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Jacobs Journal of Radiation Oncology

Research Article

Effect of Blockade of Indoleamine 2, 3-dioxygenase in Conjunction with Single Fraction Irradiation in Rat Glioma

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Received: 09-14-2015

Accepted: 11-02-2015

Published: 11-12-2015

Abstract

Glioblastoma (GBM), or WHO Astrocytoma grade IV, is the most common primary brain tumour in adults. GBM is shown to escape host immune surveillance through many paths, of which expression of indoleamine 2,3-dioxygenase (IDO), leading to induction and accumulation of regulatory T-cells in the tumour microenvironment, has been shown to be of importance. 1-Methyl tryptophan (1-MT) is an inhibitor of IDO that has been shown to have a positive effect on survival in experimental models of GBM. In this study, we evaluate the effect of combined single-fraction irradiation of 8 Gy with 1-MT treatment in Fischer rats carrying the RG2 glioma model. We also investigate expression of IDO in the RG2 model before and after irradiation.

Thirty-three Fischer 344 rats received intracranial inoculations of RG2 tumour cells, and were treated with either intraperitoneal 1-MT, 8 Gy single-fraction radiotherapy, or a combination of the two. Survival in the combined treatment group (29 days ± 0.75) was significantly better than controls (20 ± 0.99, p=0.015) and radiation only (17 ± 2.75, p=0.014). Survival was also better with combined treatment compared to 1-MT only but the difference was non-significant (18 ± 0.28, p=0.215).

Our results add to the growing base of evidence suggesting 1-methyl-tryptophan is an attractive candidate for clinical investigation in patients carrying highly malignant astrocytoma, especially in combination with radiation treatment, even in singular fraction settings.

Keywords: Glioblastoma; 1-MT; single-fraction radiation; IDO; RG2

Abbreviations

1-MT : 1-Methyl Tryptophan;
IDO : Indoleamine 2,3-dioxygenase;
GBM: Glioblastoma;
Treg : Regulatory T-cell

Introduction

Glioblastoma, classified by the World Health Organization as Astrocytoma grade IV, is the most common and one of the most aggressive primary tumours of the central nervous system, with a yearly incidence of 2-3 per 100 000 in Europe and North America. The incidence of primary brain tumours in Sweden is approximately 1350 per year, out of which a majority are glioblastomas [1]. Current standard treatment consists of surgery for attempted removal of the tumour if possible, followed by radiation therapy and concomitant Temozolomide. Prognosis for patients continues to be poor and has not improved substantially during the last decade, holding fairly steady at an approximate median survival of 15 months with treatment [2, 3]. In the face of this abysmal prognosis a pressing need for new avenues of treatment for this disease is apparent.

Targeted therapies for glioblastoma is an expanding field. Improved molecular understanding of glioblastoma has led to trials with several therapies. Inhibitors of both VEGF as well as EGFR have been explored and shown initial promise, but have had limited success in clinical trials [4]. Immune privilege in the CNS was long supported by data based on non-rejection of tissue transplanted into the CNS. However, further research has demonstrated the CNS as immunocompetent, capable of communicating with the peripheral immune system, as well as allowing antigen specific T-cells to acquire effector function inside the CNS. This has significantly expanded the field of immune therapies in the CNS, including tumour antigen vaccines, monoclonal antibodies and inhibitors of T-cell enzymes [5-7].

The immunological suppression and avoidance of glioblastomas are mediated by several factors. Expression of indoleamine 2,3-dioxygenase (IDO) is one of these. A majority of glioblastomas express IDO [8], which induces regulatory T-cell (Treg) activation in, as well as recruitment into, the tumour microenvironment [9]. Treg accumulation in gliomas is associated with poor prognosis [10, 11]. IDO is an enzyme catalyzing oxidation of tryptophan, the depletion of which inhibits effector T-cells in addition to the mentioned Treg activation, further increasing immune tolerance to the tumour mass. IDO has been shown to induce immunological tolerance to tumour tissue and other tissue in a variety of settings [12, 13]. IDO also seems to have an effect in pregnant females leading to immunological tolerance towards the fetus [14], as displayed in pregnant mice which reject their own fetuses when the IDO expression is absent [15]. Upregulation of IDO using Zebularine has been shown to result in strong tolerogenic effects in allotransplanted pancreatic islet cells in diabetic rats [16].

IDO activity can be inhibited with 1-methyl tryptophan (1-MT) in glioma cells in vitro, and has been shown to inhibit depletion of tryptophan as well as reduce the number of Tregs in glioblastoma tissue [17] as well as in human ovarian cancer [18]. 1-MT has been approved for use in metastatic malignant melanoma and is used in clinical trials for this disease. 1-MT has been shown to increase overall survival in a mouse model of glioblastoma in conjunction with chemoradiotherapy compared to chemoradiotherapy alone, by mechanism of increased complement deposition in the tumour tissue [19]. In induced colonic preneoplastic lesions in rats, 1-MT has shown to inhibit IDO activity and thereby protect against development of cancer [20]. A toxicological study of 1-MT has shown no toxicity in rats or dogs with oral administration of the drug [21].

Radiotherapy has an important place in treatment of glioblastoma patients and remains one of the most effective current clinical treatments. Evidence of its usefulness in combination with immunotherapy and immunomodulatory therapy is also growing [22]. In addition to the direct cytotoxic effects upon tumour mass, radiotherapy has been shown to induce host immunological effects towards tumour tissue. This includes up-regulation of tumour antigen presentation mechanisms as well as enhanced tumour recognition. Abscopal effects, where immune responses can be seen even in non-irradiated tumour tissue ("out-of-field") in a radiotherapy treated patient, have also been described. Radiation increases infiltration of T lymphocytes in tumours as well as increases expression of MHC [23, 24]. Depriving gliomas of immunomodulatory mechanisms like high IDO expression in conjunction with radiotherapy is therefore an attractive approach to explore in order to increase overall survival.

Finding the optimal dosage and fractioning to maximize immunological effect of irradiation is a challenge. There is no clear dose and fractionation that is clearly superior, and multiple schemes have shown efficacy [25]. This study aims to evaluate 8 Gy in a single fraction, as it causes few adverse effects, and earlier research has shown promise in the range of 5-15 Gy in a similar setting [26].

A number of different murine glioma models are in use today. This study uses the RG2 glioblastoma model, developed by Wechsler et al. in 1969 [27] and described further in 1995 by Aas et al [28]. The RG2 model has been used in a number of preclinical studies for glioblastoma treatments [29, 30].

The goal of this study is to evaluate the effect of indoleamine 2,3-dioxygenase blockade using 1-methyl tryptophan in conjunction with single-fraction radiotherapy upon overall survival in rats carrying gliomas of the RG2 cell line compared to untreated controls as well as these treatments as single therapies. In addition, we aim to evaluate the expression of IDO in cells from the RG2 cells in vitro before and after irradiation.

Materials and Methods

Animals

A total of 33 Fischer 344 rats were used in this study. Rats were

purchased from Fischer Scientific.

Rats were housed in pairs or threes in rat hutches with access to water and rat chow ad libitum. Animals were monitored daily, and those displaying signs of paresis, epilepsy, or declined general condition were euthanized. If an animal survived for 100 days without displaying symptoms, it was euthanized in accordance to the ethical permission. All euthanized animals' brains were resected and examined.

This study was approved by the animal ethics committee in Lund with permit ID M30-13 (Nittby). All efforts were made to minimize animal suffering.

Cell culturing and suspension

Cell culturing was prepared using RPMI-1640 (Sigma-Aldrich) medium with addition of 1% ml Na-pyruvate, 1% ml HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.1% ml gentamycin, as well as 10% fetal calf serum. The medium was heated to 56 °C for 30 minutes. After culturing in T75 flasks, the cells were prepared for inoculation by flushing away the medium using PBS. Trypsin was added to the liquid to loosen the cells into suspension. More medium was added and viable cells were counted. Cells were washed again to remove any potentially immunogenic calf serum, then centrifuged and the liquid removed. R0 medium was added to achieve the concentration used for inoculation, 1000 cells/µl.

Real time quantitative PCR

RT-PCR analysis was performed using the the SuperScript® one-step RT-PCR with Platinum® Taq DNA Polymerase kit by Invitrogen™. Quantitative real time PCR was performed using the SuperScript® III Platinum two-step qRT-PCR kit with SYBR® Green by Invitrogen™. Gene expression was normalized against expression of HPRT, which has been shown to be the best single reference gene for gene expression analysis in cancer research [31].

Intracranial tumour inoculation

Each rat received an intracranial inoculation of 5000 cells from the RG2 tumour line, suspended in 5 µl of nutrient solution. This was achieved under isoflurane inhalation anesthesia using a stereotactic frame with a 10 µl Hamilton syringe, at a depth of 5 mm, 2 mm laterally from the sagittal suture, and 0-1 mm anteriorly from the coronary suture, on the right side of the cranium. The cell suspension was administered at a rate of 1 µl/min, and the syringe retracted at a pace of 1 mm/min. The cranial burr hole was sealed with bone wax, and the incision closed with absorbable suture. All rats went untreated for seven days after inoculation, to allow for establishment of tumour mass.

After inoculation, rats were assigned into one of four groups: one group receiving no treatment (control group, n=9), one receiving 1-MT only (1MT group, n=9), one receiving radiation only (RT group, n=5) and one receiving both 1-MT as well as radiation treatment (RT1MT group, n=10).

Radiation therapy

In groups receiving the treatment, radiation was administered on day 7 post inoculation in a single fraction of 8 Gy. Irradiation was administered using a Gulmay Medical D3225 orthovoltage x-ray radiotherapy unit with beam energy set at 200 kV, with a 0.5 mm Cu filter. An applicator with a maximum field size of 40 mm x 40 mm was used, and the radiation field was further collimated to cover the frontal half of the brain (approximately 8 mm x 11 mm). The source-to-skin distance was 50 cm, and the absorbed dose rate was 1.2 Gy/min. During treatment, the animals were anesthetized by intraperitoneal injection of 60 mg/kg of ketamine.

1-Methyl tryptophan

In the 1-MT treated groups, a daily dose of 10 mg of 1-MT at a concentration of 4 mg/ml was administered via intraperitoneal injection under isoflurane anesthesia, for 11 weekdays (Monday through Friday) starting at day 7 post inoculation with breaks over weekends. 1-DL-MT was prepared by dissolving 4 g 1-DL-MT in 1 L 0.1 mol/L NaOH solution, after which pH of the solution was adjusted to 7.5 by addition of HCl.

Tumour histology

As an animal was sacrificed, the brain was removed and fixed in 4% formaldehyde solution. Sectioning was done using a freezing section procedure, after treating the formaldehyde-fixed brains in a 30% glucose solution for three days. A section thickness of 40 µm was used. After sectioning, staining with hematoxylin and eosin was performed to verify tumour presence in each of the sacrificed animals.

Statistical evaluation

The main outcome of focus in the study was overall survival in the different groups. Survival was measured in number of days to development of symptoms from tumour cell inoculation, and was described using medians and standard error. Using IBM SPSS 22.0, difference in overall survival distributions between groups was calculated using Kaplan-Meier analysis and log-rank (Mantel-Cox) test. *P* values of less than 0.05 were considered significant.

Results

Overall survival

Median survival in the RT1MT group was 29 days (29 ± 0.75). For the Control, 1MT, and RT groups, overall survival was 20 ± 0.99 days, 18 ± 0.28 days and 17 ± 2.74 days respectively. Ka-

plan-Meier survival curves are given in Figure 1. In the RT1MT group, one rat perished during treatment due to accidental perforation of a big blood vessel, and was thus censored in the Kaplan-Meier analysis. Another rat in the same group survived for the entire 100 day duration and was therefore censored. In this rat, no evidence of tumour mass was found in the cerebrum. Pairwise log rank comparisons between the RT1MT and the Control (χ^2 = 5.871, p = 0.015) and RT (χ^2 = 6.014, p = 0.014) groups showed significant difference in survival distribution. Comparison between RT1MT group and 1MT group showed a non-significant difference, χ^2=1.534, p = 0.215. No significant difference of survival distributions was found between the Control and the 1MT and RT group.

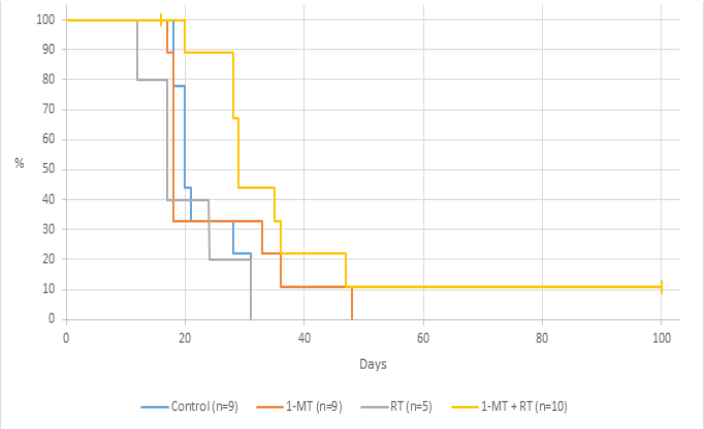

Figure 1. Kaplan-Meier survival curves for the different treatment groups. Vertical marks indicate censorship in the survival analysis.

Histopathology

Brains from the deceased animals were retrieved and sectioned. In the animal that survived for 100 days and was euthanized due to time constraints in the ethics permit, no evidence of tumour mass was found. One animal in the RT1MT group did not have a large hemispheric tumour, but rather a more posterior and smaller tumour. All other animals had a heavy intracerebral tumour burden, in line with the displayed clinical signs of brain tumour mass. Sectioning of the brains revealed that the tumours occupied most of the right hemisphere, and were solid and with a mass effect. No rat had any tumour in the left hemisphere. From a microscopical evaluation, we could not conclude that the tumours had any difference in size between the treatment groups, except for the two animals in the RT1MT group described above.

IDO expression

Using quantitative real-time PCR analysis, IDO1 expression normalized to HPRT expression (IDO/HPRT, mean values of three samples) was shown to be 142 before radiation, with a decrease to 79 after exposure to 8 Gy radiation. This is illustrated in Figure 2.

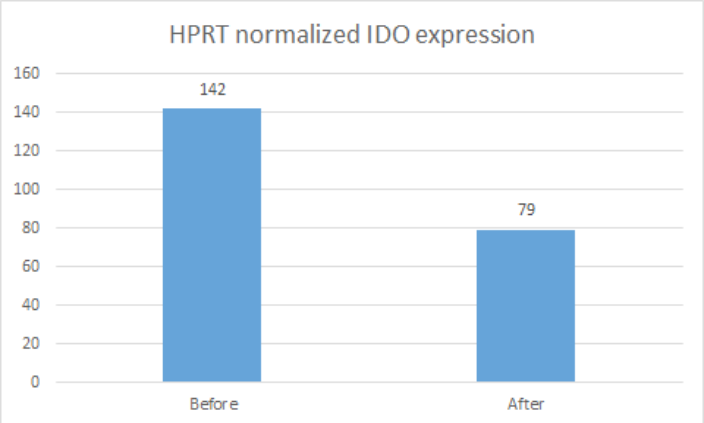

Figure 2. Expression of IDO, normalized to HPRT expression, before and after irradiation of RG2 cells.

Discussion

The results of this study show a significant overall survival advantage in rats treated with a combination of 1-MT and single fractioned radiotherapy compared to rats treated with only one of the treatment modalities. Interestingly, no significant advantage in survival was found in the singular treatment groups compared to controls. This may suggest there is a synergistic effect with IDO blockade and radiation in a singular radiation fraction setting. IDO inhibition leads to less regulatory T-cells and more effector T-cells. The synergistic effect may be that radiotherapy leads to up-regulation of tumour antigen presentation mechanisms as well as enhanced tumour recognition, enabling the immunomodulatory effect of IDO inhibition to be more effective by giving the T-cells more tumour associated antigens to react upon. This is in line with evidence suggesting an increased anti-tumour immune response as a result of radiation treatment.

In this study we also investigated the expression of IDO in vitro by cells from the RG2 glioblastoma model treated with radiotherapy compared to untreated cells. The effect of 1-MT upon expression of IDO was not measured. Further study on IDO expression may be done upon extracted tumour mass from rats receiving both treatments, since it is difficult to draw conclusions from in vitro experiments when studying an immunological reaction. This is a topic of further investigation to deepen the understanding of gliomas and the RG2 tumour model.

Investigations in 1MT in combination with irradiation has not previously been explored in rat models, and these results are helpful in order to expand the knowledge for further clinical application of this and other treatment strategies. It is also encouraging that the results of this study are in line with other research exploring the use of 1-MT in similar settings [32].

One particularly encouraging aspect of this study is the ap-

parent positive effect of the radiation treatment in this single fraction setting. Minimizing the absorbed dose lowers the risk and degree of side effects and 1-MT is a safe drug to use, and is possible to administer orally, which makes this combination an attractive approach for clinical testing in patients who have recurrent tumours and have already undergone the established treatment with surgery and concomitant Temozolamide and radiotherapy.

The results also show IDO expression in vitro seems to decrease following single-fraction irradiation. The effect however, is fairly small, and its implications difficult to evaluate. The immunomodulatory effects of radiation in cancers established in other research [23] seem to play a larger role in anti-tumour immune response, although greatly increased by IDO inhibition as our results may imply. This could suggest that gliomas depend on IDO for immune escape to a greater degree after irradiation, in spite of the decrease in expression, and are thus more vulnerable to inhibition of the enzyme. The absence of tumour mass in the animal with long term survival may be due to tumour regression, or that the inoculated cells did not manage to establish a stable tumour. Our control animals all developed large tumours and symptoms of tumour growth well in line with previous publications from our laboratory (Aas et al. 1996), where more than 200 animals were investigated and developed symptoms around day 19.4 after tumour inoculation. This strengthens the stability of our RG2 cell line, and possibly, the treatment in the rat who survived has actually been a cure. However, without imaging throughout the treatment period, this is impossible to definitively state.

The importance of tryptophan degradation by IDO in glioblastoma immune system avoidance and immunomodulatory treatment is well documented at this point [9-12, 17, 19, 33]. 1-MT has been established as a safe and non-toxic [21] agent for IDO blockade. Thought may have to be given to which isoform of the drug to use in clinical study, since some evidence suggests that 1-D-MT may upregulate a variant of IDO in cancer cells [34]. In this study we used 1-DL-MT. Study on the mother-fetus interface in mice using 1-L-MT and 1-D-MT showed that of these two, only 1-L-MT inhibited IDO and disrupted immunological tolerance to the fetus [35]. Another study showed 1-L-MT but not 1-D-MT inhibited tryptophan catabolism in human cancer expressing both IDO-1 and IDO-2 [36]. Evidence suggests that 1-MT alone may not be enough to hinder immune escape and Treg recruitment, but seems to be more promising when used as a part of a combinatory treatment. Wainwright et al. have shown a sustained antitumour response combining IDO blockade using 1-MT with blockades of CTLA-4 and PD-L1 in a mouse model [32]. Whether the effectiveness of 1-MT treatment could be increased by other routes of administration than peroral or intraperitoneal, remains to be studied. Blood-brain-barrier passage of 1-MT seems to be poor, as brain concentrations of 1-MT are much lower than in other tissue after peroral administration [21, 32]. Low BBB passage of 1-MT may warrant study on subcutaneously implanted tumours, to evaluate if concentration issues is limiting the efficacy of the drug.

Combination with other immunological treatments is another avenue of interest. Different vaccine treatments have been tried in patients carrying glioblastoma, for example a vaccine targeting EGFRvIII, and have shown to result in a survival advantage compared to similar populations [37-39]. Monoclonal antibodies, including radioisotope conjugated antibodies have also been investigated with interesting preclinical results [40]. Combining these with 1-MT treatment as well as radiotherapy is of interest for further study. Interestingly, Wainwright et al, when exploring IDO blockade in conjunction with CTLA-4 blockade and PD-L1 blockade, found that concomitant Temozolomide actually decreased overall survival, suggesting that chemotherapy may be harmful when a robust antitumour immune response has been established [32].

Whether different approaches to primary gliomas compared to secondary ones could be useful, remains to be discovered. Secondary gliomas have been shown to more often carry mutations of isocitrate dehydrogenase type 1 (IDH1)[41], and immunizations against mutated IDH1 has shown promising results in preclinical trials [42, 43]. These studies were done on models of lower grade gliomas, but may be translatable into secondary glioblastomas. IDO expression is higher in both secondary and primary glioblastomas compared to lower grade gliomas [11] and for the subgroup of secondary glioblastomas, a combination of IDH1 vaccination and IDO blockade may be of use.

In conclusion, 1-MT is by no means a silver bullet for cancers, including glioblastoma, although its potential use in a concomitant treatment regimen in gliomas, primary or recurrent, is intriguing. In this study we have displayed a survival advantage when administering 1-MT in conjunction with single fraction radiotherapy in rats carrying the RG2 glioma. Further study is also warranted in more complex combinatory treatments with for example anti-PD1 antibodies in combination with 1-MT.

Acknowledgements

We are grateful to Dr. Xing-Jun Jing for excellent assistance with cell culturing and suspension, as well as to Dr. Zhongtian Xue for assistance with RT-PCR procedures. We would also like to extend our gratitude towards the Elsa Schmitz Foundation and the Mrs. Berta Kamprad Foundation, for the financial support they have provided.

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