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Förnvik, Karolina

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KAROLINA FÖRNVIK JONSSON
DEPARTMENT OF CLINICAL SCIENCES, LUND | LUND UNIVERSITY

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Karolina Förnvik Jonsson

LUND UNIVERSITY

DOCTORAL DISSERTATION

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Faculty opponent
Professor Hrvoje Miletic, MD, PhD
The Department of Biomedicine
University of Bergen, Norway

Organization LUND UNIVERSITY Faculty of Medicine Department of Clinical Sciences, Lund Division of Neurosurgery Author: Karolina Förnvik Jonsson	Document name DOCTORAL DISSERTATION Date of issue: 2020-12-04

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Abstract

One of the major problems with malignant brain tumours, such as glioblastoma multiforme, is that despite being able to remove the major bulk of the tumour through surgery and treating the patients with chemotherapy and radiotherapy, we know that tumour cells have already spread throughout the brain. Furthermore, we now know that the glioblastoma cells effectively suppress the patients' own anti-tumour response. One key part of the immune response, the complement system, acts as a functional bridge between innate and adaptive immunity. Here we wanted to further investigate the complement system in both glioblastoma patients and in laboratory animals by looking at the role of CRP and C1-inhibitor (C1-INH). In order not to rely solely on old glioblastoma models, which have been passed *in vitro* for decades, we also developed a new glioblastoma model.

Initially we studied an experimental treatment, ITPP, which had previously shown promising results in other cancer models. This was done using the old glioblastoma model RG2. Subsequently we went on to develop a new GFP positive glioblastoma model called NS1. The NS1 cell line was then used to further investigate the role of the complement system, both *in vitro* and *in vivo*, by treating the cells and animals with anti-C1-INH and anti-CRP. The *in vivo* experiments involved intratumoral treatment of both intracranial and subcutaneous tumours. Additionally, tumour material from glioblastoma patients was examined on the gene and protein level and compared to tumour data available from public databases.

Treatment with ITPP showed no survival advantage in the RG2 model, and the route of ITPP administration did not affect outcome. The NS1 model turned out to generate infiltrative CNS tumours with perivascular growth and characteristics of a glioblastoma upon histopathological examination. With MRI tumour could easily be detected within 14 days after inoculation. We could demonstrate a significantly increased survival *in vivo* in animals inoculated intracerebrally with NS1 glioblastoma cells pre-coated with anti-C1-INH antibodies. On the contrary no effect of coating glioblastoma cells with anti-CRP antibodies prior to intracranial inoculation was seen. In the subcutaneous NS1 tumour model, intratumoral anti-C1-INH treatment showed a significant survival advantage and there was a significant decrease in tumour size over time following treatment with anti-C1-INH. Using data fram a publicly available database and our own mRNA material from glioblastoma patients, we found an upregulation of C1-INH in human glioblastoma cells. Furthermore, by using immunohistochemistry, we could demonstrate the presence of both C1-INH and CRP on glioma cells *in vitro* from humans and rats.

We could conclude that C1-INH seems to play an important role in glioblastoma. This could possibly be explained by effects on the complement system, but also other effects are possible, since C1-INH has many biological functions. The exact role and mechanisms of anti-C1-INH treatment are topics for future studies.

Key words: Glioblastoma, complement system, C1-INH, ITPP, CRP, experimental glioma models

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Karolina Förnvik Jonsson

Division of Neurosurgery
Department of Clinical Sciences, Lund
Faculty of Medicine
Lund University
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To my beloved parents
who have always helped me along the way and still do

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Abstract

Background

One of the major problems with malignant brain tumours, such as glioblastoma multiforme, is that despite being able to remove the major bulk of the tumour through surgery and treating the patients with chemotherapy and radiotherapy, we know that tumour cells have already spread throughout the brain. Furthermore, we now know that the glioblastoma cells effectively suppress the patients' own anti-tumour response. One key part of the immune response, the complement system, acts as a functional bridge between innate and adaptive immunity. Here we wanted to further investigate the complement system in both glioblastoma patients and in laboratory animals by looking at the role of CRP and C1-inhibitor (C1-INH). In order not to rely solely on old glioblastoma models, which have been passed *in vitro* for decades, we also developed a new glioblastoma model.

Materials and methods

Initially we studied an experimental treatment, ITPP, which had previously shown promising results in other cancer models. This was done using the old glioblastoma model RG2. Subsequently we went on to develop a new GFP positive glioblastoma model called NS1. The NS1 cell line was then used to further investigate the role of the complement system, both *in vitro* and *in vivo*, by treating the cells and animals with anti-C1-INH and anti-CRP. The *in vivo* experiments involved intratumoral treatment of both intracranial and subcutaneous tumours. Additionally, tumour material from glioblastoma patients was examined on the gene and protein level and compared to tumour data available from public databases.

Results

Treatment with ITPP showed no survival advantage in the RG2 model, and the route of ITPP administration did not affect outcome.

The NS1 model turned out to generate infiltrative CNS tumours with perivascular growth and characteristics of a glioblastoma upon histopathological examination. With MRI tumour could easily be detected within 14 days after inoculation.

We could demonstrate a significantly increased survival *in vivo* in animals inoculated intracerebrally with NS1 glioblastoma cells pre-coated with anti-C1-INH antibodies. On the contrary no effect of coating glioblastoma cells with anti-CRP antibodies prior to intracranial inoculation was seen.

In the subcutaneous NS1 tumour model, intratumoral anti-C1-INH treatment showed a significant survival advantage and there was a significant decrease in tumour size over time following treatment with anti-C1-INH.

Using data from a publicly available database and our own mRNA material from glioblastoma patients, we found an upregulation of C1-INH in human glioblastoma cells. Furthermore, by using immunohistochemistry, we could demonstrate the presence of both C1-INH and CRP on glioma cells *in vitro* from humans and rats.

Conclusion

We could conclude that C1-INH seems to play an important role in glioblastoma. This could possibly be explained by effects on the complement system, but also other effects are possible, since C1-INH has many biological functions. The exact role and mechanisms of anti-C1-INH treatment are topics for future studies.

Abbreviations

2,3-BPG	2,3-Bisphosphoglyceric acid
BBB	Blood-brain barrier
C1-IA	Complement1-inactivator (*also denoted C1-INH*)
C1-INH	Complement1-inhibitor
CDC	Complement-dependent cytotoxicity
CMV	Cytomegalovirus
CNS	Central nervous system
CR	Complement receptor
CRP	C-reactive protein
CT	Computed tomography
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAF	Decay accelerating factor
DAPI	4,6-diamidino-2-phenylindole
DCs	Dendritic cells
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ENU	Ethyl-nitrosourea
FDA	Food and Drug Administration
FLAIR	Fluid attenuation inversion recovery
GBM	Glioblastoma multiforme
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSCs	Glioma stem cells
H/E	Haematoxylin/eosin
HIFs	Hypoxia-inducible factors
IDH1	Isocitrate dehydrogenase 1
IDO 1	Indoleamine 2,3-dioxygenase 1
IL-1β	Interleukin 1 beta
ITPP	Inositol trispyrophosphate
KPS	Karnofsky Performance Scale
MAC	Membrane attack complex
MASP	MBL-associated serine proteinase
MBL	Mannose/Mannan-binding lectin
MCP	Membrane cofactor protein
MDSCs	Myeloid-derived suppressor cells
MGMT	O^6-methylguanine DNA methyltransferase
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
NS1	Nittby Salford 1

NSCs	Neural stem cells
PBS	Phosphate buffered saline
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PET	Positron emission tomography
PTEN	Phosphatase and tensin homolog
PTX3	Pentraxin 3
RBCs	Red blood cells
RCA	Regulators of complement activation
RT	Radiotherapy
SVZ	Subventricular zone
Treg	Regulatory T-cells
TAMs	Tumour-associated macrophages
TERT	Telomerase reverse transcriptase
TF	Tissue factor
TGF-β	Transforming growth factor-beta
TICs	Tumour-initiating cells
TMZ	Temozolomide
VEGF	Vascular endothelial growth factor

Populärvetenskaplig sammanfattning

Varje år drabbas över 1000 personer i Sverige av primär hjärntumör. Den mest aggressiva av dessa är så kallat glioblastom eller astrocytom grad IV. Standardbehandlingen består av kirurgi, strålbehandling och cellgifter, men trots detta är prognosen minst sagt dyster; överlevnaden är endast 15 månader i genomsnitt och de flesta patienter avlider inom två år efter diagnos. Vår forskning är inriktad på att försöka bromsa eller allra helst bota dessa hjärntumörer.

En anledning till att glioblastom är så svåra att behandla är att de hämmar kroppens egen immunrespons och att små tumörceller redan tidigt i förloppet hunnit sprida sig till den omgivande hjärnan. Vi har utvecklat en djurmodell som gör att man kan se de här små tumörcellerna och följa hur de sprider sig i den friska hjärnan. Dessutom har vi undersökt en del av immunsystemet som kallas komplementsystemet. Det fungerar som en brygga mellan det mer basala immunsystemet och det så kallade adaptiva immunsystemet, som är mer specifikt. Vi har sett att en hämmare av komplementsystemet tycks vara påverkad i glioblastom och vi har kunnat märka in tumörceller både från råtta och från människa med olika antikroppar och funnit att proteinet C1-inhibitor finns på cellytan. C1-inhibitor är ett protein som hämmar den så kallade klassiska vägen i komplementsystemet. Vi har även behandlat med antikroppar mot C1-inhibitorn i vår tumörmodell och funnit att det leder till mindre tumörer och ökad överlevnad hos våra råttor.

Vi har också undersökt genuttrycket hos tumörceller från patienter med glioblastom. Både i vårt eget tumörmaterial och i tumörmaterial från olika fritt tillgängliga databaser fann vi att genen för C1-inhibitorn var uppreglerad, det vill säga att det finns mer C1-inhibitor i tumörceller än i vanliga hjärnceller.

Utöver detta har vi genomfört ett flertal andra experiment. Bland annat har vi utvecklat en ny modell för elakartade hjärntumörer där man lättare kan följa tumörens infiltrativa växt in i hjärnvävnaden. Vi har kartlagt utvecklingen av tumören med magnetkamera samt undersökt vävnadsprover från tumören.

Framöver har vi tänkt att gå vidare med ytterligare studier där vi mäter olika komplementfaktorer, däribland C1-inhibitor, i blodet hos patienter med glioblastom. Fortsatt kartläggning av vilken roll C1-hämmare spelar i glioblastom vore också mycket intressant, liksom vidare studier med behandling av tumörer enligt olika regimer för att optimera effekten.

Aims of the research

Overall aim

Since patients with glioblastoma multiforme have such a poor prognosis there is urgent need for finding new treatments and ultimately a cure, which is the overall aim of our research. Finding such a drug or mechanism would drastically improve the dismal survival that glioma patients face today.

One of our approaches was to test the drug ITPP, which had previously shown promising results in other cancer types that also exhibit hypoxia. Since many of the glioma models currently used in neuro-oncological research have been through decades of culturing and thus have become progressively more homogenous and hardly resemble an aggressive glioblastoma anymore, we also set up the aim to develop a new fully immunocompetent glioblastoma model that could be used to further investigate the complement system in glioblastoma.

This part of the research is based on the theory that the classical pathway of the complement system is inactivated in glioblastoma through the upregulation of C1-inhibitor (C1-INH), and that it would be of benefit for the patients to re-activate the complement system. The main hypothesis was that we could prolong survival if we treated animals, inoculated with glioblastoma cells, with antibodies against C1-INH. We also wanted to start the search for possible mechanistic explanations to the effects observed.

Other specific questions raised

Does ITPP halt glioblastoma tumour growth?
Is CRP and/or C1-INH upregulated on gene level in glioblastoma tissue from patients as compared to non-tumour brain?
Do human and rat glioblastoma cells express CRP and/or C1-INH *in vitro*?
Does treating glioma cells with anti-CRP or anti-C1-INH *in vitro* before inoculation affect survival in animals with intracranial tumours?
Does intratumoral treatment with anti-C1-INH increase survival in rats?
How do untreated NS1 glioblastoma tumours develop in fully immunocompetent animals *in vivo*?

Brain tumours

Cell types in the brain

There are two types of cells in the human brain: neurons and glia. The question as to how many there are of each type is still one of debate and ongoing research. Scientists have challenged the prevailing notion that glia outnumber neurons and seem to agree on the fact that the glia:neuron ratio is roughly 1:1 as demonstrated by the recently validated isotropic fractionator method (von Bartheld et al. 2016) and that there is a total number of about 100 billion glial cells in the human brain.

Glial cells are further subdivided into astrocytes, oligodendrocytes, ependymal cells and microglia, which together provide important mechanisms such as structural support, metabolic support and insulation, and glioma is an overarching term used for the brain tumours of glial origin (DeCordova et al. 2020).

Astrocytes are star-shaped cells that tile the entire central nervous system (CNS) and exert many essential complex functions in the healthy CNS. One example is the interaction with blood vessels, where it has been found that astrocytes produce and release various molecular mediators such prostaglandins, nitric oxide, and arachidonic acid, that can increase or decrease blood vessel diameter and thus regulate local blood flow (Sofroniew and Vinters 2010).

Astrocytes are divided into two subtypes, protoplasmic or fibrous, on the basis of differences in their cellular morphologies and anatomical locations. Protoplasmic astrocytes are found throughout gray matter whereas fibrous astrocytes are found throughout all white matter (Sofroniew and Vinters 2010). Glial fibrillary acid protein (GFAP) has become the prototypical marker for immunohistochemical identification of astrocytes, but GFAP is not an absolute marker of all astrocytes and regional and local variability of expressions exists (Sofroniew and Vinters 2010).

Microglia represent the macrophages of the CNS and are capable of orchestrating a potent inflammatory response, but microglia are also involved in various other mechanisms such as synaptic organization, phagocytosis of apoptotic cells in the developing brain, myelin turnover, control of neuronal excitability, as well as brain protection and repair (Bachiller et al. 2018).

Types of tumours

Two types of tumours can develop in the brain: malignant and non-malignant. The malignant tumours can be further subdivided into primary tumours and secondary tumours, where the secondary tumours consist of brain metastases from other tumours located within the body such as for instance breast cancer, malignant melanoma and lung cancer. The incidence and prevalence of metastatic tumours outweighs primary tumours by 4:1 (Merrell 2012). The reason why lung and breast cancers make up the majority of the metastatic tumours is largely because of their higher prevalence in the population as compared to other types of tumours, whereas malignant melanoma has a high propensity to metastasize to the brain even if it is a less prevalent malignancy. This can be compared to for instance colorectal and prostate cancers which are less likely to metastasize to the brain (Merrell 2012).

The most common benign or non-malignant tumours in the brain are the meningiomas, which are tumours originating from arachnoid cap cells. They often grow slowly and are usually found in the fourth through sixth decade of life with a female:male ratio of 2:1 (Merrell 2012).

The malignant primary brain tumours are most often astrocytomas, which together with oligodendrogliomas traditionally have made up the glioma group based on morphological grounds, with a third mixed category of oligoastrocytoma for those cases in which tumour morphology showed characteristics of both. This classification of glioma was recently changed, and glioma diagnosis is now primarily based on molecular characteristics (van den Bent et al. 2017). The World Health Organization (WHO) has a classification of tumours of the central nervous system where tumours are histologically graded into four grades (grade I-IV). Tumour grading correlates well with tumour morphology, biology and prognosis (Akhtar et al. 2018) and in 2016 molecular parameters were used for the first time in addition to histology to grade gliomas (Louis et al. 2016). Thus, the histopathological name is followed by the genetic features, since we now know that the genetic and molecular profile of a tumour outweighs its histopathological phenotype (Iv et al. 2018).

Risk factors

There are only a few identifiable risk factors for developing brain tumours as far as we know today. Past exposure to ionizing radiation and a family history of certain genetic syndromes are known risk factors (Merrell 2012). Examples of such inherited syndromes are neurofibromatosis type 2 (can cause vestibular schwannomas), tuberous sclerosis (can cause giant cell astrocytoma), Li-Fraumeni

syndrome (can cause glioblastoma), Turcot syndrome (can cause medulloblastoma or glioblastoma) and von Hippel-Lindau disease (can cause hemangioblastomas).

There are also other documented risk factors for developing brain tumours such as exposure to vinyl chloride (Hehir et al. 1981) and Epstein-Barr virus which can cause CNS lymphomas (Akhtar et al. 2018). There have also been theories that Epstein-Barr virus could cause glioblastoma (Akhtar et al. 2018) and another suspected risk factor is human cytomegalovirus (CMV). CMV nucleic acids and proteins have been found in 90–100% of examined glioblastomas, but also in other solid cancers such as breast cancer, colon cancer, prostate cancer, medulloblastoma, neuroblastoma and rhabdomyosarcoma (Stragliotto et al. 2020). The virus has been found to have oncomodulatory effects and promotes tumour growth in a glioblastoma mouse model (Krenzlin et al. 2019). On the other hand, there is research pointing towards no detectable CMV protein or genomic material in glioma samples depending on assay sensitivity and more recent studies using PCR have also failed to detect CMV in GBM patient peripheral blood or tumour samples, thus highlighting the controversy surrounding the role of CMV in glioblastoma (Rahman et al. 2019).

Glioblastoma

Characteristics

Glioblastoma multiforme (GBM) or astrocytoma grade IV is the most common malignant primary brain tumour in adults, with a poor prognosis despite multimodality treatment comprising surgical resection, chemotherapy and radiotherapy (Stupp et al. 2005). Median survival is only 8-13 months depending on factors such as extent of surgical resection, Karnofsky Performance Scale (KPS) score and age (Lacroix et al. 2001).

One of the major problems when treating patients with glioblastoma is that, despite being able to remove the major bulk of macroscopically visible tumour through neurosurgery, tumour cells have already spread throughout the brain and even extensive resections will not cure the patient. This is due to the diffuse infiltration of GBM cells which enables the tumour to grow tentacles into the surrounding brain, making the outline of the tumour undefined, and to seed tumour cells into the brain tissue beyond the circumference of the tumorous mass itself. Together with pseudo-palisading necrosis, microvascular proliferation, extensive inter- and intra-tumour heterogeneity, genomic instability and dynamic plasticity this contributes to the malignant nature of GBM (DeCordova et al. 2020).

Theories on origin and oncogenesis

GBM usually arises as *de novo* tumours from glial cells in the brain, but can also develop from lower grade neoplastic lesions, such as astrocytoma grade I-III and are then classified as 'secondary' glioblastomas, which account for about 10% of GBM cases. The tumour is most often located in the frontal and temporal lobes of the brain, followed by the other supratentorial parts of the brain, but can also rarely occur in the brainstem, cerebellum and spinal cord (DeCordova et al. 2020).

There are various theories on the development of glioblastoma. Cancer is generally a disease of genetic mosaicism because cancerous cells harbour genetic mutations that are absent in normal cells within the same individual. In familial cancer patients, even though initial mutations exist in every cell in the body, in most cases only specific cell types progress into malignancy (Liu et al. 2011). Those cell types are

called cancer cell-of-origin. The cancer cell-of-origin in GBM still remains controversial. There are cells found in the brain that have stem cell features, these cells are termed neural stem cells (NSCs) and have been suggested to be the cancer cell-of-origin. This was based on the fact that the location of the tumours appeared to associate with the subventricular zone (SVZ), where adult NSCs reside (Liu et al. 2011).

A small subpopulation of cells within the tumour microenvironment are termed glioma stem cells (GSCs) and serve as critical regulators of tumour progression and recurrence (Ma et al. 2018). GSCs are thought to have tumour-initiating properties and as such would contribute towards self-renewal and multi-lineage differentiation. It is also thought that the complement system is involved in GSC maintenance (Bouwens van der Vlis et al. 2018). Although the origin of these multipotent cells is not clearly defined, the GSCs are hypothesized to fuel tumorigenesis with potential to induce angiogenesis and modulate therapeutic responses through their capacity to recover rapidly from conventional therapeutic stress, which leads to resistance and disease relapse in glioma patients (Ma et al. 2018). It has been found that GSCs are enriched in areas of hypoxia, in perivascular niches and at the invasive edge of the tumour (Bouwens van der Vlis et al. 2018).

Genetic mutations

Several different mutations exist in brain tumours and some are specific to GBM. Most gliomas harbour molecular alterations disrupting key signalling pathways such as for instance receptor tyrosine kinase pathways MAPK/ERK and PI3K/Akt/PTEN, as well as cell cycle and DNA repair pathways such as retinoblastoma/E2F7/p53 and pathways for metabolism, most notably isocitrate dehydrogenase (IDH) (Akhtar et al. 2018). One of the most relevant genetic mutations affecting GBM is in fact alteration of the IDH gene since it may be linked to survival due to its ability to alter the effectiveness of chemotherapy and the presence of IDH mutation may identify a better prognostic subgroup within GBM patients (Lieberman 2017).

The enzyme IDH catalyses the oxidative decarboxylation of isocitrate to alfa-ketoglutarate and reduces NAD(P)+ to NAD(P)H (Reitman and Yan 2010). IDH has two isoforms IDH1 and IDH2, where mutations in IDH1 are the most common and are mostly found in low grade gliomas, whereas loss of either or both IDH genes combined with loss of chromosome arms 1p and 19q is an indication of oligodendroglioma.

IDH mutations are present in 80% of secondary GBM, but in only 5% of primary GBM (Lieberman 2017). It has also been found that gliomas with IDH1 or IDH2 mutations respond better to chemotherapy than those without

the mutation. Thus, tumours with wt IDH are the most aggressive and since 2016 IDH-wt glioblastomas are subdivided into three types: giant cell glioblastoma, gliosarcoma, and epithelioid glioblastoma (Louis et al. 2016).

Further genetic alterations include loss of TP53 and ATP-dependent helicase (ATRX), which is a characteristic of astrocytomas (DeCordova et al. 2020).

Epidermal growth factor receptor (EGFR) is commonly overexpressed in GBM and EGFR gene amplification is seen in 50% of GBMs (Akhtar et al. 2018). The prognostic implications of EGFR amplification are still unsettled; higher levels of gene amplification appear to correlate with poor survival outcome (Lieberman 2017).

Telomerase reverse transcriptase (TERT) is an enzyme responsible for adding nucleotides to telomeres and can be reactivated in various cancers to promote onocogenesis (Bush and Butowski 2017). In GBMs TERT gene mutations usually occur in the TERT promoter region and activate the telomerase, which is otherwise inactive in normal adult cells. Mutations in the *TERT* gene occur in approximately 75% of GBM cases (Lieberman 2017).

GBMs are also often affected by copy number aberrations (CNA) such as gains and losses of genetic material. Examples include gains on chromosomes 4, 7 and 12, while deletions are commonly observed on chromosomes 9 and 10 (Akhtar et al. 2018). Chromosome 10 includes the *PTEN* gene, which encodes phosphatase and tensin homolog (PTEN), a tumour suppressor in the PI3K signalling pathway, and one of the most commonly lost tumour suppressors in human cancer. Inactivation of PTEN occurs in glioblastoma, endometrial cancer, and prostate cancer; and reduced expression is found in many other tumour types such as lung and breast cancer (Yin and Shen 2008).

The Cancer Genome Atlas (TCGA) network was among the first to conduct a major genomic study of GBM, leading to novel genomic variations being identified, such as deletions of neurofibromin gene (NF1) and parkin RBR E3 ubiquitin protein ligase (PARK2) as well as copy number variations (CNVs) of AKT serine/threonine kinase 3 (AKT3) and other single nucleotide variations (SNVs). The TCGA has then subsequently grouped GBM into three molecular subtypes: proneural (PN), mesenchymal (MSC), and classical (CL), where patients with the MSC subtype tend to have a poor survival compared to the other subtypes (DeCordova et al 2020).

To summarize, *TERT* promoter mutation, *PTEN* tumour suppressor gene mutation, and gene amplification of the *EGFR* gene are found in primary GBM, whereas mutations of *IDH1/2*, *TP53*, and *ATRX* are frequent in secondary GBM (Aldape et al. 2015). Furthermore, patients who had undergone neuro-oncological treatment were shown to have higher genetic variability in their recurrent tumours than untreated patients, meaning that the tumour seen upon recurrence is not genetically the same tumour as was diagnosed initially (Neilsen et al. 2019).

MGMT promoter methylation status

O^6-methylguanine DNA methyltransferase (MGMT) is an enzyme involved in DNA repair and MGMT promoter methylation is commonly detected in GBMs, about 35-50% (Akhtar et al. 2018). Epigenetic silencing of *MGMT* by promoter methylation compromises DNA repair and has been linked to longer survival in glioblastoma patients receiving Temozolomide. Conversely, due to an unmethylated MGMT promoter gene in the tumour of majority of patients, they may not benefit from Temozolomide therapy (Stragliotto et al. 2020). MGMT promoter methylation status has been shown to be a predictive biomarker for survival in elderly GBM patients (Lieberman 2017).

There are also other epigenetic mechanisms that have shown prognostic significance in gliomas. Tumours demonstrating hypermethylation of CpG sites throughout the genome, usually seen in younger patients, have an especially favourable prognosis (Aldape et al. 2015).

Hypoxia and ITPP

Hypoxia is a phenomenon often found in the glioblastoma setting, as well as in many other tumour types. It has been demonstrated that changes in the structural and organizational properties of extracellular matrix (ECM) favour adhesion and migration of cancer cells from the initial tumour site. An active angiogenesis equally contributes to these environmental changes with the formation of new blood vessels that supply growing cancer cells with nutrients (Macor et al. 2018). These blood vessels form in an unstructured manner and are permeable, thus allowing tumour spreading. One way of limiting tumour growth could theoretically be by controlling angiogenesis, since a major hallmark of malignant growth is this rapid formation of new blood vessels, where the driving force is thought to be the hypoxic state created by the tumour itself (Kieda et al. 2013).

The natural allosteric effector that reduces the oxygen-binding affinity of human haemoglobin is called 2,3-bisphosphoglyceric acid (2,3-BPG) and is found in red blood cells (RBCs). 2,3-BPG facilitates the release of oxygen by RBCs in tissue that needs it the most and can help prevent tissue hypoxia in conditions where it is likely to occur, such as at high altitude (Biolo et al. 2009). It has been shown that people acclimated to high altitudes have higher levels of 2,3-BPG and the same is true in pregnant women, in whom the decreased haemoglobin affinity for oxygen allows oxygen to be delivered to the growing foetus through the maternal uterine arteries where the oxygen pressure is low (Pritlove et al. 2006). Utilizing this kind of natural mechanism that already exists in the human body has been further explored in cancer research where the hypothesis has been that restoring normoxia in the tumour

will lead to halted tumour growth. Experiments have shown that using the synthesised drug inositol trispyrophosphate (ITPP) leads to increased oxygen delivery to the tumour and reduces tumour growth, thus resulting in a more favourable outcome in animal models with pancreatic cancer (Raykov et al. 2014) and liver cancer (Aprahamian et al. 2011). Interestingly, the same results have been obtained in a model with human U87 glioblastoma cells grafted and implanted onto chick chorioallantoic membrane (Sihn et al. 2007).

In yet another study it was described that ITPP treatment reduces the size of malignant melanoma in mice and leads to diminished metastatic invasion, resulting in increased survival compared to control animals (Kieda et al. 2013). The exact mechanism of action of ITPP is suggested to be by restoring normoxia in the tumour through the regulation of hypoxia-inducible factors (HIFs). The hypoxic state usually seen in GBM activates the immunosuppressive STAT3 pathway, leading to hypoxia-inducible factor-1 alpha (HIF-1α) synthesis, activation of regulatory T cells (Treg) and production of vascular endothelial growth factor (VEGF) (Brown et al. 2018). ITPP reduces HIF1α mRNA expression and HIF1α is associated with a higher number of blood vessels, tumour grade severity and treatment failure (Kieda et al. 2013). Some hypoxic tumour cells are even thought to be resistant to radiotherapy and chemotherapy through HIFs and hence counteracting hypoxia in the tumour could improve the results of radiotherapy.

Radiological characteristics

When patients present with acute neurological symptoms due to sudden changes such as for instance bleeding in the tumour a computed tomography (CT) scan is performed. This can reveal mass effects related to GBM. Magnetic resonance imaging (MRI) is needed for further radiological characterization and for preoperative planning and postoperative control. Thus, MRI is the gold standard when it comes to GBM patients, even if techniques such as amino acid positron emission tomography (PET) imaging can be used to follow the metabolism in the tumour.

Usually there are two MRI images acquired, T1-weighted or T2-weighted, which depend on the time between the radiofrequency pulse and the image acquisition, and respectively highlight tissue with a high fat content (such as for instance white matter in the brain) and a high water content (such as for instance cerebrospinal fluid). Different contrast agents are frequently used in radiology, and when it comes to MRI they are often based on chelated gadolinium. Glioblastomas often show a highlighted irregular border on contrast enhanced T1 imaging, surrounding a central non-enhancing tumour core, as a result of central tumour necrosis (Abd-Elghany et al. 2019).

Other relevant imaging series in neuro-oncology include diffusion-weighted imaging (DWI), diffusion tensor imaging (DTI), perfusion-weighted imaging, magnetic resonance spectroscopy (MRS), and functional magnetic resonance imaging (fMRI), which together allow for evaluation of both the anatomy and physiology of tumours in addition to structural and phenotypic assessment (Iv et al. 2018). Examples of applications include differentiation between tumour and abscess formation. Conventional MRI techniques cannot readily be used to distinguish necrotic tumours from brain abscesses and DWI can be of aid in these situations together with apparent diffusion coefficient (ADC) (Chang et al. 2002). DTI on the other hand is used clinically mostly for presurgical planning and intraoperative guidance (Iv et al. 2018).

When it comes to the specific radiological characteristics of gliomas, MRI using fluid attenuation inversion recovery (FLAIR) can visualise the peritumoral oedema surrounding the tumour (Iv et al. 2018). In non-infiltrative, nonglial tumours such as metastatic disease, non-enhancing hyperintense T2/FLAIR signal surrounding a tumour core is essentially synonymous with oedema, whereas in infiltrative tumours such as gliomas, the peritumoral non-enhancing hyperintense T2/FLAIR signal often consists of both oedema and infiltrating tumour cells (Iv et al. 2018). Interestingly, information about the molecular alterations of the tumours can be linked to specific radiological findings. One such example is the T2-FLAIR mismatch sign, which is characterized by a hyperintense signal on the T2-weighted sequences paired with a hypointense signal on the FLAIR sequences combined with a hyperintense peripheral rim (Corell et al. 2020). The T2-FLAIR mismatch sign has been validated as a reliable and specific marker of *IDH* mutation in astrocytomas.

It is well known that radiation therapy (RT) and chemotherapy can alter the blood-brain barrier, leading to increased vascular permeability and increased contrast enhancement even in the absence of tumour (Hojjati et al. 2018). Therefore, fractioning of the radiation is a means to allow normal brain tissue to recover between RT treatment sessions. In the GBM setting, novel targeted therapies, immunotherapies, and anti-angiogenic therapies have created the need for even more reliable neuroimaging techniques to differentiate the effects of therapy from tumour progression (Lieberman 2017). As it stands today, up to 30% of glioblastoma patients develop treatment-related injury or radiation necrosis that can mimic tumour recurrence by conventional MRI (Hojjati et al. 2018). Patients treated with chemoradiation for newly diagnosed GBM may have transient worsening in MRI findings in the first post radiation therapy follow up MRI. This phenomenon has been termed pseudoprogression and cannot be distinguished from true progression using only routine clinical MRI criteria (Lieberman 2017). In these cases, perfusion MRI can be used to differentiate between progression, pseudoprogression and pseudoresponse (van Dijken et al. 2019). Pseudoresponse can be seen in cases with antiangiogenic treatment and is defined as a decrease of

enhancement on postcontrast MRI, while the tumour in fact remains stable or even increases. Pseudoprogression typically occurs within three months after treatment termination (van Dijken et al. 2019). In 2010 the Response assessment in neuro-oncology (RANO) criteria were published, which are used to assess treatment response in GBM by using MRI and clinical information such as the use of corticosteroids. In 2015 the RANO criteria were modified to include immunotherapy response and accommodate for pseudoprogression, hence the name iRANO, short for immunotherapy response assessment for neuro-oncology (van Dijken et al. 2019). Although the use of perfusion MRI is of great aid in the clinic and widely used, currently, there is no consensus regarding which technique or combinations of techniques that have the best ability to address the diagnostic challenge of distinguishing between different post treatment responses in GBM (Hojjati et al. 2018).

Current treatment regimes

Currently glioma patients undergo both surgery, radiation therapy and oncological treatment, so there are different ways to approach the situation, but one must not forget that far from all patients can actually benefit from surgery. This can be due to the location of the tumour, the KPS score or the fact that the patient is too fragile due to other medical conditions, which makes surgical resection impossible (Müller et al. 2019). Most of these patients undergo biopsy instead and are left with radiation therapy (if possible) and chemotherapy, often using Temozolomide (TMZ). Müller et al. 2019 found a biopsy rate between 20-40% with biopsy being the preferred choice for tumours growing in the thalamus, internal capsule, splenium of the corpus callosum, and periventricular region around the occipital horns.

Surgery is ideally performed with the guidance of 5-aminolevulinic acid (5-ALA) administered preoperatively, which makes the tumour fluoresce in blue light and thus improves the extent of the resection (Stummer et al. 2006). The mechanism of action is the conversion of 5-ALA into the fluorescent metabolite, protoporphyrin IX, once it has been taken up by glioblastoma cells. Elevated protoporphyrin IX permits violet-red fluorescence visualization of malignant tumour tissue after excitation with 405 nm wavelength blue light. The preferential accumulation of 5-ALA in glioblastoma cells is due to the decreased levels of ferrochelatase (Hadjipanayis et al. 2015). Additionally, other techniques can be used when called for, such as intraoperative nerve monitoring and awake surgery, which maximize the extent of resection whilst avoiding damage to vital brain functions (Hervey-Jumper, et al. 2015).

Unfortunately, recurrence is something that needs to be taken into consideration and second surgery should be considered in patient with recurrence of a surgically

resectable tumour, given that the patient is in a good general condition and preferably that additional oncological treatment is available post-operatively, but currently no consensus exists regarding the management of recurrent GBM.

In 2005 Temozolomide (TMZ) was approved by the Food and Drug Administration (FDA) for treating GBM. It is most often given as adjuvant therapy for six months at a concentration of 150–200 mg/m^2 (Lieberman 2017).

When it comes to radiation therapy, standard care for newly diagnosed patients after surgery comprises fractionated RT in the form of 60 Gy in 2 Gy fractions over five to six weeks with concomitant TMZ. As patients over age 70 are often not suitable candidates for full 60 Gy radiation therapy, some elderly patients receive hypofractionated radiation therapy (3.4 Gy x 10 days) in addition to chemotherapy. Hypofractionated radiotherapy uses higher daily doses, which reduces the overall number of fractions and treatment time and it has been shown that hypofractionated radiation therapy has similar efficacy for survival as compared to conventional radiotherapy, particularly for individuals aged 60 and older (Khan et al. 2020).

In addition, other forms of precision radiation treatment exist, such as stereotactic radiosurgery including the so called gamma knife, or proton beam therapy.

Novel and future types of treatment

There are different types of novel treatments currently being investigated based on molecularly targeted therapeutics, personalized therapy using molecular profiling of individual tumours, and immunotherapeutic strategies (Lieberman 2017). Several previously described molecular markers such as MGMT, EGFR, IDH, 1p19q, ATRX, and TERT are now being used to classify brain tumours as well as influence management decisions in the clinic. Some of these markers are also being used as therapeutic targets in research trials (Bush and Butkowski 2017). Examples include EGFR tyrosine kinase inhibitors and VEGF receptor inhibitors.

Although most studies with EGFR tyrosine kinase inhibitors, such as erlotinib and gefitinib, have been negative, retrospective studies of outcome suggest that a subgroup of tumours with EGFR mutation and wild-type PTEN do in fact respond. Clinical trials for recurrent GBM using a bifunctional antibody targeting EGFR and a microtubule-disrupting agent have been completed, and a study adding this agent to the standard therapy is ongoing with preliminary results expected in December 2020 (Lieberman 2017).

Lomustine (also termed chloroethyl-cyclohexyl-nitrosourea or CCNU) is an alkylating agent of the nitrosourea family increasingly used in recurrent glioblastoma. Activity is largely restricted to patients with tumours with MGMT promoter methylation, since MGMT reverts one of the most relevant lesions

induced by lomustine, the formation of O^6-chloroethylguanine (Weller and Le Rhun 2020). Lomustine is lipid-soluble, which means that it is able to pass the blood brain barrier well, which *a priori* made it a reasonable candidate for the chemotherapy of intrinsic brain tumours. It is also administered orally, which is another benefit, but unfortunately haematological toxicity, most notably thrombocytopenia, often limits adequate exposure (Weller and Le Rhun 2020).

Current vaccine strategies include autologous vaccines generated from the patient's tumour at resection, peptide-based vaccines, and a new generation of vaccines using dendritic cells exposed to tumour cell RNA (Lieberman 2017). An example of the latter is vaccination against autologous cancer stem cells in glioblastoma using mRNA-transfected dendritic cells (Vik-Mo et al. 2013). This type of active immunotherapy aims to stimulate or amplify the patient's own immune response where the antigen presenting cells, often dendritic cells, are used to express the target antigen (Lampson 2013). It makes use of the underlying situation in GBM, where dendritic cells are thought to present tumour cell peptides to T cells, leading to a cytotoxic T cell response and secretion of pro-inflammatory cytokines (DeCordova et al. 2020).

Viruses can also be used in GBM therapy trials, either added as viral vectors, in which the vector encodes immunomodulatory molecular signals, or completely blocked by antiviral drugs. One such example is the administration of the antiviral drug valganciclovir to glioblastoma patients as an add on to standard therapy based on the theory that cytomegalovirus is present in glioblastoma and as such may represent a therapeutic target (Stragliotto et al. 2020).

It has turned out that GBM is very difficult to treat by trying to target the hypoxic state of the tumour or the abnormal vasculature. In recent years, much effort has been put into patient studies trying to find VEGF receptor inhibitors, but no effect upon survival has been demonstrated despite well-conducted studies with large patient cohorts (Weathers and de Groot 2015). Initially, bevacizumab (also called anti-VEGF or VEGF inhibitor) seemed to be promising, but subsequent clinical trial experience has been disappointing, where the addition of bevacizumab to chemoradiation and adjuvant temozolomide did not prolong overall survival (Lieberman 2017). Still, there is a place for bevacizumab in the clinic today with bevacizumab sometimes being able to ameliorate the radiation-induced worsening of oedema and mass effect seen in newly diagnosed GBM patients, and it has been approved by the FDA.

Glioblastoma in rats

Different rat models

Several rodent GBM tumour models have been used in experimental neuro-oncology since the mid-20th century. Examples include C6, 9L, T9, RG2, F98, BT4C, RT-2 and CNS-1 rat gliomas (Barth and Kaur 2009). These models have been used for exploration of tumour behaviour and for investigations of different treatment modalities.

One of the frequently used models, RG2, is an aggressive model with a short survival time (19.4±3.8 days) compared to other rodent models such as C6 or 9L, but also compared to U87, which is a human primary glioblastoma cell line (Aas et al. 1995). However, one of the major advantages of the RG2 model is the stable development of tumours and that it can be used in fully immunocompetent animals (Aas et al. 1995).

The C6 glioma model on the other hand, was chemically induced in an outbred strain of Wistar rats. As a consequence, inoculation of C6 cells into common Wistar rat strains resulted in an allogeneic immune response and thus lack of tumour growth (Lenting et al. 2017). The C6 cells share several tumour markers with human GBM, but differ in the expression profile of the tumour suppressor p53, coupled with reduced PTEN expression (Stylli et al. 2015).

The CNS-1 glioma cell line was established from a rat injected weekly with methyl-nitrosourea (MNU). It expresses several glioma markers, including glial fibrillary acidic protein (GFAP), whilst displaying a diffuse and infiltrative pattern of growth (Stylli et al. 2015).

The 9L and T9 gliomas are induced by repeated injections of MNU to adult Fischer rats, whereas the RG2 and F98 models are induced by an intravenous injection of ethyl-nitrosourea (ENU) into pregnant Fischer 344 rats, where the offspring then develop spontaneous gliomas (Aas et al. 1995). Resulting gliomas often carry mutations in oncogenes or tumour suppressor genes that are frequently encountered in human gliomas (Lenting at el. 2017).

The 9L glioma cell model has a sarcomatous appearance, does not express GFAP or show the diffuse infiltrative pattern of GBM cells, but it does however possess a

mutant p53 gene and EGFR overexpression as seen in many human GBM (Stylli et al. 2015).

A notable difference in the rat tumour models is the fact that T9 and 9L can be immunogenic in syngeneic hosts, whereas RG2 and F98 on the other hand are non-immunogenic in syngeneic Fischer rats. The immune competence status of the animals is important, especially when it comes to studies involving immune therapies. It is also crucial that the tumour model harbours the same genetic mutations as the human GBMs, otherwise treating random aberrations in the animal tumours could represent an effect of genomic instability and thus not represent worthwhile therapeutic targets. To summarize, glioma models need to comply with specific and more strict demands than other cancer models, and these demands are directly related to the combination of genetic alterations and the specific brain micro-environment (Lenting et al. 2017).

There are different ways of performing experiments which take the BBB and the brain micro-environment into consideration. In the rat models, different types of allografting of glioma cells can be performed: heterotopic (subcutaneous) or orthotopic (intracerebral). Using an orthotopic allotransplantation *in vivo* model though could lead to the difficulty of discriminating tumour cells from reactive cells in the vicinity of the tumour, detecting single migrating tumour cells far away from the main tumour mass, and to readily discern non-tumour cells invading the tumour.

Xenotransplantation

The term xeno means ‘foreign’ and xenotransplantation, or heterologous transplantation, refers to the transplantation of cells from one species to another. Subcutaneous xenografting of human glioma cells in immunocompromised mice is a frequently used approach to obtain preclinical proof of concept for the efficacy of targeted drugs (Lenting et al. 2017). A major drawback of these subcutaneous glioma xenograft models is the lack of appropriate CNS microenvironment, which is known to play an essential role in glioma biology. One way to accommodate for this is the use of orthotopic patient-derived xenografts in which freshly obtained surgical glioma samples are directly injected into the brains of immune-deficient mice or rats using stereotactic devices (Lenting et al. 2017). A drawback of these glioma models is that xenografting is performed in immune-deficient animals and therefore there will be a selection for the fastest growing cell clones resulting in reduced intratumor heterogeneity (Lenting et al. 2017).

The problem of rejection of human GBM xenografts in immune competent animals can be overcome by serial *in vivo* passaging in T cell compromised rats before transfer to immune competent rats (Huszthy et al. 2015) or by a transient blockade of T-cell co-stimulation in adult mice with intact immune systems at the time of

inoculation (Semenkow et al. 2017). A somewhat different approach in immunocompromised animals is also worth mentioning, where injecting T cells and other human immune cells or human hematopoetic progenitor cells into immune compromised mice leads to a situation where a lack of xenoreactivity can be established whilst human cells can be studied in the presence of a somewhat restored immune system, with reports of up to 40-60% of the human immune system components being reconstituted (Rämer et al. 2011). In the first example, human GBM cells somehow acquire the ability to avoid or suppress the host immunity and are subsequently able to engraft in immunocompetent rats without signs of an inflammatory response (Huszthy et al. 2015).

One considerable advantage using mice over rats is the access to transgenic mouse models and a plethora of knock-out models, such as for example p53 knock-out mice. Unfortunately, it is a lot more difficult to create these models in rats and knock-out rats have only been available for the last few years.

Cell culture and passages

While the immunological complexities induced by xenotransplantation have been acknowledged (Liu et al. 2011), the tendency of tumour cells grown *in vitro* to become progressively more homogenous seems often not to be considered (Stylli et al. 2015). It has been shown that the cells which have undergone sequential passage in the presence of animal serum do not show the classic appearance of human glioma and present genotypic and phenotypic deviations relative to the original cells (Stylli et al. 2015). Thus, they do not retain their original chromosomal profile and are less heterogeneous than the original tumour.

The rat glioma cell line RG2 is relatively old and has been through so many passages that it might have been altered due to decades of culturing, and therefore might not represent an adequate model for glioblastoma anymore. It is important that the animal models employed have clinical relevance. Parameters to be evaluated should include whether animals are immune competent or not, the infiltrative growth pattern of the tumour, growth rate, tumour volume resulting in symptoms and molecular characteristics.

The NS1 glioblastoma model

A syngeneic model

Considering all the difficulties and limitations of the rat glioblastoma models described above, we decided to develop our own rat glioblastoma model. Thus, the rat glioblastoma cell line NS1 is a new GFP positive tumour cell line created in our laboratory. It was initiated by treating pregnant homozygous GFP-positive Fischer 344 rats with ENU, where the offspring subsequently developed GFP-positive CNS-tumours. The NS-1 tumour cell line was established from an intra-parenchymal tumour growing in the offspring.

Since the cell line is intrinsically GFP-positive, the infiltrative growth pattern can be studied and monitored in depth when inoculated into other syngeneic rats and as such provides a fully immunocompetent animal model (figure 1 and figure 2).

The concept of utilizing GFP in brain tumour models is not new and has been profitably used before (Liu et al. 2011) but the NS1 model has the advantage that the tumour cells can be administered to fully immune competent animals without any *ex vivo* manipulation and thus tumour growth can be easily monitored and visualised. Additionally, the NS1 model displays and an infiltrative growth pattern and perivascular dissemination, which makes it an appropriate model for studying glioblastoma multiforme.

Genetic mutations

Rats inoculated with NS1 cells, either intracranially or subcutaneously, develop cell-rich tumours with an invasive growth pattern that can easily be tracked since the tumour cells express GFP. The tumours are positive for GFAP and the tumour cells also show RNA expression of wt IDH1, wt p53, IDO1 and EGFR.

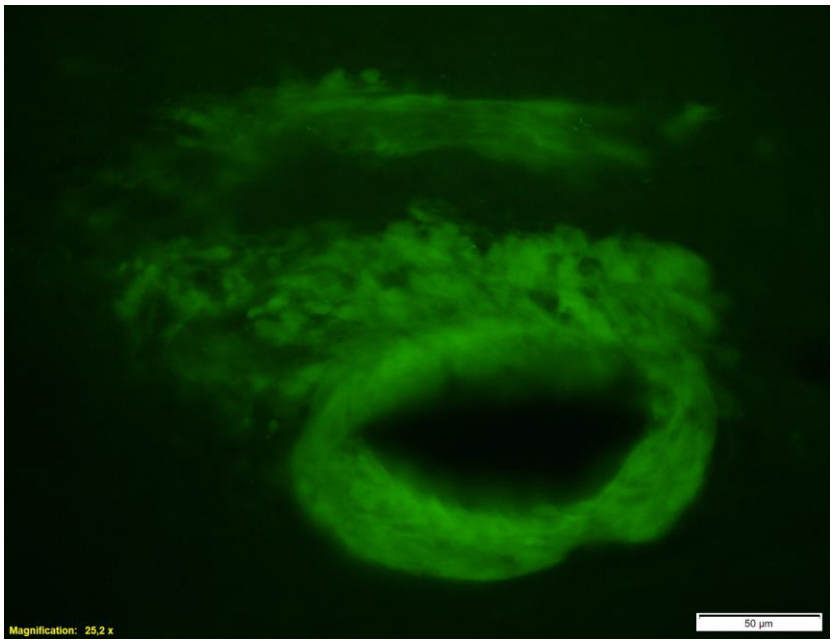

Figure 1. Autofluorescence of GFP-positive NS1 cells inoculated into the brain of syngeneic Fischer 344 rats showing perivascular growth of the tumour.

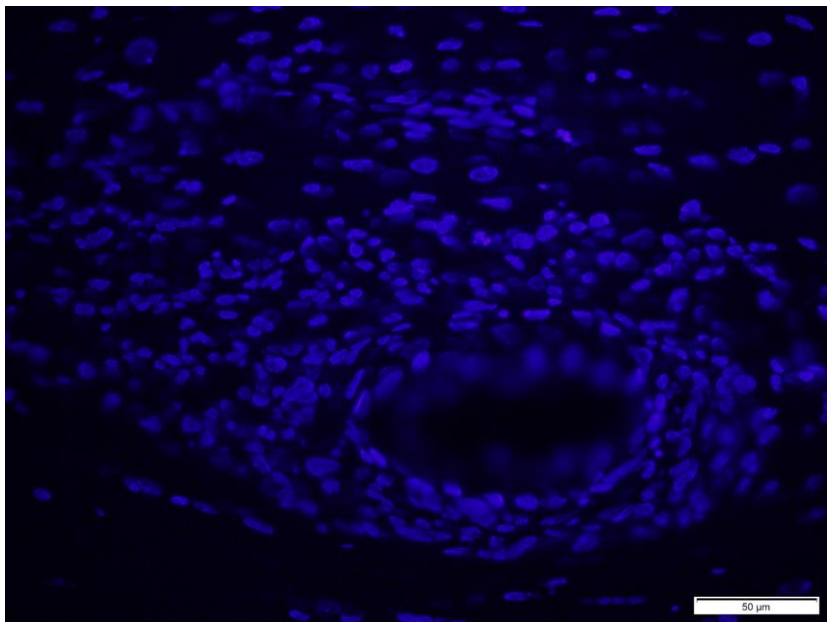

Figure 2. DAPI-staining of the corresponding section as seen in figure 1. When compared one is able to distinguish the green NS1 cells from normal brain cells.

Alterations in the immune system

Innate and adaptive immune mechanisms

Glioblastoma has the capacity to inactivate the body's immune response and tumour-induced immunosuppression in the immediate tumour microenvironment is often seen (Lieberman 2017, DeCordova et al. 2020). Tumour development should otherwise be under constant control by the immune system that recognizes cancer cells as potential threats and mounts a response leading to local recruitment of effector cells of both innate and adaptive immunity. Recently much attention has been drawn to the T-cell dependent immune response, where inactivation of CD8+ cytotoxic T-cells and increase in regulatory T-cells (Treg) has been observed. Although cell-mediated immunity has long been recognized to play a critical role in tumour eradication, the complement system is also important in cancer immune surveillance (Macor et al. 2018).

In the brain, microglia harbour the role of antigen presenters, and together with infiltrating immune cells, mostly tumour-associated macrophages (TAMs), comprise much of the non-neoplastic population in GBM, up to 30% of the tumour mass (Brown et al. 2018, DeCordova et al. 2020). GBM cells are also known to release interleukin-10 (IL-10), and as such promote tumour growth and downregulation of antigen presentation by downregulating expression of major histocompatibility complex (MHC) (Brown et al. 2018). Antigen presentation can also occur though as dendritic cells (DCs) travel outside the brain and present antigens to T-cells located in the cervical lymph nodes (CLN) (DeCordova et al. 2020).

CD4+ helper T cells can be subdivided into T helper type 1 (Th1) cells and T helper type 2 (Th2) cells. Th1 cells promote cell-mediated immune responses against intracellular pathogens, whereas Th2 cells are involved in humoral immunity. The CLN are important draining lymph nodes for the brain parenchyma and favour a Th2 response, which dampens the cytotoxic T lymphocyte response (Lampson 2013). In 2003, the forkhead box transcription factor FoxP3 was identified as a new specific marker of Treg cells and FoxP3+ regulatory T cells are critical in maintaining immune tolerance. For this reason, the regulatory T cells have also been termed "suppressor" T cells in the setting of tumour biology (Li et al. 2015).

Glioblastomas have an immunosuppressive role

Just like many other types of tumours, glioblastoma induces a tumour microenvironment characterised by immunosuppressive cytokines secreted by the tumour cells themselves, but also by microglia and tumour-associated macrophages (TAMs). These cytokines, most notably IL-6, IL-10, transforming growth factor-beta (TGF-β), and prostaglandin-E, collectively inhibit both the innate and adaptive immune systems (Lampson 2013, Brown et al. 2018). TGF-β plays a key role in contributing to the immunosuppressive GBM microenvironment by inhibiting the activation of natural killer cells, downregulating IL-2 production, and promoting FoxP3+ regulatory T-cells (Treg) (DeCordova et al. 2020).

GBM also harbours the immunosuppressive signals programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1), cytotoxic T lymphocyte antigen 4 (CTLA-4), and indoleamine 2,3-dioxygenase 1 (IDO 1) (Lieberman 2017). IDO 1 is an inducible tryptophan catabolic enzyme which normally is not expressed at appreciable levels in the CNS but can rapidly become upregulated upon inflammation (Zhai et al. 2015). It is a target for drug development since it has been shown that IDO 1 is expressed in 96 % of malignant gliomas and expression levels correlate with overall patient survival (Zhai et al. 2015). CTLA-4 is expressed by FoxP3+ Treg cells and essential for immunological self-tolerance. This is highlighted in the fact that CTLA-4 deficient mice develop aggressive autoimmune disorders (Klocke et al. 2016). GBMs express PD-L1, which binds to PD-1, and promotes Treg activity (Zhai et al. 2015). It has also been shown that the loss of the *PTEN* tumour suppressor gene enhances the expression of PD-L1 on glioma cells (DeCordova et al. 2020). PD-1 inhibitors and CTLA-4 inhibitors have been FDA approved for clinical trials involving malignant melanoma and non-small-cell lung cancer, and are currently being investigated in glioblastoma research as well, although so far without positive outcome (Lieberman 2017).

Microglia also release the pro-inflammatory cytokines IL-1β and TNF-α, which have been shown to increase tumour invasiveness *in vitro* (DeCordova et al. 2020). Regarding IL-1β it has been described that it is also released by glioblastoma cells themselves and that it induces changes affecting the glioma microenvironment in favour of increased migration and angiogenesis (Tarassishin et al. 2014*a*, Tarassishin et al. 2014*b*).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a monomeric glycoprotein secreted by macrophages, T cells, natural killer cells, endothelial cells, fibroblasts and glioblastoma cells themselves (Kohanbash et al. 2013). Any damage to brain tissue tends to cause an increase in GM-CSF and GM-CSF synthesized by the brain due to damage by a growing tumour and by the tumour itself stimulates the bone marrow to shift lineages in haematopoiesis, which is immunosuppressive and generates the relative lymphopenia characteristic of GBM (Kast et al. 2017).

The complement system

Three different pathways

Complement was discovered in the 1890s by Jules Bordet as a non-specific factor that was required along with antibodies in the serum to cause lysis of bacterial cells (Singh 2008). Since then is has been established that the human complement system is composed of over 30 soluble as well as membrane-associated proteins and comprises three biochemical pathways; the classical, the alternative, and the lectin induced pathways. Under normal conditions the complement system acts as a functional bridge between the innate and the adaptive immune responses (Carrol 2004) and activation of complement opsonises target pathogens or altered-self cells for phagocytic uptake, inducing an inflammatory response (DeCordova et al. 2020). There is growing evidence of the involvement of complement in tumour progression where deposits of complement components have been documented in several human tumours (Macor et al. 2018).

The classical pathway is predominantly activated by IgM or IgG antibodies binding to specific antigens and thus plays an important role in adaptive immunity, whereas the alternative pathway on the other hand is triggered in an antigen-independent manner by substances found on bacteria, viruses and other pathogens, thus having an important role in innate immunity (Singh 2008). The lectin pathway is also a part of the innate immune system since it is also antigen-independent and initiated by mannans, terminal mannose or N-acetyl glucosamine found on bacterial surfaces. These three pathways all converge at the level of C3 convertase and proceed to a common terminal pathway that leads to the formation of the membrane attack complex (MAC) that lyses the membranes of the foreign cells (Singh 2008).

The classical pathway is initiated by activation of the C1 complex, consisting of one molecule of C1q, two molecules of C1r and two molecules of C1s. Human pentameric IgM, IgG1, and IgG3 are known to be the most effective complement activators whereas IgG4 fails to bind C1q (Merle et al. 2015). IgA, IgD, IgE do not bind C1q and thus cannot activate the classical pathway. Structural studies have shown that C1q is a hexamer in the shape of a bouquet of tulips and binds to the Fc region of the antibody (Singh 2008).

Subsequently activated C1s in the C1 complex cleaves C4 into C4a and C4b, which together with C2a makes up the C3 convertase of the classical pathway, i.e. C4b2a,

bound to the non-self surface. The C5 convertase is then made up of C4b2a3b after the cleavage of C3 into C3a and C3b. The terminal or lytic components are C5b, C6, C7, and C8 that together make up MAC by inserting yet another three to six molecules of C9 into the membrane of the non-self cells (figure 3).

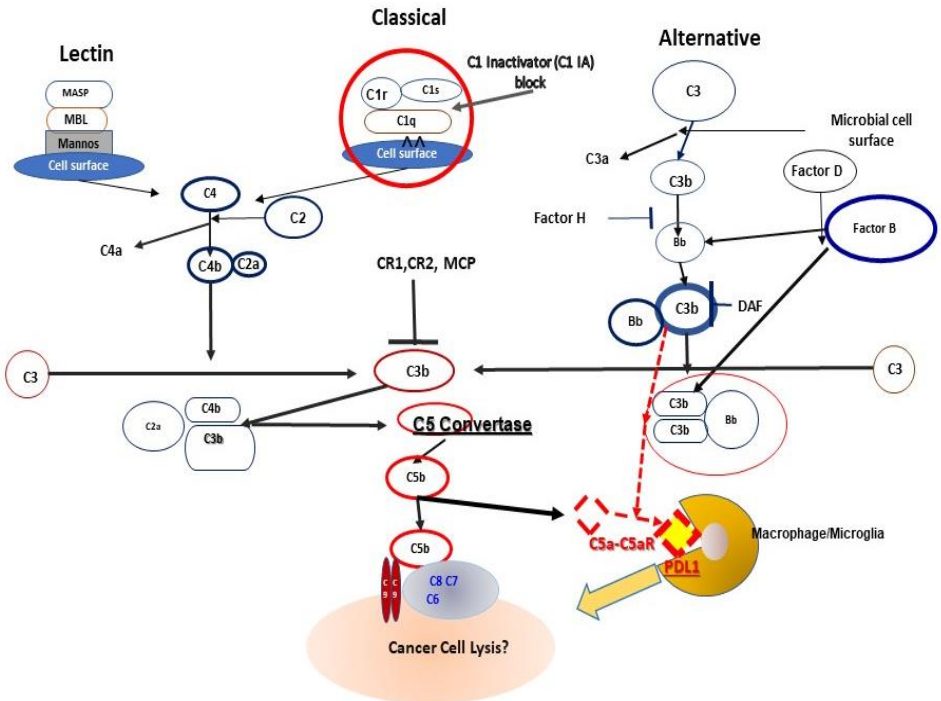

Figure 3. Overview of the complement system. Original picture kindly provided by K. Osther.

The classical pathway can be activated on cancer cells by IgM antibodies that recognize and bind to carbohydrate moieties on cell surfaces. In a similar manner expression of tumour-associated molecules on cells undergoing malignant transformation can lead to complement activation on the cell surface by the other two pathways. The lectin pathway has been implicated in complement activation on glioma cells which express high mannose glycopeptides that bind mannan-binding lectin (MBL) and trigger consumption of C4 and C3 (Fujita et al. 1995).

C3 is the most abundant complement component with a serum concentration of 1-2 mg/ml (Singh 2008). Most complement components are synthesized in the liver, but complement components can also be synthesized locally by resident and recruited cells including fibroblasts, endothelial cells, tissue specific cells, and macrophages, and are then released into the tumour microenvironment (Macor et al. 2018).

Although C3 activation is not limited to the extracellular space since intracellular C3 activation is an ubiquitous event within human cells (Bouwens van der Vlis et al. 2018).

The alternative pathway includes factors B, D and properdin where the activated form of factor B is called Bb (Singh 2008). C3b marks the start of the pathway and together with its derivatives C3d and C3dg all act as opsonins. Thus, the C3 convertase of the alternative pathway is C3bBb and the C5 convertase is C3bBb3b (figure 3). The C5 convertase is stabilized by properdin on the non-self surface.

The components of the lectin pathway are MBL, ficolins and the MBL-associated serine proteinases MASP-1 and MASP-2 (figure 3). MBL and C1q both belong to the collectin protein family whereas MASP-1 and MASP-2 are homologues of C1r and C1s (Singh 2008).

The complement system and inflammation

Inflammation is another important factor in glioblastoma pathophysiology, where the complement, clotting and kallikrein pathways are interlinked, and are all involved in inflammatory processes (Singh 2008). Local changes in the tumour tissue due to necrosis, apoptosis and inflammation, are responsible for complement activation (Macor et al. 2018).

It is known that inflammation itself and various types of diseases in the CNS can compromise the integrity of the blood-brain barrier, thereby enabling circulating immune cells to pass it and infiltrate the parenchyma (DeCordova et al. 2020). Immunoglobulins can also cross the blood-brain barrier via carrier mediated transporters by attaching to the FcRn receptor (DeCordova et al. 2020).

It is also known that the complement system regulates B cell responses through complement regulatory proteins belonging to the group called regulators of complement activation (RCA). The RCA group contains both membrane bound proteins such as complement receptor 1 (CR1, also known as CD35), complement receptor 2 (CR2, also known as CD21 or C3d receptor), membrane co-factor protein (MCP) and decay accelerating factor (DAF, also known as CD55), as well as plasma proteins such as factor H and C4b-binding protein (Seya 1995). DAF is a surface glycoprotein expressed on all peripheral blood cells and it prevents the assembly of the C3- and C5-covertases (Singh 2008). CR1 and CR2 on the other hand are found on follicular dendritic cells, which can retain C3 coated immune complexes within lymphoid follicles, thus providing a second mechanism for B cell immune response (Singh 2008).

GBM has made use of some these RCA proteins to avoid complement-mediated killing (DeCordova et al. 2020). Examples include DAF, MCP, factor H and

protectin (also known as CD59), as well as C1 inhibitor (C1-INH). Factor H is an important soluble regulator of the alternative pathway, since it competes with factor B, thus preventing the formation of the C3 convertase C3bBb, whereas CD59 binds to the C5b-8 complex and inhibits the formation of MAC at the point of C9 insertion into the GBM cell membrane (DeCordova et al. 2020).

There are three anaphylatoxins produced in the complement cascade and they can all start inflammation: C3a, C4a and C5a. C3a binds to mast cells and basophils which release histamine and other mediators (Singh 2008). Although C5a is more potent than C3a and has histamine-induced effect on vascular endothelium causing vascular permeability and degranulation of granulocytes. Binding of the anaphylatoxins to their receptors also induces multiple effects on myeloid cells such as chemotaxis for granulocytes and monocytes and upregulation of β-2 integrins (Singh 2008). C3a and C5a signal through their respective G protein coupled receptors: C3aR and C5aR (figure 3). It is thought that interaction of several downstream signal transduction pathways following C3aR and C5aR activation may aid GSC maintenance (Bouwens van der Vlis et al. 2018).

Links to the coagulation system

Both the complement system and tissue factor (TF) are activated in several serious conditions such as cancer and sepsis, and cross-talk occurs between the complement and coagulation systems (Landsem et al. 2013). TF is a transmembrane glycoprotein and the key initiating component of coagulation. Together with its co-factor, factor VIIa, it makes up the TF/FVIIa complex, which subsequently catalyses the production of factors IXa and Xa during the initiation phase of coagulation (Mackman 2009). FVII is formed upon autocatalysis and activation by other coagulation enzymes (Mackman 2009).

It is well known that GBM are associated with a high risk (10–30%) of peripheral venous thromboembolism, including deep vein thrombosis and pulmonary embolism (Magnus et al. 2013). Previously TF-bearing microparticles had been proposed to be the circulating procoagulant responsible for these thromboembolisms, but this has been questioned (Magnus et al. 2013). Several of the cascade systems are involved in the intravascular innate immune system: complement, contact, coagulation, and fibrinolytic systems, and activation of the intravascular innate immune system *in vivo* leads to thromboinflammation that can be activated by several of the system's pathways (Ekdahl et al. 2016). Especially the lectin pathway of the complement system is an emerging part of innate immunity that interacts with the contact/coagulation system (Ekdahl et al. 2016).

C1-inhibitor

The C1 inhibitor (C1-INH) is also denoted C1-IA (complement1-inactivator), C1 esterase inhibitor or serpin family G member 1, where serpin is short for serine protease inhibitor. C1-INH can act as a regulator in both the complement and the coagulation systems, and is also known to inhibit proteases of the fibrinolytic and kinin pathways (Landsem et al. 2013). Additionally, it is the most important physiological inhibitor of kallikrein, factor XIa, and factor XIIa, thus inhibiting all the enzymes of factor XII-dependent pathways which also include plasmin. C1-INH inhibition of kallikrein plays a direct role in blood pressure regulation through the production of the vasoactive peptide bradykinin in the kinin-kallikrein system (Singh 2008).

In the classical complement pathway C1-INH inactivates the pathway by irreversibly binding to the C1r and C1s proteases in the C1 complex. It is the only known physiological inhibitor of C1r and C1s. C1-INH also inactivates MASP-1 and MASP-2 since they are homologues of C1r and C1s (Singh 2008).

C1-INH is highly glycosylated, bearing both N- and O-glycans and its inhibitory activity is located in the C domain. In our experiments we could see that antibodies targeted against human C1-INH and rat C1-INH could both bind to rat glioma cells. It is known that the C1-INH protein is quite conserved between the two species with a sequence identity of 79%, which further strengthens the theory that it plays a very important role in the immune response. It has also been shown that C1-INH efficiently reduced the production of several *E. coli*-induced cytokines in both human and porcine whole blood, and that the anti-inflammatory effects were largely independent of the protease inhibitory activity of C1-INH and instead due to the fact that C1-INH is a multifunctional molecule interacting with a number of non-complement related proteins participating in the inflammatory response (Thorgersen et al. 2010).

In 1974 it was shown that malignant primary and secondary brain tumours were coated with a protein, immunologically indistinguishable from C1-INH (Osther et al. 1974). The experiments were performed by immunofluorescence cytophotometry of individual cells and we have further explored this in the glioblastoma setting.

C1-inhibitor in other types of cancer

Apart from GBM we have previously shown that C1-INH is upregulated in pancreatic cancer (Osther et al. 2019). Studies have also found that patients suffering from various malignant diseases, both solid tumours and hematologic cancers, have

significantly higher C1-INH plasma concentrations than healthy controls (Starcevic et al. 1991). The complement components C3 and C4 have been more extensively studied than C1-INH in the context of cancer, and both C3 and C4 are elevated in several different types of cancer (Pio et al. 2014).

Deficiencies of complement components

There are several diseases associated with complement deficiency, which can be both inherited and acquired. Deficiencies of complement components lead to reduced capacity of the affected host to fight off infections, especially bacterial infections, and are linked to autoimmune disorders (Singh 2008). Examples include C3 deficiency which can be life threatening due to severe bacterial infections, and deficiencies in C1, C2 or C4 which are linked to the autoimmune disease systemic lupus erythematosus (SLE).

SLE patients can have autoantibodies against different complement components. Examples include anti-C1q antibodies, which are indicative of severe disease and strongly associated with consumptive hypocomplementemia and lupus nephritis. Anti-C1q antibodies have high diagnostic specificity and are often found alongside anti-double stranded DNA antibodies (Hristova and Stoyanova 2017). It has also been found that MBL deficiency predisposes to SLE, which can be attributed to the presence of anti-MBL autoantibodies. Other autoantibodies include anti-C3 antibodies, which are thought to bind to C3b as well as C3 (Hristova and Stoyanova 2017).

Hereditary angioedema is an autosomal-dominant deficiency of C1 inhibitor. Symptoms include amongst others swelling of the arms, face and airways. Quantitative or qualitative deficiency of C1 inhibitor leads to the generation of vasoactive mediators, such as bradykinin, which promotes swelling (Bracho 2005). In some patients with congenital C1-INH deficiency both plasma C1-INH functional activity and concentration are decreased, whereas in other patients only C1-INH activity is decreased, with normal or elevated concentrations (Starcevic et al. 1991).

When it comes to treatment, C1 inhibitor concentrates and fresh frozen plasma are available for acute intervention together with airway monitoring and pain relief. New treatment agents include recombinant C1 inhibitor, bradykinin inhibitors and kallikrein inhibitors, such as the FDA-approved drug Ecallantide (Bracho 2005).

C-reactive protein

Conserved across species

The name C-reactive protein (CRP) comes from its ability to precipitate pneumococcal C-polysaccharide and the structure of the protein is a characteristic pentamer in both humans and rats. CRP is an evolutionarily conserved protein and has been found in every organism examined so far (Pathak and Agrawal 2019). Although CRP is an acute phase protein in humans, this is not the fact in rats where the normal concentrations typically range between 0.3–0.5 mg/ml (Pathak and Agrawal 2019).

Marker for inflammation

Elevated CRP levels is a phenomenon seen in many patients with glioblastoma (Nijaguna et al. 2015) and recent studies have shown that CRP measured preoperatively in GBM patients can be used as an independent predictor of GBM outcome (Pierscianek et al. 2020). Kaplan-Meier survival plots confirmed a significantly poorer overall survival in patients with CRP at or above 2.9 mg/dl (Pierscianek et al. 2020). In pancreatic cancer it has been shown that a certain genotype results in an increase in IL-1β production, which was also associated with shortened survival and increased serum CRP levels (Barber et al. 2000).

CRP binds to the surface of damaged cells and surfacebound CRP recruits C1q, which activates the classical complement pathway (Mihlan et al. 2009). CRP bound to a ligand can thus initiate the function of the C3 convertase and plays a major role in the defence against bacterial pathogens and clearance of apoptotic and necrotic cells (Volanakis 2001).

CRP is a pentraxin and shares several structural and functional properties with pentraxin 3 (PTX3) (Pathak and Agrawal 2019). Just like CRP, PTX3 also binds to C1q (Ekdahl et al. 2016). PTX3 is produced by several cells, including monocytes and DCs, and does in fact not bind to any classical pentraxin ligands other than C1q (Landsem et al. 2013). It has been shown that PTX3 expression is elevated in GBM and that it correlates with survival outcome (Wang et al. 2020).

How to move on from here

Blood Brain Barrier

In other types of cancer such as breast cancer or pancreatic cancer, novel treatment options can be readily tested and explored, whereas in the glioblastoma setting the presence of the blood brain barrier (BBB) needs to be taken into consideration. The BBB exerts strict control over the passage of substances into the brain and is designed to protect the neural tissue from toxic substances. Although the BBB is partially disrupted around malignant tumours in the brain, which leads to an increase in permeability, effectively allowing immune cells to enter the tumour, as well as contrast agents such as gadolinium used in MRI.

Apart from creating a physical barrier, the endothelial cells of the BBB express several different P-glycoproteins, which makes the BBB impermeable to the vast majority of drugs (Lenting et al. 2017).

Several different approaches to transiently disrupting the BBB have been explored in the context of potential drug delivery. There are studies reporting for example nanoparticles penetrating the BBB in healthy brain when delivered by convection-enhanced delivery (Saucier-Sawyer et al. 2016).

The use of devices such as an Ommaya reservoir could also represent a surgical way of intratumoral treatment and is sometimes used for intrathecal drug administration in the clinic (with the tip placed in the ventricular space).

Choice of journals

Publishing our work in journals that provide open access has been an active measure on our behalf since we think that it is of uttermost importance that the research is easily accessible and free of charge. This is also the thought behind the choice to publish ’negative results’. GBM is such a devastating disease and the research community should strive to collaborate rather than to compete.

Methods and techniques used

Cell culture

Rat glioma cells (NS1 and RG2) were cultured using RPMI-1640 medium with the addition of sodium pyruvate, HEPES, gentamycin, and inactivated fetal calf serum. After culturing, the cells were prepared for inoculation by removal of the medium and washed gently with PBS. Trypsin was added in order to detach the adherent cells from the flask. Additional medium was added and viable cells were counted. The cells were subsequently centrifuged and the supernatant was carefully removed to avoid any potentially immunogenic calf serum. Afterwards the cell pellet was re-suspended in serum-free medium and adjusted to achieve the desired concentration used for inoculation.

Human glioma cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with fetal calf serum, streptomycin, and sodium pyruvate. Three primary glioma cell cultures were used (AMN, DZ and GA) and the cells were regularly phenotyped.

Tumour inoculation

Two types of inoculations were performed, either subcutaneous or intracranial. In the subcutaneous model animals were inoculated with NS1 or RG2 glioblastoma cells subcutaneously on their right hind leg, performed under isofluorane inhalation anaesthesia.

The intracranial inoculations were performed using a stereotactic frame. The rats were sedated with isofluorane inhalation during the whole procedure. A hole was drilled 2 mm laterally to the sagittal suture, at the level of the coronal suture on the right side. NS1 or RG2 cells were then inoculated at a depth of 5 mm from the skull corresponding to the location of the caudate nucleus. To establish intracranial tumours, the required number of cells were suspended in 5 µl of serum-free medium. The suspension was injected at a pace of 1 µl/min, with a 5-minute pause before retraction at a pace of 1 mm/min. The cranial burr hole was sealed with bone wax and the wound sealed with resorbable suture.

The NS1 cells demonstrated GFP fluorescence prior to inoculation and the detection of GFP in tumour cells was evaluated *in vitro* using a fluorescence microscope.

ITPP treatment

The rats were administered ITPP (kindly provided by Professor Jean-Marie Lehn) either through an intraperitoneal route, an intravenous route or both routes combined. The drug was administered on days 7, 8, 15, 16, 21, 22, 28 and 29 after tumour cell inoculation using a dose of 1.5 g/kg since this dose had previously been used in models of pancreatic, melanoma and prostate cancer. Treatment was to be cancelled if any of the following occurred: neurological deficits, impaired general condition or a tumour diameter of 3 cm regarding the subcutaneous tumours. If none of these criteria were met, the rats would be observed for a total of 100 days. After 100 days, the rats would be euthanized.

Antibody treatment

Different types of antibodies were used. For the intratumoral experiments treatment was achieved using rabbit anti-rat C1 inhibitor (Covance, USA). The antibody was dissolved in PBS at a concentration of 6 mg/ml.

Antibodies were also used to coat NS1 cells before inoculation. These experiments consisted of pre-treatment with either anti-C1-INH or anti-CRP antibodies in the following manner: tumour cells were either incubated with rabbit anti-human C1-INH antibodies to coat the cells (anti-human antibodies are known to cross react with rat) or with rabbit anti-rat C1-INH antibodies, for two hours at 37˚C directly prior to intracranial inoculation. The same was done for the rabbit anti-human CRP antibodies and the rabbit anti-rat CRP antibodies. Control cells were not pre-treated with antibodies. Animals were examined and observed daily and were euthanized immediately if they started to show any neurological symptoms, upon which the brains were removed. The brains were fixed in isopenthane and cryosectioned, after which they were stained with hematoxylin/eosin (H/E).

Immunohistochemistry

Subcutaneous tumours isolated from the rats were fixed using phosphate-buffered paraformaldehyde. They were then paraffin embedded and sectioned using a

microtome. The tissue samples were incubated with rabbit anti-rat C1 inhibitor, then subsequently with secondary antibodies consisting of Alexa Fluor 594-conjugated goat anti-rabbit serum.

Intracranial tumours from rats were obtained from apparently still healthy, normally functioning animals, which were sacrificed under deep carbondioxide anaesthesia. The animals were first perfused with PBS at 4°C followed by formaldehyde with the same temperature. Brains were dissected and then cut into 3-5 mm thick, coronal slabs, visually confirmed to contain the implanted tumour. Tissue slabs were then embedded in paraffin and cut at 7 µm in the coronal plane. After being mounted on glass slides the sections were deparaffinized and every fifth stained with H/E. Individual sections were also incubated with anti-GFP antibodies.

Additionally, the presence of different types of T-cells was examined using antibodies against CD4, CD8 and FOXP3. This was done using frozen sections, which were serially cut into 40µm slices focused around the maximum tumour diameter in the coronal plane. Some sections were incubated with a rabbit antibody against GFP and subsequently treated with a biotinylated secondary antibody and ABC reagent. Oher sections were incubated with antibodies against CD4, CD8 and FOXP3 overnight and subsequently treated with a biotinylated secondary antibody and ABC reagent, using a ready-to use Vectastain ABC kit.

Human glioblastoma cells from three patients (AMN, DZ and GA) were also examined using immunohistochemistry. The cells were cultured for 1–2 days in two-chamber culture slides at 37˚C in a humidified 5% CO_2 incubator. The medium was carefully removed and the cells were fixed in paraformaldehyde. The cells were then washed and incubated overnight with either rabbit anti-human C1 inhibitor or rabbit anti-human CRP. The cells were subsequently incubated with secondary antibodies consisting of FITC-conjugated goat anti-rabbit. After washing with PBS, the cells were mounted with anti-fading vectashield mounting medium with DAPI, and photographed with a fluorescence microscope fitted with the appropriate wavelength filters. The same procedure was used for the negative controls except that either the primary antibody or the secondary antibody was omitted to verify that there was no auto-fluorescence or unspecific binding. Also, human fibroblasts were used as control cells and treated in the exact same way.

ELISA

ELISA was used to analyse CRP concentrations. In control animals (i.e. untreated animals), serum was collected on day 0, 7 and 14 after NS1 tumour cell inoculation and CRP levels were analysed using a rat C-reactive protein ELISA kit in order to follow CRP progression during tumour development. Briefly, an anti-rat C-reactive protein precoated 96 well plate was used and diluted samples were pipetted into

each well. Subsequently biotinylated antibody was added, followed by streptavidin-HRP solution after washing. Absorbance was measured and CRP concentrations calculated.

Serum analyses

Serum from experimental animals was analysed for different immunological responses. This was performed using Bio-Plex 200 analysis (Multiplex immunoassays), a Luminex Technology based on flow cytometry immunoassays. Using the Bioplex technique, expression levels of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, GM-CSF, IFN-γ and TNF-α were analysed.

Animals used and ethics statement

Female Fischer 344 rats were housed in pairs in hutches with enriched environments and with *ad libitum* access to water and rat chow. The animals were monitored daily for tumour growth and in the subcutaneous experiments, once the tumour size exceeded 30 mm or penetrated through the skin to cause open wounds, the rat was euthanized and the tumour saved for histological analysis. In the intracranial model, endpoints were defined related to the symptoms of brain tumour, such as paresis, seizures, or poor general condition. Animals displaying any of these symptoms were euthanized. If an animal should survive for 100 days without displaying symptoms, it would be euthanized in accordance with the ethical permission.

All animal experiments were approved by the Regional Ethics Review Board in Lund, Sweden, with the appropriate permit numbers, and were performed in accordance with Swedish regulations for animal experiments and the EU Directive 2010/63/EU for animal experiments. All efforts were made to minimize animal suffering. Additionally, all handling of patient material and data was approved and performed according to the guidelines of the Regional Ethics Review Board in Lund.

Gene expression analysis

Two different data sets were used for analysing mRNA gene expression levels of complement associated proteins and CRP. The first data set was derived from material from our own institution where data from 26 patients with astrocytoma grade IV was compared to material from four non-tumour brain samples. 27 k in-

house printed cDNA microarrays were used to investigate global gene expression changes. The second data set was derived from a publicly available database (GSE4290 Affymetrix Gene Chip Analysis; Sun L, Hui AM, Su Q, Vortmeyer A et al. Neuronal and gliomaderived stem cell factor induces angiogenesis within the brain. Cancer Cell 2006 Apr;9(4):287– 300. PMID: 16616334) were they investigated global gene expression levels in 77 glioblastoma samples compared to 23 non-tumour brain samples.

Magnetic resonance imaging

MRI was used for *in vivo* examination of five rats using a 3 T clinical whole-body MR system and a wrist coil. Rats were sedated using ketamine at a dose of 60 mg/kg i.p. which maintained sedation during the entire imaging procedure. Animals were placed with the skull centred in the carpal region of the coil and scanned under free breathing at 21°C. Body temperature was maintained during the scan by wrapping the body extremities in a soft tissue towel, which also restrained motion. The procedure lasted for 40 minutes in total.

For contrast enhanced MRI, gadobenate dimeglumin was administered i.p. in two animals on day 14, amounting to a dose of 2.8 mmol/kg body weight.

In addition, high resolution MRI images were obtained from two rats *post mortem*. The brain specimens were fixed in formaldehyde 14 days after tumour inoculation. Images were obtained using a 9.4 T animal MR scanner equipped with a superconducting cryocoil.

Statistical analysis

All gene expression values were log2-transformed and statistical analysis was performed using IBM® SPSS® Statistics version 20.0.0 or version 25.0.0. Alterations in gene expression levels were considered to be statistically significant for fold change > 2 and p-value < 0.05.

In vivo experiments were evaluated using either 2-tailed student's t-test assuming equal variance or Log Rank (Mantel-Cox) analysis, with $p < 0.05$ considered to be statistically significant. Overall survival was demonstrated with Kaplan–Meier curves.

Serum CRP concentrations were compared using ANOVA with post-hoc Bonferroni corrected p-value <0.05 considered to be statistically significant.

Alternative techniques that could be used

How to motivate the use of laboratory animals?

Although the use of rats gave us survival data and *in vivo* results of tumour growth we always tried to avoid the use of laboratory animals wherever possible. Examples of ways in which we did this was to perform *in vitro* cell experiments using glioma cells from patients. We also used databases with glioma material from patients to compare genetic and protein characteristics.

When it comes to working with animals several ethical issues need to be considered. There is a limit as to how long our experimental animals are allowed to live. The animals need to be well taken care of with daily inspections, an enriched environment in their hutches, which also have to be large enough, and as far as possible the rats should share cage with another rat. Suffering needs to be minimized and examples from our own work include euthanasia if the animals show signs of neurological illness or impaired general condition, which is in accordance with the ethical permissions we have for the work.

Alternative mouse models

Rats have been the laboratory animal of choice in our case since rats are bigger than mice and thus have larger brains. In an experimental setting using intracranial inoculations like we do, this means larger margins and hopefully less risk of anatomical error when performing intracranial and intratumoral procedures. Although it would be interesting to perform parts of our research in different mouse models since mice have the benefit of established knock-out schemes that could be further examined in the context of glioblastoma complement system research. Additionally, immune-deficient mice could be used for xenograft experiments where patient tumour tissue could be directly inoculated into the mouse brain without rejection.

Summary of the main results

Paper I – ITPP Treatment of RG2 Glioblastoma in a Rat Model

The drug inositol trispyrophosphate (ITPP) increases oxygen delivery to the tissue or tumour in question by shifting the oxygen binding affinity of haemoglobin, and was provided by Prof. Lehn to be evaluated in our rat glioma model. Unfortunately, the drug had no effect on survival despite showing promising results in other tumour models. This has improved our knowledge of glioblastoma and also showed how important it is to publish negative results.

The specific aim of the investigation was to establish whether ITPP halts the growth of RG2 glioblastoma tumours in rats in a similar fashion to U87 glioma cells engrafted onto chick chorioallantoic membrane or subcutaneous malignant melanomas in Balb mice. Before start of the experiments we hypothesized that ITPP could result in increased survival since glioblastomas display both angiogenesis and pathological vessels, as well as a hypoxic environment. A model comprising female Fischer 344 rats was chosen and RG2 cells were implanted either intracranially or subcutaneously. ITPP was administered by different routes (intraperitoneal, intravenous and both routes combined). Subcutaneous tumour implantation was used as a complement in order to evaluate whether the BBB restricted the passage of ITPP into the brain parenchyma or not.

Interestingly, a negative effect of ITPP was seen in the animals that received ITPP treatment on the first day after intracranial tumour inoculation, as compared to control animals and animals that received ITPP treatment one week after tumour inoculation, amongst whom there was no difference in survival. The route of ITPP administration did not affect outcome.

In the experiments with subcutaneous tumours there was a tendency towards a negative effect of ITPP since the survival was greater for the control group than the ITPP-treated group, however, the difference was not statistically significant.

Paper II – Growth pattern of experimental glioblastoma

The visualization of single cancer cells in brain parenchyma or other tissues is beyond the capabilities of standard histological techniques. This was something we wanted to explore further in our GFP-positive NS1 glioblastomas model by comparing the histological examinations with tumour characteristics seen by imaging, as well as correlating the number of intracranially inoculated cells with

time until onset of symptoms. In addition, we also wanted to study some of the immunological traits of the developing tumour by using immunohistochemical staining for CD8, CD4 and FoxP3.

By using the NS1 model stained with anti-GFP antibodies, we could observe distinctly GFP-positive cells in the brain parenchyma which on morphological grounds alone would not be classified as tumour cells in H/E stained sections. Glioblastoma cells were also found to adhere to the ependymal lining of the ventricle and GFP-positive cells were observed inside the ventricles in the shape of apparently free-floating intraventricular 'spheroids'. The number of inoculated cells was clearly related to survival and to the size of the main tumour at the site of implantation. No changes in growth pattern of the tumour as a result of increased number of inoculated cells could be observed; time until development of symptoms was the only affected factor.

Infiltration of CD4, CD8 and FOXP3 positive cells could be seen in the tumour border zone, but not in the centre of the tumour mass and not in the rest of the brain.

MRI was used *in vivo* to depict the tumours and alterations of the BBB, a common feature of pathological tumour vascularization. A gadolinium-based contrast agent was administered and the contrast uptake was homogeneous across the tumour centre with a maximum about 30 minutes after administration. The tumour burden as demonstrated by MRI correlated well with the corresponding histological volume examinations.

High resolution MRI of fixed brains could demonstrate the presence of macrophages in the hypointense tumour rim, and in the corpus callosum the jagged border demonstrated infiltration along the axonal tracts. Homogeneous contrast in the centre of the tumour indicated high cellularity.

Both the MR-images and the survival data yielded important information concerning the NS1 tumour and its growth characteristics. While the MR-images gave direct measurements of the tumour mass at different time points, the survival data represented the time until onset of symptoms leading to euthanasia. A mathematical model based on the Gompertz function was set up to calculate the tumour growth rate and tumour mass as a function of time from inoculation.

Paper III – What is the Role of CRP in Glioblastoma?

CRP is an acute phase protein in humans, and is upregulated in a magnitude of situations, examples of which include infections and certain malignant disease. Although CRP is evolutionary conserved, it does not act as an acute phase protein in all species. Rat constituting one such example.

We wanted to examine the role of CRP in both patient material and in our NS1 rat glioblastoma model, looking at gene expression levels and the presence of CRP protein on the glioma cells. We also performed *in vivo* experiments to examine the

effect of pre-coating the NS1 glioblastoma cells with anti-CRP antibody prior to intracranial inoculation.

Both NS1 rat glioblastoma cells and all three human glioblastoma cell lines were clearly positive for anti-CRP staining when examined using immunohistochemistry. Human fibroblasts were used as control cells and did not display anti-CRP staining to the same extent. Interestingly, it could be seen that CRP was confined to the cell surface/membrane of the NS1 tumour cells.

Pre-coating NS1 glioblastoma cells with anti-CRP antibodies before inoculation did not affect survival in rats with intracranial tumours.

Rat CRP serum levels were also examined during tumour growth of intracranial glioblastoma and although serum levels of CRP in the rats increased during tumour progression they never reached significantly different levels.

To evaluate whether CRP expression was upregulated on the gene level in GBM patients, we quantified the gene expression of CRP in tissue from human glioblastomas using data from two publicly available databases. We could conclude that CRP was not significantly upregulated on gene level in glioblastoma tissue from patients, even though the first set of data showed slight upregulation in the GBM samples as compared to control tissue, but only with a log2 transformed fold change of 0.3 ($p = 0.024$).

Paper IV – C1-inactivator is upregulated in glioblastoma

With these experiments we wanted to explore the theory that the complement system in glioblastoma is inactivated through the upregulation of C1-INH. To evaluate whether complement inactivation was present in human glioblastoma, we quantified the gene expression of several complement factors in tumour tissue using data from a publicly available database, as well as data from our own mRNA material from GBM patients. In addition, we performed *in vitro* and *in vivo* studies where we stained rat NS1 glioblastoma cells and human glioblastoma cells with anti-C1-INH antibodies and subsequently coated NS1 cells with anti-C1-INH antibody prior to intracranial inoculation.

We found an upregulation of C1-INH in human glioblastoma cells using our own mRNA patient material. The same results were obtained when we used data from a publicly available database. C1q, C1s and C4a were also significantly upregulated.

We could also demonstrate the presence of C1-INH on glioblastoma cells from both humans and rats *in vitro*, a phenomenon that had already been reported in the 1970s by Osther et al. when they found complement inactivator on cultured cells from human malignant brain tumours (Osther et al. 1974).

Finally, we could demonstrate a significantly increased survival *in vivo* in animals inoculated intracerebrally with NS1 glioma cells pre-coated with anti-C1-INH antibodies as compared to control animals.

Paper V – Anti-C1-inactivator treatment of glioblastoma

Building on the results from paper IV we wanted to further investigate the effect of treatment of glioblastoma with anti-C1-INH antibodies based on the theory that it would be of benefit to re-activate the complement system. Experiments were performed using the subcutaneous NS1 model. In addition, rat sera were obtained from animals treated with anti-C1-INH and analysed for IL-1β and GM-CSF.

We were able to demonstrate a significant survival advantage in rats treated with intratumoral anti-C1-INH antibody. Furthermore, there was a significant decrease in tumour size following treatment with anti-C1-INH.

Serum analyses revealed significantly decreased levels of IL-1β and GM-CSF in the anti-C1-INH treated group when compared to control animals. Immunohistochemistry also revealed decreased expression of C1-INH following treatment with anti-C1-INH.

Discussion

Different glioblastoma models

GBM is a challenging tumour to study since it is well known that the tumour comprises extensive cellular and molecular heterogeneity, both between patients and within the tumour itself. The tumour also changes character following treatment (Neilsen et al. 2019).

There are several rodent glioblastoma models presently available for neuro-oncological research. Our NS1 model takes advantage of the constitutive expression of GFP which makes it possible to study the infiltrative growth of the tumour. The NS1 model also contains several relevant genetic mutations, which makes it similar to a human glioblastoma, and presumably contains many other genetic and epigenetic alterations apart from the ones described. As such it could possibly be more useful in clinically oriented research than some of the currently available rodent models.

Striving to reproduce the clinical situation is of course of vital importance and the Gompertz growth model, which we employed in paper II, has actually been used to calculate tumour mass as a function of time in the clinical setting (Stensjoen et al. 2018). Using this model it could be estimated that a GBM tumour is approximately one year old at the time of diagnosis, with the median age of the tumour being 330 days at diagnosis (range from 156 days to 776 days). The same growth model was then employed to calculate the impact of extent of resection on gained survival time (Stensjoen et al. 2018).

GBM is thus a tumour that grows fast and its origin is complex. Using animal models can give some insight into this since ENU injection into adult animals does not produce brain tumours, only the offspring of pregnant ENU-treated animals develop tumours (Lenting et al. 2017). This somehow suggests that the ENU-induced mutations are passed on to the progeny in their developing brains and cause deleterious effects if they modulate oncogenes or tumour suppressor genes. This model may well represent the development of GBM in humans since it allows for identification of different driver mutations (Lenting et al. 2017).

Especially the subventricular zone (SVZ) has attracted attention in GBM research since this area may be the origin of tumour-initiating cells (TICs) in spontaneous tumours (Sanai et al. 2005). Apart from the SVZ, neural stem cells, which are

multipotent and self-renewing, have been isolated from amongst others the dentate gyrus and the lining of the lateral ventricles (Sanai et al. 2005). The SVZ thus contains the progenitor cells for both glial and neuronal cells, and there is considerable overlap between NSCs and GSCs (Ma et al. 2018). Interestingly, we could see suspected tumour spread in this area in our NS1 model. Our results also indicate that intraventricular dissemination of a low number of malignant cells (or maybe even TICs) may be more common than has been recognized before when using less sensitive methods for the detection of individual tumour cells.

Theories on hypoxia in glioblastoma

Oxygen can be a highly toxic substance damaging cells through oxygen free radicals. This was highlighted in the work by McCord et al. where they grew cells isolated from human glioblastoma at 7% and 20% oxygen concentration. They found that growing the cells at the more physiologically relevant level of 7% oxygen increased the self-renewal potential of the cells and as a result, the cultures also expressed higher levels of HIF2α and exhibited enhanced capacity to differentiate along both the glial and neuronal pathways (McCord et al. 2009). Drawing from these experiments and the fact that hypoxia is a common feature of GBM, one could therefore argue that a possible explanation for ITPP having a negative effect in our model could be that the RG2 glioma cells, which are very adaptive, started dividing even more rapidly when normoxia was restored by the administration of ITPP.

There could also potentially exist links to the complement system since components of the complement system have been identified to interact with HIF associated signalling pathways and could maybe act as additional effector mechanisms in HIF dependent GSC survival (Bouwens van der Vlis et al. 2018).

Glioblastoma and the complement system

Our results point towards the significance of the complement system in GBM *in vivo*. We could demonstrate a considerable survival benefit in animals intracerebrally inoculated with glioblastoma cells pre-treated with anti-CI-INH antibodies. Direct intratumoral treatment of subcutaneous NS1 glioma also demonstrated the positive effects of anti-C1-INH regarding animal survival and diminished size of treated tumours. Taken together, this strengthens the hypothesis that C1-INH is upregulated in glioblastoma, which could also be confirmed by immunohistochemistry and expression data using human glioblastoma cells. The gene analysis also showed increased expression of C1q and C1s in glioblastoma. This increase is believed to be a side effect of the inhibited formation of the $C1qr_2s_2$

complex due to the elevated level of C1-INH. Studies have shown that GBM patients have elevated serum C1q levels and C1q secreted into the tumour microenvironment leads to tumour progression by promoting adhesion, proliferation, and migration of the cells, as well as aiding angiogenesis (Macor et al. 2018). The implications of elevated C1q levels could potentially have other systemic effects as well since LPS lipid A, mitochondria, and beta-amyloid fibrils have all been shown to bind to C1q (Singh 2008) and there is evidence that the complement system plays a role in neurodegeneration, for example in Alzheimer's disease (DeCordova et al. 2020).

The positive effects of anti-C1-INH might not necessarily be attributed to or limited to its ability of re-activating the classical pathway of the complement system, but could also stem from the effects that C1-INH usually exerts on other pathways such as the kallikrein pathway, and all the other factor XII-dependent pathways.

Human glioma cells have developed molecular strategies to evade attack and eradication by the immune system and by the complement system. One example of the latter includes H2 human glioblastoma cells, which have been shown to be very resistant to complement-mediated cytolysis *in vitro*. H2 cells express the membrane attack complex inhibitor protectin (CD59) and actively produce the complement inhibitor factor H (Junnikkala et al. 2000) (figure 3).

Usually complement regulatory proteins are utilized by the body to protect healthy self-cells from complement attack. Regulation is necessary at all the major checkpoints of complement activation to shield self-cells from an over-reactive complement system. GBM express these complement regulatory proteins, which then allow the tumour cells to proliferate unchecked (DeCordova et al. 2020).

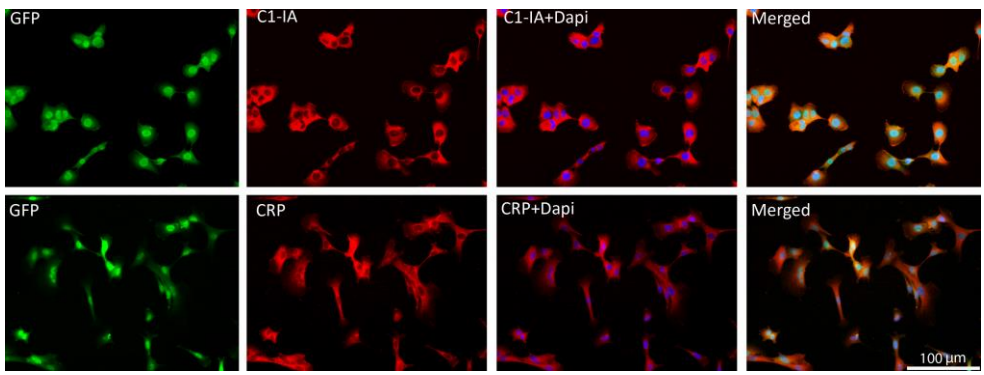

Figure 4. Top panel shows NS1 cells stained for C1-IA (second picture from the left) and then merged with Dapi staining (third from the left). Bottom panel shows NS1 cells stained for CRP (second from the left) and then merged with Dapi staining (third from the left).

Glioblastoma and CRP

Our immunohistochemical results showed that both human and rat glioblastoma cells either express or interact with CRP as indicated by the binding of anti-CRP to the cells. In the rat glioblastoma cells CRP coated the membrane in a very distinct way, and over-expression of CRP on gene level was not present in human glioblastoma tissue, suggesting that the CRP was in fact recruited from serum. Binding of CRP to the tumour could possibly be an attempt by the host to eradicate the malignant cells.

Previous research has shown that elevated levels of CRP preoperatively in patients with high grade astrocytoma is correlated to decreased survival and serum CRP levels above 29 mg/l (Pierscianek 2020) have been identified as a negative prognostic marker in GBM patients. Bearing this in mind we proceeded to target CRP with anti-CRP antibodies by coating the NS1 cells with anti-CRP prior to intracranial inoculation, but we could not observe any survival effects from adding antibodies against CRP. Even though there was an increase of serum CRP levels during tumour growth in NS1 tumour bearing rats, it was not statistically significant.

A clue regarding the role of CRP might be the fact that it can induce microglial cells to release IL-1β (Nijaguna et al. 2015). This might represent yet another link to the complement system since serum analyses of our rats revealed significantly decreased levels of IL-1β and GM-CSF in the anti-C1-INH treated group when compared to control animals. Although CRP has been shown to be an important prognostic factor in many forms of cancer, it has not been identified as a therapeutic target by itself. Instead approaches have been aimed at targeting IL-6 and IL-1β with antibodies, and only monitoring CRP levels as a result.

The efficacy of antibodies and the route of administration

There are several questions to be answered when it comes to potential treatment of GBM with antibodies. How do you administer the antibody so that it reaches the brain? In the hypothetical situation of treating GBM patients with anti-C1-INH antibody, an intrathecal route of delivery would be chosen, probably using a Rickham or Ommaya reservoir, which could be put in place during tumour resection. Although alternative and more effective ways of administration would be preferred.

Further research on the permeability of the BBB could also be of interest. In the normal state of things antibody molecules are too big to passively cross the BBB (Lampson 2013) but the BBB is dynamic and affected by both tumour growth and

different therapeutical approaches. At the time when the antibody is actually administered, it may well be that the BBB no longer impedes antibody access to the tumour site (Lampson 2013).

In our animal experiments we could see that the effect of the anti-C1-INH antibody was not due to the mechanical disruption of the tumour environment caused by the share volume being injected, nor the medium in which the antibody was suspended, as the control animals receiving PBS had a worse outcome than untreated control animals. The benefits observed could most probably be attributed to the antibody treatment itself, also with the knowledge that treating the tumour with other antibodies raised in rabbit (i.e. rabbit anti-rat antibodies such as the anti-CRP antibody described above) had no effect whatsoever.

One potential side effect of using anti-C1-INH with a systemic way of administration could theoretically be the induction of a state resembling angioedema, where the levels of C1-INH are too low. Intratumoral delivery instead of systemic use might reduce this risk.

So far the development of therapeutic antibodies against solid tumours has been limited by the difficulty to identify specific target antigens on the surface of the cancer cells, which could in theory be composed of either overexpressed self-antigens or neoantigens following tumour-specific mutations (Macor et al. 2018).

Conclusions and future perspectives

Summary of the major findings

We have demonstrated the spatial and temporal growth pattern of a new infiltrative GFP positive glioblastoma model and presented equations to calculate the tumour growth, which could potentially be of use also in other models.

We have also shown that C1-INH is overexpressed in glioblastoma on gene level and on protein level, with immunohistochemical experiments revealing both C1-INH and CRP on glioblastoma cells from patients as well as on glioblastoma cells from rat (figure 4). Additionally, treatment with antibodies against C1-INH had beneficial effects on survival.

Conclusions

Using ITPP to increase oxygen levels was not successful in the RG2 glioblastoma model; an old model which has been used for decades. Instead our new GFP positive rat tumour model represents a useful way of studying glioblastoma, and we could demonstrate perivascular dissemination and an infiltrative growth pattern. C1-INH, which inhibits the classical complement pathway amongst other functions, is upregulated in glioblastoma and treatment with antibodies against C1-INH prolongs animal survival. Systemic immunological effects following antibody administration into the tumour were observed. CRP could possibly mark glioblastoma cells, since it was seen using immunohistochemistry, but was not upregulated on gene level.

Future work

In our future work we would like to try to explain the role of the complement system in glioblastoma. Further mechanistic studies are needed and much still has to be clarified, especially regarding other possible effects of the anti-C1-INH therapy. The best routes for administration also need to be further addressed. We believe that the way forward is through collaboration and sharing of data as far as possible.

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