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Radiotherapy and IDO-inhibition for Glioblastoma

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Radiotherapy and IDO-inhibition for Glioblastoma

Radiotherapy and IDO-inhibition for Glioblastoma

Jonatan Ahlstedt

LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Glioblastoma remains an incurable and highly aggressive disease. With currently employed treatment in the clinical setting, encompassing surgery, radiation and chemotherapy, median survival remains at 13-15 months. During the last decades, several types of new treatments have been explored to increase survival or find a cure for this disease. Immunotherapy has emerged as a strategy with great clinical potential, with many different targets explored in this field. Indoleamine-2,3-dioxygenase (IDO) is one of these targets, a molecule involved in immune system regulation, inducing immune tolerance towards antigens presented in the contexts it is expressed. At the same time, the immunological effects of radiotherapy have been explored, showing that radiation has significant potential in both instigating and attenuating immune response, depending on its usage.

In this thesis, I describe the employment of a strategy of IDO-inhibition using 1-DL-methyl-tryptophan in conjunction with radiotherapy to effect immunological response toward glioblastoma in an experimental setting. I also describe the development of an experimental glioblastoma model in rats, suitable for immunotherapeutic study, by our research group. This is an ethyl-nitroso-urea induced model in homozygotic GFP-positive Fischer 344 rats transplanted to normal Fischer 344 rats. We found this model representative of human glioblastoma and describe its growth pattern, histological features, and suggest its potential for use in preclinical study. We initially describe finding a synergistic effect between single-fraction radiotherapy and IDO-inhibition on survival in an experimental glioma model. The combined treatment increased survival compared to monomodal treatment. We used this data and historical data to mathematically model the effect of treatment upon tumor growth to assist in hypothesis formation regarding radiation fractionation and dosage in an immunotherapeutic setting. This was achieved by adapting a previously described model for immune checkpoint inhibition treatment and radiotherapy, and fitting it to our available data. Following this, we found two-fraction radiation superior to single-fraction radiation when combined with IDO-inhibition in in-vivo experiments, as informed by this modeling. This significantly increased survival in rats carrying our experimental glioblastoma model. Study of gene expression datasets failed to show increased IDO-expression in glioblastoma samples compared to normal brain tissue samples, although glioma stem cells showed increased expression of IDO compared to neural stem cells.

Taken together, these findings support the idea of a synergistic immunotherapeutic effect of radiotherapy in a hypofractionated setting in conjunction with IDO-inhibiton, although much work remains to be done regarding the exploration of optimal dosage and fractionation of radiation. We look to the future of mathematical modeling of biological processes like tumor growth as promising, as well as its potential for usefulness in hypothesis generation and decision support. Furthermore, we observed no toxicity or side effects of this combined treatment, opening doors for other immunotherapeutic strategies on top of IDO-inhibition, which in other research has shown promise.

Key words: Radiotherapy, Glioblastoma, IDO, Immunotherapy, experimental glioma

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2020

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We are all going to die, all of us, what a circus!
That alone should make us love each other but it doesn't.
We are terrorized and flattened by trivialities,
we are eaten up by nothing.

-Charles Bukowski

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Abbreviations

GBM	Glioblastoma multiforme
CNS	Central nervous system
IDH1	Isocitrate dehydrogenase 1
IDO	Indoleamine 2,3-dioxygenase
RT	Radiotherapy
GFP	Green fluorescent protein
NSC	Neural stem cells
SVZ	Subventricular zone
BBB	Blood-brain barrier
CT	Computed tomography
MRI	Magnetic resonance imaging
FLAIR	Fluid attenuation inversion recovery
TAM	Tumor-associated macrophage
T_{reg}	Regulatory T-cell
CD	Cluster of differentiation
CTL	Cytotoxic lymphocyte
IL	Interleukin
MHC	Major histocompatibility complex
TAA	Tumor-associated antigen
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
CTLA-4	Cytotoxic T-lymphocyte antigen 4
Trp	Tryptophan
Kyn	Kynurenine
ENU	Ethyl-nitrosourea
GSC	Glioma stem cells

Populärvetenskaplig sammanfattning

Hjärntumörer drabbar ca 1300-1400 människor varje år i Sverige. Av de så kallat primära hjärntumörerna, de som uppstår i hjärnan eller hjärnans hinnor, är ungefär hälften en variant som benämns glioblastom. Detta är den mest aggressiva varianten, klassificerad som grad IV enligt WHO, och är ännu obotlig. Endast 3% av de som drabbas är vid liv fem år efter diagnostillfället, trots behandling som innefattar omfattande kirurgi, intensiv strålbehandling och cytostatikabehandling. Kirurgiska tekniker har generellt blivit bättre runtom i världen, vilket ökat överlevnadstiden något, men den genomsnittliga överlevnaden är runtomkring 13-15 månader från diagnostillfället.

Orsakerna till att tumören är så pass svårbehandlad har mycket att göra med dess tillväxtsätt. Glioblastom växer infiltrativt och diffust vilket omöjliggör att kirurgiskt utrymma hela tumören från hjärnan, även om den inte är lokaliserad vid livsviktiga områden. Detta medför ett stort behov av effektiv efterbehandling för att angripa resterande tumörceller i hjärnan.

En viktig aspekt i cancerbehandling generellt, och särskilt de senaste åren, är tumörens interaktion med kroppens immunförsvar. Glioblastom, liksom en del andra typer av tumörer, utnyttjar flera av kroppens egna mekanismer för att dämpa immunförsvaret, vilket annars kan känna igen tumören som kroppsfrämmande och gå till angrepp. Behandling som syftar till att påverka tumörens effekt på immunförsvaret eller att använda immunförsvaret mot tumören kallas för immunoterapi.

Denna avhandling inriktar sig till att utforska effekten av att kombinera immunoterapi och strålning i ett försök att utvinna större behandlingseffekt av strålterapi av glioblastom. Detta kan potentiellt uppnås genom att blockera ett protein, indoleamin 2,3-dioxygenas, eller ”IDO”.

I gravida däggdjur fungerar normalt IDO för att inducera immunologisk tolerans vid gränssnittet mellan fostret och placenta. Med andra ord, proteinet IDO hjälper mammans immunförsvar att förstå att avkommans proteiner och celler, som ju till hälften är främmande p.g.a. DNA från pappan, är fredliga och inte att angripa. Utan IDO stöts fostret bort och detta leder till missfall, då moderns immunförsvar betraktar fostret som främmande. Genom att glioblastom aktiverar genen för IDO kan tumören träna kroppens immunförsvar till tolerans, och därmed undgå angrepp. Denna effekt skulle man kunna föreställa sig är ytterligare skadlig när man försöker att behandla en tumör genom att provocera immunförsvaret till angrepp.

Strålning har använts som behandling mot tumörer i över etthundra år. Redan tidigt såg man en märklig effekt där en tumörmetastas som låg utanför det strålade området, också denna blev mindre efter strålning. Detta beskrevs redan 1953 av Dr. R. H. Mole, och kallas för abskopal effekt (ab – utom, scopus – syn/omfång/mål).

Orsaken var okänd så länge som till 2004, då man föreslog immunologiska effekter som förklaring. Detta beror bland annat på att när en tumör strålas, släpper den ifrån sig ämnen, s.k. antigener, som immunförsvaret kan plocka upp och därmed tränas i att känna igen tumörvävnad för angrepp.

I sedvanlig strålbehandling för glioblastom ges strålningen uppdelad i s.k. fraktioner, där ett vanligt schema är 2 Gy per dag under trettio dagar, till en total av 60 Gy stråldos mot tumören. Med tanke på den celldödande aktiviteten i kombination med immunsystemets aktivitet kan man hypotetisera att strålningen i denna typ av fraktionering inte endast angriper tumörvävnad, utan också tillströmmande immunceller som reagerar mot tumören. Detta skulle kunna tänkas minska den immuneffekt som strålningen kan ha. Därför finner vi det meningsfullt att utforska s.k. hypofraktionerad, eller ofraktionerad strålning för att minimera negativa effekter på immunförsvaret.

I denna avhandling utforskas effekten av att kombinera strålning och IDO-hämning i en experimentell modell av glioblastom som vi själva tagit fram.

I det första delarbetet utforskade vi kombinerad behandling med IDO-hämning och ofraktionerad strålning. Här lät vi försöksdjur med glioblastom få antingen behandling med den IDO-hämmande substansen 1-metyl-tryptofan (1-MT), strålning i en fraktion med total dos 8 Gy, eller bådadera. Dessa tre grupper jämfördes också med en helt obehandlad grupp råttor med samma tumör. I denna studie kunde vi se en ökad överlevnadstid i gruppen som fick den kombinerade behandlingen jämfört med endast en av behandlingarna. Vi undersökte också uttrycket av IDO-proteinet från tumörcellerna före och efter strålning. Här kunde vi se att uttrycket av IDO var mindre efter strålbehandling motsvarande den behandling som försöksdjuren fick.

Andra delarbetet presenteras en anpassning av en tidigare publicerad matematisk modell för tumörtillväxt och dess interaktion med immunbehandling och strålning. Denna modell anpassas med hjälp av historiska försöksresultat, vilket sedan extrapoleras till vidare hypoteser om strålningsfraktionering i kombination med immunbehandling.

I tredje delarbetet beskrivs vår tumörmodells egenskaper och hur den beter sig i försöksdjur. Många tumörmodeller som används i experiment är transplantat från djur som inte är genetiskt identiska till djuret som används i experimentet, och ibland inte ens från samma art. Vi har utvecklat en tumörmodell som är syngen, dvs ursprungen från genetiskt identiska djur. Detta gör att man kan använda djur med helt normala immunförsvar, vilket är viktigt när man arbetar med immunoterapi. Magnetkamerabilder och mikroskopibilder från histologiska och immunohistokemiska snitt presenteras.

I sista delarbetet prövar vi hypotesen att hypofraktionerad strålning i 8 Gy-doser kan vara bättre än en enstaka strålfraktion i samband med IDO-hämning. I ett första

försök testar vi varierande strålningsscheman och deras effekt på tumörtillväxten, där vi avslutar försöket och jämför tumörstorlek mellan grupper som mottog antingen en eller två strålningsfraktioner. Effekten tycktes stor och vi gick vidare med ett överlevnadsförsök där vi jämförde den till synes bästa behandlingen från första försöket med obehandlade kontroller. Överlevnaden i de behandlade djuren dubblerades jämfört kontrollerna.

Mycket arbete kvarstår för att föra en sådan här grundläggande förändring i behandlingsstrategi till kliniken för denna svåra sjukdom. I denna avhandling utforskar vi möjligheten att kraftigt förändra den konventionella strålbehandlingen till förmån för en mindre intensiv behandling inriktad mot att mobilisera kroppens eget immunförsvar.

Introduction

Glioblastoma

Glioblastoma (GBM), classified by the World Health Organization as Astrocytoma WHO grade IV, is the most aggressive primary tumor of the brain. Sadly, it is also the most common. Prognosis is generally very poor and mean survival from time of diagnosis is most often below 13-15 months, even with aggressive combinatory surgical, radiological, and medical treatment [1, 2]. In this introduction I will attempt to summarize the gathered knowledge of this disease and some of its aspects.

Epidemiology

The world

Globally, the incidence of glioblastoma is approximately 3.2 per 100 000 people, but varies depending on the country, with the highest incidences occurring in Western Europe and North America. GBM is uncommon in children, and the median age at diagnosis is 64. There is a difference in incidence between ethnicities, with studies showing European Americans having a higher incidence than African Americans by a factor of 2,5 [1, 3]. The reasons for this are currently unknown.

Sweden

According to the report *Cancer in Numbers 2018* (*Cancer i siffror 2018*) by the Swedish National Board of Health and Welfare (Socialstyrelsen), 1353 people were diagnosed with primary CNS tumors in 2016. Median age of diagnosis is approximately 65 years but incidence increases with age and peaks in ages 70 to 80 years for both sexes [4]. According to the statistical database of cancer curated by the National Board of Health and Welfare, 534 of these were high-grade astrocytoma (WHO Grade III or IV), and incidence is higher among men (6,27 per 100 000) compared to women (4,48 per 100 000) [5].

Risk factors

There are no known genetic predispositions for developing GBM, although GBM is more common in people with certain syndromes, such as Turcot syndrome, Li Fraumeni syndrome and neurofibromatosis type 1 [6, 7]. GBM also occurs more frequently in males than in females, although study on intracranial volume and risk for GBM has indicated a higher risk for females compared to males when intracranial volume is controlled for [8]. Exposure to ionizing radiation has been found to be the only environmental risk factor for developing brain tumors, and young age at exposure seem to increase risk [9]. Interestingly, an inverse association between atopic allergy, especially respiratory allergies and glioma risk has been found, although an explanation or mechanism behind this association has not been found [10].

Etiology and classification

A majority of GBM arise as primary, or *de novo* from glial cells in the brain. The remainder develop from lower grade neoplastic lesions, astrocytoma of grade I-III according to the WHO central nervous system tumor classification [11]. These are categorized as 'secondary' glioblastomas. Generalized disease by spreading either via cerebrospinal fluid (CSF), blood, or lymphatic vessels is very rare, but not nonexistent [12, 13]. This is likely due to the localization and aggressiveness of the tumor, often killing patients before generalization occurs. In fact, most extracerebral manifestations of GBM occur after surgical resection, in the skin near the surgical site. Localized spread in the brain is more common, with 13% of patients presenting with multifocal GBM at time of diagnosis [14].

A few key common genetic abnormalities have been identified in GBM [15] and by large genomic studies by projects such as The Cancer Genome Atlas (TCGA) [16]. Three of these are the p53 pathway, tyrosine kinase/Ras/phosphoinositide 3-kinase, and the retinoblastoma pathways. Specifically in secondary GBM, deletion of the q arm of chromosome 19 and isocitrate dehydrogenase 1 (IDH1) mutation is seen as opposed to in primary GBM [14]. Epigenetic changes are present in some GBMs in the form of methylation of the O6-methylguanine-DNA-methyltransferase (MGMT) promoter, and is associated with more favorable outcome in patients when treated with alkylating chemotherapy in addition to radiotherapy [17]. MGMT is a DNA repairing protein which breaks the DNA cross-links created by alkylating agents such as temozolomide, and the presence of methylation informs the clinical decision on treatment.

Morphologically, GBM is a heterogenous group of tumors, initially prompting the use of 'multiforme' in its name. Although grading and classification of glial tumors

still revolve around light microscopy and lineage surface markers of cells, genetic and molecular analysis has widened the spectrum of diagnoses for tumors of glial origin. The IDH-wt glioblastomas are since the 2016 WHO classification document divided into three types, giant cell glioblastoma, gliosarcoma, and epithelioid glioblastoma, the latter being the newest addition [11].

The cells of origin of GBM has been hypothesized to be neural stem cells (NSC) migrating from the subventricular zone (SVZ). The SVZ contains progenitor cells for glial and neuronal cells, which in their physiological state carry many of the factors required of the glioma cells. By accumulation of oncogenic driver mutations over time, NSC:s migrate and seed gliomas in the brain, and it has been suggested that the SVZ may even contain a reservoir of brain tumor propagating cells, causing the frequent recurrences of GBM observed in the patient population [18, 19].

The histology of glioblastoma

The glioblastoma is characterized by a highly polymorphic and heterogenous histological image. Central necrosis with surrounding pseudo-palisading cells is often seen. Cells are often poorly differentiated with nucleic atypia, and a high degree of vascular proliferation is a characteristic of a grade IV tumor. Immunohistochemistry of EGFR, IDH and Ki67 can be used for aid in classification or prognosis [20]. A low Ki67 index has an association with poorer prognosis, but does not currently influence treatment [21].

The blood-brain barrier

The blood vessels of the brain have properties unlike those of the rest of the body. These properties regulate the flow of fluid and other compounds into and out of the brain. The blood-brain barrier (BBB) is the structure of endothelial cells, basal membrane, pericytes and astrocytes forming a tightly controlled surface regulating the escape of ions and molecules from the blood into the brain extracellular space. The endothelial cells of cerebral capillaries are bound by tight junctions, and most of the molecule transport through these cells is done by active transportation. Surrounding this layer lies two basement membranes, one secreted by the aforementioned endothelial cells, and the other by surrounding pericytes. Both of these layers additionally prevent transfer of a wide range of molecules through the barrier. Around these a final layer consists of glial and neuronal protrusions encasing the vessel, providing BBB modulation and regulation of blood flow in response to neuronal/glial activity.

The blood-brain barrier function can be disrupted in many ways, and this disruption is a major factor in several neurological disease processes, both autoimmune and otherwise [22]. Glioblastomas secrete several factors both contributing to rapid neovascularization lacking in functional BBB as well as disrupting already present BBB [23].

Contrast enhancement of radiological examinations of the brain can show this BBB disruption. Glioblastomas often exhibit a “ring-enhancing” property on these examinations, displaying the escape of contrast into the brain tissue where the BBB is disrupted. Although contrast enhanced imaging often is used to plan and evaluate surgical resection, contrast enhancement does not necessarily represent the entirety of the tumor mass. Apart from the invasive spreading of GBM cells throughout the brain, the border of the tumor does not necessarily align with the contrast enhancement on radiological imaging.

Radiological characteristics

Radiology is frequently used for diagnosis, preoperative planning, postoperative control and monitoring of GBM. Most often, mass effect is seen on computed tomography (CT) imaging upon presentation. Magnetic resonance imaging (MRI) using fluid attenuation inversion recovery (FLAIR) usually displays signal abnormality, corresponding to perifocal edema or tumor infiltration [24]. On contrast enhanced T1 imaging, an irregular border contrast enhancement is seen surrounding a central non-enhancing tumor core, due to central tumor necrosis [25].

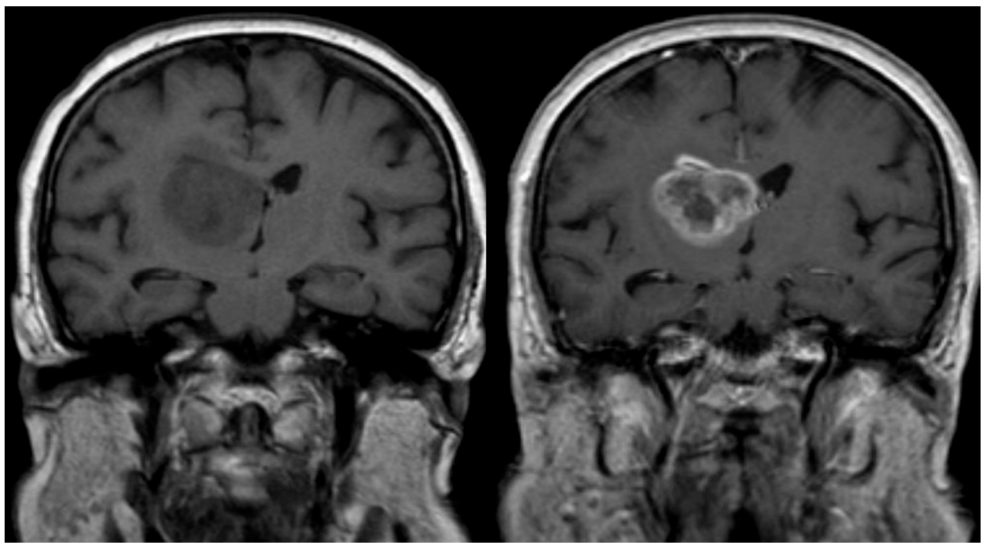

Figure 1. Coronal images of glioblastoma. Left image is T1 MRI without contrast, right image is T1 contrast enhanced. Author is Hellerhof @Wikimedia, used under Creative Commons Share Alike 3.0.

Cell migration and growth pattern

It is established that GBM growth is diffuse and even when diagnosed early in the course of the disease, microscopic metastases spread throughout the brain parenchyma, even to the contralateral hemisphere. This renders complete resection of the tumor near impossible, even with very aggressive strategies such as complete hemispherectomy. This was displayed in the famous article on hemispherectomy on a patient with glioblastoma published in 1928 by Dr Walter Dandy of the John Hopkins Hospital.

Glioblastomas are most frequently found in white matter, and cells migrate into the surrounding healthy brain, one of the greatest factors to the frequent failure of curative treatment. The tumor cells often use the perivascular space, exhibiting a more rigid extracellular matrix, to invade the surrounding brain. Paths along white matter axonal tracts are also used by glioma cells to migrate through the brain. This cell migration appears to begin early in the development of the manifest glioma, and an estimated half of patients have glioblastoma cells in the contralateral hemisphere at the time of clinical presentation.

Current clinical practice and outcome

In clinical practice, conventional treatment of glioblastoma is a combination of surgery, radiotherapy and chemotherapy. The most commonly used strategy is 60 Gy of radiation in 2 Gy fractions over 30 days with concomitant Temozolomide treatment, following surgery aimed at maximal possible resection. While long term survival is extremely rare, these measures increase survival, and current median survival with the Stupp protocol treatment is approximately 15-16 months [1, 2].

Surgery

Surgery is an important part of the initial treatment for glioblastoma, in many cases buying time for other treatment modalities to be administered and have their effect. The degree of resection is important for the outcome, as demonstrated in a study of outcomes where no residual tumor as assessed by postoperative contrast-enhanced MRI of the brain was associated with longer survival [26], as well as need for repeated surgery occurring later. Of course, tumor location may have a large impact on both outcome and possibility of macroscopically radical surgical resection. Different surgical tools are used to maximize resection. Fluorescence guided surgery by preoperative administration of 5-aminolevulinic acid (5-ALA) increases overall survival [27, 28], and tools such as preoperative fMRI, intraoperative nerve monitoring as well as awake surgery may be used to maximize resection while avoiding damage to brain functions important to quality of life [29].

Radiation

Radiotherapy has been used for cancer treatment for over one hundred years. The primary mechanisms of radiotherapy are immediate cell death as the result of DNA damage, or the production of free radicals in the tells of the target tissue. These effects also occur in surrounding normal tissue and therefore fractioning of the radiation is done to allow normal tissue to recover between during treatment.

In glioblastoma treatment, radiotherapy is administered in a highly fractionated, high dose manner closely following surgery. Unless contraindications are found, patients commonly receive a total of 60 Gy in 30 fractions of 2 Gy radiation to the tumor area over a period of 6 weeks. This radiation treatment scheme has become a standard since the work of Stupp et al [2]. When the patient is deemed susceptible to adverse events due to age or frailty, the radiotherapy regime may be altered to fit the patient.

Chemotherapy

In the standard of care, chemotherapy is added during radiotherapy, in the form of Temozolomide. Temozolomide is an alkylating agent, administered orally, that has been shown to be effective in clinical practice for primary glioblastoma [30]. Temozolomide was included in the standard of care in 2005, again after the work of Stupp et al [2]. Treatment with Temozolomide as adjuvant to radiotherapy has a positive effect on outcome in patients with hypermethylation of the MGMT-promoter, as mentioned earlier. Usually, treatment begins at the same time as radiotherapy, and may continue after the radiation treatment is complete.

Glioblastoma and the immune system

There is abundant evidence that glioblastomas interact with the human immune system in multiple ways to evade attack, with has both local and systemic effects. These effects may have a role in the lack of efficacy of novel immunotherapies, seeing as no immunotherapies to date have succeeded in effecting a cure for GBM.

The brain was long considered immuno-privileged tissue, partly because of the blood-brain barrier (BBB), preventing the traversal of molecules and immune cells into the extracellular space in the brain from the blood. It is now established that the brain is an immunologically active tissue with its own brain-associated immune cell types, microglia, which act as antigen presenters. The brain also has lymphatic connection with the rest of the body through draining cervical lymph nodes [31]. The BBB is disrupted as a glioblastoma grows bigger, effectively allowing immune cells to enter the tumor from the blood stream [32].

Glioblastomas modify the microenvironment and inhibit the response by activation of FoxP3+ regulatory T-cells (T_{reg}) and the inactivation of cytotoxic lymphocytes (CTL) in part by secreting indoleamine 2,3-dioxygenase (IDO). This mechanism is described below as it is one of the focal points of this thesis. By themselves and through interaction with the surrounding tissue, mainly tumor-associated macrophages (TAM) and microglia, GBM cells release interleukin-10 (IL-10), promoting tumor growth and downregulating antigen presentation [33, 34]. IL-10 also promotes immunosuppression by these TAMs. By secreting CD70, GBMs may induce apoptosis in infiltrating T-cells, adding to the decimation of the immune response [35]. In addition, GBM cells downregulate expression of major histocompatibility complex (MHC), lowering the exposure of tumor-associated antigen (TAA) to immune cells [32]. It has been demonstrated that GBM carries a high population of myeloid-derived suppressor cells, having the effect of lowered T-cell activation and poorer anti-tumor response [36].

Furthermore, GBM cells also evade the immune system by activating so called immune checkpoints, of which programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) are two that have rapidly become prominent in immunotherapy in cancers [37]. Immune checkpoints provide important inhibition to the immune system, preventing autoimmune reactions. CTLA-4 deficient mice develop aggressive autoimmune disorders [38], while PD-1, a receptor present on the surface of T-cells, has important functions related to both inflammatory bowel disease and multiple sclerosis [39]. Inhibition of CTLA-4 and PD-1 has shown effect in many cancers [37]. CTLA-4 is a strong down-regulator of T-cell response when these are presented with antigen. In trials with dendritic cell (DC) based vaccines, expression of CTLA-4 on CD4+ and CD8+ T-cells is strongly associated with poor outcome [40]. GBMs express the ligand to PD-1, programmed cell death ligand 1, (PD-L1), which binds to PD-1, suppressing CTLs and promotes T_{reg} activity [41].

IDO

The protein indoleamine 2,3-dioxygenase (IDO) is encoded by the IDO1 gene located on position p11.21 on chromosome 8. It was first implicated in regulating the maternofetal immune response by Munn et al in 1998, where it was demonstrated that IDO-deficient mice to a large extent suffered allogenic fetal rejection [42].

IDO functions by catabolizing tryptophan (Trp) into kynurenine (Kyn). This catabolism inhibits proliferation and activation of T-cells which in the physiological state suppresses harmful autoimmune responses [43]. The increased concentration of Kyn increases recruitment and differentiation of T_{reg} cells [44]. These T_{reg} cells modulate the stimulation by antigen presenting cells, inducing tolerance instead of intolerance towards the presented antigen. In a GBM context, this has the effect of

inducing system-wide tolerance against TAAs expressed by the tumor-associated microglia [45]. Increased age is associated with increased expression of different immunosuppressive factors in normal brains, including IDO1, which has recently been hypothesized to have an effect on suppression of tumor development and even on the efficacy of immunotherapeutic treatment [46].

In GBM, overexpression of IDO is correlated with poor outcome. It has also been shown that higher grade gliomas overexpress IDO at a higher rate than lower grade gliomas [47]. In a GBM setting, while trials using inhibitors of IDO have shown little success as adjuvant to standard of care treatment, several preclinical and early clinical results indicate a combination of IDO with immune checkpoint inhibitors and/or irradiation may have a favorable effect [48, 49]. Because of this, many studies aim towards more and more complex combinations of immune-based therapies. In recent years, the effects on immunological processes by ionizing radiation have been discovered and explored.

Immunological effects of irradiation

As mentioned above, ionizing radiation has been used for many decades in treatment of cancer, and the effect has been believed to be mostly or only through the effects on the irradiated cancer cells. As early as 1953, the abscopal effect on distant unirradiated metastases of cancers by radiation to the primary tumor was described by Dr. R. H. Mole [50]. The phenomenon stands as proof of concept of the immune mediated anti-tumor response, although the mechanisms behind this remained obscure until immunological processes were proposed and later demonstrated as the mediator of this effect [51-54].

Radiation therapy has a multitude of immunological effect in the target tissue. One is simply by causing cell death which in itself is immunogenic, releasing multiple immunostimulatory substances into the microenvironment. Furthermore, RT increases antigen presentation with MHC-1 on the surface of tumor cells [55], as well as tumor antigens and surface proteins mediating cell death by natural killer cell attack [56].

With conventional radiotherapy aimed at tumor cell death, dose-response is fairly linear with higher doses resulting in more extensive cell death, however the dose-response relationship when the goal is immunological effect is not yet clear. Many factors of combined immunotherapy and radiotherapy remain to be thoroughly evaluated, among them are the optimized radiation dosage, fractionation, and timing, along with timing and combination of immunotherapy strategies. This is a field of ongoing study not only in glioma research but in many other cancers [57].

Mathematical modelling of tumors and tumor growth

There are many uses for mathematical models in biology, and this thesis employs mathematical modeling of tumor growth. These models can be simple or complex, but essentially a model takes into account different biological processes and their effects on tumor volume over time. This often includes doubling rate of tumor cells, and other cell populations within the tumor, cell death upon treatment and dead cell clearance [58]. Intracranial tumors are a special case, considering the locale of the tumor in an enclosed space, where intracranial space and pressure becomes a limiting factor.

Even more sophisticated models are being developed. Models for modeling the invasive and migratory growth of gliomas have been presented, and could be used as support for preclinical and even clinical studies [59].

Attempts have been made to model effects of immune cell interaction with the tumor as well as effects of immunotherapy and immune checkpoint inhibition. In one of the included papers we adapt and employ one of these models (see Methods and materials).

Animal models of glioblastoma

Disease modeling is a constant challenge for preclinical scientists in many fields, becoming increasingly important as the understanding of tumor biology, immunology and tumor-host interaction increases. As with many other tumor types, there exists several different models for GBM, ranging from human to animal xenografts, to genetically engineered animal models, and induced or spontaneous syngeneic animal models [60, 61]. When modelling a glioblastoma there are certain important aspects to take into account, such as the genetic abnormalities in the model tumor compared to the often-varying genetic factors in human GBM, the interactions of the microenvironment and the tumor, and the immunological interactions between the tumor and the carrying host.

Xenograft models are patient-derived glioblastoma cells injected or transplanted into mice or rats. This can be done either orthotopically, in the brain, or subcutaneously. This requires immunodeficient animals to avoid a strong immunological reaction to the tumor tissue but has the advantage of being genetically and microbiologically identical to a human glioma. There are multiple models of this sort used in current research [62]. The immunodeficient animals required for this make it difficult to use these models in experiments around the immunological mechanisms and effects of the tumor and its treatment. This can be worked around using humanized mouse models, immunodeficient mice with a

transplanted human immune system as well as a patient-derived xenograft, making it possible, albeit complicated, to study the immune system-tumor interaction [63].

The genetically engineered animal models (most often in mouse) are animals genetically modified to display genetic abnormalities resulting in highly oncogenic mice. These may have the disadvantage of not accurately representing the relevant disease because of the selected oncogenic drivers not precisely matching that of the tumor of study. These may be difficult to predict and evaluate due to high or variable disease latency in research animals, although eliminating in vitro culturing may preserve cellular and structural heterogeneity [64].

A syngeneic model is a tumor model that arose in a specific animal, cultured in vitro and then used to transplant the tumor into genetically identical animals to achieve a predictable and repeatable tumor model. This carries the disadvantages of a patient derived xenograft in that some cellular heterogeneity may be lost during repeated culturing, but also has the distinct advantage of keeping the immune system intact and the genetic makeup of the tumor matches that of the carrier, eliminating the need for immunodeficiency. This allows for rigorous study of the tumor-host interaction on an immunological level.

The models used in this thesis are syngeneic rat glioma models, induced by administering ethyl-nitroso-urea (ENU) to a pregnant rat, and culturing any resulting nervous system tumors. This culture can then successfully be transplanted either orthotopically or subcutaneously to produce a predictable and repeatable glioma tumor model (see Methods and materials).

Aims of this thesis

To investigate the potential synergistic effect of hypo-fractionated radiotherapy in conjunction with IDO-inhibitory treatment.

To further characterize a usable and practical rodent model of glioblastoma multiforme with potential to monitor tumor growth and migration not only macroscopically but on the microscopic level.

To investigate optimal fractioning and dosing of external beam radiotherapy in an immunotherapeutic setting for glioblastoma treatment, using mathematical modeling of immunotherapy and radiation to generate testable hypotheses.

Methods and materials

Selected aspects of the methods employed in this thesis are presented and discussed below.

In vivo glioma model

There are a number of glioma models available for experimental purposes, both in mouse and rat. Many models are either xenografts or allografts, requiring in vivo trials to be done in immunodeficient animals. This is an obvious hurdle for studies in the field of immunotherapy and the immunological effects of radiation, prompting the need for genetically modified animal models or syngeneic models wherever this research is done. These have also been developed in both mice and rats [60, 61].

In the included papers, two different glioblastoma models were used. In the first, we used an older and well used rat model by the name RG2, established by Wechsler in the 1960's [65] and further characterized by Aas et al in 1995 [66]. The following papers that include in vivo and in vitro work utilize the NS1 model, which our group developed and initially presented in 2014 [67]. Both are syngeneic ethyl-nitrosourea (ENU) induced models developed in Fischer 344 rats, with the exception that NS1 was induced in homozygotic green fluorescent protein (GFP) positive rats. Pregnant rats received ENU treatment after which the offspring developed CNS tumors. The cell line was chosen for its reliable growth intracranially and subcutaneously, and its histopathological features in accordance to GBM, showing necrosis and vascular proliferation in addition to a highly infiltrative growth pattern.

The two models are similar in practical application. Rats are anesthetized and receive intracranial inoculations of glioma cell suspension aimed at the right caudate nucleus using anatomical landmarks, delivered through a syringe mounted to a stereotactic frame. This produces a reliable and reproducible tumor growth and small deviations in time until symptom development, which we in all experiments use as proxy for survival due to ethical considerations.

IDO-inhibitory treatment

As IDO-inhibitory treatment in the included papers, we use 1-DL-methyl tryptophan (1-MT). The stereoisomer 1-D-methyl tryptophan, also known as Indoximod, has been used in clinical trials for a range of cancers. There are many options for substances to use when studying IDO-inhibition, however we have chosen to continue using the DL-compound. This is partly because of ease of use, and a very low side effect profile, with low toxicity [68] and no adverse effects of the compound noted in our studies.

1-MT was initially thought to inhibit IDO1 activity but was later discovered to act as a Trp substitute for the target mTORC1. This removes the suppression of T-cell proliferation and activation that the mTORC1 pathway effects [69].

Epacadostat, navoximod, BMS-985205 and PF-06850003 are all different substances used for IDO-inhibition in preclinical and clinical research. These all share the effect of inhibiting the breakdown of Trp into Kyn in the tumor microenvironment, although by slightly different mechanistic effects and varying selectivity for IDO1, IDO2 and TDO [70]. These all have potential as clinical therapeutic substances but remain outside of the scope of this thesis.

External beam radiotherapy

In papers I and IV, animals received irradiation in 8 Gy fractions towards the tumor site. Beams were collimated to an approximately 11 mm x 8 mm area over the injection site in the frontal half of the brain. For additional details of the radiation treatment see Materials and methods of the corresponding papers.

Hypofractionated radiotherapy has been explored in lower accumulated doses, as well as dose escalation above the 60 Gy of the Stupp regimen, but not yielded any increase in overall survival [71] except in elderly individuals above 70 years of age [72]. This has been evaluated in the context of conventional therapy with surgery and temozolomide addition, but not immunotherapy. It has also been hypothesized that radiotherapy suppresses the immunological response to the tumor, by killing the immune effector cells present in the irradiated space. This is contrasted with findings that irradiation also is immune stimulating, as seen with abscopal effects. The key here seems to be radiation fractioning and timing. Many studies have used single-fraction irradiation in conjunction with an immunotherapeutic agent, such as IDO-inhibitors or PD-1 blockade. Not much research exists where the timing and dosing of radiotherapy is explored in this context [49, 73].

Modeling of tumor growth

A visualization of the interactions modeled in paper II can be seen in the figure below. The different interactions between administered radiotherapy and tumor-infiltrating immune effector cells, and the effect of IDO. The model presented by Serre et al. [74] is a model of the interaction of radiotherapy and CTLA-4 and PD-1 inhibitors in a tumor setting. We repurposed the model to describe the daily growth of the tumor volume (1), the inhibition of the tumor growth by the immune effector cells (5), the effect on the tumor cells as well as the immune cells by the radiation (3, 6), the stimulation of the immune response by TAA (4), and the inhibition of IDO effect by 1-MT administration.

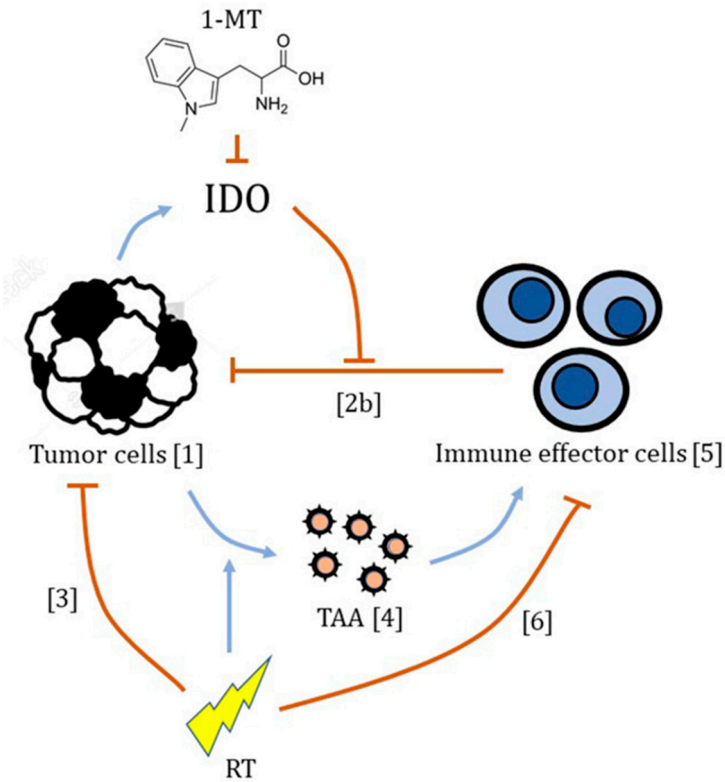

Figure 2. Illustration of mathematical model of interaction between tumor, immune system and therapeutic effects.

Results

Here, results of the papers are briefly presented. For in-depth discussion and results, please see the included papers.

Paper I: Effect of blockade of Indoleamine 2,3-dioxygenase in conjunction with single fraction irradiation in rat glioma

In this paper we find that rats carrying the RG2 model of GBM show longer survival when treated with a combination of 1-MT and a single fraction of 8 Gy radiotherapy to the tumor area, when compared to untreated controls, or animals treated with only either one of the treatment modalities. Comparison between the groups showed significantly longer survival with combined treatment compared to controls and RT treated animals, although the difference between the combined treatment and 1-MT only was non-significant (29 days ± 0.75 vs 18 ± 0.28, p=0.215). None of the single treatments increased survival significantly over untreated controls.

Paper II: Mathematical modelling of the synergistic combination of radiotherapy and (IDO) inhibitory immunotherapy against glioblastoma

The results of this paper are twofold. We fit data from the previous paper along with historical data from experiments with single-fraction RT in rats carrying RG2 GBM in a mathematical model designed to model the response and immunological effects of radiation and treatment with immune checkpoint inhibitors. This shows that a model of synergistic effect between radiotherapy and immune checkpoint inhibitors can be used to model a similar relationship between IDO-inhibition and RT, and we find that it can be fitted with available data. We then use this model to generate predictions of optimum RT fractionations and doses, which we move on to test in paper IV.

Paper III: Growth pattern of experimental glioblastoma.

In this paper a comprehensive presentation of the growth pattern and histological features of our syngeneic, GFP positive glioma model, NS1, is presented. Histological features of the model are described, with highly invasive growth pattern, perivascular infiltration, areas of necrosis. Genetic characteristics consistent with primary GBM are demonstrated. We show infiltration of CD4, CD8 and FoxP3 positive immune cells in the tumor periphery and surrounding tissue. Survival of inoculated animals showed a clear dose-response pattern with shorter survival times as number of inoculated cells increases, and volumetric measurements over time are presented in rats intracranially inoculated with 5000 cells. We propose the model as a useful tool for in vivo immunotherapeutic studies.

Paper IV: Increased effect of two-fraction radiotherapy in conjunction with IDO1 inhibition in experimental glioblastoma

Based on our own data and the literature on immunogenic radiation, we hypothesized that fractionated radiation may have greater effect on gliomas compared to unfractionated RT. We show here that two fractions of 8 Gy towards a tumor in combination with IDO inhibition gives a significant reduction in tumor volume at 18 days after inoculation with the NS1 glioma model. We show that overall survival increases significantly with two-fraction RT in combination with IDO-inhibition compared to untreated controls. Furthermore, we present immunological serum marker analysis from the animals in these cohorts, although failing to demonstrate significant difference in any of the markers analyzed but one, IL-1A, which was significantly lower in the untreated group compared to all treated groups.

We present gene expression of IDO from three available public datasets comparing glioblastoma tissue compared to normal brain tissue, or glioma stem cells (GSC) compared to adult neural stem cells. Here we see that in one of the sets, glioma tissue has significantly increased expression of IDO compared to normal brain tissue, and interestingly in the GSC set, GSC:s show a marked increase in IDO expression compared to neural stem cells.

Discussion

In this thesis, we find there is promise in the combinatory usage of IDO-inhibition combined with hypo-fractionated low dose radiotherapy in the treatment of gliomas. There are however many factors to take into consideration when contemplating these results and their potential application for future research.

Glioma models

The attentive reader will notice that two different glioma models were used in papers I and IV. This makes results slightly difficult to translate between the different papers, although no contradiction arises in the results. In the first paper a single fraction of RT was compared to controls and monomodal groups, and in the fourth a two-fraction regimen was used in the survival study and compared with untreated controls. The difference in effect on tumor size between two-fraction and single-fraction radiotherapy is illustrated in the fractionation study in paper IV, where a visible difference is seen between the single-fraction and the two-fraction regimens on tumor size at the time of experiment termination. This could be interpreted as consistent between the two models. Even so, inferences from the different results on different models should be made with care. The NS1 model we have developed continues to display the characteristics of a high-grade glioma and we see positively on its prospects as a subject for future preclinical research.

Modeling aspects

It is an attractive prospect to be able to harness models and simulations to generate data without the sacrifice of large amounts of time, drugs or research animals. Although before too many conclusions are drawn, it is important to recognize the multitude of assumptions needed to be made to fit the model to the collected data, and the limitations of that data in our study. But there are some interesting aspects of the model we employed here; we did see that the model generated an optimal number of fractions larger than one and reduced therapeutic effect as the number of fractions increased beyond four, although the model fit was generated using only

data from single-fraction trials. In the future, adding more data to further improve the modeling accuracy will possibly generate more promising hypotheses. This data, however, will be applicable to experiments in the present animal model of the disease only. Just as with all research on animal models, care must be taken to not too readily translate findings in animal models to clinical usage. New models will undoubtedly be formulated in the relevant systems, and I believe that the preclinical model will be able to inform the clinical models of disease treatment and progression.

Radiation fractionation and dosage

We have explored a rather limited range of fractions and dosages in this thesis. There is some evidence for higher dosages in single fractions when combined with immunotherapy in mice [75], however the optimal dosage and fractionation remains to be discovered. There is also big hurdle to overcome in the translation of these rodent findings to human equivalents, as simple radiation dosage translations are currently impossible. This is an area where published data and knowledge is at a very preliminary stage and there are many avenues to explore in this context, including fractionation, dosage, timing and combinations with different immunotherapeutics.

Future prospects

Radiotherapy and immunotherapy are here to stay in cancer treatment. Technological advances and minimizing of adverse effects are continually progressing and continue to generate better therapeutic results. The fractionation and dosage we explore in this thesis however is very different from the established treatment in clinical use today, and this of course raises some ethical considerations when moving into clinical trials. Many clinical trials are performed on patients after receiving conventional treatment for GBM, and no experimental trials on immunotherapy and radiotherapy have been performed on treatment-naïve patients. This makes conclusions very difficult to draw from these trials in relation to immunotherapy studies, seeing as there is an apparently complex relationship between radiation and the immune system. Radiation may be heavily immunosuppressive in the conventional schemes compared to hypofractionated treatment, which is why this regimen may require to forego conventional treatment in patients with already poor prognoses, in favor of an unknown benefit. This is however a field that deserves to be explored thoroughly in a preclinical context, as the potential for benefit is apparent for patients affected by this disease.

Conclusions

This thesis focuses on studying the synergistic effects of immunotherapy and radiotherapy in a preclinical glioblastoma setting. We find a synergistic relationship between IDO inhibition and radiotherapy in hypo-fractionated low-dose settings. We continue and find that two-fractioned radiotherapy outperforms single fraction therapy when combined with IDO inhibition. This simple combination of IDO inhibition and RT increases survival in rats carrying models of glioblastoma. This is a promising and interesting pathway towards more potent glioblastoma treatment. Even though this simple combination alone might not in the future effect a cure for glioblastoma, it is an explorative step towards a more complete and functional glioblastoma treatment that is not currently available, but that may well one day be.

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