High-dose Chemotherapy and Adoptive Immunotherapy in the Treatment of Recurrent Pediatric Brain Tumors

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Abstract
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Pediatric patients with recurrent brain tumors have a poor prognosis and limited therapeutic options. We investigated the use of high-dose chemotherapy with adoptive immunotherapy for recurrent brain tumors. Three pediatric patients with recurrent brain tumors received high-dose chemotherapy. This was followed by adoptive transfer of ex-vivo expanded T-cells. The T-cells were generated from peripheral blood after immunization with autologous cancer cells. The objectives of this study included (1) establishing the safety and feasibility of this potential treatment, (2) measuring changes in immune response after high-dose chemotherapy and adoptive immunotherapy, and (3) determining whether adoptive immunotherapy would be able to translate into a clinical response. Immune function was tested in all patients at the time of enrollment into the study. Humoral responses to recall antigens delayed-type hypersensitivity (DTH) were intact in all patients. After immunizing patients with autologous cancer cells, peripheral blood lymphocytes were harvested and activated with anti-CD3, expanded in-vitro, and infused post-autologous transplant. Patients received at least three doses of the vaccine, each consisting of an intradermal administration near a draining lymph node at biweekly intervals. Toxicity was limited and well tolerated in all patients. All three patients showed a tumor-specific immune response by serial imaging. Responses were durable at 16, 23, and 48 months, respectively.

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Introduction
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Central nervous system (CNS) neoplasms are the most common solid tumors in children [2]. During the last two decades, significant progress has been made in diagnostic procedures and treatments of these neoplasms. New techniques in imaging (CT and MRI), neurosurgery, intensive care, the introduction of intensive chemotherapy, and refined radiotherapy have had the greatest impact on prognosis. Nevertheless, the outcome of recurrent malignant primary brain tumors in children is dismal, with mortality within one year of diagnosis. The prognosis is particularly poor for pediatric patients with glioblastoma multiforme (GBM), the worst subtype of malignant primary brain tumors.

High-dose chemotherapy (HDC) with combination alkylators, including agents such as melphalan, thiotepa, nitrosureas and busulfan, followed by autologous bone marrow and/or peripheral blood stem-cell transplant (PBSCT), has been successfully used in young patients with high-risk CNS malignancies [7]. There is justification to deliver high-dose chemotherapy with stem-cell rescue in patients with brain tumors. It is based on evidence such as the sensitivity of brain tumors to chemotherapy in vitro (depending on dosage) and the penetration of chemotherapy through physiological barriers such as the blood-brain barrier by high-dose chemotherapy (HDC) [7].

Immunotherapy is an appealing treatment modality because of the potential for tumor-specific cytotoxicity. Immunotherapy in CNS neoplasms is complex, and most reported trials are based on adult patients. In several clinical trials, immunotherapy has been shown to mediate regression in patients with extra-cranial malignancies [4,5,10,19]. However, the effectiveness of this therapy in CNS tumors remains to be elicited due to several obstacles. First of all, cancer cells have been shown to be less immunogenic than other cells. Tumors can frequently interfere not only with the development of immune responses, but with the function of those

responses as well [10]. Second, tumors that are progressing exhibit numerous strategies to evade the immune system. These include both exclusion of immune cells from the tumor site and poor immunogenicity secondary to the reduction of major histocompatibility complex (MHC) expression or co-stimulatory proteins [4]. Furthermore, it has been demonstrated that cytokines that suppress tumor-specific immune responses, like transforming growth factor beta, IL-10, IL-4R, and IL-13R, are elevated in patients with CNS tumors [1,5]. Finally, if an immune response does occur in the brain, it may not be well tolerated. Nonetheless, active cellular immunotherapy can be potentially beneficial and has been shown to prolong survival in animal models.

Investigators have demonstrated in phase I trials that adoptive immunotherapy with autologous tumor cells is safe and feasible in patients with GBM [1]. It has been demonstrated that large numbers of activated T-lymphocytes can be produced from autologous peripheral blood after stimulation with IL-2 and anti-CD3. Furthermore, it has been shown that these lymphocytes can also be re-infused without toxicity [1,14]. The results of this therapy have shown to delay recurrence and prolong survival in some patients [8,14].

We have conducted a trial of HDC with PBSCT followed by AI in three patients, one with a recurrent ependyoma and two with malignant gliomas. This trial was designed primarily to assess the possibilities of inducing an immune response against pediatric brain tumors when using HDC followed by PBSCT and AI. The secondary objective of this trial was to determine whether the combination of HDC and AI would prevent tumors from recurring and prolong survival.

Materials and Methods
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Eligibility
The protocol was reviewed and approved by the institutional review board at the Karmanos Cancer Center of Detroit, Michigan. Informed consent was obtained prior to enrollment. Patients were eligible for the study if they had recurrent or refractory primary high-grade brain tumors and if they were candidates for PBSCT. Candidates had a histologically confirmed brain tumor by either neuropathology determination or tumor progression on >2 serial imaging studies, or by symptomatic progression due to tumor at the time of referral for PBSCT. Patients were required to have a performance status of greater than 60% on Karnofsky's scale or greater than 50% on Lansky's scale. Candidates were also required to have organ function at a level that was sufficient to proceed to PBSCT. Eligible patients were required to have no active infections, WBC greater than $3.5 \times 10^3/\mu L$, and platelet count greater than $100 \times 10^3/\mu L$. Antibodies for the immunoglobulins (Ig) classes IgG, IgA and IgM were monitored to determine if the patients had normal levels prior to vaccination and transplantation. Patients also underwent DTH testing (◉ Fig. 4).

Patients
Patient #1 was a five-month-old infant who initially presented with a suprasellar mass involving the optic chiasm. Histology revealed an anaplastic astrocytoma, and the patient went on to be treated with surgical debulking. Chemotherapy was initiated with cisplatin, cyclophosphamide, and vincristine. After two cycles of chemotherapy, re-evaluation revealed progression. The

patient underwent further debulking followed by enrollment in the treatment protocol.

Patient #2 was a four-year-old who presented with a posterior fossa mass. The patient underwent surgical resection and had a ventricular peritoneal shunt placed. Histology was consistent with the diagnosis of ependymoma. Chemotherapy was initiated with topotecan at $22\,mg/m^2$ for five days every three weeks. The patient went on to develop progressive disease by the fourth cycle. The patient underwent salvage therapy with surgical debulking and postoperative radiation of 6200 cGy to the posterior fossa. The patient was then enrolled on the treatment protocol.

Patient #3 was a seventeen-year-old female who presented with a headache and emesis. She was found to have a suprasellar mass and underwent surgical resection. The patient went on to receive chemotherapy consisting of carmustine, vincristine, and corticosteroids. Re-evaluation revealed progression of her disease. Salvage therapy was performed with debulking and radiation therapy of 5000 cGy to the suprasellar region. This was followed by enrollment on the treatment protocol.

Treatment plan
All patients underwent stereotactic volumetric surgical resection of their tumors. The goal was to debulk as much enhancing tumor as possible without inducing a neurological deficit. Patients #2 and #3 received external beam radiation therapy that targeted the tumor with stereotactic boost.

Tumor preparation was started with autologous tumor cells collected from patients during surgery. A small section of the solid tumor was transported to the laboratory under sterile conditions. Using standard operating procedures, the tumor was first washed with sterile RPMI 1640 media, then cut or minced into one mm fragments followed by digestion with an enzyme, collagenase type 3 (Worthington Biochemical Corp, Lakewood, NJ), overnight at 37 °C. The digested cells were washed twice with sterile RPMI 1640 media and resuspended in RPMI 1640 media. The cells were then irradiated (5000 rads) and a cell count and viability test were performed. A small aliquot was used for endotoxin and microbiology testing, and the rest of the cell suspension was frozen in freezing media (90% RPMI, 10% DMSO) at −80 °C in one mL aliquots at a concentration of at least 1×10^7 cells/mL. All cell preparation was sterile and greater than 80% viable.

Leukapheresis
Patients underwent chemotherapy-based mobilization to harvest PBSC. Cyclophosphamide ($2\,g/m^2$) and Taxol ($175\,mg/m^2$) were administered, followed by G-CSF (10 mcg/kg/day as a daily subcutaneous injection) starting on day four and continuing until the completion of leukapheresis. Leukapheresis was performed by either peripheral or central venous access using standard techniques. Peripheral blood mononuclear cells were collected beginning 24 h after the white blood count (WBC) recovered to $15 \times 10^6/mL$ and the peripheral CD34 count was greater than 20/mL. Harvesting was performed daily until a target dose of 2.5×10^6 CD34+/kg adjusted body weight was reached.

HDCT/PBSCT
High-dose cyclophophamide ($1875\,mg/m^2$/day on days −6, through day −4), cisplatin ($55\,mg/m^2$/day on days −6 through day −4) and carmustine ($400\,mg/m^2$/day) was given as previously described [20]. Autologous PBSC were infused on day 0

Table 1 Patient characteristics.

Age	Pathology	Prior Treatment	Vaccines	KPS	CD34+/kg dose	Engraftment	Preparatory Regimen	Survival
5 months	GBM	POG 9233	3	70%	5.28×10^6/kg	D+12	CDDP/BCNU/Cytoxan	D+480
4 years	Ependymoma	POG 9432	3	80%	6.21×10^6/kg	D+11	CDDP/BCNU/Cytoxan	D+ >7 years
17 years	GBM	CCG 945	2	90%	6.21×10^6/kg	D+13	CDDP/BCNU/Cytoxan	D+81

Fig. 1 Pre-transplant magnetic resonance imaging of patient #2 is shown in **A**. Post-therapy results after HDC & PBSCT and vaccination at day +120 are shown in **B** and **C**, respectively.

(○ **Fig. 5**). Patients received granulocyte colony-stimulating factor (G-CSF) subcutaneously from day +1 until engraftment.

Vaccination protocol

After autologous transplantation, patients were monitored until white blood cell count was above $2.0 \times 10^3/\mu L$ and absolute lymphocyte count was greater than 500 to begin the vaccination procedure.

At the time of vaccination, cells were quickly thawed in a 37 °C water bath and washed with sterile media. After washing, a small aliquot was removed for Gram stain. The remaining tumor cells were re-suspended in 500 μg GM-CSF (Immunex, Seattle, WA), and the mixture was divided evenly into four syringes of 0.25-mL aliquots. The mixture was then injected intradermally, giving rise to small wheals. An identical vaccination was delivered every two weeks for a total of at least three vaccinations.

Delayed-type hypersensitivity (DTH) reaction

Upon initiation of the second vaccination, the patients were tested for a DTH reaction. This was done by injecting 0.10–0.25 mL of serum-free IDMEM at a location distant from the immunization site. The diameter of the resultant wheal was measured with a caliper 24 h later. A positive response was defined as a raised indurated wheal 10 mm or more in diameter at the injection site.

Activation of T lymphocytes

Two weeks after the second immunization, mononuclear WBCs were isolated from non-moblized peripheral blood using a cell separator. Harvested cells were counted and ficolled (Ficoll Paque PLUS, Amersham Pharmacia, Piscataway, NJ), then recounted. They were inoculated into tissue culture bags (Lifecell, Nexell Therapeutics, Irvine, CA) at a concentration of 3.0×10^{10} to 5.0×10^{10} cells/mL for six to seven days at 37 °C in RPMI 1640 media supplemented with 2.5% autologous serum and 1% gentamycin. Anti-CD3 (Orthoclone OKT3, Ortho Biotech Inc., Bridgewater, NJ) was added to the media at time of inoculation into cell culture bags at a concentration of one pg/mL. After 48 h of incubation, IL-2 (Proleukine, Chiron Corp., Emeryville, CA) was added to the media at a concentration of 100 IU/mL, and cells were re-incubated for another four to five days. At the time of the harvest, the cells were washed at least twice in sterile saline, pooled into one cell suspension and counted. Aliquots were removed for endotoxin and microbiology testing. After reaching maximum density, cells were pooled and harvested into infusion bags by using a cell harvester system. After viability and cell count were determined, cells were re-infused into the patients. Re-infusion occurred over a six-hour period, and patients were observed for an additional 90 min. Patients went on to receive additional vaccines every two weeks for a total of three vaccinations. There were no adverse events that occurred due to T-cell infusion.

Response

In all three patients, tumor response was evaluated by clinical parameters as well as by serial MRIs.

Results
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Patient characteristics are summarized in ○ **Table 1**. Three patients were enrolled in the study between 1998 and 2000. All three patients were females. Their ages were five months, four years old, and seventeen years old. The patients had histologically proven GBM or other malignant gliomas, according to the criteria of the World Health Organization (WHO), and one patient had a recurrent ependyoma.

All patients underwent a surgical resection of their tumor. Two patients received external beam radiation therapy with stereotactic boost. The patients with documented recurrence and/or progression underwent surgical debulking, and they were then enrolled on the treatment protocol.

Treatment-related toxicity
HDCT/PBSCT

All patients went on to receive HDC as outlined above, followed by autologous PBSCT. This therapy was well tolerated. There were no serious adverse events that occurred. The mean CD 34+ count was 5.25×10^6/kg, and the mean time to engraftment was day +13 (absolute neutrophil count >500 for three consecutive days). Patient #2 was treated during the neutropenic phase of transplant for a Gram-positive cocci infection with intravenous antibiotics. The result was resolution without any complications.
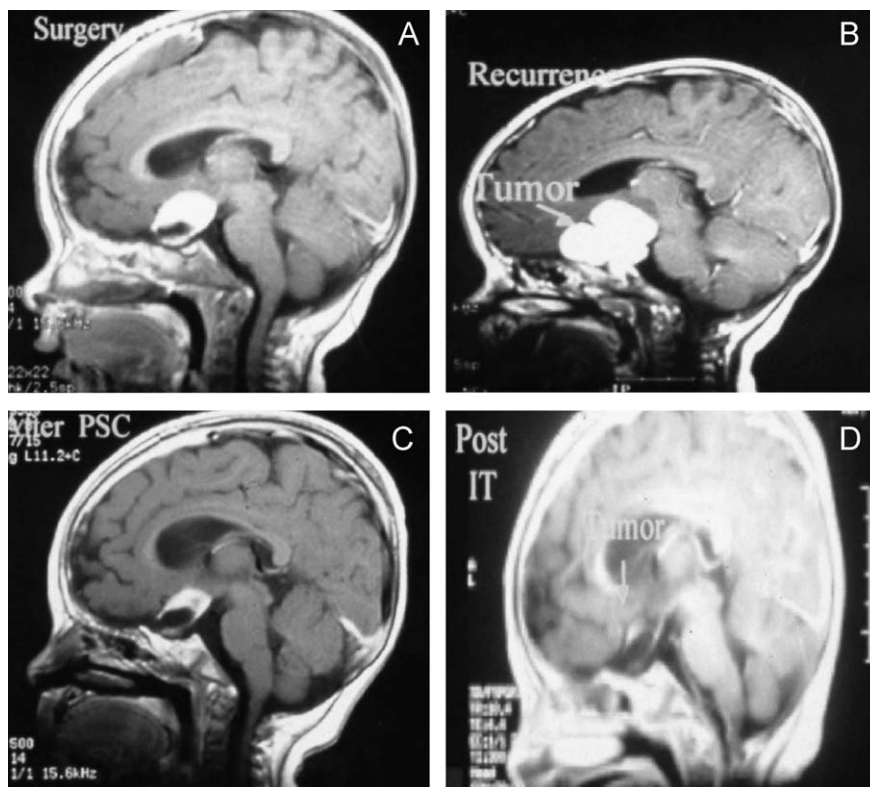
Fig. 2 Pre-transplant magnetic resonance imaging of patient #1 is shown in **A** and **B**. Post-therapy results after HDC & PBSCT at day +60 and vaccination are shown in **C** and **D**, respectively.
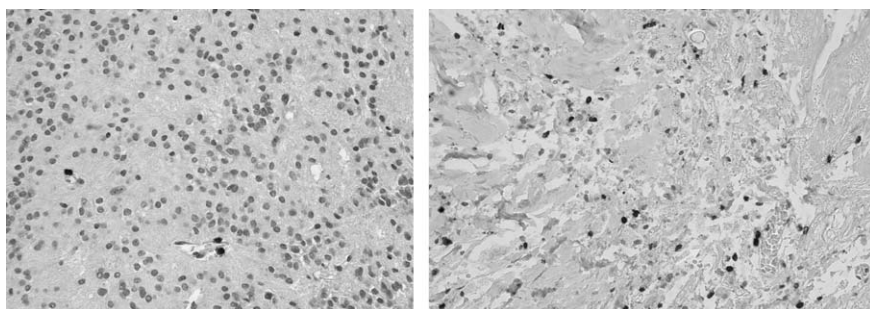

Fig. 3 Histological cross-sections from a post-mortem examination of patient #3 reveal intratumoral T-cell infiltration. The images show post-vaccination staining positive with CD3^{+}.

Safety of AI

All patients received intradermal AI, and there were no adverse reactions or effects of the AI. No autoimmune phenomena were observed, nor were there any laboratory demonstration of auto-antibodies by standard laboratory testing in our institution.

Clinical response

Two patients had major clinical and radiographic responses (see ⊙ **Figs. 1–3**). At the time of evaluation, one patient (ependymoma) was alive at seven years old, and another was alive at 23 months old. One patient expired from pneumonia at day +81. This was not felt to be related to the adoptive immunotherapy. Comparative MRIs for patients #1 and #2 are shown in ⊙ **Figs. 1, 2**. Pathology demonstrating T-cell infiltration into the tumor bed (patient three) is shown in ⊙ **Fig. 3**.

Discussion
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The primary objective of this study was to determine the safety and feasibility of HDC with PBSCT, followed by adoptive immunotherapy in pediatric patients with CNS malignancies. All patients tolerated the vaccines well without clinical toxicity. No

Fig. 4 A patient demonstrates a DTH response after the second vaccination.

auto-antibodies were produced, and no autoimmune reactions were observed. When considering this treatment, the possibility of inducing experimental allergic encephalitis, which can be deadly when using autologous cellular material, should be taken into consideration (⊙ **Figs. 4, 5**).

The secondary objectives of this study were achieved, with both the humoral and cellular arms of the immune system evaluated at baseline prior to vaccination. Clinical assessments of immunocompetency by lymphocyte subsets and total Ig isotype titers suggested that all three patients had intact immune systems. All patients had normal lymphocyte subsets and IgG levels prior to
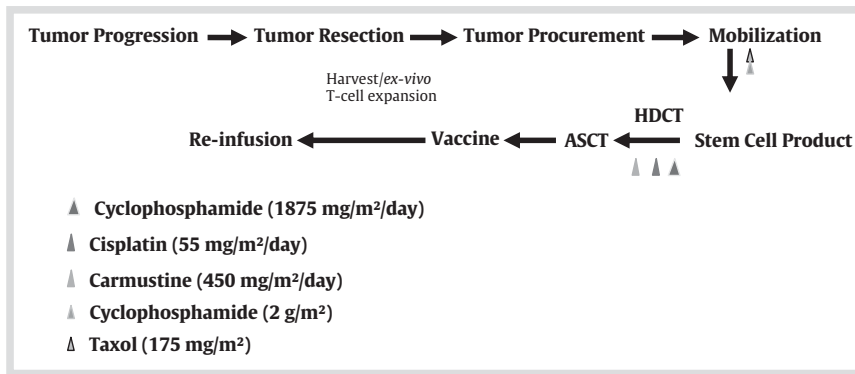
Fig. 5 Treatment scheme.

HDC and PBSCT (data not shown). All three patients tolerated HDC and PBSCT, with only one patient developing a complication secondary to infection.

In this trial, each patient's tumor was resected, and the tumor lysate was used as the source of the antigen. We speculated that this approach would have the following advantages: (1) it would provide multiple antigens for recognition by specific T cells; (2) it would accommodate for gliomas being heterogeneous (meaning there are no known defined tumor antigens expressed by all glioma cells); (3) it would not require us to grow autologous cultured tumor cells (which can be problematic with irradiated tumor tissue), since we are using tumor lysate; (4) it would allow glioma cells to potentially express artifactual antigens when cultured; and (5) it would allow the tumor lysate to potentially target multiple antigens which would obviate immune selection of single antigen-loss tumor variants.

Glioma cells have been thought to be poor in antigen presentation [1]. This is thought to occur secondary to down-regulation of co-stimulatory molecules such as transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF) and interleukin (IL)-10, or by blocking the production of pro-inflammatory molecules by increasing expression of the STAT3 protein [12, 14, 17, 18]. Even when a response is induced, tumor cells can still escape their elimination by losing targeted antigens, rendering tumor-reactive T cells anergic [12]. The biggest challenge for immunotherapy is to safely and effectively augment strategies that assist antitumor responses [3, 12]. The delivery of cell-based vaccines that exploit natural mechanisms of antigen presentation represents a promising approach for the immunotherapy of cancer [3, 12].

This strategy tests the hypothesis that ex vivo manipulation and re-injection of cellular products can induce immune responses and circumvent immune incompetence to achieve clinically significant results [3]. Previously, it has been demonstrated that subcutaneous vaccination with genetically modified cytokine-secreting tumor cells could be efficacious against intracranial tumors in a murine model [3, 15, 19]. This is evidence that an effective immunological response can be generated against intracerebral tumors [3, 15, 16]. A cancer vaccine would be successful if it recruited antigen-specific cytotoxic T-lymphocytes to the tumor site [1, 16]. In theory, cancer vaccines should disrupt immune tolerance and activate an occult T-cell population that has escaped immune tolerance [14]. Recently, there have been an increasing number of reports harnessing innate immune cells as effectors in systemic immunotherapy [1, 8, 14, 16].

Effector cells have been expanded and activated in vitro with IL-2, and then infused with high doses of IL-2 back into patients [18]. Such infusions have resulted in marked antitumor activity,

with complete remissions in a subset of patients that received this form of treatment [18]. Rapoport et al. reported their results that early T-cell infusion (D+12) after vaccination resulted in high CD4 numbers and sustained T-cell-dependent antibody response [19]. They used T-cells activated with CD3/CD28, resulting in a more robust T-cell product [19].

Of great significance was our finding that two of the three patients treated with adoptive immunotherapy demonstrated markedly prolonged survival when compared to historical controls. The strategy of this approach was reported four to five years ago in mostly adult trials. Little data has been published about this from pediatric trials. Our findings suggest the therapeutic superiority of tumor-specific cytotoxicity above craniotomy and conventional therapy in patients with recurrent gliomas. The successful use of systemic tumor responses and antigen-specific cytotoxicity will help to establish this strategy as a model in understanding the underlying mechanisms underlying of adaptive immunity against glioma.

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