Survival of Rats with N29 Brain Tumors after Single fraction 5 or 15 Gy Radiotherapy combined with Immunotherapy.

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Abstract

Intra cerebral tumors were inoculated into the brain of Fischer-344 syngeneic rats. After one week they were treated with either 5 or 15 Gy of 60Co-gamma radiation. The first immunization was given 1 hour before the radiation treatment and then two more times with 14-day intervals. Immunization was performed with 3 x 10^6 radiation sterilized tumor cells (N29) injected intraperitoneally.

Neither radiation therapy with 5 or 15 Gy nor immunization with N29 cells alone had any significant effect on the length of survival of N29 tumor bearing rats. But radiation therapy with 5 Gy combined with immunization with IFN-γ-secreting syngeneic N29 cells resulted in 63 % complete remissions and significantly (p < 0.05) increased survival for the tumor bearing rats. Corresponding combination with 15 Gy RT resulted in 50% complete remissions. There is a possibility of a synergistic effect by optimal combination of radiation therapy and immunization.

1. Introduction

Gliomas are malignant tumors of the central nervous system (CNS) derived from glial lineage. Effective cellular anti-tumor immunity for a glioma, as for all syngeneic tumors, is dependent upon the efficient activation, clonally proliferation and subsequent lyses of tumor cells by glioma-specific T cells. While it has been widely believed that CNS is an “immune privileged” site [1], it has become increasingly clear that T-lymphocytes can enter the CNS in a variety of pathological states [2, 3]. Analysis of lymphoid cells infiltrating malignant gliomas has revealed that the cellular infiltrate of these tumors primarily consists of macrophages and cytotoxic T cells (CTL), while B cells are relatively absent [4-6].

Therapeutic immunization utilizes the fact that the immune system has a potential to react against tumor antigens and that this can result in immunological control of the tumor. There is an increasing body of evidence that the activation of cytolytic T-lymphocytes have a dramatic effect on the long-term survival of cancer patients receiving traditional therapies only such as surgery, chemo therapy (CT) or radiation therapy RT [7-9]. These studies clearly demonstrate the importance of immune reactivity toward several types of tumors.

The immune response to glioma is primarily a result of the tumoricidal function of activated cytotoxic T cells (CTL), and thus a vaccination regimen may augment the effectors functions of CTL as well as increase the number of lymphoid cells within the glioma. Yet, even when it has been documented that large populations of lymphocytes do breach the blood-brain barrier (BBB) to enter CNS tumors, lyses of glioma cells and complete eradication of the neoplasm do not occur [10].

In the clinical study “Brain-Immuo-Gene-Tumor-Therapy” (BRIGTT) patients are immunized with their own tumor cells transfected with IFN-γ gene [11-18]. The cells are taken from the surgically removed tumors and are grown in culture [19, 20]. The day before immunization the karyotyped tumor cells are infected with an adenovirus expressing human IFN-γ (100 MOI). At the day after transfection, the immunization of the patient takes place soon after the cells have been irradiated with 100 Gy of Cs-137 gamma radiations. The irradiated cells cannot proliferate but they survive.
for some time in the skin of the patient, during which they produce their abnormal proteins and also the IFNγ [19, 20]. This alerts the immune system and leads to a production of activated T-lymphocytes, which have the capability of passing through the Blood-Brain Barrier. Out in the brain parenchyma, the activated T-cells are free to actively seek for the tumor cells, both in the original tumor and in the surrounding brain with its migrating “guerrilla” cells [11, 14-16].

Results from the first human treatments show that the method is safe for the patients and gives positive DTH reactions and an increase of infiltrating CD8+ and CD4+ T cells at the immunization site. Co-cultures show that the IFNγ production is higher in lymphocytes from patients with a prolonged survival. Of nine immunized patients, four survived for 19.5, 21, 26.5, and 24.5 months. The mean survival time of these nine patients (mean age, 61.4 years) is 16.4 months, as compared with the 9.7-month survival (p = 0.03, Mann-Whitney) of the 11 patients (mean age, 60.3 years) included in the study, but where the cells did not grow sufficiently well in the cultures to make immunization possible. A tenth patient (age 57) had a remarkable regression of her tumor and has also shown stronger DTH reactivity than any of the nine patients treated earlier [16].

In other studies attempts have been made to enhance the effect of immune therapy by combining immunization with other established therapeutic regimes such as radiation therapy [21-29].

In the present study we report the results of our investigations of the therapeutic effects of immunization with interferon-gamma secreting cells in combination with a single fraction of conventional radiation therapy in a rat model with N29 rat glioma tumors cells inoculated in the rat brain.

2. Material and Methods

2.1. Animals

Inbred Fischer-344 rats of both sexes, females weighing around 190 g and males 370g. The strain was maintained by continuous, single-line brother/sister mating in our laboratory. During the experiment the rats were housed in a climate controlled cabinet and stored 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).

2.2. Cell lines and culture

The rat glioma N29 was induced by transplacental administration of ethyl-N-nitrosurea to 17- to 18-days pregnant Fischer rats. By this method 80-90% of the
offspring developed tumors in the central or peripheral nervous system. The origin of the N29 tumors arose in the right hemisphere of a female offspring at 205 days after induction. The tumor is only weakly immunogenic and grows readily both intracerebrally, subcutaneously and in vitro [31].

All cells were cultured in antibiotic-free RFMI-1640 medium supplemented with 5-10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 0.5 mM pyruvate and 0.096% NaHCO₃. All cell-cultures were regularly checked for contaminating microbes by staining with the fluorescent dye Hoechst 32 258 and were examined with fluorescent microscopy. Cell cultures were maintained in culture flasks (Nunc, Denmark) and harvested by treatment with trypsin/EDTA.

Cells used for immunization were gene modified sterilized N29 tumor cells secreting interferon-γ (IFNγ). The cells were transferred from the culture flasks to 15 ml centrifuge test tubes (Nanclon) and stored in a melting ice bath before irradiation that does not allow the cells to grow during the procedure. The cells were irradiated with 137Cs γ-rays using a Gammacell 2000 (Mølsgaard Medical, Riso, Denmark) source to 70 Gy at a dose rate of 4.0 Gy/min with a cell density of 3×10⁶ cells/ml in serum free medium (IMDM-0). During the irradiation the cells were kept at room temperature. Directly after the irradiation they were placed in a melting ice bath.

The first immunization was given 1 hour before the radiation treatment and then two more times with 14-day intervals. Immunization was performed with 3×10⁶ cells tumors cells (N29) injected intraperitoneally.

2.3. Inoculation and treatment of intracerebral tumors.

2.3.1 Inoculation. Inoculation was performed by injection of 5000 N29 cells, in 5 μl nutrient solution by stereotaxic technique with a Hamilton syringe into the head of the right caudate nucleus in the brain of Fischer 344 rats. To avoid extra-cranial tumor growth, the injection site was cleaned with 70% ethanol after injection and the borehole was sealed with wax.

2.3.2. Treatment. Treatment was performed with the animals arranged into groups of controls, radiation therapy at 5 and 15 Gy, immunization and the combinations of radiation therapy with immune therapy.

Before radiation therapy, the animals were anesthetized with 5% chloral hydrate given intraperitoneally (i.p.) or Ketalar®/Rompun®, 0.55 ml/ 100g. Animals were given a single radiation treatment using a ⁶⁰Co 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 (1x1 cm²). The adsorbed dose of either 5 or 15 Gy was measured both by an ionization chamber (Fig 1) and a TLD chip placed next to the tumor in the field under the bolus.

Table 1. Groups of animals with various treatments in the various experiments with N29 tumors.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Treatment</th>
<th>Number of N29 Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Controls with no treatment</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Radiation 5 Gy</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>Radiation 15 Gy</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>Immunization</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Radiation 5 Gy + Immunization</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>Radiation 15 Gy + Immunization</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 1. Animals were given a single radiation treatment using a ⁶⁰Co 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 (1x1 cm²). The adsorbed dose of either 5 or 15 Gy was measured both by an ionization chamber and TLD dose meter.

Radiation treatment was performed using a ⁶⁰Co 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 (1x1 cm²).
The adsorbed dose of either 5 or 15 Gy was measured using a TLD chip placed next to the tumor in the field under the bolus.

Within one hour after the radiotherapy session the animals were immunized by intraperitoneal injections of irradiated (70 Gy), gene modified N29 tumor cells, secreting interferon-gamma (IFNγ). The immunization was then repeated weekly for two more times.

The animals were observed daily for symptoms of the growing tumors and when the first symptoms of paresis, apathy, red-eye lids or shaggy fur they were sacrificed and the brain was examined for tumor growth. The tumor was resected and weighted.

2.3.3. Challenging. Challenging was performed by subcutaneous injection of 200,000 N29 cells in the flank of those animals with N29 tumors that continued to live more than 400 days after the first series of treatment. Two controls with no previous treatment were inoculated as well.

3. Results

3.1. Survival

The resulting number of survivals and the mean survival time in each group of animals with intra cerebral N29 tumors treated with radiation therapy at 5 and 15 Gy, immunization and the combinations of radiation therapy with immune therapy are given in Table 2. The combined treatments were given by intraperitoneally injections of radiation sterilized (70 Gy) transfected N29 tumor cells secreting interferon-gamma (IFNγ) one hour after a single radiotherapy session with either 5 or 15 Gy of 60Co-gamma radiation. The immunization was repeated twice with two weeks interval.

Table 2 shows that there is a significant effect of a single fraction of radiation therapy of 5 Gy followed by immunization 3 times at 2 weeks interval. Neither immunization alone nor radiation therapy alone in single fractions of 5 or 15 Gy resulted in any significant therapeutic effect versus the controls. But both immunization alone, radiation therapy alone in single fractions of 15 Gy and combined with immunization resulted in significant therapeutic effect versus radiotherapy alone with 5 Gy.

Table 2. Number of Survivals and mean survival time of intra cerebral N29 tumors treated with IFNγ cell immunization (Immun), radiation therapy (RT) and their combination (RT+Immun).

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Number Survived &gt;200 d / Number animals</th>
<th>Survival time/d at 50% KM survival</th>
<th>Median Survival time/ days ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1/6</td>
<td>56</td>
<td>56 ± 28</td>
</tr>
<tr>
<td>Immun 3x</td>
<td>2/6</td>
<td>143</td>
<td>143 ± 26</td>
</tr>
<tr>
<td>RT 5 Gy</td>
<td>0/8</td>
<td>39</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>RT 15 Gy</td>
<td>2/8</td>
<td>88</td>
<td>88 ± 24</td>
</tr>
<tr>
<td>RT 5 Gy + Immun 3x</td>
<td>5/8</td>
<td>234</td>
<td>234 ± 20</td>
</tr>
<tr>
<td>RT 15 Gy + Immun 3x</td>
<td>3/8</td>
<td>99</td>
<td>118 ± 24</td>
</tr>
</tbody>
</table>

The Kaplan Meier plots of rat survival in the various groups with different treatments are displayed in the following Fig. 2 – Fig. 7. The probability of survival is displayed relative to the time after inoculation the tumor cells into the brain.

Figure 2. Probability of survival in untreated controls with inoculated N29 tumors (number of rats N=6). The dotted lines are 95% confidence intervals.
3.2. Challenge

The 14 survivors out of 48 animals with N29 tumors that survived 227 days and 4 new controls (C1) were inoculated with 200,000 N29 glioma cells in 200 μl just under the skin in the thigh of the hind leg of the rat.

In Figure 8 the primary survival rate in % is given in the lower panel. The challenged animals that didn’t develop any tumor are given in the middle panel. The fraction of animals that did not develop any tumor after challenge multiplied with the fraction of primary
survival gives the percentage of cured animal displayed in the upper panel.

Immune therapy using IFNγ secreting syngeneic N29 tumor cell only, resulted in no significant increased length of survival time. Neither radiation therapy with a single fraction of 15 Gy or 5 Gy nor immunization only had any significant effect on the survival. But a single radiation therapy session with 5 Gy immediately followed by the first immunization with IFNγ secreting syngeneic cells resulted in significant (p = 0.01) increased length of survival time with 63% complete remissions. Radiation therapy with 15 Gy combined with immunization with IFNγ secreting syngeneic cells resulted in no significant increased survival time for the N29 tumors although with 50% complete remissions.

The median survival time is assumed to be inversely proportional to the tumor growth rate. Then the concept of specific therapeutic effect can be used to quantify the effect of the various types of treatments [32].

\[
STE^{TM} = \text{median}(i) \left( \frac{T_{i}^{E} - \overline{T_{C}}}{T_{i}^{E}} \right)
\]

If N = 2i - 1 (odd) \( i = (N+1)/2 \); if N=2i \( i = N/2 \)

where

- \( T_{i}^{E} \) The individual survival time (days) in the group of exposed rats.
- \( \overline{T_{C}} \) The median value of survival time (days) of the group of unexposed control rats.

The STE is equal to 0 when the median survival time in the group of exposed rats, is equal to the median survival time in the group of control rats.

The STE is equal to 1 when the median survival time in the group of exposed is much larger than the median survival time of the group of control rats.

The STE value can also be estimated from the frequencies of complete remissions in each group of treatment.

\[
STE^{CR} = \frac{CR_{E} - CR_{C}}{CR_{E}}
\]

where

- \( CR_{E} \) The frequency of complete remissions in the group of exposed rats.
- \( CR_{C} \) The frequency of complete remissions in the group of unexposed control rats.

Figure 8. In the lower panel is given the primary survival rate in %. The frequency (%) of the challenged animals that didn’t develop any tumor, given in the middle panel, multiplied with the frequency (%) of primary survival, in the lower panel, gives the frequency (%) of cured animals that is displayed in the upper panel. The number of rats included in each group is given next to the columns.

4. Discussions

The model with N29 tumor cells intra-cerebrally inoculated in the brain of Fischer-344 syngeneic rats has been extensively used in our laboratory. In the present study one of the controls survived more than 200 days. But the other 5 animals in the control group developed tumors following the normal pattern. It is extremely rare that a control rat does not develop a tumor and survive more than 80 days after inoculation of 5000 cells into the brain. In spite of this fact we have not excluded it from the group of controls in the statistical considerations. This will thus give an unfavorable statistics in the comparison with the groups of treated animals.

One hour before a single fraction radiotherapy session (RT) with either 5 or 15 Gy of 60Co-gamma radiation the animals were given intraperitoneally injections of irradiated modified N29 tumors cells secreting either interferon-gamma (IFNγ). The immunization was then repeated two more times with 14-day intervals.
The therapeutic enhancement ratio, \(\text{TER}\), of the combined treatment of two therapeutic agents, immunization (IT), and ionizing radiation (RT), is the ratio of the specific therapeutic effect of the experimental combination of therapeutic agents \((\text{STE}_{\text{Experimental}})\) and the specific therapeutic effect of the hypothetical combination of the two independently applied agents \((\text{STE}_{\text{Independent}})\) [32].

The specific therapeutic effect of the hypothetical combination of independently applied RT and IT, is given by the equation:

\[
\text{STE}_{\text{Independent}} = (\text{STE}_{\text{RT}} + \text{STE}_{\text{IT}})
\]

\(\text{TER}\) is a measure of any synergistic or diminishing effect obtained in the combination of the two agents. It may be due to interaction of sub lethal lesions induced by both agents to produce lethal events that cause the \(\text{TER} > 1\). If the individual therapies are highly efficient by themselves there might, however, also be an over killing effect when they are combined, that reduce the effect compared to the additive action, so that \(\text{TER} < 1\). It is thus important to investigate the effect of combined treatments at various dose levels to find an optimal therapeutic regime that gives the maximum value of \(\text{TER}\).

The \(\text{STE}\) value for immunization therapy of N29 tumors is 0.7. Radiation therapy with 5 Gy in a single session resulted in a negative \(\text{STE}\) value that means no therapeutic effect at all. Although radiation therapy with 15 Gy in a single session resulted in a \(\text{STE}\) value of 0.4. Thus in estimating the therapeutic enhancement ratio (\(\text{TER}\)) for the combined treatment the \(\text{STE}\) for 5 Gy RT is set to zero. In this way the \(\text{TER}\) estimated for the combined treatment with RT 5 Gy and IT resulted in a \(\text{TER} = 1.2\). The combined treatment with 15 Gy RT and IT resulted in a \(\text{STE}\) value of 0.7 and a \(\text{TER}\) of 0.5. The \(\text{TER}\) value is somewhat lower than for combined treatment with 5 Gy RT, which might indicate that this dose level is favorable.

The \(\text{STE}\) values based on the frequency of complete remissions \(\text{STE}_{\text{CR}}\) is 1 for immunization, negative for RT 5 Gy and 0.5 for RT 15 Gy. For combined treatments the \(\text{STE}_{\text{CR}}\) values 2.8 and 1.3 are not very different between RT 5 Gy and 15 Gy respectively. In this way the \(\text{TER}_{\text{CR}}\) estimated for the combined treatment with RT and IT resulted in a \(\text{TER} > 2.8\) and 0.9 for 5 and 15 Gy respectively. Thus there is an indication of a synergistic effect based on complete remissions at 5 Gy.

The long term (> 200 days) survivals after the first treatment were challenged by injection of tumor cells on the flank. The percentage of challenged animals that didn’t develop any tumor on the flank, multiplied with the fraction of primary survivals gives the percentage of permanently cured animals. In the group of immunized animals 17% of the tumors were cured, and in the groups of combination with 5 Gy RT the cure rate was 25% and with 15 Gy RT it was 13% respectively.

Our results show that radiation therapy combined with immunization by s.c. injection of irradiated syngeneic tumor cells induces a significant anti-tumor response to i.c. implanted N29 and glioma in Fischer-344 rats support the finding of Graf et al. (2002). [21].

Another mechanism explaining the synergistic effect might be that treatment with RT induces DNA-repair and production of gene products such as GADD34 which might enhance the expression of tumor antigens [33].

In the present study we have focused on the effect of treatment with RT on the survival time and frequency of complete remissions. The variation in therapeutic enhancement factor with the absorbed dose indicate that there might be an absorbed dose level that gives still better results. Studies on the effect of more RT fractions and the search for the optimal time between fractions are also areas of interest for future research.

5. Conclusions

The most effective of the studied therapeutic regime for N29 tumors was shown to be a single fraction of radiation therapy of 5 Gy immediately followed by immunization which was repeated twice during the following two weeks. This regime resulted in a significantly prolonged survival and 63 % complete remissions. Corresponding combination with 15 Gy RT resulted in 50% complete remissions. Neither immune therapy, nor radiation therapy alone with 5 or 15 Gy, resulted in any significant therapeutic effect.

The combined treatment with 5 Gy RT and immunotherapy resulted in a therapeutic enhancement ratio (\(\text{TER} > 1\)), and for 15 Gy \(\text{TER}\) was 0.5. These values indicate that there might be a synergistic effect of optimal combination of radiation therapy and immunization.

6. Acknowledgement

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7. References


