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Radiation Immune Modulation Therapy of Glioma

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1. Introduction

Since Roentgens discovery of the X-rays 1895, radiation therapy (RT) has been one of the most successful modalities used to treat cancer (Rontgen 1995). The experimental radiation treatment of glioma, however, took place first in 1938 (Bailey & Brunschwig 1938). Since then advances in radiation technology have expanded the role and value of using ionizing radiation in diagnosis, imaging and therapy of glioma. But despite substantial technical improvements in the current treatment modalities the survival rate for glioma patients is still very low (Barnholtz-Sloan, et al. 2007). Although the recently addition of temozolomide to conventional fractionated radiotherapy for newly diagnosed glioblastoma has resulted in an increased time of survival (Stupp, et al. 2005).

Immunotherapy utilizes the fact that the immune system has a potential to react against tumour antigens and that this can result in immunological control of the tumour. There is an increasing body of evidence that the activation of cytotoxic T-lymphocytes (CTL) has a positive effect on the long-term survival of cancer patients receiving traditional therapies such as surgery, chemo- or radiation-therapy (Nakano 2001; Prall 2004; L. Zhang, et al. 2003). It has been clearly demonstrated that tumour immune reactivity is of importance in treatment of several types of tumours (Shankar & Salgaller 2000). The immune response to glioma is primarily a result of the cell-killing function by the activated cytotoxic T cells (CTL). The aim of vaccination regimes is to enhance the effectors functions of CTL and the number of lymphoid cells within the glioma. But even if immune therapy cause large populations of lymphocytes to enter CNS tumours, total eradication of the glioma do not occur. This is partly due to the immunosuppressive factors produced by the glioma, which result in non-functioning CTL (Roszman, et al. 1991).

Traditional fractionated radiation therapy decrease the number of radiation sensitive T cells and damping the immune response of immunotherapy. Thus the interest in combining radiation therapy and immunotherapy has so far been very sparse. The use of sterotactic techniques with single radiation exposure or hypo-fractionated radiation therapy, however, does modulate the immune response and increases the therapeutic outcome (Lee, et al. 2009; Wersäll, et al. 2006). This radioimmuno modulatory effect of radiation opens for a new approach in glioma therapy by the combination of radiation- and immune-therapy.

Currently, there is a growing interest in combining radiation with other kinds of therapy, of which some are immunotherapy, to treat a broad range of malignancies (Chakraborty, et al.

2004; Gulley, et al. 2005; Sharp, et al. 2007). There is also an ongoing pre-clinical search for methods to enhance the therapeutic response of malignant glioma by combining immunotherapy with single fraction or hypo-fractionated radiation therapy (Demaria, et al. 2005a; Graf, et al. 2002; Lumniczky, et al. 2002; Newcomb, et al. 2006; B. R. R. Persson, et al. 2002; B. R. R. Persson, et al. 2003; B. R. R. Persson, et al. 2010; B. R. R. Persson, et al. 2008). The clinical trials using this approach, however, are still very sparse.

This chapter will summarize the aspects of the interaction of ionizing radiation with the immune system and its immunomodulatory effects and its implications for glioma therapy (Friedman 2002). Preclinical studies of the combinational approaches of radiation and immune therapies, which results in high fractions complete remissions of glioma in animal models, is reviewed. Various clinical studies towards combination of radiation- and immune-therapy for treatment of glioma are summarized in a final section.

2. Immune response of glioma

2.1 T cell infiltration in tumours and prognosis

Many tumours are potentially immunogenic and exhibit tumour-specific immune responses in vivo (Curiel 2008; Curiel, et al. 2004). Tumour-specific antigens are released from the tumour cells and then captured by antigen presenting dendritic cells (Huang, et al. 2010). Dendritic cell migration brings tumour antigen to the lymphoid organ where the antigen presentation stimulates immature T cells to become either "cytotoxic" CD8(+) T-cells (CTL), "helper" CD4(+) T-cells or memory T-cells (Fig. 1). Lymphocytes and some innate immune cells (macrophages, natural killer cells) migrate to the tumour in order to kill and eliminate tumour cells. Patients with high infiltration of lymphocytes in their tumours have usually found to have a better prognosis of survival.

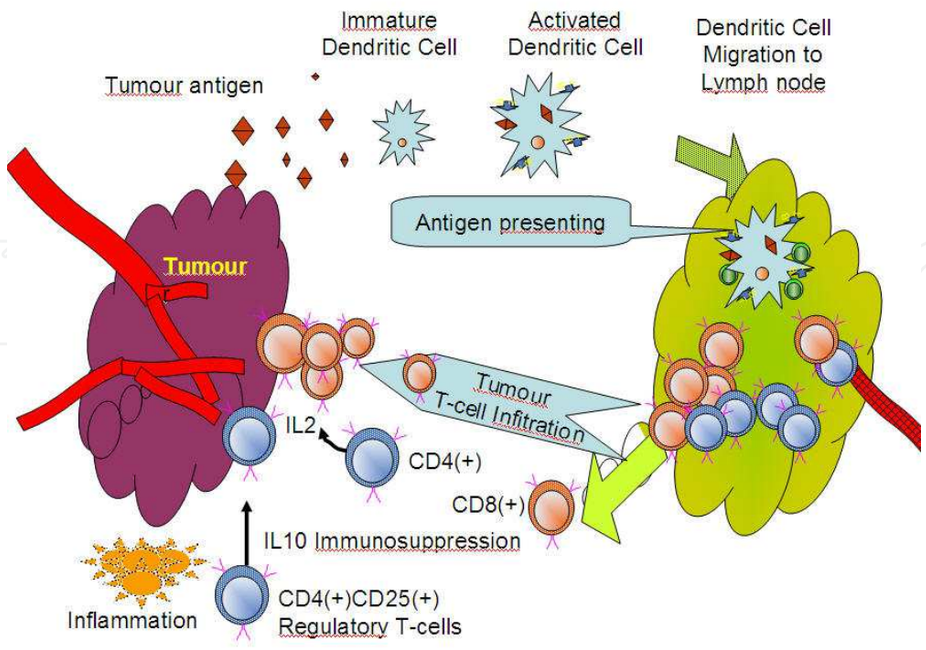

Fig. 1. Tumour immune response

Tumour infiltrating lymphocytes (TILs) of various subtypes represent the host-to-tumour reaction. Anti-tumour immune response is mediated by infiltrating CD8(+) T cells which have been shown to lyses tumour cells directly via recognition of the major histocompatibility complex class I (MHC-I) present on most tumour cells. But some tumours, which have low or none expression of MHC-I, are not affected by the CTL. Tumour infiltrating CD4(+) helper T cells seems to play a role in regulating and amplifying tumours response by priming tumour-specific cytotoxic CD8(+) T cells, as well as macrophages involved in clearance of dead tumour cells (Toes, et al. 1999; Vesalainen, et al. 1994).

In Fig. 1 is shown how tumour antigens are captured by antigen presenting cells such as dendritic cells, which migrate to regional lymph nodes. There they present the antigen to T-cells which differentiate into CD8(+) cytotoxic T-cells, CD4(+) helper T-cells, and memory T-cells. The cytotoxic CD8(+) T-cells (CTL) are transferred to the tumour in order to kill the tumour cells. The CD4(+) release IL2 which help the CD8(+) T-Cells to proliferate. But the CD4(+) can also form CD4(+)CD25(+) regulatory T-cells which excrete IL10 to suppress the activity of the CD8(+) cytotoxic T-cells.

The number of tumour infiltrating lymphocytes can be used as prognostic factor for several types of cancer (Cho, et al. 2003; Rauser, et al. 2010; Schumacher, et al. 2001; Zingg, et al. 2010). But in malignant glioma the use of tumour infiltrating lymphocytes as a prognostic factor seems to be more complex. The overall reports on tumour-infiltrating CD8(+), CD4(+) T-cells and major histocompatibility complex class I (MHC-I) expression in malignant glioma do not yield consistent correlation with clinical outcome (Dunn, et al. 2007). There seems to be factors present in patients with glioma that suppress the action of tumour infiltrated lymphocytes, and it has been demonstrated that glioma cells can actively paralyze T cell migration by the expression of Tenascin-C (Huang, et al. 2010).

Regulatory CD4(+)CD25(+)FoxP3(+) T cells (Treg) have been shown to play a major role in suppression of the immune response to malignant glioma. In human CNS tumor samples both CD4(+) and Treg infiltration have found to be significantly increased throughout the time of metastatic tumor progression. Thus immunotherapeutic strategies for treating metastatic CNS tumors must fight against Treg (Sugihara, et al. 2009). In an experimental GL261 intracranial tumor model, it was shown that depletion of CD25(+) regulatory T-cells (Treg) using anti-CD25 antibodies enhance the efficacy of DC immunotherapy (Maes, et al. 2009).

Infiltration of myeloid suppressor cells (MSC) is another factor inhibiting the function of the CD8(+) T cells, which results in tumour progression (Graf, et al. 2005). Other studies indicate that glioma seems to secrete factors such as TGF_{β} and prostaglandins (PGE2) that depress the cell-mediated immunity by down regulating the function of infiltrated CD8(+) T-cells and monocytes (Dix, et al. 1999; Farmer, et al. 1989). This might be one of the reasons why anti-tumour response of the immune system is decreased in patients with primary glioma (Brooks, et al. 1972).

2.2 Radio-immune-modulating effects by local irradiation

Recent studies have shown that local single fraction radiotherapy stimulates the immune response by enhancing the antigen presentation of MHC class I (Liao, et al. 2004). The mechanism underlying these effects is probably at the level of the proteasome in the cytoplasm of the tumour cell, which are essential for production of antigenic peptides for

loading onto MHC class I molecules. The proteasome in tumour cells is a sensitive target for radiation, resulting in decreased processing of endogenous self antigens. The processing of tumour antigens is, however, increased by radiation, which enhance the accumulation of antigen/MHC class I complexes on the cell surface (Pajonk &Mcbride 2001).
Radiation therapy also causes an increase in production of the cytokine IFNγ in the target region which up-regulates low levels of MHC class I, creating a tumour microenvironment conducive for CD8(+) T cell infiltration and their recognition of tumour cells (Lugade, et al. 2008).
It has been demonstrated that antigen presentation by MHC class I is increased for many days by single fraction radiation therapy. The most pronounced effect was recorded at 7 days after irradiation with an absorbed dose of 8 Gy. This might be one of the reasons why the efficacy of tumour immunotherapy is most effective in combination with single fraction radiation therapy (Reits, et al. 2006). Maximum loading of the tumour micro-environment with cancer antigen occurred 2 days after radiation therapy and coincided with the optimal time for CD8(+) T cell transfer (Bin Zhang, et al. 2007).

2.3 Radiation effecting dendritic cells DC function

It has been demonstrated that the radiation modulation of MHC-I mediated antitumor immunity also depends on the antigen presenting pathways of the dendritic cells (Liao, et al. 2004). The dendritic cells either initiate an effective cytotoxic response against antigen-bearing cells, or produce tolerance, depending on the context in which those antigens are presented (Zou 2005). It has been shown that cell death caused by radiation therapy release tumour antigen, which facilitates an effective cytotoxic response of the dendritic cells (Hatfield, et al. 2005). Radiation therapy activation of dendritic cells (DC), induce secretion of interleukin-1 beta (IL-1_{β}), which is required for the adequate polarization of IFN_{γ} producing CD8(+) T-cells (Aymeric, et al. 2010).

3. Preclinical experience of glioma-radio-immune-modulatory therapy

In the Lund clinical study, named "Brain-Immuno-Gene-Tumour-Therapy" (BRIGTT), patients were immunized with their own tumour cells, cultivated from their surgical specimens and transfected with human IFNγ gene (Salford, et al. 2002). The cells taken from the surgically removed tumour were grown in culture. The day before immunization the karyotyped tumour cells were infected with an Adenovirus expressing human IFNγ. At the day after transfection, the immunization of the patient takes place soon after the cells have been irradiated with Cs-137 gamma radiations to an absorbed dose of 100 Gy (Baureus-Koch, et al. 2004). By subcutaneous (s.c.) implantation of these cells in the arm of the patient it is expected that the host immune system is activated against the tumour. The activated CD8(+) T-cells will pass the BBB and attack the cancer cells present at the primary tumour site as well as the distant metastases "*guerrilla cells*" (Salford, et al. 2006; Salford, et al. 2001; Salford, et al. 2002; Salford, et al. 2004; Siesjö, et al. 1993; Visse, et al. 1999). Results from the first eight human treatments in the phase 1—2 BRIGTT study show that immunization with transfected tumour cells is safe for the patients and improves survival (A. Persson, et al. 2005; Salford, et al. 2005; Salford, et al. 2011; Salford, et al. 2004).
In order to further enhance the effect of this immunotherapy we investigated the effect of combining it with a single fraction radiation therapy in an animal model. The results of

these preclinical experiments, which were performed already 2001, showed that a single fraction of RT combined with immunotherapy resulted in a significantly increased survival time of rats with intra-cranially implanted N29 or N32 glioblastoma. Further there were significant numbers of complete remissions of the most infiltrative N29 tumour implanted in Fischer-344 rats (B.R.R. Persson, et al. 2010). Other researchers have also reported substantial tumour regression by single fraction radiation therapy combined with various regimes of immune therapy (Bradley 1999; Chakraborty, et al. 2003; Demaria, et al. 2005a; Friedman 2002; Garnett, et al. 2004; Graf, et al. 2002; Lumniczky, et al. 2002).

3.1 The Lund experience of combined single fraction RT and Immunization with IFN-γ secreting tumour cells

3.1.1 Animals and tumour cell lines

Fischer-344 rats were maintained by continuous, single-line brother to sister mating in the laboratory at Lund. During the experiments rats of both sexes, females weighing around 190 g and males 370 g respectively, were housed in a climate controlled cabinet. Otherwise they were kept in Macralon cages provided with food pellets and water *ad libitum*. All experimental animal procedures were approved by the Animal Ethical Committee in Malmö/Lund (Lunds tingsrätt, Box 75, 22100 Lund Sweden).

All cells were maintained in culture flasks (Nunc, Denmark) and harvested by treatment with trypsin/EDTA. The culture medium was antibiotic-free RPMI-1640 medium supplemented with 5-10% foetal calf serum, L-glutamine (2 mM), HEPES (10 mM), pyruvate (0.5 mM) and $NaHCO_3$ (11 mM). The cell-cultures were regularly checked for contaminating microbes by staining with the fluorescent dye Hoechst 32 258 and examined with fluorescent microscopy. If *Mycoplasma* infection was indicated the cultures were discharged or treated with *Mycoplasma Removal Agent* (Hoechst, Germany) twice with 7 days interval, and repeatedly confirmed free of infection.

The tumour cells (N29 or N32) used for immunization were interferon-gamma (IFN-γ) gene modified to enhance secretion of IFN_γ. The cells were cultured for one week, washed twice, and suspended in serum free medium (IMDM-0) to a cell density of $2 \cdot 10^4$ cells/ml. Just before immunization the cells were transferred from the culture flasks to 15 ml centrifuge test tubes (Nanclon) and stored on melting ice to prevent the cells to grow during the procedure. Irradiation of the cells was performed during 20 minutes at room temperature to an absorbed dose of 70 Gy by using a ^{137}Cs gamma-ray source (*Gammacell 2000;* Mølsgaard Medical, Risø, Denmark) (Siesjö, et al. 1996; Sjögren, et al. 1996; Visse, et al. 1999).

3.1.2 Inoculation and treatment of intracerebrally tumours

Inoculation was performed by injecting 5 000 tumour cells in 5 μl nutrient solution into the head of Fischer 344 rats, using a stereotactic technique with a Hamilton syringe. To avoid extra-cranial tumour growth, the injection site was cleaned with 70% ethanol after injection and the borehole was sealed with wax. The animals were arranged into 6 groups, which included: controls, RT with either 5 or 15 Gy, immunization with IFN-γ gene modified tumour cell, and RT with either 5 or 15 Gy combined with immunization (Table 1).

Animals were given a single radiation treatment using a ^{60}Co radiotherapy unit (Siemens Gammatron S) with a source-skin distance (SSD) of 50 cm and the maximum absorbed dose rate 0.65-0.70 Gy/min. The radiation field size was collimated to cover the brain. The adsorbed dose of either 5 or 15 Gy was measured both by an dose-meter diode and TLD

dose meter. A sheet of tissue equivalent bolus, 5 mm thick, was placed over the head for radiation build up.

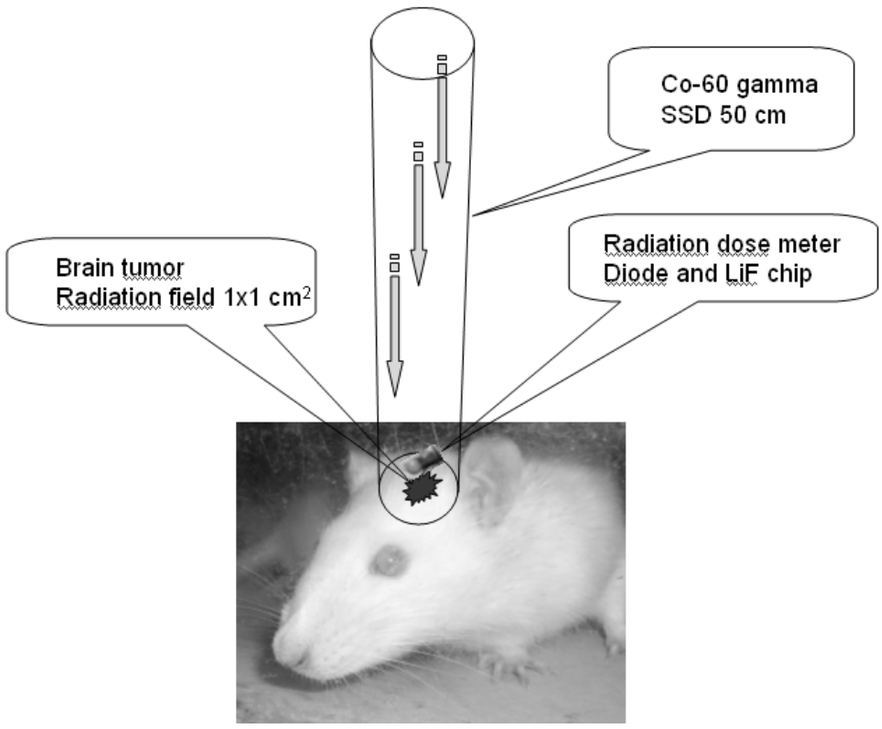

Fig. 2. Radiation therapy was performed at day 7 after inoculation with the animals anesthetized with 5% chloral hydrate given intraperitoneally (i.p.) or Ketalar®/Rompun®, 0.55 ml per 100g. The animals were given a single radiation exposure using a ^{60}Co radiotherapy unit (Siemens Gammatron S) at a source-skin distance (SSD) of 50 cm with a maximum absorbed dose rate of 0.70 Gy/min. The radiation field (1 cm^2) was collimated to cover the brain (Fig. 2). The delivered adsorbed dose of either 5 or 15 Gy was measured both by an dose-meter diode and a Lithium fluoride (LiF) TLD chip placed next to the tumour in the field under the bolus.

The animals were immunized by intraperitoneally administration of 3 x 10^6 IFN-γ gene modified N29 or N32 tumour cells, which immediately before had been irradiated with 70 Gy ^{134}Cs gamma-radiation. The first immunization was performed within one hour after the radiotherapy session at day 7. In the rats still alive it was repeated at least two more times at days 21 and 35.

Group No.	Treatment	Number of N29 Animals Experiment A	Number of N32 Animals Experiment B	Number of N32 Animals Experiment C
1	Controls with no treatment	6	9	3
2	Radiation 5 Gy	8	7	
3	Radiation 15 Gy	8	6	6
4	Immunization	6	7	6
5	Radiation 5 Gy + Immunization	8	7	
6	Radiation 15 Gy + Immunization	8	7	6

Table 1. Number of animals in the groups of various treatments used in the experiments with either N29 or N32 tumours. The various experiments A, B and C respectively, were performed at different occasions

Following symptoms of the rats were used as signs of progressing tumour growth:

- keeping their heads turned to one side,
- rotating or losing weight,
- unwillingness to move,
- shaggy fur and
- reddening of the eyes and nose.

The rats were examined daily and when the animals developed symptoms, they were euthanatized and the brains were stained for histopathological examination.

None of the rats, which were inoculated with N32 tumour cells, survived longer than 30 days. But in the group inoculated with N29 tumour cells, surviving animals could be observed for more than 170 days. In this group of animals with N29 tumours, re-challenge was performed with $2 \cdot 10^5$ N29 glioma cells in 200 μl, administered just under the skin in the thigh of the hind leg. Fourteen out of the originally 46 rats, and 4 extra control rats with no previous treatment were inoculated.

3.1.3 Survival of rats with intracerebrally implanted N29 tumours

In Table 2 are given the fractions of animals intracerebrally implanted with N29 tumour cells, which were surviving more than 170 days: Controls; IFNγ cell immunization (IMU IFNγ), single fraction radiation therapy (RT with either 5 or 15 Gy), and their combinations (IMU IFNγ + RT with either 5 or 15 Gy). RT and first immunization was performed at 7 days after inoculation. Immunizations were then repeated for at least two more times at days 21 and 35. In the 2nd column of Table 2 are given the numbers of animals survived more than 170 days, versus the number in each group of animals with intra cerebral N29 tumour. In the 3rd column is given the number of tumours appeared, relative to the number of animals that were re- challenged, including the 4 extra controls.

In the last column of Table 2 is given the number of re-challenged animals without tumour versus the original number in each group. Those animals, which resisted re-challenge, seem to have been cured from their primary glioma.

Type of treatment	Fraction of Animals Survived >170 d	Fraction of animals with tumour in the re challenged survivors	Fraction of Cured animals
Controls	1/6	5/(1+4+)	0
IMU IFNγ 3x	2/6	1/2	1/6
RT 5 Gy	0/8	-	0
RT 15 Gy	2/8	2/2	0
IMU IFNγ 3x + RT 5 Gy	**6/8*)**	**4/6**	**2/8**
IMU IFNγ 3x + RT 15 Gy	3/8	2/3	1/8

*) p=0.03; +) extra controls

Table 2. The fraction of living rats in the various groups with different treatments, followed during 170 days after inoculation of N29 tumour cells in their brain, number of tumours after re-challenge, and fraction of cure.

By using Fisher exact probability test the results show that treatment with 5 Gy radiation therapy combined with immunization resulted in significantly increased number of survivals versus controls (p = 0.03). But neither immunization alone nor radiation therapy alone with single fractions of 5 or 15 Gy resulted in any significant therapeutic effect versus the controls.
The combination of radiation therapy with immunization compared with radiotherapy alone, however, resulted in significant survival fraction at both 5 Gy and 15 Gy, with p-values <0.01** and p <0.05* respectively.
The number of living rats in the various groups with different treatments, followed during 170 days after inoculation of N29 tumour cells in their brain, is displayed in Fig. 3 for each group respectively.
In Table 3 is given the median survival time and the p-values of two-sided non-parametric Mann-Whitney test versus the control. Immunization with N29 cells significantly increased

Type of treatment	Num. Rats	Median survival time (days)		Mann-Whitney 2-tailed versus Control	Tumour weight g	
Control	6	82	± 46		0.39	± 0.22
IMU IFNγ	6	132	± 44	P=0.04 *	0	
RT 5 Gy	8	46	± 14	NS	0.25	± 0.25
RT 15 Gy	8	93	± 35	NS	0,24	± 0.14
IMU IFNγ + RT 5 Gy	**8**	**153**	**± 31**	**P=0.003 ****	**0**	
IMU IFNγ + RT 15 Gy	8	119	± 35	P=0.03 *	0.01	± 0.01

Table 3. Number of rats; mean survival time and tumour weight at time of death of rats with intra cerebrally implanted N29 tumours treated one week after inoculation, with IFNγ cell immunization, radiation therapy (RT) and their combination. Immunization (IMU-IFNγ) was repeated for at least two more times at days 21 and 35. The rats were observed during up to 170 days after inoculation.

the survival time by 60% (p=0.04). Radiation therapy alone with 5 Gy, however, did not significantly increased the survival time. But immunization combined with 5 Gy radiation therapy resulted in a significantly increased survival time with 87% (p=0.003). Radiation therapy alone with 15 Gy did not significantly increased the survival time. But 15 Gy RT combined with immunization increased the survival time with 45% (p=0.03).

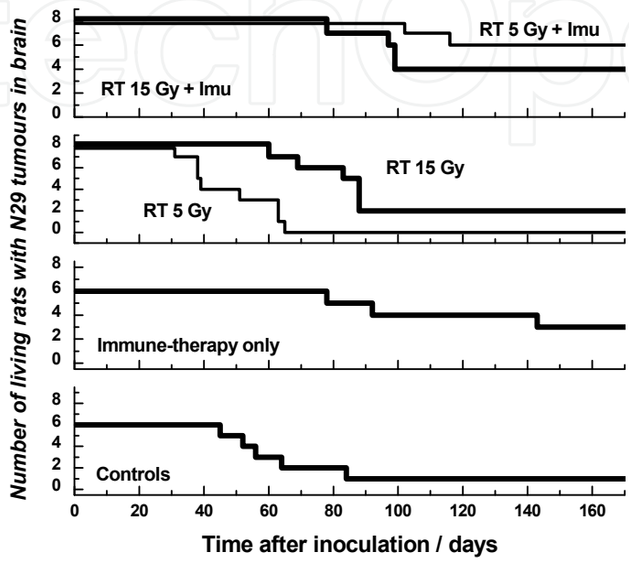

Fig. 3. Survival plot of intra cerebral implanted N29 tumours: Controls (Lower panel), immunization with syngeneic N29 tumour cells (2nd panel); radiation therapy (3rd panel) and combinations of radiation therapy and immunization (upper panel).

3.1.4 Survival of rats with intracerebrally implanted N32 tumours

The pooled results of the two experimental series (B and C in Table 1) with rats implanted with N32 tumours are displayed in Table 4. The results are given in terms of the mean survival time and weight of tumour at the time of death for each group animals. None of the rats with N32 tumours survived more than 30 days and thus no re-challenging could be done.

The survival of all rats with implanted N32 tumours were followed during 30 days and the results in the various groups of rats with different treatments are displayed in Fig. 4.

For the N32 tumours given a single fraction radiation therapy with 15 Gy resulted in significant increase of survival time with about 20% ($p<0.001$). The combination of 15 Gy single fraction radiation therapy with immunization of IFN-γ secreting syngeneic cells resulted in increased survival time by about 40% ($p<0.001$), although there were no complete remissions. But neither immunization with IFN-γ secreting syngeneic cells alone, nor radiation therapy with a single fraction of 5 Gy alone, or in combination with immunization, resulted in any increase in survival time of the N32 tumours in rats.

There is no significant difference in the weight of tumours in the different groups. Although the average growth rate of the N32 tumours treated with 5 Gy radiation therapies combined with immunization was decreased by 30% compared with the controls.

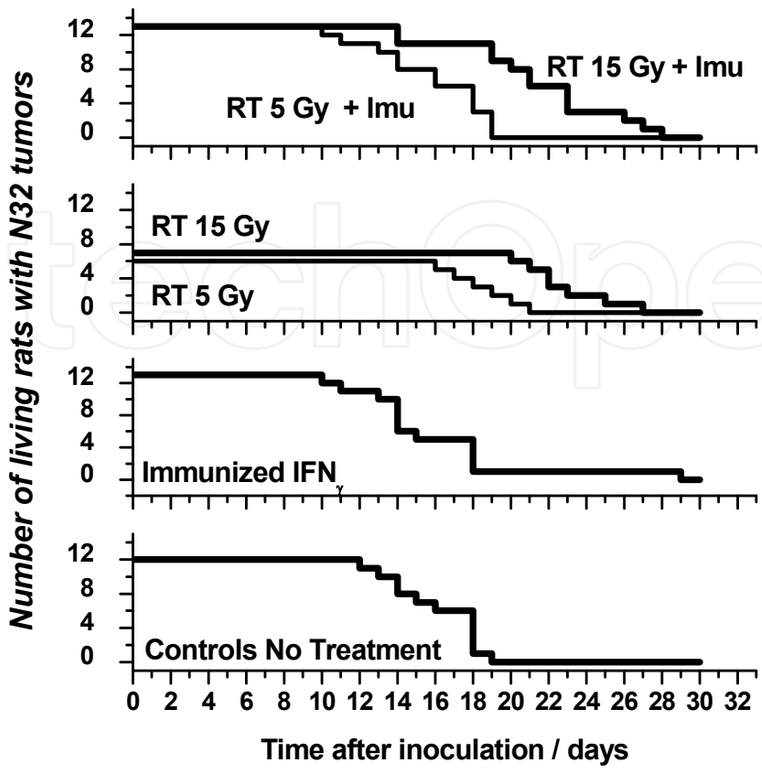

Fig. 4. Survival plot of intra-cerebral implanted N32 tumours: Controls (Lower panel); Immunization with syngeneic N32 tumours cells (2nd panel); radiation therapy (3rd panel), and a combination of radiation therapy and immunization (upper panel).

Type of treatment	Num. Rats	Median Survival time days		Mann-Whitney 2-tailed test versus Control	Tumour weight g	
Control	12	19	± 3		0.19	± 0.16
IMU IFNγ	13	19	± 6	NS	0.25	± 0.23
RT 5 Gy	6	19.5	± 2	NS	0.18	± 0.10
RT 15 Gy	13	23	± 2	P<0.001***	0.16	± 0.13
RT 5 Gy +IMU IFNγ	7	19	± 2	NS	0.14	± 0.09
RT 15 Gy + IMU IFNγ	**13**	**27**	**± 3**	**P<0.0001*** **	**0.30**	**± 0.28**

Table 4. Number of rats, mean survival time, and the significance of Mann-Whitney 2-tailed test versus control is shown in columns 2-4. In the last column is given the tumour weight at time of death of intra cerebral N32 tumours treated with syngeneic IFNγ transfected tumour cells (IMU IFNγ), radiation therapy (RT) and their combination (RT + IMU IFNγ).

3.1.5 Summary of the LUND experiment

The results of the Lund experiments reveal that a single fraction radiotherapy session of 5 or 15 Gy combined with immunization by i.p. injection of irradiated syngeneic tumour cells induces a significant anti-tumour response to intra cranial implanted glioblastoma tumours

in Fischer-344 rats. In the rats, which were inoculated with N32 tumour cells, the combination of 15 Gy single fraction radiation therapy with immunization of IFN-γ secreting syngeneic cells resulted in increased survival time by about 40% ($p<0.001$). But none of these rats survived longer than 30 days. In the group inoculated with N29 tumour cells and treated with 5 Gy RT combined with immunization the survival time was significantly increased by 87% ($p=0.003$), and 75% of the animals survived for more than 170 days. The difference in response of N29 and N32 cell lines indicate that there is difference in immune response in different clones of glioma.

3.2 The Hungarian experience of single fraction RT and Immunization with (GM-CSF, IL-4, IL-12) in a mouse glioma (Gl261) brain tumour model

In Hungary a study was performed in a mouse glioma (Gl261) brain tumour model with single fraction radiotherapy combined with administration of cytokine-producing cancer cell vaccines (Lumniczky, et al. 2002). Their brain tumour bearing mice were treated with various cytokine producing vaccines made by in vitro transduction of Gl261 tumour cells with different genes such as: IL-4, IL-6, IL-7, GM-CSF, TNF_{α}. Immunotherapy alone with vaccines producing either IL-4 or GM-CSF resulted in complete remission in 20–40% of the mice. By combining immunotherapy using (GM-CSF, IL-4, IL-12) producing vaccines with local tumour radiotherapy (single fraction 6 Gy X-ray radiations) about 80–100% of the glioma-bearing mice were cured. The high efficiency of the combined treatment was maintained even under suboptimal conditions when neither of the individual modalities alone cured any of the mice (Lumniczky, et al. 2002). Their results are in good agreement the survival rate of 75% ($p<0.05$) achieved in the Lund study of N29 tumours in rats treated with IFN-γ secreting vaccine combined with 5 Gy single fraction RT (B. R. R. Persson, et al. 2010).

3.3 The U.S. experience of radiation therapy combined with vaccination of mice with Glioma or mammary carcinoma

3.3.1 Combining radiation therapy with blockade of the CTLA-4 pathway

The cytotoxic T lymphocyte-associated protein CTLA-4 is involved in the immune regulatory mechanisms that control the early stage of the T cell response. It has previously been demonstrated that blockade of the CTLA-4 protein enhance anti-tumour responses both in experimental systems and in clinical trials (Chambers, et al. 2001; Egen, et al. 2002).

In a mouse model of the poorly immunogenic metastatic mouse mammary carcinoma 4T1, however, neither anti-tumour response nor survival-time was affected by using an anti-CTLA-4 monoclonal antibody for blocking the CTLA-4 protein. But anti-CTLA-4 monoclonal antibody administration combined with one 12 Gy fraction of radiation therapy, inhibited the growth of the primary irradiated tumour. Also the survival-time of the mice was significantly increased by this combined treatment (Demaria, et al. 2004; Demaria, et al. 2003; Demaria, et al. 2005b).

Another investigation of the effects of systemic CTLA-4 blockade with monoclonal antibody (9H10) to CTLA-4 employed in a mice model with well-established glioma, showed that CTLA-4 blockade confers long-term survival in 80% of treated mice (Fecci, et al. 2007). Thus the combination of local RT with CTLA-4 blockade might be applied as radio-immune-modulating therapeutic strategy also against glioma.

3.3.2 Combination of radiation therapy and vaccination of mice with glioma

In a study of combining radiation therapy and vaccination, mice with intracerebrally established invasive GL261 glioma were treated with two fraction of radiation therapy (2x4 Gy) to the whole brain, peripheral vaccination with cells transfected to secrete granulocyte-macrophage colony-stimulating factor GM-CSF and their combination (Newcomb, et al. 2006).

Less than 10% increase in survival time was observed in mice given radiation therapy or vaccination alone. But by combining radiation therapy and vaccination a highly significant increase in the survival time, with about 40-80%, was observed. The surviving animals showed acquired antitumor immunity by rejecting challenge tumours (Newcomb, et al. 2006). These results are in good agreement with the results of (75 %) long term survivals and acquired antitumor immunity in N29 rats treated with the combination of radiation and immune therapy with cells secreting IFN_{γ} (B. R. R. Persson, et al. 2010).

3.3.3 Combination of radiation therapy and anti-CD137 antibodies in treatment of mice with glioma

The immune response induced by CD137 monoclonal antibodies (BMS-469492, Bristol-Meyer Squibb) directed to the co-stimulatory molecule CD137 has shown to generate effective antitumor responses in several animal models and in clinical trials (Ascierto, et al. 2010; Mazzolini, et al. 2007; Nam, et al. 2005).

Treatment of murine lung (M109) and breast (EMT6) carcinoma with CD137 monoclonal antibodies BMS-469492 generate tumour growth retardation of 3 days in M109 tumours and of 12.5 days in EMT6 tumours. In combination with radiation therapy, however, the tumour responses were enhanced in both tumour models (Shi & Siemann 2006).

A recent study in mice with intracerebrally established invasive GL261glioma applied the combination of radiotherapy with anti-CD137 antibody directed to the co-stimulatory molecule CD137 (Newcomb, et al. 2010). The mice were treated with two fractions (2x4 Gy) radiation therapy to the whole brain. Non-specific rat IgG or anti-CD137 mAb was administered either alone or in combinations with RT.

Type of treatment	Median survival time days	Number of > 120 days survivals out of 9 rats
IgG	31	0
anti-CD137	42	0
RT (4Gy×2) alone	No data	No data
IgG + RT (4Gy×2)	37	2
anti-CD137 + RT (4Gy×2)	114	6

Table 5. Median survival time of rats, with 9 animals in each group, after the different types of treatments (Newcomb, et al. 2010).

The results summarized in Table 5 show that the combination of radiation (4 Gy×2) with anti-CD137 therapy resulted in complete tumour eradication and prolonged survival in six of nine (67%) mice with established brain tumours ($p < 0.001$). Five of the six long-term survivors in the combination group demonstrated acquired antitumor immunity by

rejecting challenge tumours. Antitumor immunity was associated with an increased number of tumour-infiltrating lymphocytes (TILs) in brain tumours and increased tumour-specific production of IFNγ. Since anti-CD137 therapy is already used in clinical trials it was suggested to be studied further in combination with local hypo-fractionated (2x4 Gy) radiation therapy for clinical translation (Newcomb, et al. 2010).

4. Clinical studies of combining radiation and immune therapy

The expression profiles of CD4(+) and CD8(+) T cells and T_{reg} from patients with newly diagnosed *glioblastoma multiforme* are quite different when compared with normal healthy volunteers (Learn, et al. 2006). But how various absorbed dose or various fractionation pattern or methods of radiation delivery can affect T-cell populations and alternative regulatory molecules in glioma patients is still under debate (Chiba, et al. 2010; Teitz-Tennenbaum, et al. 2008; Verastegui, et al. 2003).

4.1 Effects of concomitant temozolomide and radiation therapies on WT1-specific T-cells in malignant glioma

Like many other solid tumours, glioma have been found to express a protein characteristic for Wilms' tumour 1 (WT1) (Hashiba, et al. 2007). A peptide based immunotherapy targeting the WT1 gene has successfully been used in patients with recurrent glioma. The clinical response indicates that CD8(+) cytotoxic T lymphocytes (CTLs) are the main effectors of this WT1 vaccination (Oka, et al. 2004). A phase II clinical trial of the WT1 vaccination for patients with recurrent malignant glioma resulted in a partial response rate of 9.5% but none complete response. The median length of period with progression-free survival was 20 weeks (Izumoto, et al. 2008).

In planning for a clinical trial of WT1 vaccination involving patients with newly diagnosed malignant glioma, it is also aimed to combine concurrent radiation /TMZ therapy with WT1 immunotherapy. The critical question is, however, if the depletion of lymphocytes caused by the current standard radiation/TMZ treatment is a drawback for a combination with WT1 immunotherapy. Therefore a clinical study was performed in order to determine how the concomitant radiation/TMZ therapy affects the WT1-specific T-cells and other T-cells in terms of their frequencies and total numbers. This study concluded that, even after the decrease of the absolute numbers of lymphocytes, the fraction of WT1 specific T-cells was stable. They concluded that it may the possible to apply WT1 immunotherapy after the end of 6 weeks of radiation/TMZ therapy (Chiba, et al. 2010).

In another clinical study of 8 patients with primary glioma it was found that concomitant radiation/TMZ therapy integrated with autologous dendritic cell-based immunotherapy was feasible and well tolerated. The median progression-free survival (PFS) was 75% and at 6 months and 50% at 18 months. The median time of survival for all patients is 24 months. One patient was still free from progression or recurrence at 34 months (Ardon, et al. 2010).

4.2 Treatment recurrent malignant glioma with hypo-fractionated radiotherapy combined with immune therapy

A single fraction of high dose radiation therapy has been demonstrated to dramatically increase the priming of T-cell in draining lymphoid tissues, which increased the action of the CD8(+) T cells and lead to reduction and eradication of the primary tumour or distant

metastasis. This immune response, however, is abrogated by conventional fractionated RT or adjuvant chemotherapy (Lee, et al. 2009). So far only preclinical studies of hypo-fractionated radiation therapy in combination with immune therapy have been performed. The results are however encouraging and clinical trials using this therapeutic regime is urgently needed for both primary and recurrent glioma (Newcomb, et al. 2006; B. R. R. Persson, et al. 2002; B. R. R. Persson, et al. 2010; B. R. R. Persson, et al. 2008).

Henke et al. (2010) found that retreatment of recurrent high-grade glioma with hypo-fractionated radiation therapy with 20 Gy given over 1 week seems to be feasible even after a previous complete course of radiotherapy (Henke, et al. 2009). Thus it should be feasible to consider hypo-fractionated radiotherapy with about 8 Gy in one or two fractions to recurrent glioma in combination with immune therapy.

4.3 Treatment of newly diagnosed glioma with fractionated radiotherapy combined with vaccination therapy

An autologous formalin-fixed tumor vaccine (AFTV) has been prepared from formalin-fixed and/or paraffin-embedded glioma tumor tissue obtained upon surgery and premixed with original adjuvant materials. In a clinical pilot study, AFTV inoculations of 12 patients took place at least 4 weeks after prior primary conventional glioma treatments were concluded. Of these 12 patients, four responded to the AFTV therapy: one showed a complete response, one showed a partial response, two showed minor responses, and one had stabilization of disease. The median survival period was about 11 months from the initiation of the AFTV treatment. But three of these patients survived for 20 months or more after AFTV inoculation (Ishikawa, et al. 2007). In a subsequent phase I/IIa clinical trial, the AFTV was inoculated in 24 patients with newly diagnosed glioblastoma multiforme, in combination with conventional fractionated radiotherapy. The treatment protocol in that study included aggressive tumor resection, fractionated radiotherapy, 2 Gy per fraction, up to a total dose of 60 Gy, and 3 concomitant courses of AFTV administered with an interval of one week during the last 3 weeks of irradiation. The median duration of overall survival was 21.4 months (95% CI 13.8–31.3 months). The actuarial 2-year survival rate was 40%. These results demonstrate that vaccine treatment in combination with fractionated radiotherapy may be effective in patients with newly diagnosed glioblastoma (Muragaki, et al. 2011). Since the previous pilot study with AFTV therapy only, also has shown a good response, the outcome of the phase I/IIa clinical trial might have been even better if it has been combined with hypo-fractionated radiation therapy as described in the previous paragraph 4.3.

5. Summary and conclusion

Many pre-clinical models have proven that one or two radiotherapy fractions with a total absorbed dose in the range of 5 - 16 Gy in combination with immune therapy result in enhanced therapeutic response to glioma. This finding opens for the possibility of clinical testing of new challenging therapeutic regimes for glioma, based on a combination of immune-therapy and hypo-fractionated radiotherapy. A regime of one or two radiation sessions with a total radiation target dose in the order of 8 Gy in combination with clinically proven immunotherapy seem so be adequate (De Vleeschouwer, et al. 2008; Gulley, et al. 2005; J. Nemunaitis, et al. 2006a; J. J. Nemunaitis, et al. 2006b; Newcomb, et al. 2010; B. R. R. Persson, et al. 2010; Salford, et al. 2006; Salford, et al. 2004).

Although the total lymphocyte count decrease as a consequence of the current radiation/temozolomide therapy, it seems not affect the frequency of antigen specific T-cells, which suggest that combination with immunotherapy might be successful (Ardon, et al. 2010; Chiba, et al. 2010).

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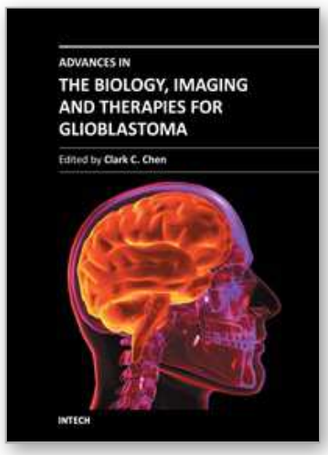

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