Immunotherapy for Malignant Gliomas

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ABSTRACT

Malignant gliomas are the most common type of primary brain tumor. They show characteristics of extreme aggressiveness as well as being invasive to the surrounding brain. Current treatment options include chemotherapy, surgical resection, irradiation, brachytherapy, and immunotherapy. Malignant gliomas are not always responsive to these treatments, which accounts for the recurrence of the gliomas after therapy and an associated poor prognosis. This project focuses on an innovative type of immunotherapy for the treatment of malignant gliomas in rats. The study involves implanting the RG-2 glioma cell line into the right striatum of the rat brain. The progressive growth of the tumors will be monitored weekly with the use of magnetic resonance imaging (MRI). Then a combination of immunostimulatory agents, lipopolysaccharide (LPS) and interferon-gamma (INF-g) will be injected directly into the tumor. The combination treatment of LPS and INF-g has been chosen in order to stimulate tumor cytotoxicity of microglial cells in an effort to enhance the natural immune response against the tumor. MRI was also used to observe the progression and hopefully regression of the tumor. It was found that the treatment was highly effective in one of the animal subjects.

Key words: Lipopolysaccharide, Interferon-gamma, Magnetic resonance imaging, immunotherapy, microglia, glioma

INTRODUCTION

Gliomas, the most common primary tumor of the central nervous system (CNS), are tumors that arise from glial cells, such as astrocytes, oligodendrocytes, ependymal cells, and choroid plexus cells (Parney et al., 2000). Malignant gliomas show characteristics of extreme aggressiveness as well as being invasive to the surrounding brain. Current treatment options include chemotherapy, surgical resection, irradiation, brachytherapy, and immunotherapy. Malignant gliomas are not always responsive to these treatments, which accounts for the recurrence of the gliomas after therapy and an associated poor prognosis. For example, a patient with a glioblastoma multiforme faces a mean survival time of less than one year (Parney, et. al, 2000). Two-thirds of all diagnosed malignant gliomas are the glioblastoma form (Hildebrand et. al., 1997).

CNS immunology

The brain has always been thought of as an immunologically privileged organ, which has been linked to the aggressive and lethal characteristics of gliomas. However, recent studies have demonstrated that microglia may be able to produce an immune response against malignant tumors (Morikomo et. al, 1992). Microglia are the main immune effector cells of the CNS that are activated rapidly, express major histocompatibility complexes (MHC) molecules, and release cytokines and chemokines (Graeber & Streit, 1990). They have been identified as the antigen-presenting cells of the CNS that share common antigens with macrophages and are functionally related to
phagocytic macrophages (Hickey & Kimura, 1988). In vitro studies showed that when resting microglia are activated by interferon-gamma (INF-γ) they become fully competent to present antigens to lymphocytes (Fathallah-Shayk et al., 1998). In addition, microglia are recognized as tumor cytotoxic effector cells (they have tumor killing capacity). Studies have identified an abundance of microglia within and surrounding human and experimental glioma (Parney et al., 2000). It is unclear if there is any microglia infiltration of the glioma despite their overwhelming presence (Morimura, 1990). The microglia do not seem to be producing a protective immune response. Studies have shown that tumor cells release transforming growth factor (TGF-β) which is an immunosuppressive cytokine (small immunoregulatory proteins that function as intercellular communication molecules) (Morioka et al., 1992). TGF-β is consistently overexpressed in gliomas and has been identified as the main contributor to glioma immunosuppression. Northern blotting revealed high concentration of TGF-β RNA within tumor cells (Keifer, 1994). Since there are an immense amount of microglia found in and around gliomas, it is thought that TGF-β is preventing the activation of the microglia to produce cytotoxic effects. The paracrine immunosuppression model has been used to demonstrate the interaction between microglia and gliomas. Microglia can produce tumor necrosis factor (TNF-α) to kill glioma cells when activated. TNF-α is a cytotoxic agent that attacks target cells (glioma cells). This activation is mediated by TGF-β, which is produced by both gliomas and microglia. The additional glioma derived TGF-b produces a decrease in TNF-α thereby suppressing microglial immune response. Streit et al have demonstrated through in vitro studies that the combination of lipopolysaccharide (LPS) from E.coli and Interferon-gamma (INF-γ) can act as immunostimulatory agents to induce the anti-tumor activity of microglia. When microglia are stimulated by INF-γ they have been shown to mediate tumor-cell cytotoxicity and are phagocytic (Fathallah-Shaykh et al., 1998). When cultured rat microglia was stimulated with LPS/INF-γ there was a significant increase in TNF-α and nitric oxide (NO). NO has also been identified as a cytotoxic agent. In addition, these agents increased the expression of other immune cells, which further enhances the cytotoxic ability of the microglia.

RG-2 glioma model

The RG-2 glioma model has been shown to be an excellent mimic of the human glioblastoma (Aas et al., 1995). The RG-2 glioma is a highly malignant brain tumor characterized by immense vascularization, and aggressive invasiveness. The RG-2 model was derived from Fischer-344 rats. An in vivo study was performed to focus on an innovative type of immunotherapy for the treatment of malignant gliomas. It was hypothesized that a combination treatment of two immunostimulatory agents (LPS/INF-γ), which was injected directly into the tumor, would stimulate tumor cytotoxicity of microglia cells and enhance the natural immune response against the tumor. A regression in the tumor size should be observed, indicating the effectiveness of the treatment.

MATERIALS AND METHODS

Subjects

Adult, male Fischer 344 rats weighing approximately 200g were purchased from Harlan Industries (Indianapolis, IN). This experiment used three rats referred to as BT-1, BT-2, and BT-3.
The animals were group housed in clear polycarbonate cages (9”x18”x7”) located in room LG-120 at the Brain Institute at the University of Florida. The study was carried out in compliance with the rules set forth in the NIH Guide for the Care and Use of Laboratory Animals and was approved by the University of Florida’s Institutional Animal Care and Use Committee (IACUC).

**Glioma cell culture**

Rat glioma (RG-2) cell line has been obtained from Dr. Keith Black at UCLA. The cells were grown in 75cm² flasks in 10ml of complete media. The cultures were maintained in an atmosphere containing 5% CO2 and 95% humidity. After two days, the cells were harvested and counted for inoculation.

**Surgery: Tumor Inoculation**

The animals were first anesthetized with xylazine (15mg/kg i.p.) and ketamine (80mg/kg i.p.). They were then placed into a stereotactic apparatus and a midline scalp incision was made with a scalpel. The bregma was identified, a burr hole was drilled and the dura was exposed. The burr hole was drilled 3mm to the right of bregma. A Hamilton syringe was then lowered 5mm deep into the right striatum. The glioma cells were then injected over a time period of five minutes. Fourteen thousand cells suspended in 8μl phosphate buffered saline (PBS) were injected. After the cells were injected the, the syringe was withdrawn and the wound was closed using stainless steel wound clips. The animals were then placed on a heating pad until they recovered from the anesthesia. All three animals were operated on the same day and were injected with same cell batch. The same procedure was used when injecting the immunostimulatory agents (LPS/INF-γ). The treatment consisted of 4μg LPS and 400 units INF-γ suspended in 8μl PBS. Only BT-3 received the treatment at twenty-two days and twenty-nine days post inoculation.

**Magnetic Resonance Imaging**

Magnetic Resonance Imaging (MRI) was performed at the AMRIS facility at the McKnight Brain Institute at the University of Florida using a Bruker 4.7T/33cm instrument. The brains were imaged with a multislice T2 weighted sequence, fast spin acquisition, contiguous 256x256 images with a field of view (FOV) of 5cm x 3cm. This sequence was followed by a 3-dimensional T1 weighted image. The same protocol used for the T2 weighted images was used for the T1 images. The 3-D sequence was then repeated again after an injection of gadolinium (Gd). Gd is a contrasting agent that provides enhancement of the tumor. The rats were kept under isoflurane anesthesia during the imaging process. MRI was also used to determine the necrotic index (NI) of the tumor in BT-1.

\[
NI = \frac{Necrotic\ Center + Peripheral\ Necrotic\ Area}{Total\ Tumor\ Area}
\]

The necrotic areas and total tumor area were manually outlined and determined with the use of the computer software program paravision. BT-1 and BT-2 were imaged twelve days post surgery. BT-3 was imaged a total of eight times. Tumor volumes for BT-3 were determined at each imaging session.

**Histological Procedures**
BT-1 and BT-2 were sacrificed twelve days after inoculation, and BT-3 was sacrificed eighty-four days after inoculation. They were sacrificed by perfusion with 4% paraformaldehyde in 0.1M PBS, and the brains were then removed and fixed for 24 hrs. Vibratome sections of 50μm thickness were cut through the tumor site in the coronal plane. The sections were then stained with a Nissl cresyl violet or isolectin to identify the presence of microglia. A correlative study between the histopathology and the MRI results was also performed to determine the effectiveness of the treatment.

RESULTS

BT-1 and BT-2 developed very large tumors within twelve days after surgery and they had to be sacrificed. They were displaying several behavioral abnormalities such as extreme weight loss, discolored fur, and crusty eyes, which was likely produced by the intracranial pressure caused by the tumor. The figure below shows BT-1, which was taken twelve days after inoculation. A large intracerebral glioblastoma is present in the right hemisphere of the brain. Dark areas within the middle of the tumor and surrounding the tumor are present indicating areas of necrosis (death of cells or tissue). Significant amounts of necrosis are characteristic of advanced, late stage tumors as seen in BT-1.

![Figure 1. MR images of RG2 glioma in BT-1 twelve days post inoculation.](image)

The necrotic index was determined for the RG2 glioma in BT-1. It was found that 41% of the tumor
Figure 2. MR images of necrotic index determination of RG2 glioma in BT-1. Taken twelve days post inoculation.

The histology of BT-1 was correlated with the MR images. The histology shows areas where the Nissl stain was not absorbed, these are areas of necrosis. Fatigued or dead cells do not absorb Nissl stain.
Figure 3. Low-power photomicrograph of a coronal section stained with cresyl violet from the RG2 glioma in BT1. Note areas of necrosis. Dark areas in the MR image are areas of necrosis correspond to the areas of necrosis in the coronal section (third image from the left in the first row from Figure 1.

The tumor in BT-3 grew at a slower pace than the tumors in BT-1 and BT-2. The growth of the tumor was monitored and after twenty-two days a large tumor in the right hemisphere of the brain that extended from the striatum to the lateral ventricles and the thalamus was present. BT-3 did not show any behavioral abnormalities indicating an optimal time to treat the animal. BT-3 was then treated with LPS and INF-g and then imaged five days later. After one treatment the tumor was reduced in size by approximately 50%. A second treatment was given and the regression of the tumor was monitored with the MRI. The last imaging session was taken on August 9, 2001 and there was no tumor detectable. The figure below shows that after two treatments the tumor has been reduced significantly. The first column is a horizontal plane image and columns 2, 3, and 4 are coronal plane images. The first and second columns show a T2 weighted image. The third column shows T1 weighted image before Gd injection and column four shows T1 weighted image after Gd injection. The first row is from June 22, 2001, the second row is from June 27, 2001, the third row is from July 9th, 2001, and the fourth row is from August 9, 2001.

Figure 4. The effects of immune stimulation by LPS/INF-γ on RG2 glioma as observed by MRI.

The volume of the tumor in BT-3 was determined at each imaging session and is shown below in table 1. The animal was treated on June 22, 2001 when the tumor volume was 1.705cm3. After five days, the tumor volume was reduced to 0.3761cm3. A second treatment was given on June 29, 2001, which reduced the tumor volume to an undetectable size by August 9, 2001 (not shown in Table 1).
Table 1
Volume Measurements of RG2 glioma in BT3 over time

<table>
<thead>
<tr>
<th>Time</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>31-May</td>
<td>N.D.</td>
</tr>
<tr>
<td>18-Jun</td>
<td>1.2085</td>
</tr>
<tr>
<td>22-Jun</td>
<td>1.7052</td>
</tr>
<tr>
<td>27-Jun</td>
<td>0.3761</td>
</tr>
<tr>
<td>9-Jul</td>
<td>0.1794</td>
</tr>
<tr>
<td>18-Jul</td>
<td>0.0538</td>
</tr>
<tr>
<td>25-Jul</td>
<td>0.0546</td>
</tr>
<tr>
<td></td>
<td>0.0784</td>
</tr>
</tbody>
</table>

The corresponding graph also shows the tumor volume over time and yellow arrows indicate the treatment points.

Figure 5. Changes in tumor volumes in BT3 with time. Times of treatment are indicated by yellow arrows.

Histology was also performed on the brain of BT-3 after it was euthanized on August 24, 2001. Examination of the sections revealed no remaining tumor tissue. There was a large cavity where the tumor was once present and it was surrounded by activated microglial cells.
DISCUSSION

The BT-1 and BT-2 developed massive tumors that began to spread from the striatum towards the lateral ventricles and the thalamus. The tumors were extremely aggressive, showed high levels of vascularization, and areas of necrosis. This evidence indicates that RG2 gliomas are a good model for the human glioblastoma form.

Previous studies have shown great numbers of microglia within and around glioma tissue, however, the microglia do not seem to be producing any immune response against the tumor. In vitro studies have been able to show that microglia tumor cytotoxicity can be stimulated by the combination of LPS/INF-γ, but the effects of this combination in vivo has not be demonstrated. The results seen in BT-3 indicate that microglia cytotoxicity can be stimulated in vivo by LPS/INF-γ. After two treatments of the LPS/INF-γ combo, the tumor in BT-3 became undetectable by MRI. The corresponding histology also showed an empty cavity where the tumor was and it was surrounded by numerous activated microglial cells. These results are best explained by the idea that the combination treatment stimulated the immune active state of microglia and caused an increase in NO and TNF-α levels, thus suppressing the effects of TGF-β. The treatment also activated T cells and NK cells.

Future studies will be performed with more animals and different dosages. In addition, microglia from the glioma will be isolated to test for levels of cytotoxic agents.

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REFERENCES


