Effects of Concomitant Temozolomide and Radiation Therapies on WT1-specific T-cells in Malignant Glioma

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Objective: Immunotherapy targeting the Wilms' tumour 1 gene product has been proven safe and effective for treating malignant glioma in a phase II clinical study. Currently, radiation/temozolomide therapy is the standard treatment with only modest benefit. Whether combining radiation/temozolomide therapy with WT1 immunotherapy will have a negating effect on immunotherapy is still controversial because of the significant lymphocytopaenia induced by the former therapy. To address this issue, we investigated the changes in frequency and number of WT1-specific T-cells in patients with malignant gliomas.

Methods: Twenty-two patients with newly diagnosed malignant glioma who received standard radiation/temozolomide therapy were recruited for the study. Blood samples were collected before treatment and on the sixth week of therapy. The frequencies and numbers of lymphocytes, CD8+ T-cells, WT1-specific T-cells, regulatory T-cells, natural killer cells and natural killer T-cells were measured and analysed using T-tests.

Results: Analysis of the frequency of T lymphocytes and its subpopulation showed an increase in regulatory T-cells, but no significant change was noted in the populations of T-cells, WT1-specific T-cells, NK cells and NKT cells. Reductions in the total numbers of T-cells, WT1-specific T-cells, NK cells and NKT cells were mainly a consequence of the decrease in the total lymphocyte count.

Conclusions: Radiation/temozolomide therapy did not significantly affect the frequency of WT1-specific T-cells, suggesting that the combination with WT1 immunotherapy may be possible, although further assessment in the clinical setting is warranted.

Key words: Wilms' tumour 1 – glioma – temozolomide – cancer immunotherapy – WT1-specific T-cells

INTRODUCTION

Currently, the standard treatment for malignant gliomas is surgery, followed by external radiation and chemotherapy. For patients with newly diagnosed glioblastoma, concurrent radiation therapy and temozolomide (TMZ) chemotherapy, followed by adjuvant TMZ therapy for at least 6 months, offer only a modest benefit, with a median survival of 14.6 months and a 2-year survival rate of 26.5% (1). Thus, glioblastoma is still considered an intractable disease and extensive clinical trials recruiting various modalities are ongoing.

Peptide-based cancer vaccination is a novel form of therapy and recent advances have resulted in the identification of a large number of tumour-associated antigens. One of these is the product of the Wilms’ tumour 1 (WT1) gene (2), which is thought to function as an oncogene rather than a tumour-suppressor gene (3). It has been shown to be over-expressed in leukaemia and various types of solid cancers (4,5) and to be an attractive target antigen for immunotherapy against these cancers (6).

We have been conducting a phase I/II clinical trial to examine the safety and efficacy of WT1-based vaccination.
for patients with myeloid malignancies, including acute myeloid leukaemia and myelodysplastic syndrome (7,8), and a variety of cancer types including malignant gliomas (9). We have performed a phase II clinical trial of WT1 vaccination for patients with recurrent malignant glioma and have recently reported the safety and efficacy of WT1 vaccination for 21 patients with recurrent glioblastoma (9).

Immunologically, CD8+ cytotoxic T lymphocytes (CTLs) are thought to be the main antitumoural effector of WT1 vaccination. In a study conducted by Oka and colleagues (7), a direct relationship was observed between the frequency of WT1-specific CTLs after WT1 vaccination and clinical response.

As a consequence of the favourable outcome obtained from our phase II clinical trial of WT1 vaccination for recurrent glioblastomas, a trial involving patients with newly diagnosed malignant glioma is now being contemplated. The proposed plan is to combine concurrent radiation/TMZ therapy with WT1 immunotherapy. However, one of the main disadvantages of this plan is the lymphocytotoxicity brought about by the current standard radiation/TMZ treatment. It has been reported that lymphocytopenia is encountered in 4–60% of patients (10–12). Thus, before this strategy can be put into clinical application, the important question of whether concomitant radiation/TMZ therapy affects the antitumour immunity of WT1 vaccination needs to be addressed.

In our literature search, no articles concerning the lymphocytotoxic effects of radiation/TMZ therapy on subpopulations of T lymphocytes, specifically WT1-specific T-cells, have been reported. This study aims to determine how concomitant radiation/TMZ therapy affects the WT1-specific T-cells and other T-cells in terms of their frequencies and total numbers.

PATIENTS AND METHODS

PATIENT POPULATION

Blood samples were obtained from patients with newly diagnosed malignant gliomas who underwent standard treatment based on the recommendation by Stupp and colleagues (1) at Osaka University Hospital from May 2007 to January 2009. Briefly, the patients received fractionated conformal radiotherapy for a total dose of 60 Gy in 30 daily fractions of 2 Gy. Concomitant chemotherapy consisted of oral TMZ at a daily dose of 75 mg/m² from the first to the last day of radiation therapy. After a 4-week break, patients began to receive adjuvant oral TMZ (150–200 mg/m²) for 5 days every 28 days. Prior to sampling, written consent was obtained from all patients included in the study. Blood samples were taken prior to the administration of radiation/TMZ therapy and on the sixth week of concurrent radiation/TMZ therapy. A third blood sample, taken during the adjuvant phase of TMZ therapy, was collected from some patients.

The white blood cell count and the proportion of lymphocytic cells from all blood samples obtained prior to the start of treatment were first assessed. Thereafter, peripheral blood mononuclear cells (PBMCs) were extracted and their components further assayed to determine the frequency and number of T-cells, WT1-specific T-cells and their phenotypic composition, regulatory T (Treg) cells, natural killer (NK) cells and natural killer T (NKT) cells. The procedure was then repeated for blood samples taken after sixth week of treatment and the same parameters were evaluated.

The human leukocyte antigen (HLA)-A genotype of patients was also examined. The presence of WT1 protein in the tumour was detected by immunohistochemistry using a standardized staining protocol (5). Briefly, formalin-fixed tissue sections were prepared from the resected tumours. Sections were microwaved for 15 min in citrate buffer (pH 6.0) for antigen retrieval and incubated with anti-human WT1 mouse monoclonal antibody 6F-H2 (Dako Cytomation, Inc., Carpinteria, CA, USA; diluted 1:50). The WT1 reaction was visualized with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (Wako, Osaka, Japan). The sections were then counterstained with haematoxylin. Control positive staining was evaluated with Wilms’ tumour tissue and control negative staining was evaluated with normal brain tissue.

This study was conducted with the approval of the Ethics Review Board of Osaka University Hospital.

WT1 PEPTIDE/HLA-A*2402 TETRAMER ASSAY OF WT1-SPECIFIC T-CELLS

WT1 peptide/HLA-A*2402 tetramer assay of WT1-specific T-cells was performed to calculate the frequency of WT1-specific T-cells in PBMC as previously described (9). First, frozen PBMCs were thawed and stained with PE-conjugated HLA-A*2402-WT1 235–243 tetramer (MBL, Nagoya, Japan) in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline containing 5% foetal bovine serum) for 30 min at 37°C. Subsequently, these cells were stained with five colours of fluorescence-labelled monoclonal antibodies (all from BD Pharmingen, San Diego, CA, USA): allophycocyanin (APC)-anti-CD3 antibody; fluorescein isothiocyanate (FITC)-anti-CD4, 14, 16, 19, 56 antibody; APC-cy7-anti-CD8 antibody; phycoerythrin (PE)-cy7-anti-CCR7 antibody; and PE-texas Red-anti-CD45RA antibody. The cells were incubated with the antibodies for 20 min on ice in the dark. Then, these cells were washed twice with FACS buffer. The samples were analysed using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA) and data analyses were performed using CellQuest software (BD Biosciences). A total of 10⁶ events per sample were collected. CD3+ and CD4+CD14–CD16–CD19–CD56– fractions were considered representative of T-cells. CD8+ and WT1 tetramer+ T-cells were considered representative of WT1-specific T-cells. Moreover, WT1-specific T-cells were further divided
into four types based on their differentiation level: naïve (CD45RA^CCR7^), central memory (CD45RA^-CCR7^), effector memory (CD45RA^-CCR7^-) and effector (CD45RA^CCR7^-).

**TREG, NK AND NKT CELL DETERMINATION**

Frozen PBMCs were thawed and stained with four colours of fluorescence-labelled monoclonal antibodies (all from BD Pharmingen): APC-anti-CD4 antibody, peridinin-chlorophyll protein complex (PerCP)-anti-CD3 antibody, PE-anti-CD127 antibody and PE-cy7-anti-CD25 antibody. The cells were incubated with the antibodies for 20 min on ice in the dark. Then, these cells were washed twice with FACS buffer. A total of 10^5 events per sample were collected. CD3^+CD4^+CD25^high-int CD127^low cells were regarded as Treg cells.

Frozen PBMCs were thawed and stained with two colours of fluorescence-labelled monoclonal antibodies (both from BD Pharmingen): PerCP-anti-CD3 antibody and PE-anti-CD56 antibody. The cells were incubated with the antibodies for 20 min on ice in the dark. Then, these cells were washed twice with FACS buffer. A total of 10^5 events per sample were collected. CD3^-CD56^+ cells were considered as NK cells, whereas CD3^-CD56^- cells were considered as NKT cells.

**STATISTICAL ANALYSIS**

All statistical analyses were carried out using paired T-tests or unpaired T-tests. All probability values <0.05 were considered statistically significant and all statistical computation was performed using StatMate III software (ATMS, Tokyo, Japan).

**RESULTS**

**PATIENT CHARACTERISTICS**

Blood samples from 22 patients with newly diagnosed malignant glioma were collected before treatment and after the completion (sixth week) of combination radiation/TMZ therapy. PBMCs were successfully extracted from all blood samples taken. Blood samples from four patients were further analysed during the adjuvant phase of TMZ therapy.

Table 1 shows the profiles of the 22 patients (10 male and 12 female; mean age, 51.0 years; range, 19–77 years) included in the study. Histological diagnoses were as follows: anaplastic astrocytoma (AA) in 2 patients, anaplastic oligoastrocytoma (AO) in 3 patients and glioblastoma in 17 patients. All patients received radiation therapy (60 Gy) and TMZ (120–140 mg/day) as recommended by Stupp and colleagues (1). Expression of the WT1 protein in the glioma was noted to be positive in 19 patients and negative in 3 patients. Twelve patients were HLA-A*2402-positive and expressed the WT1 protein.

**EFFECT OF RADIATION/TMZ THERAPY**

Before the start of the treatment, the average lymphocyte count in the peripheral blood was 1300 cells/mm^3. Blood samples taken on the sixth week of radiation/TMZ therapy showed that the count significantly decreased to 631 cells/mm^3 (P < 0.001) (Table 2). On further quantification, no significant difference (P = 0.156) was seen in the mean frequency of T-cells before treatment, 17.1% (range, 8.1–37.0%), and after treatment, 18.5% (range, 7.6–35.7%) (Fig. 1A). On the other hand, the mean number of T-cells significantly decreased to 123 cells/mm^3 (range, 3–316 cells/mm^3) from the initial 225 cells/mm^3 (range, 77–536 cells/mm^3) (Fig. 1B).

The mean frequencies of WT1/HLA-A*2402 tetramer-positive T-cells were 0.196% (range, 0.087–0.405%) prior to treatment and 0.256% (range, 0.062–0.830%) 6 weeks after treatment. Figure 2A gives us a graphical representation of the frequencies before and after treatment in 12 patients. A remarkable increase in the frequency can be seen in two
patients (Cases 12 and 15), but for the rest no significant change was noted. Overall, these findings did not reach statistical significance. In terms of the mean number of WT1-specific T-cells, a decrease was noted from 0.378 (range, 0.138–0.677 cells/mm$^3$) to 0.220 cells/mm$^3$ (range, 0.096–0.670 cells/mm$^3$), although, this was also found to be not significant ($P = 0.011$) (Fig. 2D). Figure 2C shows us the individual data on mean WT1-specific T-cell number for the 12 patients, only one patient (Case 15) showed a reverse trend. With reference to Fig. 2A and C, it is evident that one patient (Case 15) had an increase of both the number and frequency of WT1-specific T-cells. Further proving this point is the FACS histogram of this patient shown in Fig. 2B.

Figure 2E shows a diagram of the phenotypic classification of WT1-specific T-cells before and after therapy. No significant change was found in the composition of the WT1-specific T-cells after treatment.

Extended analysis of the data from four patients (Cases 7, 11, 13 and 15) with HLA-A*2402, who provided blood samples during the adjuvant phase of TMZ therapy, revealed three different patterns in terms of frequency and number of WT1-specific T-cells: (i) a gradual increase (Cases 7 and 13); (ii) an abrupt increase followed by a decrease (Case 15) and (iii) a gradual decrease (Case 11) (Fig. 3B and C). Noteworthy is that, in these cases, the total number of lymphocytic population showed a gradual increase after concomitant radiation/TMZ therapy, possibly indicating recovery from myelosuppression (Fig. 3A).

Analysis of Treg cells from extracted PBMCs showed that the mean frequency before (4.83%) and after (7.50%) treatment reached statistical significance ($P < 0.01$) (Fig. 4A). On the other hand, the mean number of Treg cells before (25.3 mm$^{-3}$) and after (18.1 mm$^{-3}$) treatment failed to reach statistical significance ($P = 0.064$); however, when plotted against each other, a downward trend can be observed (Fig. 4B). Further analysis of the frequency of Treg cell subpopulation from six patients (Cases 7, 11, 13, 15, 16 and 17), with or without HLA-A*2402, during the adjuvant

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WT1, Wilms’ tumour 1; TMZ, temozolomide; WBC, white blood cell; NE, not evaluated.

aWT1-specific T-cells were measured in HLA-A*2402-positive patients (12 patients).
phase of TMZ therapy, generally seemed to fit into three models: (i) a decrease (Cases 7, 15, 16 and 17); (ii) an abrupt increase followed by a decrease (Case 13) and (iii) no change (Case 11) (Fig. 3D).

For both NK and NKT cells, the decrease in mean frequency before and after treatment did not reach statistical difference (Fig. 4C and E). However, when the mean numbers of NK and NKT cells before and after treatment were analysed, a statistically significant decrease was noted (P < 0.001) (Fig. 4D and F).

**DISCUSSION**

Malignant gliomas are the most common primary brain tumour in adults and its treatment involves a combination
of surgery, radiation therapy and chemotherapy. Despite this, the outcome remains uniformly fatal. In our institution, we have tried a novel treatment approach using WT1 peptide vaccination in patients with recurrent glioblastoma, with favourable results (9). Whether the addition of WT1 peptide vaccination to the current treatment standard will improve survival of these patients is yet to be determined.

Treatment modalities that induce lymphocytopenia are known to deprive patients of the benefits of active antitumour immunization. The current standard approach, a combination of radiation and TMZ therapies, used in patients with newly diagnosed malignant glioma has been shown to induce significant lymphocytopenia. This study was conceptualized to address the concern regarding the relationship between the standard therapy and WT1-specific T-cell population in this subset of patients and, ultimately, to serve as preclinical groundwork for future studies involving these treatment modalities.

In this study, a significant reduction in the mean number of WT1-specific T-cells after 6 weeks of radiation/TMZ therapy was observed. This occurred as a consequence of the marked reduction in the total number of lymphocytes. However, the mean frequency of WT1-specific T-cells increased (0.196 vs. 0.265%), although it did not reach statistical significance. As seen in Fig. 1A, individual frequencies before and after treatment were well conserved and in some cases, even increased. In one patient (Case 15), the increased was noted to be exceptionally high. It is assumed that, in cases of increased frequencies, glioma cells damaged by radiation/TMZ therapy might have released WT1 protein and its fragments, which were then taken up by antigen-presenting cells (APCs) such as dendritic cells, resulting in the generation of WT1-specific T-cells. WT1 peptides through the processing mechanisms in APCs are presented on the cell surface in association with HLA class I or class II molecules. The T-cell epitope induces the direct production of WT1-specific T-cells and the helper epitope

Figure 3. Frequency of WT1-specific T-cells (A), number of total lymphocytes (B), number of WT1-specific T-cells (C) of four cases and frequency of Treg cells of six cases (D) are shown during the adjuvant temozolomide phase.
induces the production of WT1-specific helper T-cells, which aid in the induction or activation of WT1-specific T-cells. As for Case 15, despite the marked increase in both number and frequency of WT1-specific T-cells, other lymphocytic response was in sync with the rest of the sample population (e.g. decrease in total number of lymphocytes and increase in frequency of Treg cells). The cause of this phenomenon is unclear but in terms of clinical outcome, retrospective investigation showed that the patient followed the 'typical' course for an AA (data not shown).

Although beyond the scope of the study, we tried to determine the relationship between frequency of WT1-specific T-cells and patients' prognosis. No apparent relationship between the two was noted statistically. This particular finding should be interpreted cautiously as the sample population is limited (12 HLA-A*2402 positive patients) and the histological diagnosis are varied [10 glioblastoma multiformes (GBMs), 1 for both AA and AO].

Evidence, as of the moment, only points to a definite persistence of WT1-specific T-cells after chemo/radiotherapy and that it could be activated by the WT1-specific vaccination. Other findings should be further verified on future studies.

To date, we cannot find any articles related to the dynamic analysis of T-cells, including their composition, and WT1-specific T-cells after treatment with combined radiation/TMZ therapy or TMZ alone. There are, however, some studies on combined chemotherapy and immunotherapy in which some analyses of lymphocytes were conducted. Heimberger and her colleagues (13) published a case of glioblastoma treated with radiation/TMZ therapy followed by a peptide vaccine. After the standard initial radiation/TMZ therapy for 6 weeks, the patient received concurrent TMZ therapy and a tumour-specific vaccine targeting epidermal growth factor receptor variant III (EGFRvIII). Although there was some monthly fluctuation in the white blood cell count, specifically the CD4 and CD8 counts, no cumulative decline was observed. They concluded that, despite conventional dogma, chemotherapy and immunotherapy can be delivered concurrently without negating the effects of...
immunotherapy (13). A number of other similar trials regarding combination of EGFRI-III-targeted vaccine (CDX-110) with radiation/TMZ therapy were reported (13–16). Preliminary experience with this vaccine yielded a median progression-free survival of 16.6 months in patients with glioblastoma and is currently under investigation in several phase III trials (16).

Another trial on immunotherapy was conducted by the team of De Gast (17) involving patients with metastatic melanomas. In that study, TMZ therapy followed by combined immunotherapy with granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-2 (IL2) and interferon (IFN)α was delivered to the patients. After 5 days of TMZ administration, depression of T-cells occurred, but it did not prevent the activation of CD3, CD4 or CD8+ T-cells or the expansion of NK cells. Experimental murine brain tumour immunotherapy (13). A number of other similar trials demonstrated, through detailed analysis of T lymphocyte subsets, that TMZ even enhances the antitumour immunity of dendritic cell vaccination.

With regards to the frequency of Treg cells in our patient population, we noted an increase in Treg cells after radiation/TMZ therapy, from 4.83 to 7.50% (Fig. 4A). These values subsequently decreased or plateau’d during the adjuvant phase. A similar phenomenon was observed by Sampson and colleagues (17) in their trial using combined radiation/TMZ therapy and EGFRI-III-specific vaccine for the treatment of glioblastoma. In their study, the Treg levels increased from 5.2 to 11.8% after radiation/TMZ therapy but were maintained in subsequent therapies. In contrast, a recent report by Banissi and colleagues (20) showed that the standard TMZ dose did not significantly alter the frequency of Treg cells in rat glioma models. As to what brought about this discrepancy warrants further studies. This may be attributed to several factors including (i) sample population used (human vs. rat), (ii) materials utilized (peripheral blood vs. spleen) and (iii) therapeutic intervention employed (radiation/TMZ vs. TMZ alone).

The role of Treg cells in immune tolerance of cancer has been studied in many animal models. Despite this, the exact part it plays in tumour rejection is still an enigma. As to the clinical implication of our study, if we are going to abide to the premise that Treg depletion is required for an effective immunization, the finding in this study may serve to help decide the timing of immunization. As Treg population gradually decrease or stabilize beyond the concomitant phase, logically, maximal therapeutic benefit from immunotherapy would be achieved if the vaccine is given beyond this time period.

In this study, we have provided evidence that the frequency of WT1-specific T-cells remains stable, even after its absolute numbers decrease, despite the myelosuppressive effects of concomitant radiation/TMZ therapy in patients with newly diagnosed malignant glioma. It is recommended that examination of the frequency of other peptide-specific T-cells be made to further enhance our understanding and give a better rationale for the combined radiation/TMZ and WT1 vaccination therapy.

Similarly, regarding the timing of WT1 vaccination, further studies are needed. It may be more suitable to start WT1 vaccination after the end of 6 weeks of radiation/TMZ therapy and be given concomitantly with TMZ during the adjuvant phase, rather than starting during the intensive phase of the treatment because, aside from the reason mentioned above, tumour-specific immunity is reported to be induced effectively by the tumour-specific stimulation during homeostatic proliferation (13,21).

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Conflict of interest statement

None declared.

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