been shown to exist between human proteins and proteins from other species [16]. Moreover, use of artificial pathogen invasion signals, such as CpG motifs, or other innate immunity agonists, initiates and augments antigen-specific immune reactions [17], and may break tolerance to self-tumour antigens, mimicking microbial infections during immunotherapy or

vaccination [18,19]. Alternatively, xenogeneic antigen from endothelial cells is able to break immune tolerance against autologous angiogeneic endothelial cells [14]. This suggests that self-tolerance to tumours may be broken by cross-reactivity against a homologous foreign antigen.

In the present study, we combine the principles of immune-based allorecognition and administration of syngeneic tumour antigen to overcome tolerance to selftumour- associated antigens and to develop a novel approach to the treatment of tumours. It is well known that genetically identical individuals can accept tissue from one another, while tissue transplanted into heterozygous individuals will produce an immune response and eventual tissue rejection. Recognition of intact, same-species, nonself major histocompatibility molecules, on the surface of donor cells results in direct, immune-mediated elimination, is referred to as acute allograft rejection [20,21]. Indirect allorecognition results from recognition of donor histocompatibility molecules that are internalized, processed, and presented by self-MHC molecules on host antigen presenting cells. After xenotransplantation, tissues or cells are transferred across species, which causes even faster rejection by processes analogous to those seen in allografts. Hence, identical twins and genetically close family members are less likely to reject transplanted tissue since they have similar HLA loci [22]. This is based on the fact that the MHC class I genes are expressed co-dominantly, and in most cases are inherited in intact form without recombination [23]. Therefore, homozygous, syngeneic rats could theoretically accept a brain tumour from a homozygous donor. However, more critically, they would reject a brain tumour from a heterozygous donor based on direct or indirect allo-immune rejection [20,21]. MHC class I molecules play an important role in the immune surveillance of tumours by monitoring of mitochondrial DNA integrity. One of the roles of MHC I molecules is to eliminate cells carrying mitochondrial mutations [6]. Human glioma cells carry multiple mutations in both the mitochondrial DNA and in the mitochondrial complex [24]. Hence, gliomas of the same histological type/grade are likely to carry similar mutations in their DNA and have similar abnormal surface proteins associated with both MHC class I molecules and the cell membrane. Experimental data suggests that not only MHC class I molecules are involved in immune surveillance against cancer, but also that the altered phenotype of the MHC class I molecule is linked to a variety of different tumours. Therefore, if two heterozygous individuals develop a tumour of a similar type and histological grade, then transplantation of tumour tissue from one individual to another will not only induce rejection of the transplanted tissue, but may also prime the immune system to peptides shared between these tumours and other tumours sharing similar peptides.

In this paper we show *in vivo* proof of principle experiments demonstrating that allogeneic tumours can be used to vaccinate against an established syngeneic tumour, resulting in inhibition of tumour growth or complete tumour elimination. Application of this technique in human patients may not only lead to eventual rejection of the primary tumour, but may also lead to a lasting immunologic memory, preventing the patient from developing tumour recurrence.

Materials and methods

Cell lines and cell culture

The cell lines used in this experiment were the rat glioma cell lines (9L, C6, RG2), and the human glioma cell lines (U87, LN229). All lines were obtained from the American Type Tissue Collection (ATTC), and grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% heat-killed fetal calf serum (FCS), 5% penicillin—streptomycin, and Hepes buffer in a humidified incubator at 37 ◦C in a 5% CO_2 atmosphere.

Cell lysate antigen preparation

1.0×10^6 cells were placed in a 5-ml tube in culture medium and centrifuged for 5 min at 2.5×10^3 rpm. The supernatant was discarded and 150 l of sterile distilled water was added to the tube. The cell/water solution was mixed well and transferred to a 1.0-ml Eppendorf tube and centrifuged at 1.0×10^4 rpm for 10 min. The supernatant was not discarded and this preparation was used for cell lysate injections.

***In vivo* studies**

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California. All rats were maintained in a specific pathogen free (SPF) environment. For the experiment, we used Sprague—Dawley (SD) and Fisher 344 rats. All rats were males and between the ages of 4—6 weeks. Rats were obtained from Harlan (Indianapolis, IN). In the subcutaneous tumour model, C6 and 9L were collected using only DMEM to wash them from the tissue culture flasks. Syringes were then prepared containing 100,000—150,000 cells suspended in 150µl.

Sprague—Dawley rats were divided into two major groups (Table 5.1). SD-A (three rats) were injected with the 9L allogeneic cell line, while SD-B (nine rats) were implanted with the C6 glioma, a syngeneic like glioma cell line for SD rats. SD-A rats, which never formed tumours, were tested for immune memory by challenging them with syngeneic C6 cells (100,000 cells). They were re-challenged with 500,000 C6 cells 10 days later, and checked for formation of a flank tumour.

Once a palpable flank tumour developed in the SD-B rats, they were further divided into two groups. The control group (SD-B1; n = 5 rats) received no injections. In the therapeutic treatment group (SD-B2; n = 4) rats were injected with a combination of allogeneic 9L cells, allogeneic 9L lysate, and syngeneic C6 lysate. On day 27, four of the five SD-B1 were sacrificed. At this time, one of the control rats, rat number (#) 9, started receiving the same treatment protocol as SD-B2 rats.

Fisher rats were also divided into two major groups (Table 5.2). The control group (Fisher-A; three rats) were injected with the allogeneic C6 cell line. They initially formed tumours that were subsequently rejected. They were tested for immune memory by challenging them after 40 days with 100,000 syngeneic 9L cells followed by a rechallenge 10 days later with 500,00 cells and checked for tumour growth.

In the therapeutic group (Fisher-B; n = 8), rats were first implanted with the syngeneic 9L cell line. Once a palpable flank tumour developed in the Fisher-B rats, they were further subdivided into three subgroups. Fisher control group (n = 3) rats

Table 5.1. Experimental design of animal studies in Sprague—Dawley rats

Immunization	Vaccine	Group size	Tumour challenge	Outcome
Prophylactic (group A)	Allogeneic 9L cells (100,000 cells)	n = 3	Syngeneic C6 (100,000 cells) at 20 days after 9L ''immunization'' and C6 500,000 cells, again 10 days later	Immediate, complete allogeneic 9L and subsequent syngeneic C6 tumour rejection
Therapeutic (group B2)	Allogeneic 9L cell lysates (50,000 cells), syngeneic C6 cell lysates (50,000 cells), and 9L allogeneic cells (50,000 cells). Rats #5—8 and later #9	n = 4—5 (rat #9)	C 6 (100,000 cells)	Complete C6 tumour rejection
Control group (group B1)	Saline or no injections rats #1—4 and initially #9	n = 5—4 (rat #9)	C 6 (100,000 cells)	Progressive C6 tumour growth

received injections of syngeneic 9L cells, syngeneic RG2 (rat glioma) cells, or medium only (Fisher B1). One Fisher treatment group (n = 5) rats received a combination of allogeneic C6 cells only or allogeneic C6 cells and lysate (Fisher B2), or xenogeneic human glioblastoma cell lines U87 and LN229 cells (Fisher B3) (Table 5.2).

Tumour growth analysis

All tumours were detected and confirmed through visual inspection and palpation. Once discovered, the area around the tumour was further exposed by careful shaving with an electric razor. At the time of injection, tumour size was measured in millimeters using Vernier calipers. Measurements were taken in the cranial/caudal (length), superior/inferior (height), and medial/lateral (width) direction. Tumour volume was calculated by length×width×height×0.5. The mean tumour volume for each treatment group was calcu lated. For SD rats the tumour volumes of the treatment groups were compared to relevant control groups at 27 days postinjection, and for Fisher rats at 35 days, using the Student's t-test calculation as described before [25]. Differences were considered significant if a p value was <0.05.

Harvesting subcutaneous tumour tissue for immunohistochemistry

All experimental animals were euthanized with an overdose of pentobarbital. Tumours were removed and dissected under sterile conditions, cut into four pieces and stored at −80 ◦C. All tumour sections were cut at 7 µm and stained by immunohistochemistry as described before [26]. Briefly, tumour samples taken from the Fisher 344 rats were frozen in optimum temperature compound (OTC) and cut into 7 µm sections on a cryostat. These sections were dried, fixed with acetone, and washed well with PBS for 1—2 min. Blocking was done using the immune serum from the species

Table 5.2. Experimental design of animal studies in Fisher rats

Immunization	Vaccine		Group size	Tumour challenge	Outcome
Prophylactic (group A)	Allogeneic	Allogeneic C6 cells (100,000 cells)	n = 3	Syngeneic 9L (100,000) cells at 40 days after C6 ''immunization'', and 500,000 9L cells, again 10 days later	Minimal 9L tumour outgrowth and ultimate rejection
Therapeutic (group B1) ''control group''	Syngeneic or medium	100,000 cells RG2 cells (rat #1), 100,000 cells 9L cells (rat #2) or medium only (rat #3)	n = 3	Syngeneic 9L (100,000) cells Non-reduced tumour growth	
Therapeutic (group B2)	Allogeneic cells/ lysates and/or syngeneic lysates	Mixture of (150,000) C6 allogeneic cells (rat #4) and C6 (100,000) allogeneic cells plus allogeneic lysate (100,000 cells) (rat #5). And syngeneic 9L cell lysate (100,000 cells) (rat #8)	n = 3	Syngeneic 9L (100,000) cells	Reduced 9L tumour outgrowth, except for rat #8
Therapeutic (group B3)	Xenogeneic cells	9L syngeneic lysate (100,000 cells) plus U87 cells (50,000) plus LN229 cells (50,000) (rat #6) U87 (50,000) plus LN229 (50,000) xenogeneic cells (rat #7)	n = 2	Syngeneic 9L (100,000) cells	Reduction in tumour size

the secondary antibody was obtained from. Slides were washed thoroughly again and then stained with primary antibody against either CD4, CD57 (Nora Castro Lab Ltd., Burlingame, CA), CD8, dendritic reticulum cells (DRC) (Dako Corporation, Carpenteria, CA), CD20, or CD68 (Ventana, Tucson, AZ). Slides were washed again and a secondary biotinylated antibody was added. They were rinsed again and placed in a solution of 3% hydrogen peroxidase and nine parts 1% sodium azide in PBS. Slides were then rinsed and ABC was added for 30—40 min. They were washed with PBS and developed using diaminobezidine tetrahydrochloride and counterstained. Photographs of all slides were taken by light microscopy.

Results

Immunization with allorejected, non-syngeneic tumours in both Fisher and Sprague—Dawley rats primes for prophylactic immunity against syngeneic tumour challenge.

Most experimental studies of glioblastoma make use of small laboratory animal models. The most frequently used immunocompetent host models employ two different strains of rat, the Sprague—Dawley and the Fisher 344 rats [27]. C6 is a syngeneic-type cell line for the SD rats, while the 9L and RG2 cell lines are syngeneic for the Fisher 344 rats [27,28]. In a prophylactic setting we examined whether the SD and Fisher 344 rats initially injected with an allogeneic cell line would be able to reject a syngeneic cell line. SD rats were seeded with the allogeneic 9L cell line (SD-A). Each of the SD rats completely rejected the 9L tumour without visible or palpable tumour growth. Twenty days later, all ''immunized'' SD rats, were injected in the contra-lateral hind flank with syngeneic C6 tumour cells, that readily formed a tumour in naïve SD rats, using 100,000 cells first, and a fivefold higher tumour cells (500,000 cells) 10 days later. The rats were monitored every 3 days for any sign of visual or palpable tumour growth. In these SD rats, no visual or palpable tumour developed. Remarkably, at 360 days, all SD rats immunized with the allogeneic tumour cells remained tumour free (data not shown).

A similar procedure was used to prophylactically immunize three Fisher rats. They were injected with the allogeneic C6 cell line (Fisher-A). Although initially wellcircumscribed tumours did form, they were subsequently rejected within 40 days. These animals were subsequently seeded with 100,000 syngeneic C6 cells first, and a fivefold higher amount of C6 cells (500,000 cells) 10 days later. The rats were monitored every 3 days for any sign of visual or palpable tumour growth. In these Fisher rats, a relatively small (<1 cm×<1 cm×1 cm) growth developed at the injection site. This growth was noticeable only after palpation, and became progressively smaller and completely undetectable by 10 days. At 360 days, all immunized Fisher rats remained tumour free (data not shown).

These results demonstrate that in both strains, allorejection of non-syngeneic tumours induces effective prophylactic immunity against syngeneic tumour challenge.

Allo-response-based therapeutic vaccination against C6 tumours in Sprague—Dawley rats

In order to assess anti-tumour immunity in a therapeutic situation, SD rats (n = 9) were each injected with the C6 cell line, which resulted in undiminished tumour growth in untreated animals. All SD rats developed visible tumours within 10 days. At this point, five rats were kept as a control group (SD-B1), while the remaining four rats were placed into treatment groups (SD-B2). On day 27, rats #1—4 were sacrificed and an attempt was made to ''rescue'' rat #9. At this time, rat #9 entered the treatment group and started to receive the same therapeutic vaccine injections as given to the SD-B2 group.

The uncontrolled tumour growth in the control group and the diminished tumour growth in the treatment group are depicted in Fig. 1. In the treatment group

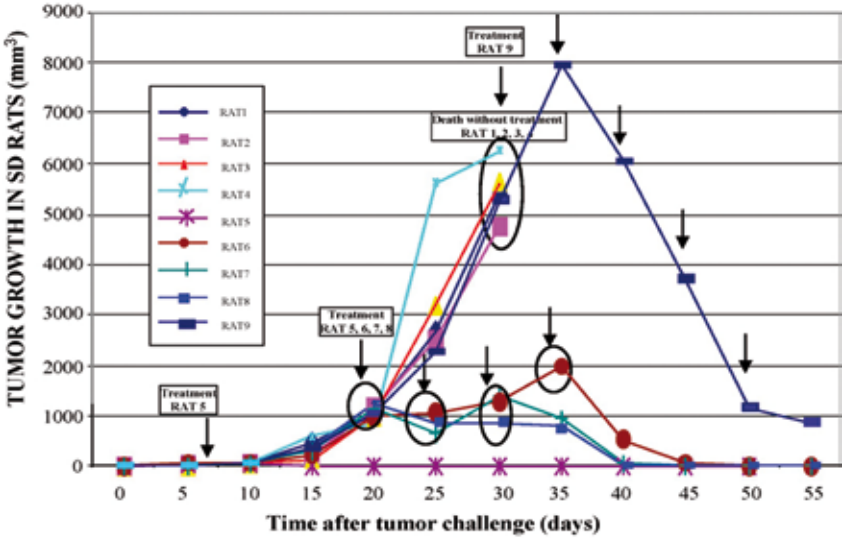

Figure 5.1. Graph charting tumour progression in nine SD rats with subcutaneously implanted syngeneic tumour (C6). Rats were placed in either control or treatment groups as previously described. Tumour progression was determined through measurements of tumour volume (mm3). Rats #1—4 received no treatment after C6 tumour implantation. Rats #5—8 received one or more therapeutic vaccination(s) with allogeneic 9L cells and lysates with syngeneic C6 lysate. Rat #9 was allowed to form a relatively large tumour before it was transferred to the treatment group to become immunized similar to rats #5—8.

(SD-B2, rats #5—8), individual rats were immunized with a mixture of allogeneic and syngeneic lysates, as well as allogeneic 9L cells per subcutaneous (s.c.) injection. One rat (#5) was treated very early. After 5 days, it had a palpable flank tumour and received only one therapeutic injection, contra-lateral to the tumour, of a mixture consisting of allogeneic 9L lysates (50,000), syngeneic C6 lysates (50,000), and 9L allogeneic cells (50,000). Remarkably, within 5 days after injection, the tumour resolved. Rats #6—8 (SD-B2 rats) all developed visible tumours within 18 days post-injection. At this time, they each received a first injection of a mixture containing 50,000 allogeneic 9L lysate cells, plus 50,000 syngeneic C6 lysate cells and 50,000 9L allogeneic cells. These injections were repeated on days 23 and 28. Rat #6 received an additional treatment at day 33, 15 days after initiation of immunotherapeutic treatment. The untreated rats (SD-B1, rats #1—4) were sacrificed 27 days post-injection because of their tumour size. When compared to the tumour progression in the untreated rats (rats #1—4), rats #5—8 (SD-B2) eventually showed complete resolution of their tumours by day 50.

Rat #9 began the experiment within the non-treated group, and then was treated after sacrificing rats #1—4 (day 27). Rat #9 received five injections every 5 days with a mixture of allogeneic 9L lysates (50,000) plus C6 syngeneic lysates (50,000) and 9L allogeneic cells (50,000). This animal was sacrificed for histological analysis at day 55, when the tumour size had reduced to 11% of the size measured at the initiation of immunotherapeutic immunization.

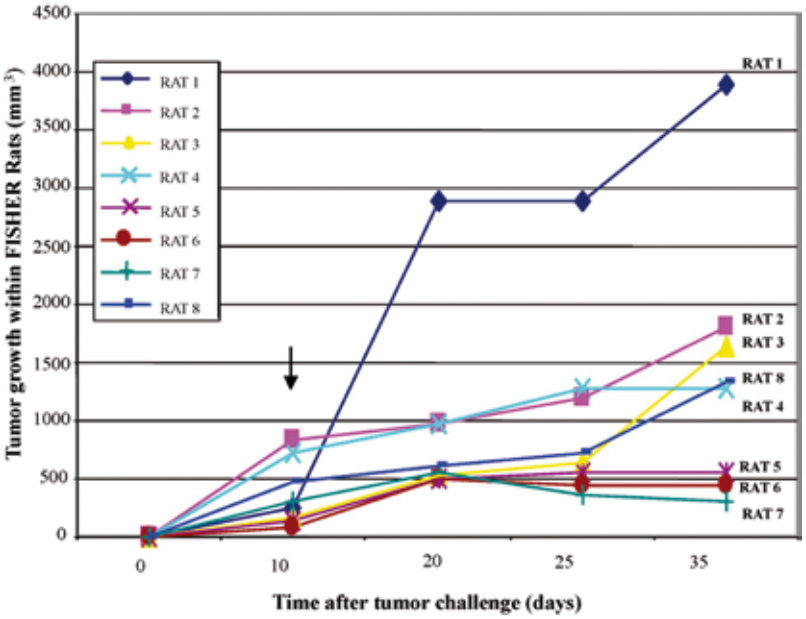

Figure 5.2. Graph charting tumour progression in Fisher 344 rats with subcutaneously implanted syngeneic tumour (9L). Control rats were injected with syngeneic RG2 cells (rat #1), syngeneic 9L cells (rat #2), or medium alone (rat #3). Rat #1 formed an extremely large tumour. Treatment group rats received allogeneic C6 cells alone (rat #4), allogeneic C6 cells and lysate (rat #5) (group B2), syngeneic 9L lysate and xenogeneic U87 and LN229 cells (rat #6), or xenogeneic U87 and LN229 cells alone (rat #7) (group B3). Rat #8 was treated with syngeneic 9L cell lysate alone. Tumour progression was determined through measurements of tumour volume (mm^3).

Allo-response-based therapeutic vaccination against 9L tumour growth in Fisher 344 rats

Fig. 2 shows tumour growth and response to the immunotherapeutic treatment of eight Fisher rats (Fisher-B) implanted with 9L cells. Rats #1—3 (Fisher B1) received therapeutic contra-lateral flank injections at day 10, with either syngeneic RG2 (100,000 cells; rat #1) or 9L (100,000 cells; rat #2), or medium alone (rat #3). There was notable reduction in tumour growth over time, while a more pronounced tumour growth was noted in the RG2 treated rat (#1). By contrast, rats #4—7 (Fisher B2 and B3) were immunized therapeutically with, either C6 allogeneic cells only (rat #4), a mixture of C6 allogeneic cells and C6 allogeneic lysate (rat #5), U87 and LN229 xenogeneic cells only (rat #7) or mixed with 9L cell lysate (rat #6). In particular rats #5—7 showed a significant reduction in tumour outgrowth ($p < 0.05$), while rat #8, receiving 9L syngeneic lysate only, demonstrated no inhibition of tumour growth (Fig. 2).

All Fisher 344 rats were sacrificed at day 40, when some of the rats started to develop hind limb paralysis. The tumours from each of these rats were removed and processed for immunohistological staining of immune cells. Within the tumours of the positive treatment groups we noted significantly greater numbers of CD4, CD8, B-lymphocyte (CD20), macrophages (CD68), and dendritic cells when compared to the control tumours (Fig. 3).

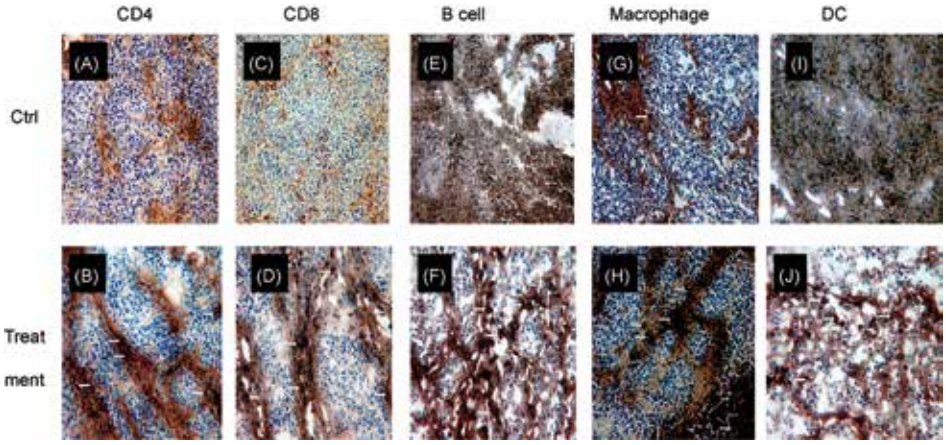

Figure 5.3. Representative tumour sections taken from control (A, C, E, G, and I) and treated (B, D, F, H, and J) Fisher 344 rats. Sections were cut at a thickness of 7 m and, according to the previously described protocol [27], stained with an antibody directed against either the CD4 receptor (A and B), CD8 receptor (C and D), B-lymphocytes (CD 20) (E and F), macrophages (CD 68) (G and H), or a dendritic cell marker (DRC) (I and J). Small white arrows indicate the location of cells staining positively for the respective marker. The magnification of both control and treatment sample is 40×.

Discussion

Anti-tumour immunotherapy based on an effective therapeutic vaccine, with an acceptable safety profile, is the greathope for cancer treatment. A vaccine will theoretically programthe patient's immune system to attack malignant, andeven metastasized, tumour antigen-expressing cells, and ideally trigger immunological memory to provide a durableanti-tumour immune response. To achieve this goal, many different vaccination strategies are currently being investigatedin animal models and clinical trials. Examples include immunizations based on patient-derived dendritic cellsloaded *in vitro* with tumour antigens or peptide fragments [29,30], virus-modified or cytokine transfected autologousor allogeneic tumour cells [31], plasmid DNA and viral orbacterial vector delivering genetically encoded tumour antigens,as well as the more classical antigen in adjuvant strategies.

In the present study, we demonstrate in two rats strains that allorejection of non-syngeneic tumours induces effective prophylactic immunity against subsequent syngeneic tumour challenges. In addition, we show that for established syngeneic tumours, therapeutic immunization with different mixtures, containing either allogeneic cell lysates plus syngeneic cell lysates, and allogeneic cells, evokes effective reduction in tumour growth in SD rats. Similarly, in Fisher rats, established tumour growth can be inhibited significantly by therapeutic immunization using either allogeneic or xenogeneic cells only, or a vaccine containing xenogeneic cells in a combination with lysates of syngeneic tumour cells. By contrast, immunization of Fisher rats with syngeneic cells

or syngeneic lysate alone failed to reduce tumour outgrowth. Our results support the conclusion that it is feasible to program effective tumour antigen-specific responses as a result of anti-allogeneic or xenogeneic cell immunization. In general, cell- or cell lysate-based tumour vaccines may be more attractive when compared to single antigen or polypeptidebased vaccines, since they theoretically evoke a broader multi-targeted therapeutic response. Due to the polyclonal immune response induced, they are less likely to result in therapeutic escape than most cancer treatments in use today.

In the prophylactic setting the SD rats rejecting the 9L tumour (SD-A) and the Fisher 344 rats rejecting the C6 tumour (Fisher-A) were reinjected in the contra-lateral flank with a higher dose of 500,000 cells of syngeneic cell line (9L for Fisher and C6 for SD). Both strains remained tumour free at 360 days. These results suggest that the injection of allogeneic cell lines evokes protection against subsequent challenge with syngeneic cell lines, demonstrating that the injection of the allogeneic cells lead to an immune response and the development of immune memory. Since C6 and 9L cell lines likely share critical tumour antigens, the development of C6 tumours is inhibited. The observed time line difference between SD and the Fisher rats in terms of allogeneic tumour rejection (SD rats rejected the 9L cell line without development of a tumour, while Fisher 344 rats took about 40 days to completely reject the C6 tumour), may be explained by a less effective immune response in Fisher rats. This may possibly result from less 9L immunogenicity, or is due to reduced susceptibility of 9L cells to immune attack; 9L is a gliosarcoma cell line, while C6 is a glioma cell line. On the other hand, C6 cells may be more immunogenic for SD rats than the 9L cells for Fisher rats.

In the more demanding therapeutic setting, untreated SD rats injected with C6 gliomas developed significant tumours within 5—15 days. These tumours grew without rejection, and rats had to be sacrificed eventually due to unacceptable tumour size and limb paralysis. The treated SD rats initially developed C6 tumours at comparable rates and sizes as the control group. However, these tumours gradually decreased in size and were no longer detectable 25 days after the initiation of therapeutic vaccination with a mixture of allogeneic and syngeneic cells and syngeneic cell lysates. Strikingly, even rat #9, rescued relatively late from the untreated control group, showed significant reduction in the size of tumour after treatment began. Together, these results demonstrate that repeated subcutaneous injection of this cocktail leads to a reduction in tumour size by triggering immunological awareness, likely directed at tumour antigens shared between the syngeneic and allogeneic cells.

There is some debate in the literature about the C6 cell line and whether or not it is syngeneic to any strain of rat [27]. However, even if the cell line may not be strictly syngeneic, it developed into subcutaneous flank tumours in SD rats without rejection. Those rats not given treatment were sacrificed when tumour size became incompatible with life. All of the Fisher 344 rats developed flank tumours at 15 days. Unlike the SD controls, the Fisher controls either received injections with two different syngeneic cell lines (9L and RG2) or with medium alone. There was no inhibition of tumour growth in these rats. This demonstrates that the injection of whole syngeneic tumour cells does not evoke an effective anti-tumour immune response, as a result of immunological tolerance to syngeneic cells. Indeed, when these rats were sacrificed, tumour sections did not stain positively for CD4, CD8, macrophages, B-lymphocytes, and dendritic cells. By contrast, the tumours in the treated Fisher 344 rats showed

different growth profiles. Rat #4 received allogeneic C6 cells only and showed growth inhibition after day 35. Especially, rats #5—7 showed decreased tumour growth when compared to syngeneic or medium treated controls. Rat #5 was treated was treated with both allogeneic C6 cells and lysate. Rat #6 was treated with 9L syngeneic lysate and xenogeneic U87 and LN229 cells. Rat #7 was treated with xenogeneic LN229 and U87 cells. Rat #8, which was treated with 9L syngeneic cell lysate, had an initial delay in tumour growth. However, this effect was not lasting, as by day 25 the tumour was similar in size to controls. These data suggest that syngeneic lysate may exert a temporary protective effect, however, a lasting protective effect was noted more clearly for allogeneic cells plus lysate, and for cell injections involving the xenogeneic U87 and LN229 tumour cell lines. Interestingly, the protective effect of the allogeneic cells appeared more pronounced when a lysate was added as compared to whole cell preparation only, as suggested by comparing rat #4 versus #5.

When tumour-rejecting rats were sacrificed, their tumour sections stained generally more positive for CD4, CD8, macrophages, B-lymphocytes, and dendritic cells, in contrast to the control group, which had tumours with intact architecture and a paucity of all of the above-mentioned immune cells. These results demonstrate that immunocompetent rats, which develop syngeneic tumours without rejection, show less or no immune cell infiltration, suggesting an escape from immune recognition due to immune ignorance [32].

When comparing the therapeutic vaccinations in SD and Fisher rats it is also worthy to note that while all of the treated SD rats rejected the C6 tumour, none of the Fisher rats has complete tumour remission within 40 days. This may be explained by the fact that syngeneic lysate was not added to either the allogeneic or the xenogeneic cells in Fisher rats, as it was done in the SD rats. Hence, addition of syngeneic lysate may significantly contribute to tumour rejection and will be examined in follow-up studies.

Collectively, pooled results from these experiments confirm that experimental vaccines based on allogeneic or xenogeneic cells only or combined with syngeneic cell lysates, are safe and protective in early and advanced malignant glioblastoma. These results lead us to conclude that ‘‘non-self’’ injections of allogeneic cells and/or allogeneic lysate, as well as xenogeneic cell lines, can break self-anti-tumour tolerance. These cells likely contain antigen determinants shared with the syngeneic tumour, leading to a reduction in tumour growth. The exact immunological mechanisms underlying the observed anti-tumour immunity remains to deciphered in further studies. Although these were small pilot treatments, in a limited number of animals per therapeutic effect, the inhibition of tumour growth within the treatment groups was statistically significant when compared with control or untreated animals. Our results support the viability of this cancer vaccine strategy as an adjuvant treatment to prevent tumour relapse in cancer patients.

The impact of these data may be far reaching when translation to patients is possible to certain degree. Glioblastoma multiforme is the most common and malignant of all gliomas, and cannot be cured by surgery, radiation therapy, chemotherapy, with 75% of patients dying within 18 months of diagnosis [33]. The use of allogeneic/syngeneic/or xenogeneic cell lines and lysates may lead to a reduction in tumour size and perhaps rejection, thereby increasing survival. In the future, allogeneic cell lines and lysates may also be used as vaccine components for other cancers.

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CHAPTER 6

Exploring the Therapeutic Efficacy of Glioma Vaccines Based on Allo- and Syngeneic Antigens and Distinct Immunological Costimulation Activators

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Summary

The efficacy of a various immunotherapeutic immunosation stragedies for maligant blioma brain cancer was evaluated in the syngeneic CNS-1 Lewis rat glioma model. A prototype glioma cancer vaccine, which was composed of multivalent antigens derived from allogeneic and syngeneic cells and lysates, formed the prototype preparation of antigens. These antigens reflect the autologous antigens derives from the patient's surgically removed tumour tissue, as well as allogeneic antigens form glioma tumour tissue surgically removed from donor patients. This antigen mixture provides a broad spectrum of tumour associated antigens (TAA) and helps to prevent escape of tumour immune surveillance when given as a vaccine. This antigen preparation was administered in a therapeutic setting with distinct single or multiple co-stimulation-favouring immunostimulants and evaluated for inhibition of tumour growth. Our prototype vaccine was able to arrest progression of the tumour growth when co-delivered in a specific regimen together with the costimulating multi-TLR agonist, Bacille Calmette Guerin (BCG) and interleukin-2, or with the Toll-Like receptor (TLR) 7/8 activator resiquimod.

Introduction

Glioblastoma multiforme (GBM) is an invasive malignant tumour of the central nervous system. Conventional therapy options include surgery, radiation, and chemotherapy, but with them the prognosis for GBM patients is limited to a mean survival time of only 14.6 months [1]. Immunotherapy is emerging as a novel complementary treatment option for a variety of malignancies including GBM. The use of successful passive immunotherapies based on the administration of immune elements, such as antibodies has proven very successful against various types of cancer. Well-known examples include antibodies that target tumour expressing receptors for epidermal growth factor (EGF), called Herceptin, those that target HER-2 [2], and those that are directed against angiogenic, tumour blood vessel growth-promoting vascular endothelial growth factor (VEGF), known as Avastin [3]. Recently, the antibody directed against an immune response inhibitory molecule, called cytotoxic T lymphocyte associated protein 4 (CTLA4), known as Yervoy®, has shown promising clinical efficacy in melanoma patients [4].

Apart from these passive antibody-based therapies, a range of active immunotherapies are in late stage development and are close to reaching approval as standard of care. These clinical studies clearly demonstrate that the immune system is able to discriminate cancer cells from normal cells following recognition of tumour associated antigens (TAA). Indeed a recent approval for a prostate cancer vaccine named Provenge®, was obtained from the FDA in April 2010, for the treatment of asymptomatic or minimally symptomatic metastatic, castrate-resistant (hormone-refractory) prostate cancer [5]. This recent approval has rejuvenated interest in the field as a whole.

In the present study we demonstrate a prototype brain cancer vaccine against gliomas which is composed of multivalent antigens derived from allogeneic and syngeneic cells and lysates. Our prototype is reflected in a clinical situation by autologous antigens derived from the patient's surgically removed tumour tissue. In addition, glioma tumour tissue surgically removed from donor patients provides a second source of allogeneic antigens that can be isolated and subsequently stored for later use. This material provides a new source of TAA that may display HLA-restriction that may overlap with that on the patient's glioma. They may serve to enhance the overall immune response. If processed under Good Manufacturing Conditions, it may provide an "off-the shelf" application. Relevant unique or shared TAAs overexpressed by tumour cells are present among thousands of irrelevant immunotolerant non-tumour associated antigens. The broad range of TAAs is preferred over vaccines with a mono- or oligo-valent antigenic content. These vaccines will prevent escape of tumour cells due to antigenic loss, or active MHC downregulation. In addition, a tumour antigen mixture also circumvents the use of monovalent synthetic peptides, which are restricted for use in patients with defined HLA types. By including TAAs of allogeneic origin, we additionally trigger allogeneic immune reactions. The haplotypes of CNS-1 and RG2 are fairly close, i.e., RT1l vs RT1lvl, yet provide a mismatch that may be considered abberant self, and also induce an alloresponse. This may provide better protection due to the "non-self" immune recognition of these antigens.

Although allogeneic tumour antigens may provoke immune responses to non-self antigens in classical allogeneic immune rejections, glioma tumours, once established, are known to actively suppress the host's immune system, by well

characterized mechanisms [6], which often leads to subsequent evasion of immune surveillance. We therefore decided to test various signalling and costimulation-favouring immunostimulants in combination with our prototype vaccine antigen formulation for anti-tumour activity in an aggressive rat glioma brain tumour model, CNS-1, that is syngeneic in Lewis rats.

Here we demonstrate that our prototype vaccine is able to arrest progression of tumour growth when co-delivered in a specific regimen with the costimulatory-enhancing multi-TLR agonist, Bacille Calmette Guerin (BCG) adjuvant with interleukin-2, or with the TLR 7/8 activator resiquimod.

Materials and Methods

Tumour model

Glioma cells: Both CNS-1 tumour cells and RG2 (ATCC, CRL-2433) rat glioma cells were cultured in Dulbecco's modified Eagle (DMEM) medium containing 10% bovine fetal serum (FBS), 5% antibiotics Penicillin, Streptomycin, and amphotericin B (Hyclone) in 175-mm2 flasks. The CNS-1 haplotype is RT1l and RG2 is RT1lvl.

Animals: Rat CNS-1 cells (2×10^5 cells/200 ul) were implanted subcutaneously (SC) using a 21 gauge needle into the right flank of 8-12 week-old (300 gram body weight) male Lewis rats. For each treatment group and control, 4-8 rats/group were used. All animal studies were approved by an independent ethical committee.

Monitoring tumour growth

The sizes of the CNS-1 tumour volumes were measured using a caliper three times per week on Mondays, Wednesdays and Fridays to monitor the effects of each treatment group.

Completion of experiment

Tumour implanted rats were sacrificed if they showed signs of discomfort, as defined by the ethical committee. For example if they appeared moribund due to weight loss, lethargy, ru(ed fur, or when tumours showed ulceration. A mixture of Rompun and ketamine was used for anesthesia, followed by a dose of sodium pentobarbital for euthansia.

Vaccine

The vaccine antigen preparation was composed of a mixture of haptenized CNS-1 cells and RG2 cells (1×10^e6 CNS-1 syngeneic and 1×10^e6 RG2 allogeneic glioma cells), together with lysates produced from 3×10^e6 CNS-1 syngeneic cells and 3×10^e6 allogeneic RG2 glioma cells. The haptinization method has been described before [7]. This vaccine preparation was kept as a constant factor and given in combination with various other anti-tumour or immunostimulatory agents as specifed in Figures 6.1A & 6.2A. The TLR7/8 agonist resiquimod was co-administered 3 times a week on Mondays, Wednesdays and Fridays.

Chemicals and Reagents

Immunomodulators and potentiators

Rats were subcutaneously (SC) injected in the flank, contralateral to the tumour-implanted side, with resiquimod (R848) (Invitrogen, TLRL-R848), a Toll-like receptor 7/8 agonist, at a dose of 100 µg/ kg, corresponding to 30 µg/dose, three times per week on Mondays, Wednesdays and Fridays.

In a parallel arm of the experiment we evaluated the effect of cyclophosphamide (CY) administration, in diverse regimens and dosing as specified, on CNS-1 glioma development. Cyclophosphamide (CalBiochem, 239785) was given at 30 mg/kg 3 times per week, or at 100 mg/kg once every week (See also Figure 6.1A and 6.2A).

We also tested the vaccine antigen preparations combined with Bacilles Calmette-Guérin, interleukin-2 (IL-2), or a B7.1 fusion protein.

The Bacilles Calmette-Guérin (BCG), substrain Connaught, (Immunocyst®, Sanofi Pasteur) is an agonist of TLRs 2, -4 -9, and was used at 2 × 10^{e}5 CFU per dose.

Interleukin-2 (IL-2) was injected daily at a dose of 75,000 IU/day from Monday to Friday starting on the day of vaccine injection.

A)

		Week 1							Week 2							Week 3		
		Mo	Tu	We	Th	Fr	Sa	Su	Mo	Tu	We	Th	Fr	Sa	Su	Mo	Tu	We
G1																		
G2	Cy100			X														
G3	B7	X	X	X	X	X												
G4	BCG	X				X			X				X				X	
	Cy30			X														
	Cy80															X		
	IL-2	X	X	X	X	X			X	X	X	X	X				X	X
	Vaccine	X				X			X				X				X	
G5	B7	X	X	X	X	X												
	BCG	X				X			X				X			X		
	Cy30			X														
	IL-2	X	X	X	X	X			X	X	X	X	X			X	X	X
	Vaccine	X				X			X				X			X		
G6	BCG	X	X	X	X	X			X				X			X		
	IL-2	X	X	X	X	X			X	X	X	X	X			X	X	X
	Vaccine	X	X	X	X	X			X				X			X		

B)

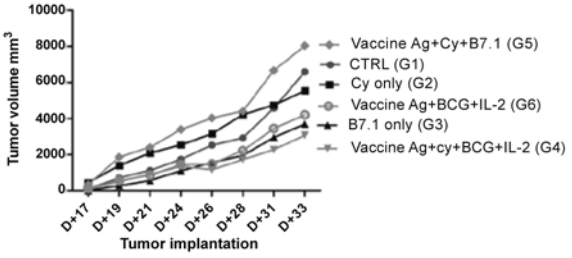

Figure 6.1: A vaccine was prepared from allogeneic and syngeneic antigens and administered in a fractionated regimen together with BCG and IL-2 to determine the effects on CNS-1 glioma growth. A) Treatment schedule representing one cycle of the various immunotherapeutic injections. B) Average values of tumour growth as a function of time post-implantation for the different treatment groups.

A B7.1 fusion protein consisting of the extracellular domains of human B7.1 and the Fc portion of human IgG1, called B7.1-Fc, was produced as described [8]. This protein induced complete regression of Colon 26 tumours in a mouse model and slowed tumour growth dramatically in mice with established poorly immunogenic RENCA and Madison109 tumours [8]. The B7.1-Fc protein was diluted in sterile PBS at 250 µg/200µl concentration.

For statistical analysis, we used ANOVA nonparametric testing followed by student's *t* tests to compare groups. P values of < 0.05 (*), $p < 0.01$ (**), $p < 0.001$ (***) were considered statistically significant.

Results

Efficacy of a BCG-containing vaccine administered at day 17 after implantation

CNS-1 tumour cells (2×10^5 cells / 200 µl) were implanted in syngeneic male Lewis rats and after 17 days were either left untreated (control group; $n = 4$) or administered with a therapeutic immunomodulatory regime or some of its components, as specified in the treatment schedule of Figure 6.1A.

The vaccine antigen preparation consisted of a mixture of haptenized 10^e6 syngeneic and 10e6 allogenic RG2 glioma cells together with lysates produced from 3×10^e6 synegeic CNS-1 cells and 3 × 10e6 allogenic RG2 gliomacells ($n = 4$). When monitoring tumour growth over time with a caliper, we observed no significant difference in tumour volumes between the control group (G1) and the rats receiving cyclophosphamide (CY) only at a dose of 100 mg/kg (G2) (Figure 6.1). From other experiments we had learned that the vaccine antigen preparation showed no anti-tumour efficacy by itself under the conditions described (for illustration see also figure 6.3, the antigen only group indicated by closed circles). By contrast, when this vaccine antigen preparation was administered together with the multiple TLR agonist BCG plus IL-2, an inhibition of tumour growth was noted (G6). Similarly, the same treatment schedule supplemented with CY (30 mg/kg on day 19, and 80 mg/kg on day 31) also inhibited tumour growth (G4). Remarkably, also five daily injections of B7.1-FC fusion protein alone (Monday to Friday, starting day 17 post implantation), showed some inhibition of tumour growth (G3), though not statistically significant relative to control groups. However, when combining the beneficial vaccine preparation plus CY (30 mg/kg) treatment with an additional regimen of B7.1-Fc antibody injections no inhibition of tumour growth was noted (G5).

A BCG-containing vaccine shows better eThcacy amongst the diverse vaccine prototypes tested

In a subsequent experiment, CNS-1 tumour cells ($2 \text{ X } 10^5$ cells / 200 µl) were implanted in 8-12 week-old syngeneic male Lewis rats and were either left untreated (control group; $n = 4$) or administered with a therapeutic immunomodulatory treatment regimen or some of its components, as specified in the treatment schedule depicted in Figure 6.2A. Instead of waiting until day 17 post tumour implantation, we now started at day 10 after tumour inoculation.

As before, the vaccine antigen preparation consisted of a mixture of

A)

		Week 1							Week 2						
		Mo	Tu	We	Th	Fr	Sa	Su	Mo	Tu	We	Th	Fr	Sa	Su
G1	/														
G2	B7		X	X	X	X									
	Cy30								X		X	X			
	Vaccine									X			X		
G3	B7	X	X	X	X	X									
G4	Cy30								X		X	X			
G5	Cy30	X		X	X				X		X	X			
	vaccine		X			X				X			X		
G6	AC				X							X			
	SC					X							X		
	AL		X							X					
	SL			X							X				
	Cy100	X													
	BCG+IL-2					X							X		
G7	Cy30	X		X	X				X		X	X			
	Ly		X			X				X			X		

B)

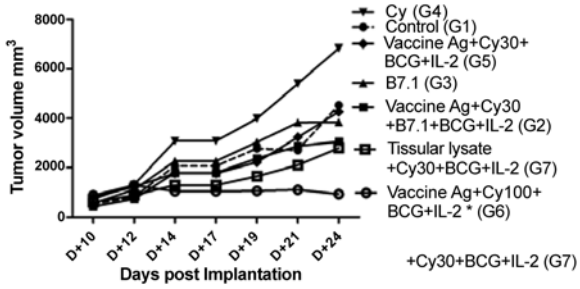

Figure 6.2: A vaccine was prepared from allogeneic and syngeneic cells administered in a fractionated regimen together with BCG and IL-2. A) Treatment schedule representing the cycle of the various immunotherapeutic injections. AC, allogenic cells, SC, syngeneic cells, AL, allogeneic lysate, SL, syngeneic lysate, Ly, is lysate only. B) Average values of tumour growth as a function of time post-implantation for the different treatment groups.

haptenized syngeneic CNS-1 and allogeneic RG2 glioma cells (10^e6 each) with lysates produced from syngeneic CNS-1 cells and allogeneic RG2 glioma cells (3×10^e6 each) (n = 4). When monitoring tumour growth over time with a caliper, we observed no significant difference in tumour volumes between the untreated control group (G1) and the rats receiving low dose cyclophosphamide (CY, 30 µg/dose) in week two (G4), B7.1-Fc fusion protein only (G3), B7.1-Fc protein plus CY (G2) or vaccine plus CY only (G5) (Figure 6.2A). By contrast, when this vaccine antigen preparation was administered together with the multiple TLR agonist BCG plus IL-2 and CY (100 µg/dose), a significant inhibition ($p < 0.05$) of tumour growth was noted (G6). This vaccine preparation was administered in a fractionated schedule starting with allogeneiic cells on the first day, followed by syngeneic cells the second day, and allogeneic and syngeneic lystates on the third and fourth days, respectively (G6). Remarkably, also *ex vivo* tumour tissue isolated from a syngeneic established CNS-1 tumour plus CY (G7) showed some inhibition of tumour growth (although not statistically significant) relative to control groups (Figure 6.2B).

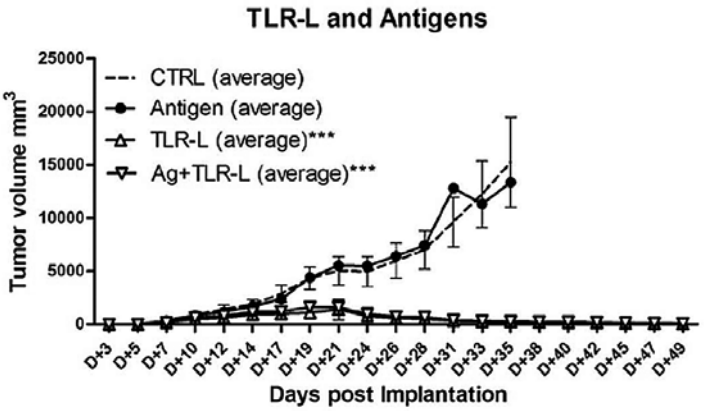

Figure 6.3: A vaccine was prepared from the standardized allogeneic and syngeneic cells administered with or without the TLR7/8 agonist, resiquimod, injected 3 times a week on Mondays, Wednesdays and Fridays. Average values of tumour growth as a function of time post-implantation for the different treatment groups.

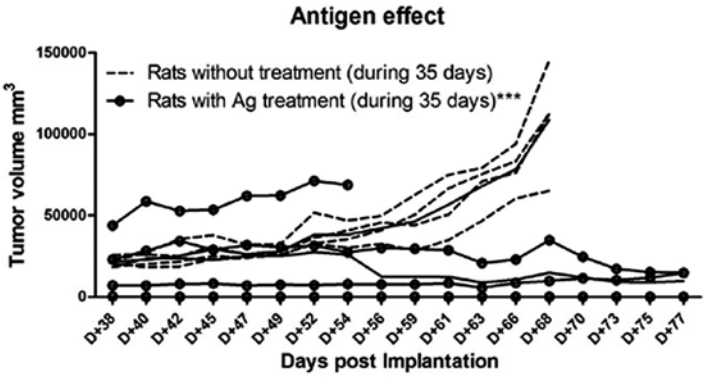

Figure 6.4: Individual tumour growth curves as a function of time post-implantation.

Therapeutic treatment with a vaccine containing immunostimulatory TLR7/8 agonist shows marked inhibition of glioma tumour growth

In view of the beneficial effect of vaccination in the context of the multi-TLR agonist BGC, we decided to evaluate the effect of a TLR7/8 agonist in our CNS-1 tumour model. CNS-1 tumour cells (2 X 10^5 cells /200 µl) were implanted in 8-12 week-old syngeneic male Lewis rats and either left untreated (control (CTRL) group; *n* = 8) or treated at day 10 after implantation with a vaccine antigen preparation consisting of a mixture of haptenized syngeneic CNS-1 and allogeneic RG2 glioma cells (10^e6 each) with lysates produced from syngeneic CNS-1 and allogeneic RG2 glioma cells (3 × 10^e6 each) (*n* = 8), given alone or combined with the TLR7/8 agonist, resiquimod (R848). This vaccine preparation was given 3 times a week on Mondays, Wednesdays and Fridays. When monitoring tumour growth over time with a caliper, we observed no significant difference in tumour growth between control and vaccine antigen only treated rats (Figure 6.3). By contrast, when this antigen preparation was administered together with a TLR7/8 agonist (30 µg/dose) significant inhibition (p, 0.001, ***) of tumour growth, with complete tumour regression was noted (Figure 6.3). Remarkably, administration of TLR7/8 agonist alone in the same regimen also inhibited tumour (p, 0.001, ***) (Figure 6.3).

Inhibition of tumour progression with large established glioma tumours by a vaccine containing the TLR7/8 immunostimulant

As mentioned above, Lewis rats treated with a vaccine without immunostimulatory TLR7/8 agonist showed little inhibition of tumour growth relative to untreated control rats, while the TLR7/8 containing vaccine strongly suppressed tumour growth when given 3 times per week starting at day 10 after implantation. We therefore decided to examine the effect of therapeutic vaccination of animals which showed no inhibition of tumour growth at day 34 after implantation. We administered the TLR7/8 containing vaccine to animals with large (12-14,000 mm^3) tumours, starting at 38 days after tumour implantation. The TLR7/8 agonist-containing vaccine was injected 3 times a week on Mondays, Wednesdays and Fridays, in both untreated control rats (*n* = 4) and rats immunized with idential antigen only (n=4). We noted in both groups a slower rate of growth of gliomas with large tumour volumes, but the TLR7/8 containing vaccine was best able to evoke a significant (p < 0.001, ***), arrest of tumour volume growth in the rats that were treated before with the antigen only vaccine preparation, relative to rats which were untreated (Figure 6.4). As well, no signs of vaccination-induced adverse effects or toxicity were observed, confirming data from another study that immunotherapy is well tolerated with limited toxicity [9]. These untoward effects point to the relative safety and tolerability of vaccines.

Discussion

In the present study we show that a prototype vaccine, consisting of a mixture of allogeneic and syngeneic glioma cells and their lysates, administered together with either a multiple TLR2, -4 and -9 agonist BCG [9], or the TLR7/8 agonist resiquimod, is able to inhibit CNS- 1 glioma brain tumour growth in syngeneic Lewis

rats. For early stage tumours, a complete regression of tumour growth volume was noted, while for large established 38-day old tumours (14,000 mm^3 volume), inhibition of tumour growth was noted for the TLR7/8 containing vaccine. These data confirm the well-know phenomenon that large glioma tumours are more difficult to control relative to smaller tumours. Nevertheless, if the latter is most relevant to the large tumour burden that may be present in individuals with gliomas in unresectable sites (e.g. parietal or tempero-parietal tumours), instituting vaccination with TLR agonists may be valuable in providing additional quality survival time.

During glioma tumour development a proportional increase in local and systemic immunosuppression has been documented [6,9,11,12]. Gliomas are known to contain T regulatory cells, which normally act as suppressor cells, regulating homeostatis and preventing autoimmune reactions. However, regulatory T cells also inhibit T effector functions, activated naturally by vaccination [13,14], and thereby facilitating tumour immune evasion and subsequent tumour progression. At the molecular level this is associated with an increase in production of immune inhibitory cytokines including transforming growth fcator (TGF)-beta, and/or interleukin-10 [15]. In general, immunosuppression can be counteracted by either strong stimulation of proinflammatory immune pathways, by blocking immune inhibitory elements triggered by the tumour itself, or by a combined "push-pull" approach [16]. We therefore evaluated well-known costimulation-enhancing immunostimulants, such as TLR-agonists [17], and inhibitors of immune system signals, such as what the B7.1 and cyclophosphamide immunomodulators provide. They were administered in conjunction with our standard vaccine antigen preparation.

We noted beneficial antitumour activity with daily injection of the B7.1Fc fusion protein starting at day 17 after tumour implantation as a monotherapy. This soluble costimulator protein was fused to the Fc portion of the antibody. It has been developed and tested for the immunotherapy of solid tumours in mouse tumour models [8]. This B7.1-Fc protein was found to be capable of activating T cells *in vitro* and *in vivo* [8]. B7.1 is able to engage with two known receptors. CD28, which triggers a stimulatory signal to activate naive T cells after binding by B7.1 (4), and its counterreceptor, CTLA-4, which triggers a negative signal to stop T-cell activation. Since B7.1 has high affinity to CTLA-4, soluble B7.1-Fc may block CTLA-4 signalling instead of cross-linking CTLA-4, thereby sustaining the activation of tumourspeci fic T cells [18,19]. Crosslinking of T cell activating CD28 may be less important than blocking the inhibitory CTLA-4, since activated T cells recognizing tumour antigens require less costimulation [20]. When B7.1 antibody was combined with other immune system activating treatments, we observed no synergistic activities, which illustrates that the timing and choice of modalities during combined immunotherapy is criticical for a beneficial antitumour effect.

Also, cyclophosphamide, when dosed carefully, has been demonstrated to facilitate immunotherapy of established tumours through the elimination/inactivation of suppressor T cells [21- 23]. In our investigations, a protocol of cyclophosphamide (CY) only, administered at a dose of 100 mg/kg given on day 19 post transplantation did not affect tumour growth (Figure 6.1), while three lower doses of 30 mg/kg each starting on day 17 given within a week period, did not show a beneficial anti-tumour activity (Figure 6.2). By contrast, CY treatment alone may even exacerbate tumour growth, relative to the untreated control group, which may likely result from suppression of endogenous anti-tumour immune reactions in parallel to inadequate direct tumour

growth inhibition, resulting in a net increase in tumour growth volume. Nevertheless, when combined with other immune adjuvants or vaccines, beneficial effects were noted despite coadministration of CY. The best efficacy was noted in treatment schedules containing the vaccine antigen preparation plus BCG and IL-2. Bacillus Calmette- Guérin (BCG) is a mycobacterium which contains various agonists of Toll-like receptor 2 (TLR2) and TLR4 in its cell wall skeleton (CWS), and also BCG DNA which acts as a TLR9 agonist [10,24]. Intravesical immunotherapy with BCG, as monotherapy, causes significant reductions in cancer tumour progression and is currently the standard treatment for superficial bladder cancer [25]. However, the exact mode of action of successful BCG therapy remains elusive, although massive cytokine expression and influx of innate immune cells have been documented, which may be involved in tumour elimination.

Apart from BCG, we also examined the effect of the TLR7/8 agonist resiquimod as an adjuvant in a separate animal study. TLR7/8 agonists exert pleiotropic effects on various immune cells which leads to stimulation of proinflammatory cytokine and chemokine production, as well as up-regulation of costimulatory molecules on antigen presenting cells [26]. The TLR7/8 agonist resiquimod has been shown to act as a vaccine adjuvant in investigational vaccine models [25,27], and to generate clinical-grade mature DCs [28].

In our CNS-1 model, the TLR7/8 agonist resiquimod showed superior antitumour effects against early tumour growth and remarkably, administration of TLR7/8 agonist alone inhibited tumour growth. The exact mode of action for the observed resiquimod-mediated anti-tumour immunity needs to be defined in detailed follow-up studies. Most likely the observed resiquimod-based immunotherapy, even in absence of additional tumour antigens, is able to activate a spontaneous, natural, innate anti-tumour immune response, that under normal circumstances is unable to control tumour growth. Hence, such dedicated followup studies need to address to involvement of anti-tumour killer macrophages or NK cells, or IFNs for the resiquimod-induced glioma growth regression. In addition, the TLR7/8 vaccine preparation evoked an arrest of tumour volume growth in rats carrying large tumours that were treated before with the antigen only vaccine preparation.

Although the presented results are promising the should always be interpretaed with caution. Subcutaneous tumours may be exposed to markedly distinct immune conditions than in the brain, and so the physiologic as well as clinical relevance has to be validated in further studies.

Our vaccine preparation contains multiple tumour associated antigens (TAA) prepared from syngeneic and allogeneic cells. Although the precise identity of antigens is unknown we chose this antigen preparation for our vaccination strategy, rather than one or a few molecular targets, in order to target multiple TAAs simultaneously. This approach minimizes the chances for classical tumour-escape of immune control, as a result of selective growth of antigen-loss variants. With this approach we guaranteed that multiple relevant TAAs gain enhanced exposure to the immune system, while the self-antigens in this mixture are neglected as a result of immunological tolerance. By including cell associated antigens, we increased the chance of crosspresentation by MHC class I molecules for priming of cytolytic CD8+ T cells. By including TAAs of allogeneic origin, we additionally trigger allogeneic immune reactions. The haplotypes of CNS-1 and RG2 are fairly close, i.e., $RT1^{l}$ vs $RT1^{lvl}$ thus providing a mismatch that may be considered abberant self, and also induce a vigorous alloresponse. Allogeneic

(major histocompatibility complex MHC-mismatched) tumour cells have shown better protection in a number of models likely due to the “non-self” immune recognition of these antigens [29-31].

Immunotherapy against TAA theoretically carries the risk of autoimmune reactions. Autoimmunity is possible, especially in view of our manipulations aimed at blocking immune response inhibitors coupled with the use of immunostimulating adjuvants. In the present studies, however, we have not observed any adverse reactions using our animal well-being scoring system. These observations are in line with well-documented observations, that therapeutic tumour vaccination is, in general, well-tolerized with minimal or no adverse events.

In conclusion, we noted beneficial antitumour activity of daily injection of the B7.1-Fc fusion protein as monotherapy, and with vaccine preparations that were administered with BCG and IL-2 only, or with the TLR7/8 agonist resiquimod. Altogether these data illustrate the importance of a beneficial immunomodulatory protocol. Different immunomodulators may result in distinct antitumour efficacy. Hence, we conclude from our data that only specific combinations of the right antigenic mixtures in conjunction with a suitable immunopotentiator is able to arrest aggressive glioma tumour growth in this experimental tumour model. Our data highlight the need for continued, more extensive exploration into such combinations.

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CHAPTER 7

Therapeutic efficacy of a glioma vaccine based on allo- and syngeneic antigens coadministered with granulocyte macrophage colony stimulating factor (GM-CSF) after a low dose cyclophosphamide regimen

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Summary

In an aggressive rat glioma tumour model, we demonstrate therapeutic efficacy of a prototype brain cancer vaccine, which is composed of multivalent antigens derived from allogeneic and syngeneic cells and lysates. We decided to test our prototype anti-tumour vaccine antigen formulation in combination with the locally administered immunostimulatory cytokine granulocyte colony-stimulating factor (GM-CSF), which is approved as a prescription medication in humans. This vaccine prototype suppressed tumour growth resulting in complete regression, and immunological memory against secondary tumour inoculation.

In a clinical situation our therapy is reflected by a vaccine composed of autologous antigens, derived from the patient's surgically removed tumour tissue, which is administered in conjunction with antigens from glioma tumour tissue surgically removed from allogenic donor patients. This allogenic tumour material provides a second source of antigens that can be isolated and subsequently stored in a tissue bank for "off-the shelf" use. Such a therapeutic vaccine can be administered post-standard surgery and radiation plus chemotherapy in order to prevent tumour recurrence.

Thus, we demonstrate that our prototype therapeutic vaccine, when co-delivered in a specific regimen together with the GM-CSF as immunological adjuvant, is able to arrest progression of glioma tumour growth, when therapeutically administered following low-dose cyclophosphamide.

Introduction

Active immunotherapy against cancer represents an exciting treatment option, involving the stimulation of the patient's immune system against tumour antigens. However, therapeutic immunization against malignant glioma brain tumours, glioblastoma multiforme (GBM), is a formidable challenge. Although, natural brain parenchyma infiltrating CD8-positive T cells have been detected in these brain tumours [Yang *et al*., 2011; Schneider *et al*., 1992) and even anecdotal rejection following bacterial infection (Bowles and Perkins, 1994), GBM, once established, normally evades immune detection, as a result of decreased MHC antigen expression and active suppression of local and systemic immune reactions (Fecci *et al*., 2006). Apart from tumour-mediated immune suppression the patient's immune reactivity is further suppressed by iatrogenic chemotherapy (J. Ohlfest personall communication) and due to post-surgical corticosteroid treatment. In combination this tilts the balance towards an immune suppressive state (Fadul *et al*., 2011) as evidenced by significant lymphopenia, with a decrease in total CD4+ T cells and a functional increase in regulatory T cells.

Glioblastoma mutiforme (GBM) is the most common and most aggressive malignant brain tumour known as gliomas, with a very poor prognosis due to marginally effective standard therapy, involving tumour-debulking surgery, followed by radiotherapy and chemotherapy. This cancer is very difficult to treat and most patients die within a year due to tumour recurrence. Consequently, novel therapies are highly demanded.

Successful post-operative immunotherapy enabling immune recognition and destruction of residual or recurrent tumour cells would provide an enormous clinical value. Induction of a vaccine-induced immune response by adaptive immune lymphocytes initially requires efficient presentation, by antigen presenting cells, of tumour associated antigens (referred to as signal 1) together with costimulatory signals (called signal 2). Most TAAs are inherently poorly antigens and require an adjuvant to break immunological tolerance (Schijns and Lavelle, 2011). The studies described here included GM-CSF as an immunological adjuvant, which is able to facilitate both signal 1 and signal 2. The cytokine GM-CSF is well-known in supporting dendritic cell (DC) recruitment and development; hence enabling improved antigen uptake and subsequently the magnitude of antigen presentation. In addition, it stimulates maturation of DC, characterised by expression of co-stimulatory molecules (signal 2), as effective antigen–presenting cells for T cells (Min *et al*., 2010). GM-CSF is one of the most frequently used cytokines as immunological adjuvant in different types of cancer vaccines (Warren and Weiner, 2000; Chang *et al.*, 2004; Parmiani *et al.*, 2007). GM-CSF is commonly used today in the production of DCs from *ex vivo* isolated peripheral blood monocytes for the clinical use in DC cancer vaccines (Inaba *et al.*, 1992; Schuler *et al.*, 2003). GM-CSF has also been shown to stimulate peripheral blood monocytes *in vitro* to become cytotoxic for the malignant cells (Grabstein *et al.*, 1986).

GM-CSF has been administered either as recombinant protein in conjunction with tumour antigens or as a transfected gene therapy product in cells genetically engineered to secrete biologically active GM-CSF (Jinushi and Tahara 2009; Parmiani *et al.*, 2007; Dranoff *et al.*, 1993). GM-CSF is also a component that is used in the first Food and Drug Administration-approved therapeutic cancer vaccine Sipuleucel-T against prostate cancer (Cheever and Higano, 2011). Notably, opposite immunological

functions have been reported for this cytokine (Parmani *et al.*, 2007; Clive *et al.*, 2010). For example, a high concentration of this cytokine may recruit immunosuppressive myeloid suppressor cells and depress the immune response (Serafini *et al.*, 2004). Hence, as for most cytokines, the timing and dosing seems crucial for optimal clinical use. However, the significance of data in pre-clinical and clinical settings, demonstrating the beneficial adjuvant effects of GM-CSF in a variety of cancer vaccine approaches, make GM-CSF an attractive vaccine adjuvant because of its immune modulation effects and low toxicity profile. The safe pharmacological use of GM-CSF in patients is well-established, which makes it very attractive and feasible for clinical use.

The present study shows complete rejection of malignant gliomas as a result of active therapeutic immunization with a mix of allogeneic and syngeneic glioma tumour antigens in conjunction with low dose GM-CSF, following low-dose of cyclophosphamide treatment. The strong efficacy of this prototype immunotherapy supports future implementation in clinical studies.

Materials and methods

Tumour Model

Glioma cells Both CNS-1 tumour cells and RG2 (ATCC, CRL-2433) rat glioma cells were cultured in Dulbecco's modified Eagle (DMEM) medium containing 10 % bovine fetal serum (FBS), 5 % antibiotics Penicillin, Streptomycin, and amphotericin B (Hyclone) in 175-mm^2 flasks. The CNS-1 haplotype is $RT1^{l}$ and RG2 is $RT1^{lvl}$.

Animals Rat CNS-1 cells (2 X 10^5 cells/200 µl) were implanted subcutaneously (SC) using a 21 gauge needle into the right flank of 8-12 week-old (300 gram body weight) male Lewis rats. For each treatment group and control, 6 rats/group were used. All animal studies were approved by an independent ethical committee.

Monitoring Tumour Growth The sizes of the CNS-1 tumour volumes were measured using a caliper three times per week on Mondays, Wednesdays and Fridays to monitor the effects of each treatment group.

Completion of experiment Tumour implanted rats were sacrificed if they showed signs of discomfort, as defined by the ethical committee. For example if they appeared moribund due to weight loss, lethargy, ruffled fur, or when tumours showed ulceration. A mixture of Rompun and ketamine was used for anesthesia, followed by a dose of sodium pentobarbital for euthansia.

Vaccine The vaccine antigen preparation was composed of a mixture of haptenized CNS-1 cells and RG2 cells (1 X 10^e6 CNS-1 syngeneic and 1 X 10^e6 RG2 allogeneic glioma cells), together with lysates produced from 3 X 10e6 CNS-1 syngeneic cells and 3 X 10^e6 allogeneic RG2 glioma cells. After a low-dose cyclophosphamide (CY), given 3 days before the first vaccine injection, this vaccine preparation was given subcutaneously in combination the immunostimulatory cytokine GM-CSF (Prospec, USA), which was co-administered together with the vaccine in a dose of 30 µg/kg (corresponding to about 10 µg/shot) according to the treatment schedule depicted in Figure 7.1.

Chemicals and reagents

Immunomodulators and potentiators

Rats were subcutaneously (SC) injected with the vaccine preparation in the flank, contralateral to the tumour-implanted side. As a control Cyclophosphamide (CY; CalBiochem, 239785) was given once, at 30 mg/kg, on day 10 post tumour implantation, in combination with GM-CSF injections without vaccine. Rat-GM-CSF was purchased as an E. coli expression product from Prospec (USA, East Brunswick, NJ). We also tested the vaccine antigen preparation combined with cyclophosphamide (CY) only or with CY plus GM-CSF. Figure 7.1 shows a treatment schedule representing one cycle of the various immunotherapeutic injections.

Statistics For statistical analysis, we used ANOVA nonparametric testing followed by students t-tests to compare groups. P values of < 0.05 (*), $p < 0.01$ (**), $p < 0.001$ (***) were considered statistically significant. (NB statistics needs to be done on final data).

Results

Except for the untreated control group (CTRL), all animals received a low dose of cyclophosphamide (CY) in order to deplete immunosuppressive T regulatory cells (Daenen *et al.*, 2009) as depicted in the treatment schedule of Figure 7.1.

		Cycle 1																			
		Week 1							Week 2							Week 3					
		Mo	Tu	We	Th	Fr	Sa	Su	Mo	Tu	We	Th	Fr	Sa	Su	Mo	Tu	We	Th	Fr	Sa	Su
A																						
B	Cy30	X																				
	Cells				2			1			3			2			3			1		
C	Cy30	X																				
	GM-CSF				X			X			X			X			X			X		
D	Cy30	X																				
	GM-CSF				X			X			X			X			X			X		
	Cells				2			1			3			2			3			1		
E	Cy30	X																				

Figure 7.1. Treatment schedule representing one cycle of the various immunotherapeutic injections. The Monday in week 1 is day 10 post implantation. One cycle takes about 2.5 weeks and ends at day 28 post tumour implantation. The numbers refer to the cellular components, with 1) allogeneic RG-2 cells, 2) syngeneic CNS-1 cells, 3) allogeneic C6 cells. The second cycle D31-D49 (= 2nd cycle). Treatment stopped at D=49 and the secondary challenge was given on D59.

We noted complete tumour regression only in the group of animals that received the vaccine in conjunction with GM-CSF (Figure 7.2). In the groups receiving Cy only, CY plus GM-CSF, or CY plus vaccine, some delay in measurable tumour growth was observed relative to the untreated control groups

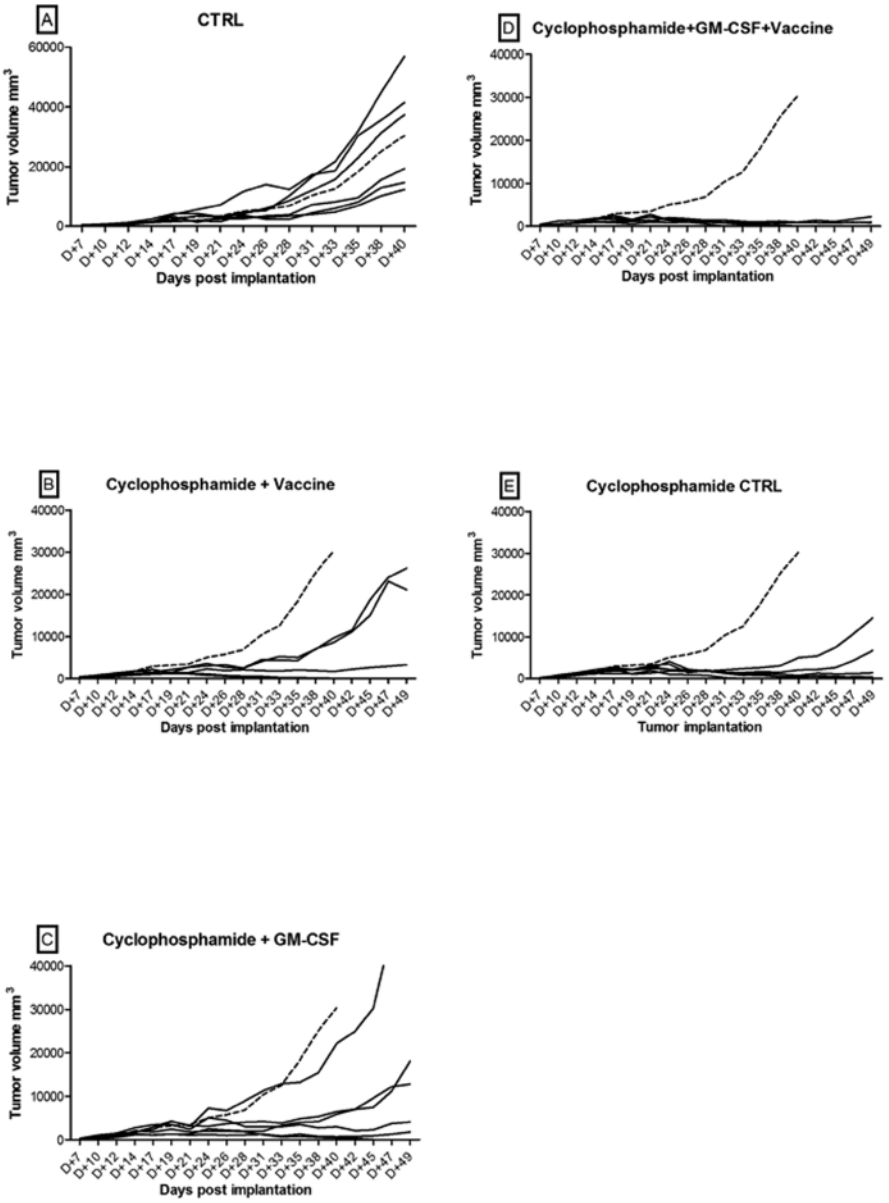

Figure 7.2. Average values of tumour growth as a function of time post-implantation for the different treatment groups. Rats were randomly injected with the CNS-1 tumours and divided into 4 groups (n=6). Control groups (CTRL) were either left untreated (A), or treated with with low-dose cyclophosphamide (CY) followed by vaccine antigen injections, that were prepared from the standardized allogeneic and syngeneic cells (B). Another group was treated with with low-dose CY and GM-CSF only (C). Another group received the same vaccine preparation given in combination with GM-CSF following the low-dose CY (D). The treatments were administered with 3-day intervals (see figure 7.1). Average values of tumour growth for individual rats as a function of time post-implantation for the different treatment groups.

Immune anti-tumour memory against glioblastoma

In the animals that all survived the primary glioma implantation within the vaccine plus GM-CSF group (*n* = 6 out of 6), and in the group of animals that was able to reduce and delay the tumour growth following CY treatment (*n* = 6 from original group of 6), we tested antitumour immunity after re-inoculation of a secondary glioma challenge in the neck. It was compared to a naive (age-matched) control inoculated tumour group (*n* = 6). We noted that only rats with a measurable primary tumour, as depicted in Figure 7.3, developed a secondary tumour. Hence, 5 out of 6 rats from the complete vaccine plus GM-CSF therapy group develop no secondary tumour after re-challenge (Figure 7.4c), while in the CY only group 3 out of 6 rats developed a secondary tumour as depicted in Figure 7.4B. It proved that the secondary tumour challenge evoked tumour growth only in animals that showed residual tumour volumes following the primary inoculation; i.e. in 3/6 animals the CY group, and only 1/6 in the GM-CSF plus vaccine group (Figure 7.4). The growth of the primary tumours, following the inoculation of the secondary tumour implantion, is likely due to less immune strength within a certain immune effector population as a result of less numbers of effector cells or less functional capacity.

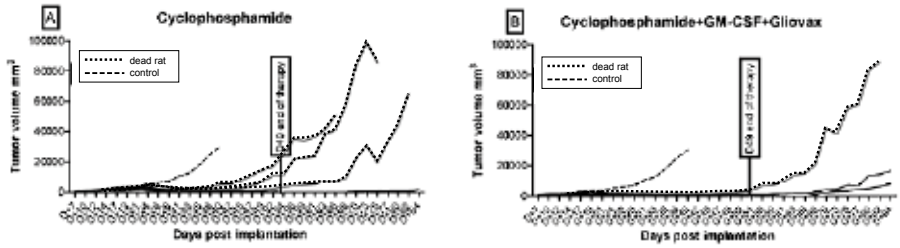

Figure 7.3. Primary tumour growth in animals initially controling tumour implantation in the CY only group (A) and vaccine plus GM-CSF group (B). At 10 days after treatment arrest (D49), all animals received a secondary tumour implantation; i.e. at day 59 post primary tumour implantation. Tumour volumes of animals which had to be sacrificied are indicated in red.

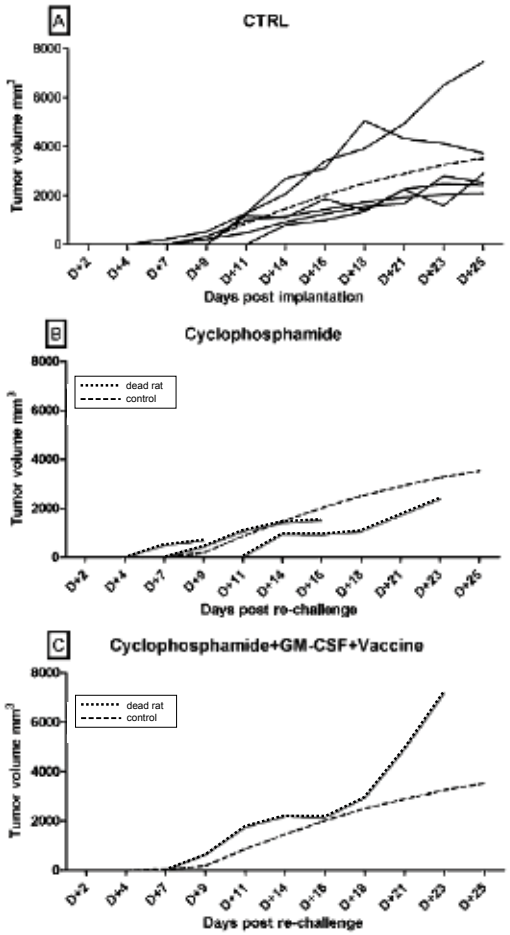

Figure 7.4. Secondary tumour growth in naïve untreated rats (A), rats controlling the primary tumour exposure following CY treatment (B), and controlling primary tumour growth after CY treatment followed by vaccination in conjunction with GM-CSF (C).
The second tumour was implanted in the neck at day 59 post primary tumour implantation, i.e. at 10 days after treatment arrest (D49). Tumour volumes of animals which had to be sacrificied because of primary tumour volumes are indicated in red. In group A all animals (n=6) devlop tumours, while in 3/6 rats develop measurable tumours in group B, and only 1/6 rats develop the secondary tumour in group C.

Discussion

In the present study we confirmed the proof-of-concept for using mixed whole tumour tissue-derived allogenic and syngeneic antigens in an immunization therapy against glioblastoma, when administered together with GM-CSF as immunological adjuvant. We show that this treatment induces tumour regression,

which becomes visible as a reduction in tumour growth rate after about 2 weeks of initiation of immunotherapy, resulting in non-detectable tumour volumes after a peak volume at 21-24 days after the start of tumour growth. This anti-tumour responses resulted in immunological memory, since the majority of animals that controlled the tumour after treatment with vaccine plus GM-CSF, also rejected a secondary tumour without noticeable tumour growth.

Except for the untreated control group, all animals were pretreated with a low-dose CY in order to deplete the immunosuppressive regulatory T cells (Rozados *et al.*, 2010). This dose proved partially effective in reducing tumour growth, either as a result of direct cytotoxic tumour killing or reduced immunosuppression and/or reduced angiogenesis, or a combination of all of these effects. However, following therapeutic immunization using an antigen preparation based on cells and lysates of synegeic CNS-1 tumour cells and allogeneic rat glioma cells (C6 and RG-2) in conjunction with the immunological adjuvant GM-CSF we observed complete tumour regression.

In a follow-up experiment we addressed the critical elements and dosing of the therapeutic regimen, by selectively changing the dose of antigen, and cyclophosphamide and the type of adjuvant, while leaving other therapeutic elements unaltered. It was noted that the dose of the antigen preparation could be lowered to 50 %, without a clear reduction in efficacy if other components (vaccine plus GM-CSF) were unaltered (data not shown). We also noted that selective reduction in cyclophosphamide dose by 50 % still provided protection in combination with the unaltered vaccine plus GM-CSF, while treatment with 50 % of cyclophosphamide only (15 mg /dose) did not exert antitumour efficacy. However, a further reduction in cyclophosphamide (7.5 mg/dose) failed to evoke antitumour responses in the context of the vaccination (data not shown). In addition, we tested the particulate saponin adjuvant (AbISCO, Isconova, Sweden) as alternative adjuvant for GM-CSF and observed a similar, though somewhat reduced, antitumour efficacy (not shown).

In a clinical situation our therapy is reflected by a vaccine composed of autologous antigens, derived from the patient's surgically removed tumour tissue, which is administered in conjunction with antigens from glioma tumour tissue surgically removed from allogenic donor patients. This allogenic tumour material provides a second source of antigens that can be isolated and subsequently stored in a tissue bank for "off-the shelf" use use. The allogenic TAAs may display partial HLA-matching with the patient. The mismatching HLA molecules serve to enhance the overall alloimmune response. Relevant unique or shared TAAs overexpressed by tumour cells are present among thousands of irrelevant immunotolerant non-tumour associated antigens. The broad range of TAAs is preferred over vaccines composed of a mono- or oligo-valent antigen. The multivalent vaccine will prevent or reduce escape of tumour cells, due to antigenic loss, or active MHC downregulation. In addition, a tumour antigen mixture is preferred above the use of monovalent synthetic peptides, because of the restricted use of peptides in patients with defined HLA types only.

GM-CSF is a frequently used cytokine known to augment immune responses for protein of peptide based vaccines (Disis *et al.*, 1996), as well as tumour vaccines consisting of cells engineered to secrete GM-CSF (Dranoff *et al.*, 2002). Intradermal or subcutaneous inoculation of GM-CSF results in an increase of class II-positive cells, and "conditions" the inoculation site to enhanced responses to antigen (Disis *et al.*, 1996). This cytokine has been used as a haematopoietic growth factor in

patients undergoing chemotherapy, and is well tolerated (Armitage *et al.*, 1992). When given as adjuvant in the skin it is known to recruit and activate antigen presenting cells, including epidermal Langerhans cells (Kaplan *et al.*, 1992). GM-CSF showed positive effects relative to other cytokines, or in synergy with other cytokines, in preclinical rat and mouse glioma vaccine studies (Herrlinger *et al.*, 2004; Jean *et al.*, 2004; Soiffer *et al.*, 1998). Despite a wealth of positive reports on the use of GM-CSF, in a randomized trial in melanoma patients GM-CSF administration caused early death and reduced DTH responses following immunization with allogeneic tumour cells and BCG, relative to the arm without GM-CSF treatment (Faries *et al.*, 2009). Also reduced CD4 and CD8 responses against a melanoma peptide vaccine were observed in the arm receiving GM-CSF, relative to the non-GM-CSF receiving arm (Slingluff *et al.*, 2009). These studies illustrated that the dose and schedule are very important parameters in the use of GM-CSF during immunotherapy.

Immunological protection against gliomas has been ascribed to cell mediated immune reactions involving cytolytic CD8+ T lymphocytes (Ghulam *et al.*, 2009). In various preclinical models depletion of these cells by anti-CD8 treatment has demonstrated their critical role in vaccine-mediated antitumour immunity (Barcia *et al.*, 2009; Maes *et al.*, 2009). Hence, in future studies we will examine the influence of these lymphocyte populations on protective antitumour efficacy in depletion- or adoptive transfer studies.

In conclusion, we noted beneficial antitumour activity of a vaccine preparation that was repeatedly administered together with GM-CSF following a low-dose cyclophosphamide injection 3 days before immunotherapy.

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CHAPTER 8

General Discussion

8.1 Preclinical studies

The hypothesis pertinent to this thesis is that glioma tumours can be therapeutically targeted by gene therapy and/or immunotherapy in order to eliminate or delay tumour progression. In our gene therapeutic approach, we observed that chronic expression of the C-terminal fusion of IsK with EGFP (enhanced green fluorescent protein) led to cell death of more than 50% of transfected U87-MG human astrocytoma cells as early as 2 days after transfection. Our results are consistent with activation of apoptotic pathways following IsK-mediated increase in K+ efflux. These data are in line with numerous encouraging results for gene therapy-based approaches, including those targeted to glioblastoma (Curtin *et al.*, 2005). However, one of the largest challenges for gene therapy to become efficient is the need to selectively and efficiently infect/transfect all malignant cells and/or to evoke a strong bystander immune response effect directed at neighboring cells in order to eliminate the non-infected tumour cells (Su *et al.*, 2005). Obviously, much more scientific effort is still necessary to turn gene therapy into a commonly used medical treatment (Fischer, 2000). We therefore changed our gene therapy approach to more attractive and promising (in our opinion) immunotherapeutic strategies for brain tumours, which are currently emerging as highly potential clinical options.

Immunotherapy is a new, promising therapeutic approach that can specifically target tumour cells following administration of tumour-antigens together with immunostimulation (Hofman *et al.*, 2010; Vauleon *et al.* (2010), or by administration of immunostimulants alone, resulting in a boost of endogenous anti-tumour immune reactions. Interestingly, we found a strong therapeutic antitumour efficacy for the innate immune response modifier Resiquimod, even as a stand-alone treatment. Resiquimod is a nucleoside analog structurally related to Imiquimod (R-837), which is licensed by the Food and Drug Administration (FDA) in the United States as active, topical treatment for genital warts, and superficial basal cell carcinoma (Urosevic *et al.*, 2003) and which likely acts via the activation of Langerhans cell migration (Urosevic *et al.*, 2005). The precise mode of action of Resiquimod in our setting requires further investigations.

Resiquimod (R-848) is an immune response modifier, and has antiviral and antitumour activity (Urosevic *et al.*, 2003). It is used as a topical cream in the treatment of skin lesions such as those caused by herpes simplex virus and as an adjuvant to increase the effectiveness of various vaccines. It has several mechanisms of action, such as being an agonist for toll-like receptor 7 and 8 and an upregulator of the opioid growth factor (OGF) receptor (McLaughlin *et al.*, 1999).

In our rat model we administered Resiquimod by subcutaneous injections. Unfortunately, Resiquimod is not licensed by regulatory authorities for parenteral administration, likely because of observed adverse systemic reactions. Also, local immune deficiencies resulting from R-848 administration have been noted, associated with a rapid and almost complete depletion of leukocytes from the blood (Gunzer *et al.*, 2005).

Remarkably, our data demonstrate that immunotherapeutic parenteral treatment of established glioma tumours by Resiquimod, as defined in the protocol,

significantly improves anti-brain tumour immunity in a way that leads to immune memory. In parallel treatment groups, we also observed that high dose cyclophosphamide (CY) treatment, although initially effective as a chemotherapeutic agent, is deleterious to the maintenance of long-term antitumour immune memory. High-grade malignant gliomas are genetically unstable, heterogeneous and highly infiltrative, and all these characteristics lend glioma cells extremely resistant to conventional therapies. Immunosuppressive factors in the tumour microenvironment and effector immune cell suppressor populations may contribute to the overall immune suppression. Treg cells constitute a fairly low percentage (approximately 5 to 15%) of peripheral blood mononuclear cells (PBMC) and variable numbers have also been detected in a wide variety of tumours. The observation that glioma cells, unlike normal neurons and glia, express relatively abundant levels of HLA class I indicated that gliomas might be amenable to local adoptive immunotherapy with HLA-restricted alloreactive cytotoxic T lymphocytes and therefore there is a need for more comprehensive understanding of the factors involved in determining the sensitivity of glioma cells to adoptive T cell immunotherapy (Gomez and Kruse, 2006).

We found in different rat glioma models that a certain composition of antigens derived from syngeneic tumour cells and their lysates when therapeutically co-administered with allogeneic cells and their lysates, is able to confer anti-tumour immune responses and to cause tumour regression. For the syngeneic C6 model in Sprague Dawley (SD) rats therapeutic injections of allogeneic cells alone were sufficient to trigger tumour regression. This immunization approach may prove useful as an adjuvant therapy after resective surgery and standard radiation plus chemotherapy (temozolomide) (Stupp *et al.*, 2005) in future malignant glioma treatment protocols, or as a stand-alone therapeutic tumour vaccination following surgical resection of recurrent tumours where no second-line treatment is available.

Most tumour associated antigens (TAAs) are inherently poor immunogenic antigens and require an adjuvant to break immunological tolerance (Schijns and Lavelle, 2011). In the syngeneic CNS-1 Lewis rat glioma model, we found that in order to cause tumour regression, specific innate immune response stimulating substances were required as immunological adjuvants. In our hands BCG and IL-2 (chapter 6) as well as the Toll-Like receptor (TLR) 7/8 activator Resiquimod (chapter 4) showed potent activity.

Microglia/macrophages appear to be the predominant immune cell populations infiltrating gliomas (~1% of total cells). Although expressing substantial levels of Toll-like receptors (TLRs), these cells do not appear to be stimulated to produce pro-inflammatory cytokines (TNF-α, IL-β, or IL-6). *In vitro*, lipopolysaccharides could bind TLR-4, but could not induce microglia-mediated T-cell proliferation. Instead, there was a prominent population of regulatory CD4 T cells (CD4+CD25+FOXP3+) infiltrating the tumour. We conclude that while microglia cells present in glial tumours may have a few intact innate immune functions, including their capacity to be stimulated via TLRs, secrete cytokines, upregulate costimulatory molecules, and in turn activate antitumour effector T cells. However, this is not sufficient to initiate efficacious active anti-tumour immune responses. Furthermore, the presence of regulatory T cells may also contribute to the lack of effective immune activation against malignant human gliomas (Hussain *et al.*, 2006). Consequently, proper immune system activating immunostimulants are considered essential to evoke durable active anti-tumour immune reactions (Schijns and Lavelle, 2011).

Finally, we demonstrate that our prototype therapeutic vaccine, when co-delivered in a specific regimen together with the cytokine GM-CSF as immunological adjuvant, is able to arrest the progression of glioma tumour growth, when therapeutically administered following a low-dose cyclophosphamide regimen. GM-CSF is an attractive vaccine adjuvant because of its proven immune modulatory effects and low toxicity profile (Disis *et al.*, 1996). The safe pharmacological use of GM-CSF in patients is well-established, which makes it feasible for clinical use. As a result of these preclinical findings the use of GM-CSF has been included in the first clinical studies based on the prototype allogeneic plus autologous combination vaccine, that have been recently been submitted and approved for an Investigational New Drug application (IND).

Critical appraisal

Although our preclinical results are very encouraging they should be interpreted with caution. For example, glioma tumours transplanted subcutaneously can be easily monitored for growth, but the microenvironment of an intracranial tumour may be more complicated than the microenvironment of a transplanted tumour. For example, the intracranial tumour may have additional immune suppressing effects. Such immunotherapeutic effects on intracranial tumour growth are subject of our future investigation. In addition, the preclinical setting of our model may not exactly mimic the clinical situation, which involves standard surgery followed by radio- and chemotherapy.

However, the results of our preclinical investigations demonstrate the feasibility of active immunotherapy in glioblastoma. In general, it is difficult to compare our immunotherapeutic strategy with those of other published successful treatments. However, we have identified several very exciting immunotherapeutic strategies resulting in complete responses of glioma rejection. Especially encouraging is the demonstration of several novel therapeutic strategies, based on the use of particular innate immune system stimulating substances, including a TLR7/8 agonist. Among them the GM-CSF-adjuvant vaccine may translate into feasible application in patients.

8.2 Outlook of clinical strategies for immunotherapeutic glioblastoma treatment

Research in various rodent systems enabled us to gain insight into critical aspects of therapeutic intervention strategies against glial tumours (chapters 4, 5, 6, and 7). These investigations provide the pre-clinical basis for the development of a therapeutic glioblastoma vaccine for patients diagnosed with GBM. The exact immunological mechanisms underlying the observed anti-tumour immunity, for example innate and adaptive cell-mediated or humoral reactions, remain to be researched in more details and determined in ongoing and future studies. These first clinical data will be extremely informative for the safety of this new therapeutic medical product. However, from our preclinical studies we were able to reach conclusive data needed to justify our rationale for the composition and design of our in progress clinical evaluation of our innovative immunotherapeutic immunization strategy against GBM.

We further plan to extend this strategy—if clinically successful—to the solid tumours where effective strategies are lacking.

Recent promising clinical anti-glioma immunotherapies

Novel treatments for brain cancer (GBM) require a multimodular approach, which encompasses several mechanisms of action, including different modes of cell death and immune stimulation. Preferably, these novel therapies should work synergistically with the best treatment options currently available; i.e., surgery, radiotherapy and chemotherapy.

During the last few years, anti-cancer immunotherapy has significantly advanced. There are very encouraging results for immunobased therapies against lymphoma, leukemia, melanoma and renal cancer (Hudis *et al.*, 2007; Ananthnarayan *et al.*, 2008; Hodi *et al.*, 2010; Buonerba *et al.*, 2011). The methodology used is either through a simple vaccination of cells and radiated lysates of autologous cancer or through the injection of isolated lymphocytes in association with specific cytokines or using monoclonal antibodies. Any therapeutic approach using cells and aiming to replace, repair, increase or modify the biological activity of an impacted organ, is called cellular therapy. Our proposed approach is 'cellular immunotherapy', a form of cellular therapy utilizing the cells of the immune system.

Promising reports of active immunotherapy for glioblastoma patients have been described in literature. For example, an early clinical trial performed by Mahaley and co-workers (Mahaley *et al.*, 1983) showed favorable data. Each patient received subcutaneous inoculations with one of two human glioma tissue culture cell lines (D-54MG or U-251MG) monthly, with 500 micrograms of bacillus Calmette-Guérin cell wall (BCG-CW) being included with the first inoculation as an immunostimulant. Each patient also received levamisole, 2.5 mg/kg 3 days per week every other week. Radiotherapy and chemotherapy with BCNU, which were the chemotherapy standard of care at that time, begun after the first month of immunization. Patients who were inoculated with the U-251MG cell line have showed a longer survival time compared to those inoculated with the D-54MG cell line (p less than 0.0590) or compared to 58 historical cases of glioma patients treated with levamisole, radiation therapy, and chemotherapy alone (p less than 0.02). No evidence of allergic encephalomyelitis was noted clinically, nor was any gross or microscopic evidence of such pathology obtained upon autopsy of three of these patients.

However, the majority of immunotherapy clinical trials for GBM still remained in the Phase I or I/II stage. No phase III clinical trials have been completed at this time. Another prominent example is DCVax®-Brain, an immunostimulant cancer vaccine, based on experimental autologous cellular therapy, produced by the Northwest Biotherapeutics, Inc. (Liau *et al.*, 2005). DCVax-Brain vaccine is manufactured using a patient's dendritic cells loaded with a tumour cell lysate prepared from surgically resected tumour tissue. The clinical data from a cohort of 141 newly-diagnosed glioblastoma patients treated in a Phase II study is still being collected, however, assessments from their Phase I trials suggest overall safety, with delayed time to disease recurrence and increased survival, especially in glioblastoma patients with stable disease at entry [see

http://www.nwbio.com/clinical_dcvax_brain.php]. Indeed, for those patients treated in the Phase I trials, the company is reporting that the median survival is 33.8 months, with 9 of 19 patients still alive at 8-82 months from initial surgery.

The company ImmunoCellular Therapeutics, Ltd (http://www.imuc.com/) has supported a Phase I study using the product "ICT-107" for glioblastoma. This also a dendritic cell-based vaccine loaded with synthetic peptide antigens that targets multiple glioma associated antigens (Yu *et al.*, 2004; Wheeler and Black , 2009). In a June 2010 IMUC company report it was stated that the median overall survival had not yet been reached at the 26.4 months analysis point, with 12 out of 16 treated newly diagnosed patients alive (http://www.imuc.com/pdf/Brain-Cancer-Vaccine-Looks-Promising-in-Small-Trial-1.pdf#zoom=100). See also Hu *et al.*, 2011, and Yu *et al.*, 2011)

Other clinical trials involving immunotherapy for brain gliomas can be found at www.clinicaltrials.gov by refining search terms using key words at that site.

8.3 Novel immunotherapeutic approaches presented on this thesis

Our preclinical data have been performed with the aim to define the most appropriate composition of our antigens and the most favorable method of immunization. The details of our in-progress clinical strategy for active immunotherapy against GBM are described in detail below.

Tumour associated antigens (TAA)

Upon recognition and rejection of the allogeneic tumour preparation, the immune system of the patient will develop an immune response by recognizing cancer cell associated-proteins, including the so-called *tumour associated antigen (TAA)*, and consequently reject the patient's own tumour. These tumour-associated antigens are antigens expressed by tumour cells and not/less (or very occasionally) by the normal cells (Lewis & Houghton, 1995). They can result from single-point mutations within open reading frames of the genome and giving rise to unique antigens expressed by the tumour cells of a single patient. They represent the genuine tumour-specific antigens. Moreover, tumours may show an abnormal expression of antigens that are normally associated with development and differentiation or with viral products. These abnormally expressed antigens are not unique to a single tumour but are found in many tumour types and across different patients. They are also named tumour-associated antigens (TAA).

In our strategy, the target antigens are not well defined (and they don't need to be). But this multi-faceted approach increases the chances to induce a strong and broad immune response to multiple tumour antigens. Another advantage is that in the whole tumour antigen preparation, TAA are presented in combination with

both major histocompatibility complex (MHC) class I and II antigens (Kakimi *et al.*, 2009). These extracts and lysates are directly recognized as a source of antigen acting as an allograft, in order to trigger a strong immune rejection, and boosting a specific immunization against TAA.

Tumour lysates and cells

Tumours can differ greatly from each other on a genetic level (Hanahan and Weinberg, 2000). Hence, a typical, single biopsy reflects only part of a tumours molecular signature (Gerlinger et l., 2012). Since high-grade gliomas (HGGs), including GBM, are a heterogeneous group of primary brain tumours, we prefer poly-antigenic immunization over the monoantigenic approach. We are convinced that achieving clinically relevant antitumour immunity requires simultaneous stimulation of immune reactions against a wide range of tumour-associated antigens. Therefore, instead of a single-agent-targeted immunotherapy based on just one or a few proteins or peptide antigens, produced synthetically or by recombinant heterologous expression, we prefer a multi-modular antigen approach based on syngeneic lysates and cells, providing a "personalized" set of perfectly matching target antigens of the patient, mixed with lysates and cells from allogeneic tumours. This mixture of antigens is not defined, but expected to overlap to a large degree with the specific tumour antigens of each and every GBM patient. This strategy enables triggering of an immune response against a broad array of tumour antigens, having the ability to replicate not only the cellular heterogeneity at the time of treatment initiation, but also to predict tumour TAA variation at the time of possible recurrence. In addition, the tumour lysate is not human leukocyte antigen (HLA)-restricted and is available in most cases for each patient undergoing surgery, allowing treatment irrespective of the patient's HLA type. The inclusion of alloantigens will also trigger allo-immune reactivity, a classical allograft-directed immune responses typical for non-matching major histocompatibility between graft cells and the host.

The antigens are prepared freshly from the surgically removed tumour tissue from the patients and the tumour bank-derived allogeneic tissue from the GBM tumour donors. This prevents selection of certain cell types by *in vitro* culture and allows the introduction of extracellular proteins as immunological targets.

Allogeneic antigens

In the case of an allogeneic immune response, an additional phenomenon occurs, the allorecognition (reviewed in Fabre, 2001; also in Gervais, 2009). Allorecognition is the mechanism responsible for allograft rejection. It is well known that unprimed T lymphocytes from one individual react with unusual strength against MHC antigens of other members of the same species, a phenomenon named "allo-agression". This process is based on a unique aspect of T-cell biology, the direct T-cell allorecognition. It reflects the capacity of T lymphocytes to recognize intact allogeneic MHC molecules on the surface of foreign cells. It is a powerful mechanism of T-cell activation, since about 1-10% of an individual's T lymphocytes will respond to the foreign MHC antigens of another individual. By comparison, the frequencies of T-cell

precursors for “normal” environmental antigen (e.g. a virus protein) are of the order of 1/10.000 or 1/100.000.

Allorecognition

The use of allogeneic immunization in cancer therapy has several important mechanistic implications. These include:

1) Cross-reactivity: The vast number of T-cells reacting with allogeneic MHC molecules will also have a specificity for normal and environmental antigens presented in association with self MHC molecules, among which self TAAs. Therefore, T-cells activated by direct recognition of the allogeneic MHC class I and II molecules, may also cross-react with TAAs presented within self MHC molecules on the patients'-own tumour cells.

2) CD8 and CD4-positive T cells are both implicated in the allorecognition phenomenon. CD4 T cells activated by direct recognition of MHC class II molecules during immunization may act as providers of T helper activity, triggering and sustaining a TAA specific immune response against the patient's own tumour cells. In organ transplant rejection, this powerful activation of T helper cells is responsible for an early antibody response against the transplant. This is particularly important in tumour therapy, as it could theoretically bypass the need for presentation of TAA within self MHC class II molecules to activate T helper cells, and could induce a powerful cellular and humoral immune reaction against tumour cells displaying TAA within MHC class I antigens.

3) In keeping with the above, it is worth mentioning that CD4 T-cell responses are not only necessary but may also be sufficient for allograft destruction. Mitchinson *et al.* (1987) have studied the requirements of T-helpers for the development of CD8 T-cells directed against MHC antigens. They showed that co-expression of helper and cytotoxic determinants by the same antigen presenting cell represents the most efficient mode of T-helper cell activation.

4) Allogeneic responses have the potential to generate a milieu rich in cytokines sustaining both innate immune reaction and promoting T-cell response by providing T-cell costimulatory ligands. For example, allogeneic HLA molecules have been shown to act as ligands for KIR and consequently activate NK cells that exert cytolytic activity against mismatched tumour cells, and thereby provide additional help to bystander immune reactions by secreting high levels of cytokines (Chewning *et al.*, 2007). Also, CD1d-dependent NKT cells have also been described in the modulation of an alloreaction as helper cells for concurrent T cell responses (Patterson *et al.*, 2005).

In summary, the rationale for the use of our allogeneic tumour tissue antigens in our immunization approach is briefly listed below:

- The induction of a combined immune reaction induced by the combination of both lysates of tumour cells and of entire cells prepared from autologous and allogeneic origin. This combination allows inducing of an immune reaction against a variety of whole cell and lysate antigens that are present at the different development stages of tumors within

the same type of glioma tumour, instead of an immune reaction against antigens limited only to antigens presented by cells or within lysates.

The immunological phenomena resemble a "graft rejection" due to the presentation of allogeneic cells, which are HLA-incompatible. In addition, autologous cells become more immunogenic as a result of chemical marking (haptenization). This mixture of antigens has the advantage that it exposes the patient's immune system to a larger variety of tumour antigens, which increases the chance to trigger one of the essential immune effector cell populations, such as for example a cytotoxic T population. In a recent *in vitro* study using allogeneic dendritic cells, Gervais *et al.* (2009) showed a reduction of the Treg population in the mixed lymphocyte reaction following priming with selected allogeneic DCs and revealed an additional mechanism for breaking tolerance and sustaining the anti-tumour immune reaction.

- The presence of the totality of the "original" tumour cells. Indeed, contrary to what has been done until now using tumour cell products, our isolated tumour cells are not cultured, but directly filtered and conditioned for vaccine storage. This production aspect is particularly important, because in *ex vivo* tumour cultures only a percentage (about 20%) of the tumour cells survive the switch towards a culture medium environment. Hence, the culture procedure reduces considerably the genetic and immunologic variety present in freshly isolated tumour cells. So, avoidance of an *in vitro* cell culture step facilitates the broadness of tumour target antigens in the final preparation, and, hence, the broadness of induced immune power against the target tumour.

- The availability of a critical quantity of product material. In contrast to immunizations based on cell lines only, the autologous/allogeneic biopsy-based immunizations depends on the tumour size isolated during surgery from the patient and the donor. By using allogeneic donor tumour tissue, a theoretical limitation in product material is partially circumvented, since a large part of the finished product can be obtained from our glioma tumour tissue bank.

Bystander immune activation

In the patient, "immunogenic" TAA are thought to be derived from tumour cells by active secretion, cellular necrosis or apoptosis. Tumour necrosis can be triggered by natural killer cells (NK cells), which are activated by cells presenting "non-self" motives, in our case, TAA associated with abnormal class I molecules or not associated with any MHC class I molecules. These released TAAs are engulfed by circulating, short lived, dendritic cells, which carry them to draining lymph nodes. The complex TAA-HLA Class I or II molecules are also phagocytosed and processed by resident, long-lived, DCs which can present these TAA-HLA class I –II complexes to CD8-positive cytolytic lymphocytes (class I restricted) and to CD4-positive T lymphocytes (class II restricted), which are able to activate in turn CD8 cells and B cell for the production of specific CTLs and antibodies, respectively, and provide the long term memory specific for the TAA. It

is important to remember that TAA have to be co-expressed with specific HLA alleles in order to be presented on the membrane of APCs: they are HLA class I and II restricted, and activated CD8 and CD4 cells, respectively.

Collectively, it is apparent that allorecognition can be used as a potent mechanism to stimulate a TAA-specific immune reaction against one patient's tumour cells. Hence, it is proposed that the intradermal injection of a preparation composed of a mix of 'non-self and self' haptenized and irradiated cell lysates, as well as 'non-self and self' haptenized and irradiated cells, can break the self anti-tumour immune tolerance, as these cells/lysates are likely to contain antigenic determinants in common with those expressed by the patients tumour.

Intradermal immunization may have the best efficacy

Applying vaccines to the upper layers of the skin stimulates a powerful immune system response, which is stronger than the traditional approach of injecting a vaccine into muscle, according to a growing body of research (Kupper and Fulbrigge, 2004). Immune system cells stationed in the skin, such as resident innate immune cells (Langerhans cells (LCs), dermal dendritic cells (DCs) and mast cells), recognize the antigen components in the context of dander and stranger signals and stimulate downstream activation cascades. Activated Langerhans cells and dermal DCs are stimulated to mature and emigrate from the tissue to the draining lymph node, carrying antigen for presentation to naive and memory T cells. Moreover, recently important immune system cells called "resident memory T cells" have been found in the skin (Jiang *et al.*, 2012).

Two different regimens for "conditioning" the inoculation site have been compared: GM-CSF was administered either intradermally (ID) or subcutaneously (SC) for five injections while a foreign antigen, tetanus toxoid (TT), was given at the beginning or the end of the immunization cycle in both the ID and the SC cohort. ID immunization was more effective than SC at eliciting TT specific immunity. In addition, GM-CSF ID, administered as a single dose with the antigen, compared favourably with the complete Freund's adjuvant (CFA) and alum in eliciting TT specific antibody and cellular immunity (Disis *et al.*, 1996).

In summary, we are proposing a personalized-medicine strategy, using freshly prepared, non-selected, patient-derived autologous tumour tissue (a source of multi-antigenic, antigens able to evoke a broad, non-HLA-restricted immune reaction) administered together with allogeneic tumour antigens (which trigger an allogeneic immune response). Both components will be delivered through the skin in order to evoke a stronger immune reaction, as suggested by our preclinical data.

Safety

Importantly, we now demonstrate that this product is safe and protective in early and advanced malignant glioblastomas in rats, as observed in pre-clinical studies and as published for example in Stathopoulos *et al.*, 2008 (Chapter 5).

Early clinical data

The Food and Drug Administration (FDA), Center for Biologicals Evaluation and Research (CBER), USA, has recently approved (March 7th, 2012) our Single Patient Investigational New Drug application (IND). The IND allowed the administration of our immunotherapy to a patient with GBM, grade IV, with a progressive tumour after second-line treatment with bevacizumab (Avastin) treated at University of California, Irvine (UCI).

In parallel, an application has been filed in Belgium to use our immunotherapy treatment under hospital exemption, engineered as defined by the CE N° 1394/2007 and as approved by EMEA. According to the description of the rule, for a drug prepared non-routinely, under controlled conditions of manufacturing (the sterile room of the Tissue bank), and used in the same European country (Belgium), in a clinical environment under the responsibility of a medical doctor following medical prescription, specifically for the patient (using autologous and immunostimulating allogeneic tissue).

Summary of clinical results in May 2012

Methods: Eligible patients who were over 18 years of age and had histologically confirmed WHO grade IV malignant glioma were treated under IRB-approved protocols. Only patients that have failed the standard of care regimens were treated. Primary end points are toxicity and 6-month progression-free survival (PFS) while secondary endpoints are median overall survival (OS) and radiographic response.

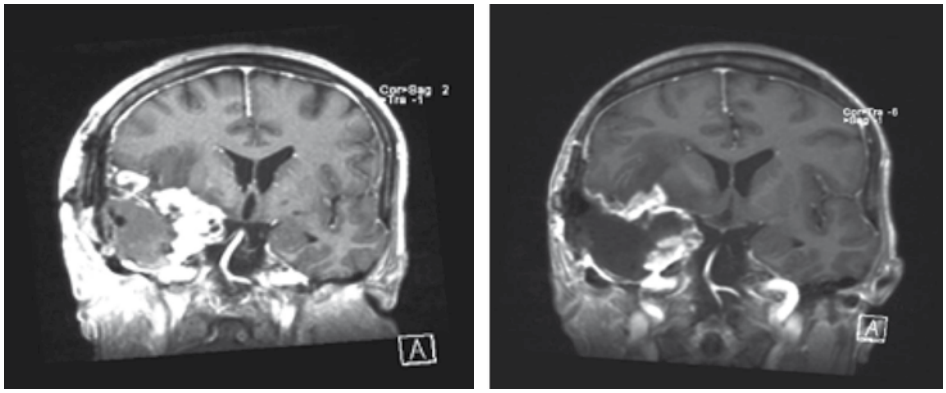
Results: To date, there were a total of 5 patients enrolled, three on a phase 1 protocol at South Cliniques of Luxembourg-Belgium, one on compassionate/ single patient IND protocol at UC Irvine Medical Center, and one at University of Southern California (USC). Median age was 52, with 2/5 female patients. The average KPS was 70 (50-80). Four patients are still alive, they are clinically and radiologically stable. One patient shows after three cycles of vaccination spectacular tumour regression which is observed after an average of 8 (4-12) weeks. One patient died of intra-cerebral hemorrhage may be due of the Bevacizumab, 4 weeks after the first treatment. The toxicities were mild: 2/5 patients developing grade 2 headaches and 5/5 grade 2 local erythema at the injection site.

Conclusion: Based on the most recent studies, the 6 months progression free survival rate is 16% for the patients with recurrent GBM who failed Bevacizumab. Our preliminary data suggest that ERC-1671 might be superior to active treatment with other chemotherapy agents in that clinical setting. Updated results will be presented at the SNO 2012 meeting.

Case report:

A woman presented with headache and nausea, which proved a right-sided temporal brain tumour as confirmed by brain magnetic resonance imaging (MRI), without any other neurological deficit. On 1st October 2010 she underwent primary brain surgery (right front-temporal craniotomy and total tumour resection) and pathological examination confirmed the GBM diagnosis.

From November 2010 to February 2012 – oncological treatment: Start-up with Temodal (TMZ) concomitant with radiotherapy (concomitant phase): TMZ administered orally, in a dosage of 125 mg/d daily for 42 days, in association with focal radiotherapy. The patient handles the first phase well, the treatment continues. Monotherapy phase with Temodal – 6 cycles: (four weeks after finishing the concomitant treatment phase with TMZ + RT). In February 2012 the patient presents a large tumour recurrence, at the original right temporal site with an infiltrative hypercaptating formation, associated with an important perilesional edema, exercising a mass effect on the right lateral ventricle. The tumour recurrence demonstrates that previous standard treatments had failed. From February 6th to February 24th 2012: hospitalized for headaches and speech impairment of Wernicke motor aphasia type, without any other neurological deficit, in connection with a right cerebral temporal tumour. After a secondary, subtotal surgical tumour resection, she was treated by our immune intervention approach, under hospital exemption, engineered as defined by the CE N° 1394/2007 and as approved by EMEA. Secondary surgery occurred on 10 February 2012 and MRI scans were made on February 20th, 2012 (see Figure 8.1, left panel), revealing that the tumour size after surgery was approximately 62,5 cm^3. Immunotherapeutic ATMP treatment was started on March 1st, 2012. According to published literature her expectancy of life is 0.4-1.1 month for recurrent Glioblastoma and prognosis without further treatment; Park *et al.*, 2010).



Day 8 before vaccination (Feb 20, 2012)

15 days post vaccination (= March 1) (March 15)

Figure 8.1. MRI scans 8 days before (left) and 15 days after the start of the vaccination schedule (right).

It proved that the tumour showed regression of about 50 % after immunotherapy. The tumour has since been shrinking. See also follow-up MRI scans of Figure 8.2 and 8.3, and another patient example in figure 8.4 after one treatment cycle.

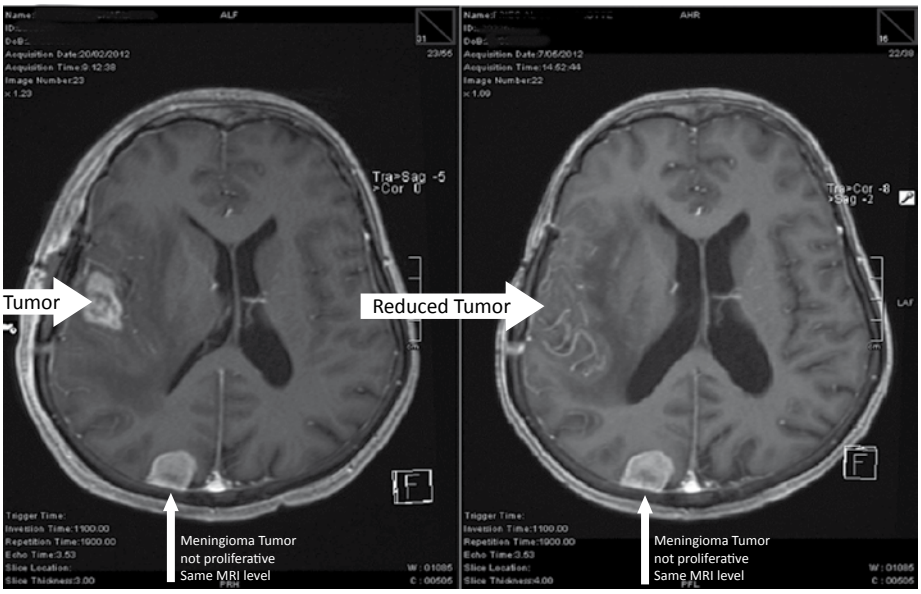


Figure 8.2. MRI scans of 20Feb2012 (left) versus 07May2012 (right), following 3 treatment cycles (coronal view)

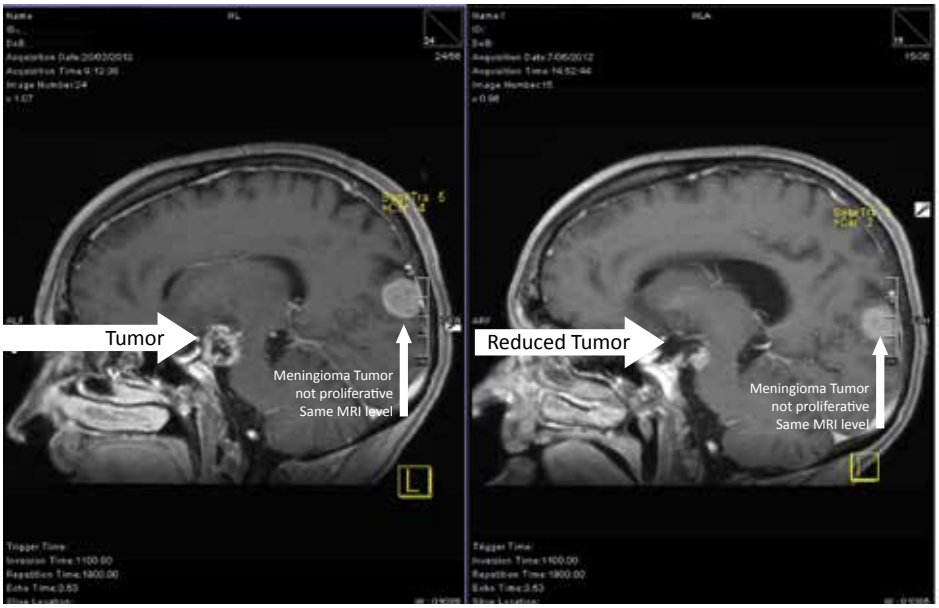


Figure 8.3. MRI scans of 20Feb2012 (left) versus 07May2012 (right), following 3 treatment cycles (MRI scan, axial view)

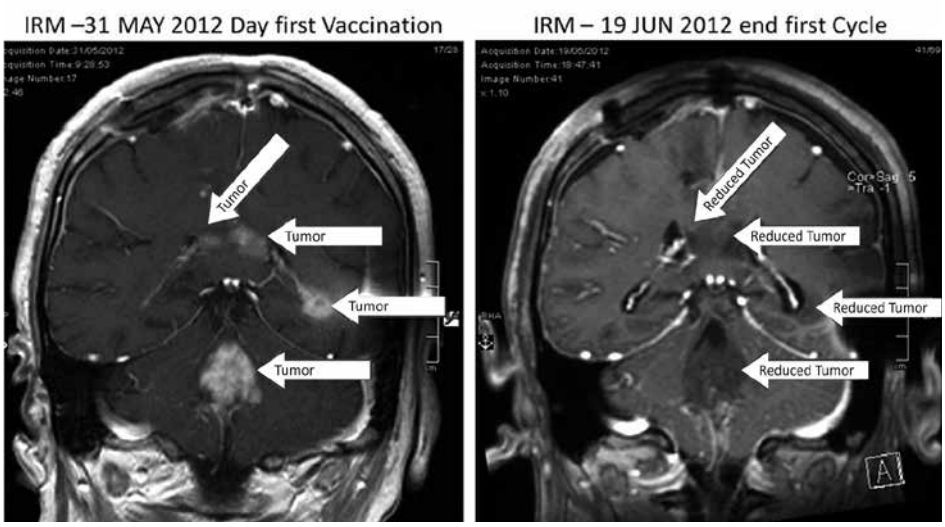


Figure 8.4. MRI scans of another patient made on June 19, 2012 (left) versus April 31, 07May2012 (right), following 1 treatment cycle (MRI scan, axial view).

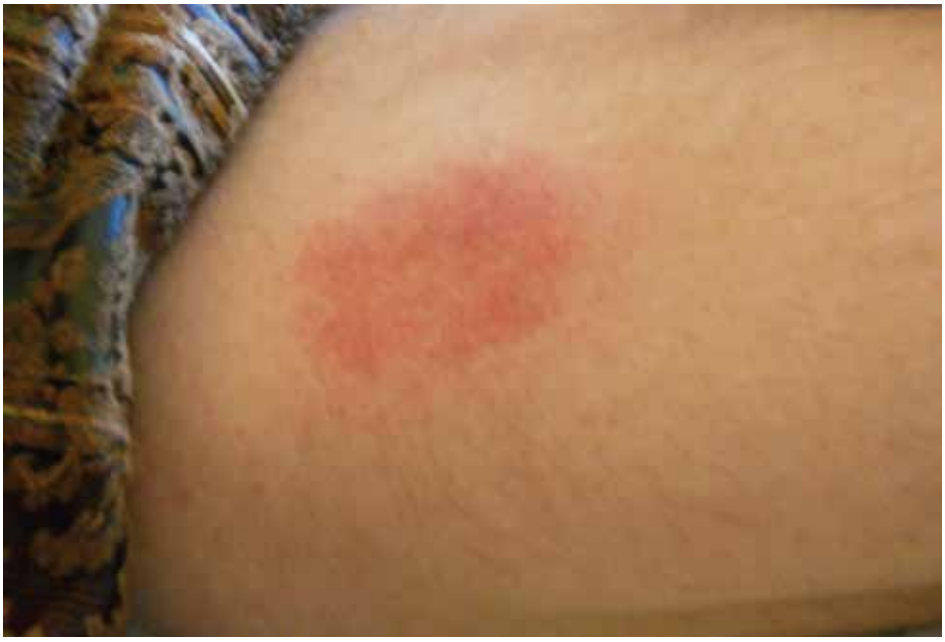


Figure 8.5. Typical delayed type hypersensitivity reaction at the injection site.

Safety

The treatment related adverse effects were well-tolerated. They consisted of erythema, induration and swelling of the inoculation sites (Figure 8.5).

In conclusion

Immunotherapy is a promising tool to treat cancer. Similar to existing situations for difficult indications like HIV, TB, malaria, a multi-modular strategic treatment approach is required. Especially a mixture of allogeneic and autologous antigens is required to provide a spectrum of antigens to harness T cell immunity preventing tumour escape. For boosting immune responses it is advisable to include a rationally chosen adjuvant/immunostimulant. The rat glioblastoma model has proven to be very useful to select and establish a proof-of-concept for a highly efficacious immunotherapeutic strategy in patients.

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Annex:

Selective Therapeutic Targeting of Malignant Glioma Stem Cells

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Introduction

Glioblastoma Multiforme (GMB) is the most common primary brain tumour with an incidence of approximately 10,000 new cases annually in the United States[1]. Despite gross total resection, and treatment with radiation and chemotherapy - temozolomide followed by bevacizumab at the time of recurrence -, the median survival has improved only to 18 months in the last decade [1,2]. Once the GBM recur, they are universally fatal, with survival less than a few weeks, despite aggressive treatment [3].

The main cause for GBM recurrence is not precisely known, but recent research suggest that it involves the development (and/or persistence) of subpopulations of tumour cells with resistance to treatment [3]. GBM is characterized by the presence of multiple glioma cell populations [4,5] organized in a stem cell hierarchical order with different stages of differentiation [6]. The cell population situated at the apex of the cellular hierarchy - the glioma stem-like cells (GSC) have been the proposed reason for the recurrence of malignant gliomas after treatment [6-8].

It is hypothesized that glioma cancer stem cells (GSC) can arise either from neural stem or progenitor cells [9] after the accumulation of mutations in oncogenic pathways such as NF1, p53 and PTEN [10] (see Figure A.1). They have the ability to self-renew and to initiate brain tumours[9]. In addition, they express neural stem cell markers and are multipotent [9]. They constitute only a minor subpopulation of the entire tumour. The progeny of GSC are progenitor like cells and differentiated cells, which divide rapidly, and form the bulk of the gliomas[9]. Several surface markers associated

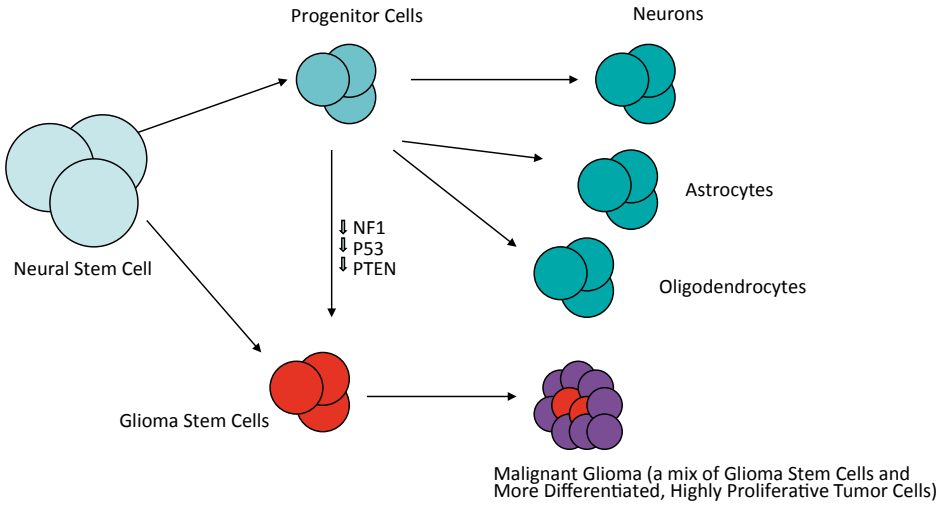

Figure A.1: ***Glioma Stem Cell Hypothesis: Malignant Transformation of Neural Stem/Progenitor Cells***. Under certain conditions such as acquiring a constellation of oncogenic mutations in various pathways (NF1, PTEN or p53), neural stem cells can become glioma stem cells. NF1=Neurofibromin 1, PTEN=Phosphatase and tensin homolog.

with GSC are known [9]. These markers, which include CD133/prominin, Musashi homolog, nestin and A2B5 [3,9], are useful for the isolation and enrichment of GSC.

Previous experiments indicate that "non-stem" cells can produce tumours in orthotopic xenograft models only when implanted in very high numbers [11]. These experimental tumours lack the classical malignant glioma behaviors—namely, an invasive phenotype, vascular proliferation-, and a limited capacity of tumour initiation [11]. However, orthotopic implantation of a small number of stem-like cells in appropriate animal models generate aggressive growth of tumours [12,13], bringing further support to the stem cell hypothesis for glioma formation [14].

In this review we will first summarize the current treatment modalities used for glioblastoma treatments and evaluate their effectiveness in controlling and eradicating the GCS. We will review the mechanisms involved in GSC-driven tumour proliferation, invasion and resistance to treatment and consider potential therapeutic targets that might hinder or block these oncogenic pathways. Finally, we will discuss potential avenues to target GSC in order to decrease the tumour burden and potentially provide a cure for this type of cancer.

A. Current Therapeutic Modalities: Glioma stem cells resist the current, first-line treatments for malignant gliomas - radiation and temozolomide (TMZ).

The standard of care for malignant gliomas after resection is temozolomide (TMZ) plus radiation therapy. A phase III clinical study showed a survival benefit by adding TMZ to postoperative radiation treatment [2,15]. The upregulation of O^6-methylguanine-DNA methyltransferase (MGMT) expression counteracts the effects of alkylating agents [16], such as TMZ. *In vivo*, the expression of MGMT correlated well with the resistance of malignant gliomas to TMZ treatment [17]. Ultimately, almost all the patients relapsed after treatment with radiation and temozolomide, with a progression free survival of zero at 5 years [18].

The main mechanism through which radiation damages and kills glioma cells is the induction of breakage of the hydrogen bonds within the DNA strands, altering the base pairs, inducing substitutes, destruction of sugars and forming dimers. However, GSC are resistant to radiation [13]. The mechanisms involved in this radio-resistance are the following:

1. Selective cellular growth arrest, or quiescence, is described as the primary means by which the GSC evade the radiation and TMZ chemotherapy induced cellular damage [9,19]. During quiescence, GSC are maintained in growth arrest by various cellular processes and signalling pathways. Once the radiation and TMZ chemotherapy is completed, the arrested GSC can again become actively proliferating leading to fatal tumour recurrences [7,13,20,21].

2. Activation of checkpoint proteins is a major mechanism for radio-resistance of GBM. The most common checkpoint proteins overexpressed in GSC are ChK1 and ChK2 kinases [13]. These proteins are also expressed in high quantities in tumours isolated from population of cells enriched by radiation, such as CD133+ GSC populations [13]. Early analysis of tumour samples found that patients who had a high percentage of CD133+ GSC had a worse outcome than those patients with a lower percentage of CD133+ GSC [13]. Since the CD133+ GSC population was enriched after radiation suggested activation of DNA repair machinery in these cells. Further investigations showed that CD133+ cells were also able to repair the damage to their DNA more effectively than the CD133- GCS [13]. Treating CD133+ GSC with a specific ChK1 and ChK2 kinase inhibitors sensitized them to radiotherapy, suggesting that one of the main pathways for resistance to radiation therapy was the activation of the DNA damage checkpoint response in GSC [13].

Temozolomide (TMZ) is an alkylating agent that methylates the DNA thereby inhibiting DNA replication and cell proliferation. The GSC are resistant to TMZ, via multiple mechanisms of resistance [8].

1. GSC have higher levels of expression of O^6 – methyl – guanine – DNA – methyltransferase (MGMT) [14] . MGMT is a DNA repair protein which reverses the alkylation in the O^6 position of guanine and thus compensates for the DNA methylation effects [16]. *In vivo*, the expression of MGMT correlates well with the resistance of malignant gliomas to TMZ treatment [17]. Bleau *et al* showed that in a mouse model of glioma, treatment with TMZ increases the population of GSC [14] . However, GSC with normal levels of MGMT were resistant to TMZ [22] while only the MGMT negative GSC were killed by TMZ [23]. In the clinical setting, the patients who had methylation of the MGMT promoter – which leads to silencing of the promoter and prevents gene expression - benefited with the addition of TMZ to their treatment [15]. Chen *et al* showed that a restricted cell population propagates glioblastoma growth after TMZ chemotherapy [8]. A relatively quiescent GCS population was identified after TMZ chemotherapy, that subsequently produced the highly proliferative cells [8].

2. Another mechanism of resistance to TMZ treatments is expression of multiple drug transporting proteins including ABC (ATP binding cassette) transporters which expel out of the cell the chemotherapeutics. Hirsmachmann –Jax *et al* showed that a side population (SP) of cancer stem cells isolated from tumours and identified by flow cytometry contains these transporters and resists cytotoxic drug treatment. They also showed that ABCG2 and ABCA3 transporters were increased in GCS [24]. We showed that GSC have 10 times higher levels of ABCG2 than NSC [3]. Multidrug resistance 1 (MDR1) protein was also over-expressed in TMZ resistant CD133+ GSC [25]. CD133 was initially described as a marker which resisted the uptake of fluorescent markers, which coincidentally resemble chemotherapeutic drugs [26,27].

3. The evasion of the cell-death pathway is another important mechanism of resistance developed by GSC. By losing various proteins and overexpression of other proteins, GSC can modulate their response to chemotherapy and select the most resistant phenotype [9]. One mechanism of avoidance of cell-death pathways is the deletion of phosphatase and tensin homolog (PTEN) in glioma stem cells.[14,28] PTEN suppresses

the Akt phosphorylation in this pathway, by reversing the PI3K phosphorylation, thus inhibiting cell proliferation [28]. Therefore, loss of PTEN leads to uncontrolled activation of Akt and tumour growth [14,28]. Bleau *et al* showed treatment with TMZ selects for GSC that lack the PTEN gene [14]. This loss leads to activation of Akt, resistance to apoptosis, tumour progression, and poor prognosis for the patient.

4. Several other mechanisms also contribute to the resistance to TMZ treatment. The apoptosis pathway is heavily exploited by GCS [29]. Anti-apoptotic genes, such as Bcl-2, were found to have higher expression in the GSC population which was resistant to TMZ [29]. The addition of XIAP inhibitors increases sensitivity to chemotherapy of the previously chemotherapy resistant GSC [30], thus suggesting that activation of antiapoptotic factors such as XIAPs, are involved in resistance of stem cells to TMZ chemotherapy.

5. Constitutive activation of the Notch pathway also increases the oncogenic potential of these cells and maintains their stem cell status [31]. *Wang et al* showed that blocking Notch pathway leads to increased sensitivity of GSC to radiation [32]. By upregulating PI3K/AKT levels and increasing the levels of Bcl-2 family proteins, the Notch pathway produces radioresistance in GSC. The knockdown Notch models increase the sensitivity of GSC to radiation [32].

6. Insulin like growth factor binding protein 2 (IGFBP2) can mediate the activation of AKT pathway leading to resistance to radiation and conventional chemotherapy [33]. IGFBP2 upregulates metalloproteinase-2 and CD24, which increases the ability of GSC to invade adjacent tissues [33]. *In vitro* inhibition of IGFBP2 in GSC lead to decreased AKT activation, increased GSC sensitivity to radiation and chemotherapy, as well as decreased stem cell gene expression [34].

B. Therapeutic targets that may hinder or block GSC-driven tumour proliferation, invasion and resistance to treatment.

1. Tumour angiogenesis and glioblastoma stem cells

GSC induce vascular endothelial growth factor (VEGF) over-expression which increases angiogenesis, leads to new formation of blood vessels to sustain tumour growth and therefore increases blood vessel density [7,9,35] (Figure A.2). Vascularization plays a significant role in the providing nutrition and oxygen for gliomas [9].

Bevacizumab (Avastin) is a humanized monoclonal antibody, that binds and inhibits the activity of VEGF both *in vitro* and *in vivo* [36,37]. Several groups showed that anti-VEGF therapy specifically blocked GSC pro-angiogenic effects [9]. Bevacizumab has been tested in clinical trials for GBM patients, and was approved by the FDA for patients who experienced first or second recurrence of GBM [38].

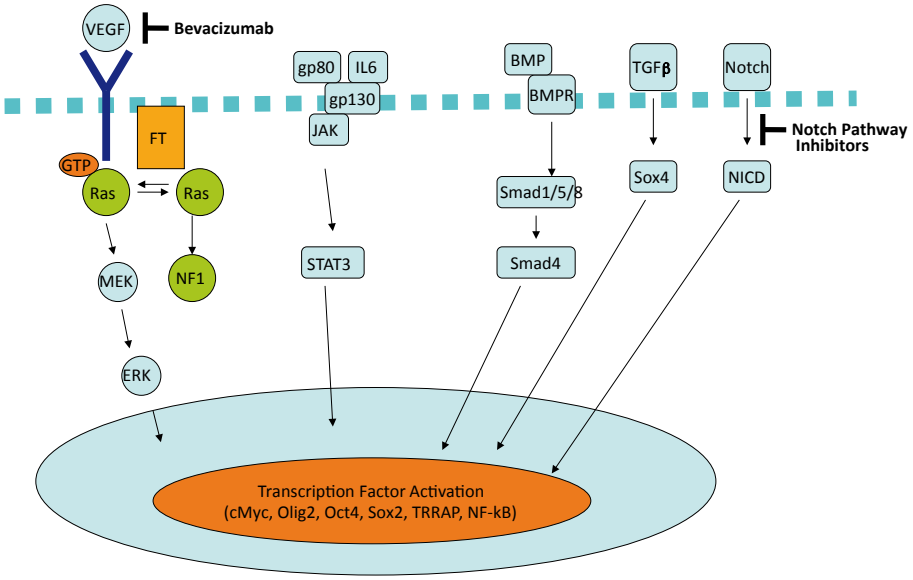

Figure 2: ***Signalling Mechanisms Important in Glioma Stem Cells and Potential Therapeutic Targeting (Part 1).*** Multiple stem cell and oncogenic pathways are involved in GSC-driven tumour proliferation, invasion and resistance to treatment. The most actively targeted by therapeutic agents are angiogenesis (VEGF inhibition) and differentiation (Notch inhibition). VEGF=Vascular endothelial growth factor, FT=Farnesyltransferas, RAS=Rat sarcoma gene family, MEK=Mitogen-activated protein kinase, ERK=Extracellular regulated kinase, gp60/80=60 kD/80 kD glycoprotein, JAK=Janus kinase, STAT3=Signal transducer and activator of transcription 3, BMP=Bone morphogenetic proteins, SMAD=Homolog of both the Drosophila protein, mothers against decapentaplegic (MAD) and the Caenorhabditis elegans protein SMA (small body size), TGFβ=Transforming growth factor beta, Sox=Sry-related HMG box, NICD=Intracellular domain of Notch, cMYC=Myelocytomatosis viral oncogene, Olig2/Olig4=Oligodendrocyte lineage transcription factor 2/4, TRRAP=Transformation/transcription domain-associated protein, NF-κB= nuclear factor kappa-light-chain-enhancer of activated B cells.

VEGF expression is controlled by the hypoxia induced factors (HIFs). Under hypoxic conditions both GSC and non-stem cells gliomas express VEGF, but the expression was higher in the GSC group [39,40]. Li *et al* found the HIF-1α and HIF-2α separately controlled VEGF expression in GSC [39]. Hypoxic conditions also select for the GSC fraction of the tumour cells and can also induce expression of the certain stem cell markers [37,41,42].

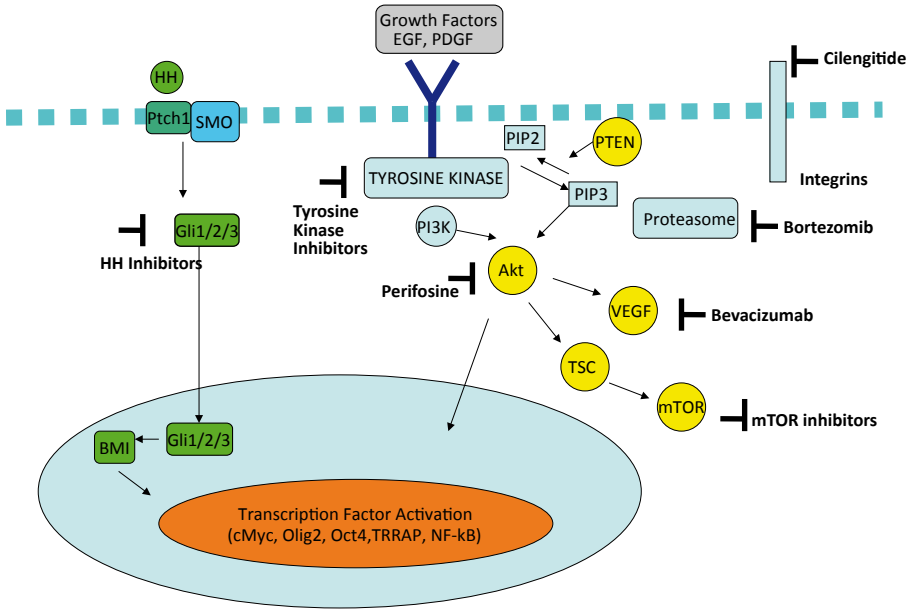

Figure 3: ***Signalling Mechanisms Important in Glioma Stem Cells and Potential Therapeutic Targeting (Part 2).*** Aberrant Growth Factors Expression, Adhesion Molecules Modulation as well as Protein Degradation and Differentiation Pathways are involved in GSC-driven tumour proliferation, invasion and resistance to treatment. The most actively targeted by therapeutic agents are aberrant growth factor receptor expression (EGF, PDGF, VEGF), adhesion (integrins), protein degradation (proteasome inhibitors) and differentiation (Hedgehog inhibitors). HH= Hedgehog, Ptch1=Patched homolog 1, SMO=Smoothened, Gli1/2/3=Glioma protein 1/2/3, EGF=Epidermal growth factor, PDGF=Platelet-derived growth factor, PI3K= Phosphatidylinositol 3-kinase, PIP2/3=Plasma membrane aquaporin 2/3, PTEN=Phosphatase and tensin homolog, Akt=Protein Kinase B (PKB), VEGF= Vascular endothelial growth factor, TSC=Tuberous sclerosis complex 1 gene, mTOR=Mammalian target of rapamycin cMYC=Myelocytomatosis viral oncogene, Olig2/Olig4=Oligodendrocyte lineage transcription factor 2/4, TRRAP=Transformation/transcription domain-associated protein, NF-κB=Nuclear factor kappa-light-chain-enhancer of activated B cells.

2. Hypoxia inducible factor HIF-1α maintains the tumourigenic potential of gliomas stem cells and increases treatment resistance

Hypoxia is common in tumour growth, and for many years it was believed to inhibit tumour growth. However, recent studies indicate that hypoxia actually contributes to tumour growth and proliferation. In malignant gliomas, hypoxia was found to promote angiogenesis, tumour growth and radioresistance [43]. Hypoxic niches play an essential role in the maintenance of GSC [39,40,43]. These "hypoxic niches" were also involved in the maintenance of normal stem cells [9]. In embryonic stem cells, hypoxia maintains the self-renewal potential and prevents the differentiation of neural stem cells. Yoshida *et al* demonstrated that hypoxia enhanced production of induced pluripotent stem cells (iPSC) [44]. GBM frequently display numerous GSC around the areas of necrosis [39]. CD133 is also a marker for hypoxic stress [45]. This may be the reason why some GBM GSC might be selected for in the hypoxic regions.

In addition, hypoxia increases the expression of GSC markers [37,43]. Previous work indicates that hypoxia increases CD133+ GSC [46,47]. In hypoxic conditions, GSC activate HIF-1α thus increasing their self-renewal ability and anti-differentiated status of GSC [48]. Notch signalling is important in hypoxia, as it maintains the cells in an undifferentiated state. Activation of Notch signalling pathway occurs by recruiting HIF-1α at Notch responsive promoters [49]. In addition, multiple HIF regulated genes were reported to be expressed in GSC, but not in glioma tumour cells [48]. Studies showed that losing factors such as HIF-2α for example, leads to a significant decrease in both GSC proliferation and self-renewal in cultures, and decrease in tumourigenic potential in animals [48]. In addition expression of HIF-1α and HIF2α was found to lead to an increase in oncogeneity of GSCs [43]. Therefore HIF-1α family proteins represent a solution for targeting GSC populations. There are a number of HIF1a targeted agents such as polyamides, quinols and naphthoquinone spiroketal analogues, shikonin derivatives, epidithiodiketopiperazines, and two representative drugs: echinomycin and bortezomib. Only bortezomib was tried in clinical studies of GBM patients with limited results [50].

3. Signalling pathways in GSC not only lead to resistance to chemotherapy, but also induce new tumour formation and lead to recurrence

Malignant gliomas are highly infiltrative tumours displaying both radiation and conventional chemotherapy resistances. They frequently recur locally despite gross total surgical resection. As described above, cellular quiescence of the gliomas stem cells allows them to evade the TMZ chemotherapy and radiation induced damage. Several signalling pathways, including Notch, Hedgehog and transforming growth factors such as TGFβ, are involved in maintaining quiescence in GSC that helps these cells evade systemic treatments (Figure A.2).

3.1 Notch signalling pathway increases the oncogenic potential of GSCs

The Notch signalling pathway is believed to maintain the cellular quiescence of GSC [51]. This pathway was implicated in the self-renewal potential of the stem cells, inhibiting differentiation and protecting the cells from excessive usage of the proliferative potential [51]. In T cell acute lymphoblastic leukemia and in lymphoma, Notch was implicated in maintaining the cancer stem cells [52]. In gliomas, the Notch pathway activating mutations were associated with purified CD133+ GSC [53]. It is believed that in GSC Notch activation makes the cells quiescent and helps them escape radio-chemotherapy treatment [9]. These cells are then released from their quiescent dormancy and enter the cell cycle and differentiate into tumours [9]. In glioblastoma sphere cultures treated with gamma secretase inhibitors (GSIs), the CD133+ GCS were depleted and the putative GCS markers were downregulated (CD133, nestin, BMI1, Olig2) [54]. In these experiments, treatment with GSIs leads to the inhibition of tumour sphere formation *in vitro* and to reduced tumour size in the xenograft model [54]. Further investigations showed that Notch signalling blockade depletes oncogenic GSC through reduced cells proliferation and increased cell apoptosis associated with decreased levels of AKT phosphorylation and STAT3 [31] Inhibition of the Notch signalling pathway can also target the endothelial cells in malignant gliomas [55]. Hovinga *et al* showed that when the tumour endothelial cells were eliminated from a GBM explant, there was a simultaneous decrease in self-renewal of the tumour stem cells [55]. Combination treatments with Notch blockade and radiation therapy resulted in a decrease in proliferation and self-renewal of the tumour explants [55]. Thus the Notch pathway links angiogenesis and GSC; this allows a dual targeting approach for future treatments. RO4929097 is a gamma secretase inhibitor of Notch signalling which has been used in Phase I trial in patients with advanced solid malignancies with promising results [56] and could have immediate relevance for GBM.

3.2. The Hedgehog – GLI is also involved in maintaining the oncogenic potential of gliomas stem cells

Hedgehog (HH) –GLI signalling pathway regulates self – renewal and tumour-forming abilities in CD133+ GSC [57]. Blockade of the HH pathway using cyclopamine depletes GSC [22]. The homeobox gene, Nanog, was recently identified as a mediator for HH-GLI and contributes to the expansion of the CD133+ GSC population, and maintaining glioblastoma growth [57]. It has also been shown that a loss of p53 contributes to up-regulation of Nanog by activating HH pathway and by negatively regulating the activity and level of GLI [57]. GLI was also found to upregulate Notch and downregulate BMP signalling, which is a pro-differentiation action on stem cells [58]. GANT61 is a preclinical molecule targeting the GLI1 and GLI2 in the HH –GLI pathway causes apoptosis in myeloid leukemia cells. [59]

3.3. Epidermal growth factor receptor (EGFR) signalling pathway is involved in maintaining the oncogenic potential of gliomas stem cells

Epidermal growth factor receptor (EGFR) signalling pathway is involved

in maintenance of GSC with a malignant phenotype [60]. Approximately 40-60 % of GBM tumours exhibit EGFR amplification along with a high EGFR protein expression levels [61]. The EGFR activation initiates the phosphatidylinositol-3-kinases (PI3K)/ Akt pathway. PI3K are lipid kinases that phosphorylate lipid phosphatidylinositol (4, 5)-bisphosphate (PIP2) to generate phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) [62,63]. PIP3 recruits AKT to the cell membrane, which enhances cell growth, survival, and proliferation of the cells. EGFR variant III (EGFR vIII) possesses a hierarchical model of expression, which was restricted to epigenetic mechanisms which are characteristic of GSC [64]. Inhibition of the EGFR pathway via tyrosine kinase inhibitors induced apoptosis in CD133+ GSCs [65,66], while inhibition of AKT activity lead to suppression of self-renewal in GSC and CD133+ GSC apoptosis [67]. *In vitro* studies showed that it is possible to target only GSC and not NSC, due to lower levels of EGFR expression on the NSC surfaces [3]. Clinical trials targeting the EGFR variant III (EGFR vIII) have had promising results [68,69] in the GBM patient population.

3.4. Transforming growth factor beta (TGFβ) enhances the tumour formation ability of glioma stem cells

Transforming growth factor beta (TGFβ) is a very powerful cytokine demonstrably involved in many cellular processes including embryonal development, cell growth, differentiation, morphogenesis, wound healing and immune system regulation [70]. When expressed within GSC, the expression of TGFβ is associated with self-renewal and tumourigenic potential of GSC. This occurs by the induction of LIF through Smad complex binding to LIF promoter [71] via the activation of JAK-STAT pathway [71]. Therefore decreasing the secretion of TGFβ and inhibiting the JAK-STAT pathway can lead to a decrease in self renewal and tumourigenic potential in GSC [71].

TGFβ signalling maintains the tumourigenic capacity of GSC via induction of SOX2 expression, which was promoted by induction of SOX4 [72]. SOX4 is a direct target gene of TGFβ signalling, and SOX4 associates with SOX2 enhancer region, promoting its expression [72]. Silencing SOX2 leads to decrease in oncogeneity and self-renewal in GSC[73]. The undifferentiated phenotype of GSC is one of the key criteria for retaining the tumourigenic potential of these cells [72,73].

3.5. Platelet derived growth factor receptor (PDGFR) augments GSC tumourigenic capacity

PDGFR plays an important role in the developing and adult brain. This protein is also over expressed in GBM at the transcriptional level. *In vivo* studies correlated abnormal PDGF signalling with glioma formation [74]. In animal studies, PDGF blocked neuroblast generation and enhanced neural stem cell proliferation in the subventricular zone with formation of glioma like hyperplasia [75]. PDGFRβ was recently reported to be highly expressed in GSC [76]. Pharmacological inhibition of PDGFRβ decreases GSC self-renewal potential, survival, tumour growth, and invasion [76]. Imatinib, a tyrosine kinase inhibitor, which inhibits the PDGF signalling pathway, reduces the ability of GSC to differentiate [77].

3.6. The c-Myc oncogene maintains the tumourigenic potential in glioma stem cells

The oncogenic transcription factor c-Myc, is involved in activating the expression of many genes through several mechanisms, such as recruitment of histone acetylase, chromatin remodeling factors, and interaction with basal transcription factors [78]. Guney *et a*l showed that c-Myc inactivation induces telomere independent senescence [78]. C-Myc is highly expressed in GSC, and c-Myc knockdown reduces cell proliferation, induction of cell apoptosis and loss of oncogenic potential [41]. In addition, inactivation of p53 and PTEN – tumour suppressor genes – leads to increased expression of c-Myc and increase in oncogeneity of GSC [79]. Inhibitors of c-Myc were found to induce cells cycle arrest and apoptosis in acute myeloid leukemia cells [80]. To date, there are no c-Myc inhibitors used in the treatment of malignant gliomas.

3.7. Bmi1 contributes to the GSC maintenance and transformation

Bmi1 is an epigenetic silencer gene.[81] Under normal circumstances it is involved in determination of the differential of stem cell in several tissues [81] and it is a positive regulator of neural stem cells [81]. *Bruggeman et al* demonstrated that Bmi1 was involved in malignant transformation of both neural and differentiated astrocytes [82]. Bmi1 is over-expressed malignant gliomas [82]. To date efforts are on the way to identify small molecule inhibitors to Bmi1.

3.8. Over-expression of chemokine receptors leads to GSC migration.

Over-expression of chemokine receptors such as CXCR4, is a common mechanism related to GSC migration [26]. For glioma cells to migrate, a complex combination of multiple molecular mechanisms is needed, including alteration of tumour cell adhesion molecules, secretion of proteases, modification of actin cytoskeleton, and acquisition of resistance to apoptosis (by affecting PI3K, Akt, mTOR, NF-kappaB regulated pathways), and autophagy (programmed cell death type II) [83]. *In vitro* CXCR4 inhibition synergizes with cytotoxic chemotherapy in gliomas [84]. Inhibiting the migration of GCS contains the tumour and prevents invasion. There are several CXCR4 inhibitors, developed for use specifically in HIV patients, but they have not yet been tested malignant gliomas.

3.9. Adhesion molecules play an important role in migration and thus are important in maintaining the oncogenic potential of glioma stem cells

Adhesion molecules play an important role in nervous system development, and in neural migration and differentiation [85]. One such molecule is L1CAM [85], which regulates neuronal cell growth, survival and migration and axonal outgrowth and neurite extension during development. It is expressed in gliomas and other cancers and makes it a good potential cell surface target [19]. The L1CAM knockdown expression in GSC disrupted the sphere formation ability of GSC, suppressed

tumour growth while inducing apoptosis [19]. L1CAM is found in association with CD133+ cells, while CD133– cells are L1CAM negative [19]. Studies involving lentiviral shRNA targeting of L1CAM disrupted neurosphere formation induced apoptosis and inhibited growth of CD133+ GSC [19]. Currently there are no drugs targeting specifically the L1CAM molecule. However, based on *in vitro* results, this is seems to be an attractive target for glioma growth inhibition.

Integrins, another class of adhesion molecules, are cell surface receptors that are expressed during development and mediate development specific events by binding matrix ligands [86]. Integrin 6α was shown to be important in the neural migration during olfactory development [86]. Recently it was shown that integrin 6α is highly expressed in GSC [87] and that by directly interacting with laminin on endothelial cells increases the oncogeneity of GSC. Disruption of the interaction and targeting of integrin 6α inhibits self-renewal, and proliferation and tumour formation potential [87]. Cilengitide is a specific inhibitor of αvβ3 and αvβ5 integrins and it was tested in phase II clinical trials with promising activity against glioma [88].

4. CD133 is used for the identification and enrichment of GSC populations and is also a potential target for therapy

CD133 is a cell surface protein found on the surface of a population of GSC. CD133+ GSC are tumourigenic and have proliferative activity [89], and that the presence of CD133+ cells is an independent risk factor for tumour recurrence and inversely correlates with patient survival in patients with malignant gliomas [90]. Liu *et al* found that purified CD133+ GSC express a series of genes which are associated with undifferentiated, slow-growing, migrating and an anti-inflammatory and anti-angiogenic phenotype [53]. Multiple studies have shown that CD133+ gliomas cells are resistant to chemotherapy [46,91] potentially because CD133+ GSC express higher levels of proteins associated with chemotherapy resistance, such as the DNA repair protein MGMT [14] and the drug transporter gene ABCG2/BCRP(breast cancer resistance protein) [24]. CD133+ cells also have high mRNA levels of other apoptosis inhibitors, including FLIP, Bcl-2, Bcl-X and some IAP family genes [26].

5. Overcoming GSC immune surveillance escape-Immunomodulation therapy in the treatment of glioblastoma multiforme

Earlier studies in gliomas showed that CD133+ cells did not express detectable Major Histocompatibility Complex (MHC) class I or natural killer (NK) cell activating ligands [89], thus these cells were resistant to adaptive and innate immune surveillance. Incubating GSC with interferon gamma significantly increased the percentage of CD133+ cells that expressed MHC class 1 and natural killer ligands [89]. In addition, when the CD133+ cells were pretreated with interferon gamma, they became sensitive to NK cell-mediated lysis *in vitro* [89]. GSC can be attacked using active immunotherapy by designing vaccines that stimulate the host's intrinsic immune response to the tumour. The initial immunotherapies against gliomas

included irradiated whole tumour cell inoculation engineered to secrete cytokines [92], or combined with cytokine secreting cells [93] or cytokines alone [94]. TR2-01849 is a specific antibody to CD133 protein, specifically designed to target glioblastoma stem cells. Various immune strategies, such as adjuvants, heat shock proteins, γδ T cell treatments have been used as immunomodulators of the immune system in the treatment of GBM [95].

5.1.

A newer method of triggering the active immunity is the development of a dendritic cell (DC) vaccine, which used patient-derived professional antigen presenting cells (APC), such as dendritic cells, to initiate the tumour-specific T-cell response when reinjected in the patients [96-100]. Using this procedure, the lysates of GSC produced a more robust immune system response than using lysates of GBM cells. The DC conditioned with GBM GCS lysates present a wide variety of antigens to T cells to stimulate effective anti-tumour immunity and it is believed that DC vaccination strategies using GSC lysates generate more effective stronger immune responses against a series of more specific epitopes. It has been known that in monoclonal gammopathy patients can develop an immune response against specific epitopes such as SOX2 [101]. In the case of gliomas, Pellegatta et al showed that the use of GSC lysates elicited a strong T cell immune response [102]. In addition, DC vaccination using the antigens/lysates derived from the “mesenchymal subtype” of GBM (which has a very poor prognosis when compared to the pro-neural GBM [103,104]) produced a better survival than those other types of GBM treated with their respective lysate-pulsed DC; i.e., classical or pro-neural types [105].

6. miRNA can modulate tumour cell proliferation, invasion, apoptosis and senescence

The miRNA are small, non –coding RNAs, which down-regulate gene expression post transcriptionally during different cell processes such as apoptosis, differentiation and development [106]. miRNA were originally identified as potential tumour suppressor molecules which induces apoptosis in neuroblastoma cells [107]. The miRNA expression in gliomas is associated with GSC maintenance and growth [106,108]. For gliomas miR-34a was down-regulated when compared to normal brain [106,108]. Mutant p53 expressing glioma tumours had lower levels of miR-34a than wildtype p53 tumours. The presence of miR-34a within those gliomas inhibits their proliferation pathways, cell survival, migration and invasion [106,108]. Transfection of cells with miR-34a inhibited oncogene expression such as c-Met, Notch-1 and Notch2 [106,108]. miR-34a expression induces gliomas stem cells differentiation and thus has the potential to be used in targeting the oncogenic pathways. Currently there are no clinical trials which use the miRNA concept. However, as technology advances, it is very likely that miRNA could be used as a treatment strategy.

Discussion and Conclusions

A significant challenge in designing treatment against GSC is to avoid damage to the neural stem cells and progenitor cells, which share many genetic similarity and antigen expression profiles with GCS [7]. The adult human stem cells serve important functions in tissue repair after injury, especially after traumatic brain injury, ischemic and tumour associated or treatment induced destruction [9]. These normal neural stem cells (NSC) also possess some intrinsic activity against tumour cells, they are attracted by glioma cells *in vivo* and they induce apoptosis and inhibit glioma stem cell growth [109]. The neural stem cells and the progenitor cells are, however, more sensitive than GSC to temozolomide and carboplatin chemotherapy [3]. Hypoxic conditions and EGFR expression seem to be most characteristic to GSC and not to NSC. We previously showed that Bortezomib, a proteasome inhibitor, and Erlotinib (ERL), an EGF tyrosine kinase inhibitor, decrease the viability of GSC, but not to affect NSC.

New research also shows the need to target both the GSC and the bulk of the glioma cells. In their new paper, Chen et al indicated that if one targets only the GSC, the mice still die due to their tumour burden [8]. Therefore, there is a need for more effective drugs or immunotherapeutic strategies that target both GSC and the more differentiated glioma cell mass. More clinical trials focused on combination therapies against multiple oncogenic pathways involved in GSC maintenance, division and differentiation are still needed.

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Summary

Summary

The hypothesis pertinent to this thesis is that glioma tumours can be therapeutically targeted by gene and/or immunotherapy in order to eliminate or delay tumour recurrence leading to significant morbidity and mortality.

In our gene therapeutic approach, described in **Chapter 2**, we observed that chronic expression of the C-terminal fusion of IsK with EGFP (enhanced green fluorescent protein) led to cell death of more than 50% of transfected U87-MG human astrocytoma cells as early as 2 days after transfection. Our results are consistent with activation of apoptotic pathways following IsK-mediated increase in K+ efflux.

However, we abandoned the gene therapy approach because of the more attractive immunotherapeutic intervention strategies for of brain tumours, which is currently emerging as a highly potential clinical option as reviewed in **Chapter 3**.

Interestingly, as described in **Chapter 4**, we found a strong therapeutic antitumour efficacy for the innate immune response modifier Resiquimod, even as a stand-alone treatment, eventually leading to immunological memory against secondary tumour challenges. In parallel, we observed that cyclophosphamide treatment, although effective as chemotherapeutic agent, may be deleterious to maintenance of long-term antitumour immune memory. Our data also demonstrates that immunotherapeutic parenteral treatment of established glioma tumours by Resiquimod, as defined in the protocol, significantly improves anti-brain tumour immunity in a way that leads to immune memory, which is superior to cyclophosphamide treatment alone. Our studies have thereby identified a promising novel antitumour immunotherapy which may lead to clinical benefit.

In **Chapter 5**, we describe our finding that, in multiple rat glioma models, a certain composition of antigens derived from syngeneic tumour cells and their lysates when therapeutically co-administered with allogeneic cells and their lysates is able to confer anti-tumour immune responses and tumour regression. For the syngeneic C6 model in SD rats therapeutic injections of allogeneic cells alone were sufficient to trigger tumour regression. This immunization approach may prove useful as a post-surgery adjuvant therapy in future cancer treatment protocols, or even as a stand-alone therapeutic tumour vaccination.

In another syngeneic rat glioma model, described in **Chapter 6**, we found that for regression of CNS-1 glioma tumours in Lewis rats specific innate immune response stimulating substances were required as immunological adjuvants. In our hands BCG and IL-2, the Toll-Like receptor (TLR) 7/8 activator Resiquimod, and the cytokine granulocyte-macrophage colony stimulating factor (GM-CSF), showed potent activity.

Finally, as described in **Chapter 7**, we demonstrate that our prototype therapeutic vaccine, when co-delivered in a specific regimen together with the cytokine GM-CSF as immunological adjuvant, is able to arrest progression of glioma tumour

growth, when therapeutically administered following low-dose cyclophosphamide. GM-CSF is an attractive vaccine adjuvant because of its proven immune modulatory effects and low toxicity profile. The safe pharmacological use of GM-CSF in patients is well-established, which makes it feasible for clinical use. The use of GM-CSF has been included in the first clinical studies that have been approved for an Investigational New Drug application (IND) for Single patient use in the U.S..

Samenvatting

Samenvatting

In dit proefschrift werd de hypothese getoetst dat gliomatumouren dusdanig therapeutisch behandeld kunnen worden door middel van gen- of immunotherapie dat eliminatie of vertraging van tumourgroei optreedt resulterend in significante verlaging van morbiditeit en mortaliteit.

In de gen-therapeutische aanpak, beschreven in **hoofdstuk 2**, werd waargenomen dat chronische expressie van het C-terminale fusie-eiwit van IsK met EGFP (enhanced green fluorescent protein) resulteerde in meer dan 50% celdood van de getransfecteerde U87-MG humane astrocytoom cellen binnen 2 dagen na transfectie. Deze bevindingen duiden op activatie van apoptotische routes in tumourcellen als gevolg van een IsK-gemedieerde toename in K+ efflux.

Omdat een immunotherapeutische interventiestrategie voor glioom-hersentumouren op dat moment meer belovend leek werden deze gen-therapeutische studies niet verder doorgezet. In **hoofdstuk 3** wordt deze immunotherapeutische aanpak als behandeloptie beschreven in een overzichtsartikel.

In **hoofdstuk 4** beschrijven we een duidelijke therapeutische antitumour activiteit voor het middel Resiquimod, een zogenaamde “innate immune response modifier”, zelfs wanneer het werd toegediend in de vorm van een zogenaamde “stand-alone” behandeling. Deze behandeling resulteerde bovendien in een effectief immunologisch geheugen, zoals duidelijk werd door het uitblijven van tumourgroei na een latere secondaire blootstelling aan de tumour. In deze studie bleek dat de behandeling met het chemotherapeutische en immunosuppressieve agens cyclofosfamide eveneens een goede initiële antitumour werking bewerkstelligt. Echter na blootstelling aan een secondaire tumour bleek er een negatief effect op het lange-termijn antitumour immunologisch geheugen. Hieruit blijkt dat Resiquimod, volgens het beschreven protocol, een significant anti-glioom immuniteit induceert, leidend tot immunologisch geheugen, en dat dit middel superieur is ten opzichte van cyclofosfamide behandeling.

In **hoofdstuk 5** worden studies beschreven in verschillende rat glioom-modellen, waarbij een bepaalde samenstelling van het antigeenmengsel, afkomstig van syngene tumourcellen en hun lysaten tezamen met therapeutisch toegediende allogene cellen en hun lysaten, in staat is om een anti-tumour immuunrespons op te wekken en tumourregressie te induceren. Voor het syngene C6 model in SD ratten bleken therapeutische injecties van allogene cellen alleen al voldoende om tumourregressie te bewerkstellingen. Een dergelijke immunisatie-strategie lijkt belovend als postoperatieve adjuvant-therapie in toekomstige kankerbehandelingen, of zelfs als een “stand-alone” therapeutische tumourvaccinatie.

In een ander syngeen glioom rattenmodel, beschreven in **hoofdstuk 6**, werd waargenomen dat regressie van CNS-1 glioom-tumouren in Lewis ratten opgewekt kon worden door middel van specifieke immuunstimulerende substanties, zogenaamde “immunologische adjuvantia”, die gericht werken op cellen van het aangeboren immuunapparaat. In dergelijke studies vertoonden met name BCG en IL-

2, de Toll-Like receptor (TLR) 7/8 activator Resiquimod, en het cytokine granulocyte-macrophage colony stimulating factor (GM-CSF), een duidelijke anti-tumour activiteit indien toegediend in combinatie met tumourantigenen bestaande uit cellen en hun lysaten.

In **hoofdstuk 7** laten we zien dat ons prototype therapeutisch vaccin, in combinatie met een specifiek regime van het cytokine GM-CSF als immunological adjuvant, in staat is om de progressie van glioom-tumourgroei te remmen, indien therapeutisch toegediend na een lage dosis cyclofosfamide. GM-CSF is een attractief vaccin-adjuvant vanwege zijn bewezen immunomodulerende effect en zijn lage toxiciteit. Het veilige farmacologische gebruik van GM-CSF is al duidelijk aangetoond in patiënten, hetgeen de haalbaarheid voor klinisch gebruik vergemakkelijkt. Het cytokine GM-CSF is daarom in de eerste klinische studies toegepast die onlangs zijn goedgekeurd voor een zogenaamde “Investigational New Drug application (IND)” voor het gebruik in individuele patienten in de Verenigde Staten van Amerika.

In **hoofdstuk 8** bediscussiëren wij de resultaten van ons onderzoek in relatie tot de internationale literatuur, speculeren we over een mogelijk werkingsmechanisme en vatten we de conclusies van ons onderzoek samen. Hierbij tonen wij ook de eerste resultaten van de klinische toepassing van een tumourvaccin, gemaakt volgens de gegevens van deze studie, bij patiënten met glioblastoma multiforme.

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I thank Corinne Foltz that she supported me in the past and the present.

Curriculum vitae

Curriculum vitae

Apostolos Stathopoulos was born in Volos Magnisia, Greece, on June 16th, 1971. He obtained his Medical Bachelor Science degree in 1996 at the University of Mons Hainaut Belgium, and his degree in Medicine (M.D.) in 2000 at the Free University of Brussels (U.L.B.) Belgium. From 2000-2006 he did a Neurosurgery Residency at University of Liege (U.L.G.) Belgium. In 2005 he fulfilled a 6 month Neuro-oncology Fellowship at the University of Southern California.

In 2006 he became Chief of the Neurosurgery department C.S.L (Cliniques du Sud Luxembourg Belgium). In 2008 he founded as CEO and President "Epitopoietic Research Corporation" (ERC) in Belgium, a company developing an immunotherapy (GBMvax) against glioblastoma multiforme (GBM) brain cancer. In 2009 he became head of the Department of Neurosurgery at Vivalia Hospitals Province de Luxembourg in Belgium. Since 2011 he is also Assistant Professor (Clinical assistant professor of Neurosurgery) at Keck School of Medicine University of Southern California U.S.A).

Other Experience and Professional Memberships

2008 Creation of Epitopoietic Research Corporation (ERC).

2004 The European Association of Neurosurgical Societies EANS European Examination August 2004.

1998 Certificate in "In Vitro Practical Techniques for Neuroscience". Bristol U.K., July 1998.

Honors

2011 Assistant Professor (Clinical assistant professor of neurosurgery Keck School of Medicine University of Southern California U.S.A).

2010 Wallonia Belgium Grant 2,5 M Euro for 30 months clinical trial 0/I/II vaccine against Glioblastoma.

2009 Wallonia Belgium Grant 1,390200 Euro for 30 months basic and in vivo immunomodulation research.

2006 Grant awarded by Bayer from the Belgium Society of Neurosurgery for "Brain tumour subcutaneously implanted and rejected by immunomodulation in two strains of rat" on March 18, 2006.

2000 Awarded Doctorate in Medicine, Surgery and Obstetrics with Distinction, June 27, 2000

2000 Prize awarded by Rotary Club Val Duchesse (Brussels) for Gene transfer into rat and human ventral mid-brain tissue by means of a recombined vector based on adeno-associated virus (AAV) on May 23, 2000.

2000 Dissertation: Gene transfer into rat and human ventral mid-brain tissue by means of a recombined viral vector based on adeno-associated virus (AAV), May 15, 2000 Highest distinction.

1999 D.R.I. (Department of International Relations, Free University of Brussels) Grant for Clinical student Internship at Harvard Medical School Brighams and Womans hospital Childrens hospital Boston MA, USA.

1998 Youth Travel Fund of the Federation of European Biochemical Societies (FEBS) Travel grant to take part in FEBS course: In vitro Practical Techniques for Neuroscience Bristol U.K.

List of publications

List of publications

1. L.Tenenbaum, M. Hamdane, M. Pouzet, B. Avalosse, **A. Stathopoulos**, F. Jurysta, C. Rosenbaum, CO. Hanemann, M. Levivier, and T. Velu (1999): Cellular contaminants of adeno-associated virus vector stocks can enhance transduction. Gene Therapy 6, 1045-1053.

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To Marie Stathopoulos ...

... My Daughter.

The only real failure in life is the failure to try Anonymous