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Human umbilical vein endothelial cell vaccine therapy in patients with recurrent glioblastoma

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We aimed to assess the clinical efficacy of glutaraldehyde-fixed human umbilical vein endothelial cell (HUVEC) vaccine for the treatment of patients with recurrent glioblastoma. Patients of a HUVEC vaccine group received intradermal injections of 5×10^7 HUVEC weekly during the first month, and every 2 weeks from the second month, until progression of the disease was observed. Salvage treatment consisted of multimodal chemotherapy, radiation, including gamma-knife therapy, and/or repeated surgery, when feasible. Hazard ratios for death were calculated using a Cox model. A total of 17 patients with recurrent glioblastoma were enrolled in this study. All the patients received the initial treatment consisting of maximal safe surgical resection, followed by radiotherapy of 50–80 Gy or more, with concomitant and adjuvant chemotherapy consisting of temozolomide or nimustine (ACNU). A total of 352 vaccinations were performed for the patients of the HUVEC vaccine group (median number of vaccination = 11 doses; range 3–122 doses). The median progression-free survival and overall survival were 5.5 and 11.4 months, respectively. The median overall survival from the diagnosis was 24.3 months. The HUVEC vaccine therapy significantly prolonged the tumor doubling time and contributed to reducing the tumor growth rate. Hematological adverse reactions due to chemotherapy were recognized: one patient experienced grade III leukocytopenia and one showed grade II lymphocytopenia. Associated with the HUVEC vaccine therapy, a delayed-type hypersensitivity-like skin reaction developed at the injection site. The HUVEC vaccine therapy effectively controlled disease progression, without evident adverse effects, except for a delayed-type hypersensitivity-like skin reaction at the injection site. (*Cancer Sci* 2013; 104: 200–205)

Glioblastoma (GBM) is one of the most devastating human tumors. Even with optimal surgical resection and standard chemoradiotherapy, GBM always recurs, and no specific treatment exists for recurrent GBM. Unfortunately, the median survival following recurrence is 5–7 months.[1] GBM is a highly vascular tumor with high expression of vascular endothelial growth factor (VEGF).[2,3] Bevacizumab (BEV), a humanized monoclonal antibody to VEGF, which inhibits tumor angiogenesis, consequently decreasing the intratumoral blood flow,[3–7] had been expected to reduce the volume of recurrent tumors at the time the drug was approved for clinical use and clinical studies were started. However, in patients with recurrent GBM, BEV has only limited clinical benefit. Although BEV causes a strong decrease of contrast enhancement on magnetic resonance images, vascular remodeling induced by BEV, which makes tumors more hypoxic and glycolytic,[8,9] might result in increased invasiveness of tumor cells into the normal brain tissue. Enhanced tumor cell infiltration after anti-angiogenic treatment has been reported in other tumor models.[4,9–11]

Human endothelial cells in culture share some properties with the angiogenic endothelium, such as the high expression of CD51 and CD105.[12] We have been focusing on human umbilical vein endothelium cell (HUVEC), which, under culture with VEGF and basic-fibroblast growth factor, has specific properties of angiogeneic endothelium, such as the high expression of the platelet endothelial cell adhesion molecule (CD31), integrin alpha-V precursor (CD51) and endoglin (CD105), as confirmed by flow-cytometry.[12,13] We have also confirmed the expression of these surface markers on the vascular endothelium of GBM and colorectal cancer. Based on these results, we tested and confirmed the effectiveness of glutaraldehyde-fixed allogeneic endothelial cells as a vaccine against solid tumors (in an animal model). In addition, we commenced a clinical trial of allogeneic HUVEC as a vaccine for the treatment of angiogenic solid tumors (malignant brain tumors and colorectal cancer) in humans. Patients with recurrent malignant brain tumors had better clinical response than those with metastatic colorectal cancer. This might be mainly dependent on the smaller size of the targeted tumor lesions of the patients with malignant brain tumors.[12] In the present study, we aim to assess the clinical efficacy of glutaraldehyde-fixed HUVEC vaccine for the treatment of patients with recurrent GBM.

Materials and Methods

Study design. We investigated 25 consecutive patients with recurrent malignant gliomas. There was no limit on previous regimens or salvage treatments. The HUVEC vaccine therapy was approved in 2002 by The University of Tokyo Investigational Review Board (No. 506), according to the "Good Clinical Practice for Medical Devices" guidelines, as well as the "Pharmaceutical Affairs Act in Japan." Informed consent, in accordance with the Declaration of Helsinki, was obtained from each patient or from a legally authorized representative before inclusion in the HUVEC vaccine therapy.

Patient population. The patient eligibility criterion was as follows: (i) presence of histopathologically-confirmed glioblastoma; (ii) the standard of care involving surgery followed by chemoradiotherapy already performed; (iii) recurrence of the disease despite treatment; and (iv) no corticosteroid use at enrollment. There were no restrictions in regards to Karnofsky performance scale (KPS).

Vaccine preparation and treatment plan. HUVEC were isolated from healthy donors at delivery, with informed consent, and cultured on 0.1% gelatin (w/v)-coated dishes in EC-SFM (Life Technologies, Grand Island, NY, USA), as described in our previous manuscript. HUVEC were fixed with 0.025% glutaraldehyde (v/v) and stored at −80°C in single

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dose aliquots, containing 5×10^7 cells/mL in physiological saline for injection.[12] The patients received intradermal injections of 1.5 mL vaccine in the upper arm weekly during the first month, and every 2 weeks subsequently, until progression of the disease was observed.

Efficacy and safety assessment. Patients who had received at least one dose of the HUVEC vaccine were included in the present study. MRI evaluations were performed at enrollment and every 3 months. Tumor progression was diagnosed based on the reports by neuroradiologists. Tumor response and safety were assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST version 1.1)[14] and the common terminology criteria for adverse events (NCI-CTCAE version 4.0),[15] respectively. Immune response was evaluated by examining patients' peripheral blood mononuclear cells and sera, which were taken monthly.

Statistical analysis. Progression-free survival (PFS) was calculated from the start of HUVEC vaccine therapy to date of progression or last follow up, whereas overall survival time was defined as the time between HUVEC vaccine therapy and death or last follow up. For tumor doubling times, the following equation was used: Tumor doubling time = log2/3 × {(time between the HUVEC vaccine therapy and death or last follow up)/[log (the tumor diameter at each follow-up period) − log (the residual tumor diameter after repeated resection)]}.[16] A paired *t*-test was performed to determine statistically significant differences between the two sets of measurements.

The Kaplan–Meier analysis was applied for the survival analyses, and statistical significance was calculated using the log-rank test. With regard to survival time, multivariate analyses were performed using a forward Cox's proportional hazard model adjusted for the following 15 clinical variables: age, sex, tumor location (frontal/temporal/parietal/occipital), primary tumor diameter, extent of initial resection, initial radiation dose, time from initial resection to treatment, KPS at recurrence, repeat resection (yes/no), residual tumor diameter after repeat resection, recursive partitioning analysis classification (Class IV–VII) by Carson *et al.*,[17] seeding at recurrence (yes/no), salvage radiotherapy (gamma knife/Boron neutron capture therapy /none), salvage chemotherapy (temozolomide and/or interferon-beta/none), and cortical steroid use during the study (yes/no). A two-sided *P*-value <0.05 defined statistical significance in all models. All statistical analyses were conducted using IBM SPSS version 19.0 software (SPSS, IBM, Somers, NY, USA).

Results

Patient characteristics. The 25 consecutive patients were enrolled in the HUVEC vaccine therapy at the Department of Neurosurgery, the University of Tokyo Hospital, in the period between August 2002 and August 2011. Among them, eight patients were excluded because four had anaplastic glioma (World Health Organization grade III), including anaplastic oligodendroglioma, three had pontine glioma, and one had pineoblastoma. The median survival time for recurrent anaplastic oligodendroglioma was 14.5 months (95% CI, not calculated), and the median survival for recurrent pontine glioma was 10.0 months (95% CI, 6.5–13.5). Consequently, 17 patients with recurrent GBM (15 primary and 2 secondary) were enrolled in this study.

All patients had received the initial treatment consisting of maximal safe surgical resection, followed by radiotherapy of 50–80 Gy or more, with concomitant and adjuvant chemotherapy consisting of temozolomide or nimustine (ACNU); 12 received standard radiotherapy of 60 Gy, three received doses of 50–54 Gy and two received high-dose radiotherapy of 80 Gy. All patients progressed in despite of previous temozolomide or nitorourea chemotherapy; 14 had the standard schedule of temozolomide (200 mg/m^2 days 1–5, repeated every 28 days) and three received ACNU (ACNU 100 mg/body day 1, repeated every 2 months) or ACNU/VCR (ACNU 100 mg /body day 1 and VCR 1 mg/body day 8 and day 15, repeated every 2 months).

At recurrence, they received the currently available salvage treatment: chemotherapy, radiation consisting of gamma-knife therapy or boron neutron capture therapy, and/or repeated operation when feasible. KPS was evaluated at recurrence: median KPS = 60%. No patient received prior BEV treatment. Corticosteroids were used by four patients during the study. The patient and tumor characteristics are outlined in Table 1.

Immune response and clinical response. A total of 352 vaccinations were performed: median number of vaccination = 11 doses, range 3–122 doses. Immunological screening was performed to confirm the immune response against HUVEC. ELISA revealed specific immunoglobulin response against HUVEC membrane antigens. In addition, HUVEC-specific CTL responses were detected using a gamma interferon enzyme-linked immunospot assay 1 month or later after the start of the treatment. Patients' cellular effectors specifically lysed HUVEC, but not non-endothelial control cells.[12] Chromium-release cytotoxicity assay revealed a specific cellular immune response against HUVEC (data not shown).[12]

Among 17 patients, progression disease occurred in four patients within 3 months (Fig. 1). An additional nine patients progressed slowly over several months. Three patients remained stable within 6 months and one patient had a partial response. The radiological response rate (cases with complete or partial response) was 5.9%.

Progression-free survival and tumor volume doubling time. The median progression-free survival (PFS) was 5.5 months (95% CI, 3.1–7.9 months) (Fig. 2). The 6-month PFS rate was 47.1% (95% CI, 25.5–69.7%), and the 12-month PFS rate was 23.5% (95% CI, 9.1–48.6%). The mean tumor volume doubling time at recurrence was calculated as 25.3 ± 23.6 days (range, 3 –77 days; median = 17 days, n = 17). After enrollment, measurements of the tumor volume doubling time were completed by 17 patients at 3-month follow up, 16 patients at 6-month follow up, nine patients at 9-month follow up, and five patients at 12-month follow up. The tumor volume doubling time at 3 months after enrollment was calculated to be fast (16–89 days) in 11 patients, slow (206–577 days) in five patients and negative (−94 days) in one patient, suggesting a shrinking tumor. The tumor doubling time, except for the shrinking tumor, was compared with that at recurrence: the mean tumor doubling time at 3-month follow up = 134.7 ± 166.6 days (n = 16) versus the mean tumor doubling time at recurrence = 22.6 ± 21.6 days (n = 16); paired *t*-test P = 0.012. The mean tumor doubling time after 6 months of treatment was also significantly elongated compared to that at recurrence: the mean tumor doubling time at 6-month follow up = 169.5 ± 262.5 days (n = 15) versus the tumor doubling time at recurrence = 23.9 ± 21.7 days (n = 15); paired *t*-test P = 0.047. After 6 months, however, the tumor volume doubling times were shortened in six patients. Fig.3 shows the time course of the mean diameter of recurrent tumors at each follow-up period. The recurrent tumors were stable in size for 6 months after enrollment, which reflected the results of tumor volume doubling times.

Overall survival. The overall survival was 11.4 months (95% CI, 7.9–14.9 months) (Fig. 44). The 6-month overall survival (OS) rate was 88.2% (95% CI, 63.2–97.0) and the 12-month OS rate was 47.1% (95%CI, 25.5–69.7%). At the time of analysis, 14 patients had died of tumor progression and three patients were alive (29.2, 34.0 and 66.5 months since treatment started). One patient who survived more than 60 months after recurrence continued to receive the HUVEC vaccine therapy every month. The 5-year OS rates were 17.6% (95% CI,

Table 1. Patient's characteristics

Characteristics	Number of patients (n = 17)
Age, mean ± SD (years)	46.9 ± 10.2
<50	8
>50	9
Sex	
Women	7
Men	10
Tumor location	
Frontal	8
Parietal	4
Temporal	3
Occipital	2
Primary tumor diameter, mean ± SD (cm)	4.1 ± 0.9
<4	4
>4	13
Extent of initial resection, mean ± SD (%)	75.9 ± 30.7
>95	6
<95	11
Initial radiation dose, Gy	
50–60	3
60	12
80	2
Time from initial resection to treatment	
Median, months	9.9
95% Confidence interval	6.3–13.5
<6 months	6
>6 months	11
KPS at recurrence	
90–100	1
70–80	6
50–60	10
Repeated resection at recurrence	
Yes	6
No	11
The diameter at the recurrence, mean ± SD (cm)	3.4 ± 1.1
The residual tumor diameter after repeated resection, mean ± SD (cm)	2.5 ± 1.5
<4	13
>4	4
Recursive partitioning analysis classification	
Class 4	1
Class 5	9
Class 6	4
Class 7	3
Seeding at recurrence	
Yes	7
No	10
Salvage radiotherapy	
Gamma knife	4
Born neutron capture therapy	2
None	11
Salvage chemotherapy	
Temozolomide and/or interferon-beta	7
None	10
Corticosteroid use during the study	
Yes	4
No	13

KPS, Karnofsky performance scale.

5.8–42.7%). The median time interval from the primary resection to the date of death or last follow up was 24.3 months (95% CI, 17.7–30.9).

Survival according to clinical variables was provided in Table 2. There was a statistically significant difference in OS between five patients <50 years of age and 12 patients >50 years of age: the median OS was 7.8 months (95% CI, 5.4–10.2 months) versus 14.2 months (95% CI, 9.8–18.6 months), respectively (P = 0.012). There was also a significant difference in OS between 11 patients with frontal or temporal tumors and six patients with parietal or occipital tumors: the median OS was 12.7 months (95% CI, 10.3–15.1 months) versus 6.9 months (95% CI, 4.8–9.0 months), respectively (P = 0.040). There were no significant differences in survival between six patients who were enrolled less than 6 months after the initial surgery and 11 patients who were enrolled at 6 months or more after the initial surgery (P = 0.8). There was also no difference in OS between four patients who used corticosteroids and 13 patients who did not (P = 0.8).

Among the 15 clinical variables, however, multivariate survival analysis using Cox's proportional hazard model showed that age was the only clinical variable that lengthened overall survival: adjusted hazard ratio = 0.865, (95% CI, 0.785–0.952); P = 0.003. Seeding at the recurrence was not a prognostic factor (P = 0.4): seven patients with seeding = 12.7 months (95% CI, 6.0–19.4 months) versus 10.9 months (95%CI, 6.7–15.1 months) = 10 patients without seeding.

Safety. The regimen was well tolerated. Delayed-type hypersensitivity (DTH)-like skin reaction developed at the injection site in 14 of 17 patients. However, except for this kind of skin reaction, no other types of adverse effects associated with the HUVEC vaccine therapy were observed. Of 14 patients who underwent salvage chemotherapy consisting of temozolomide and interferon-beta, one patient experienced grade III leukocytopenia, and one showed grade II lymphocytopenia: both continued the chemotherapy with a dose reduction of 25%. Other side effects, such as hemorrhage at the site of the tumor and a high level of protein in the urine, were not recognized.

Discussion

The HUVEC vaccine therapy was feasible for the patients with recurrent GBM and a low KPS score. In the present study, the radiological response rate was much lower (5.9%) than that for BEV (more than 50%),[4] but the patients had relatively long survival time: the median PFS and OS were 5.5 and 11.4 months, respectively. The median OS from the diagnosis was 24.3 months. The median OS compares favorably with that reported for other salvage therapies. The median OS for patients with GBM treated with temozolomide at first relapse was approximately 8 months.[18] Similarly, a phase II trial of BEV-alone or the BEV-plus-irinotecan in patients with recurrent GBM demonstrated that the median OS was 9.2 and 8.7 months, respectively.[5–7]

According to the report by Carson, prognostic factors for recurrent GBM include age, KPS, corticosteroid use, and shorter time from original diagnosis to recurrence.[17] In this study, age was the only prognostic factor, but the patients over age 50 survived longer than those under age 50. It could depend on the bias of patients' background: 10 patients (59%) had poor neurological function (KPS of 50–60%), and four patients (24%) had used corticosteroids during the study.

Because patients with recurrent GBM were clinically deteriorated, and previously treated with multimodality therapy consisting of surgery and chemoradiotherapy, treatment options were limited to palliative surgery when feasible, radiotherapy, including stereotactic radiosurgery, and chemotherapy. Because immunotherapy is associated with low risk of toxicity, it is a

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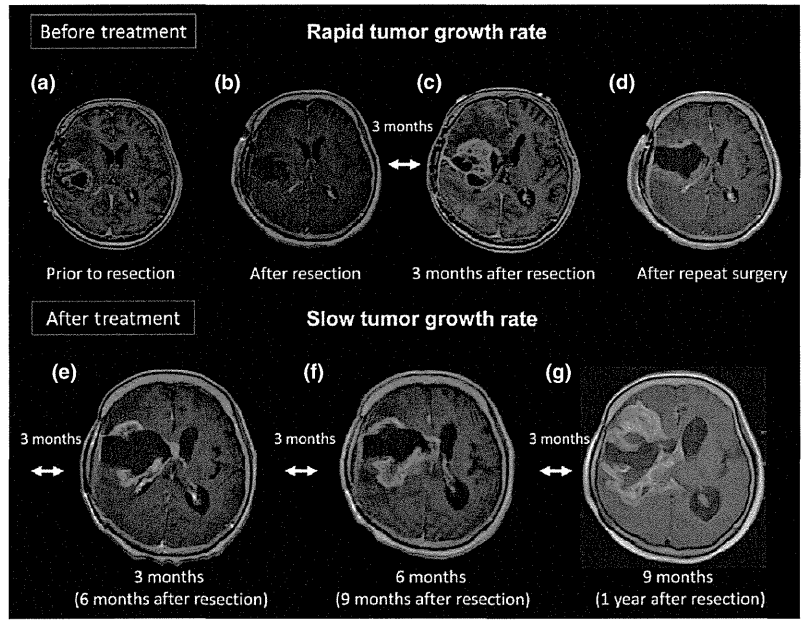

Fig. 1. Serial magnetic resonance image of a patient who showed a rapid progression. The patient recurred within 3 months and repeated surgery was performed. Karnofsky performance scale at recurrence was 60%. After repeated surgery only the HUVEC vaccine therapy was continued. The tumor kept growing, but the tumor growth speed seemed to be slowed by the HUVEC vaccine therapy.

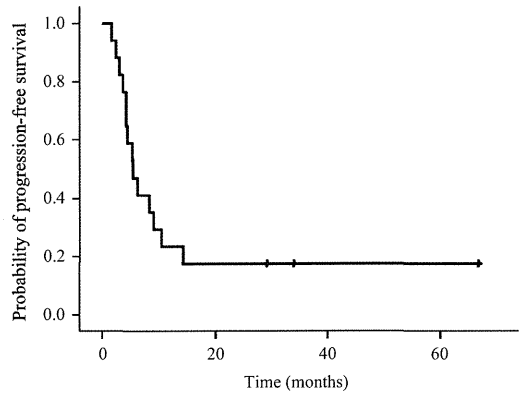

Fig. 2. Kaplan–Meier progression-free survival.

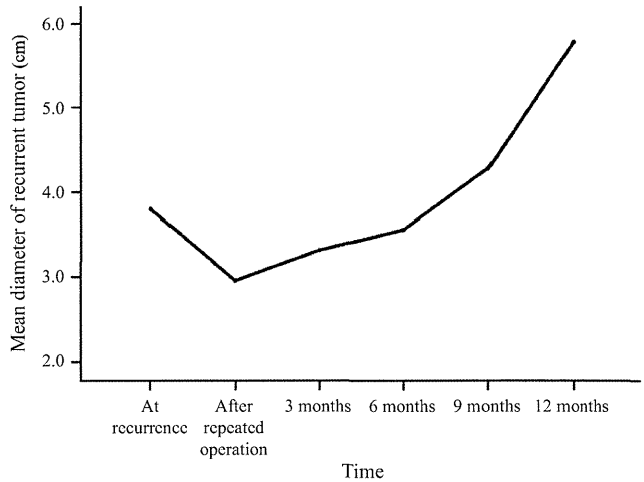

Fig. 3. The time course of the mean diameter of the recurrent tumors.

promising treatment strategy for recurrent GBM. Wilms tumor 1 peptide vaccine therapy for patients with recurrent GBM showed promising results: the 6-month PFS rate was 33.3% and the median OS was 9.2 months. This treatment, however, is limited to patients with the HLA-A *2402 phenotype.[19]

We established the HUVEC vaccine therapy for patients with progressive malignancy.[12] The HUVEC vaccine therapy was designed to target antigens specifically or preferentially on human tumor endothelium, such as CD31, integrin alpha v beta 3 and CD105: CD31 can be isolated from glioblastoma specimens;[20] integrin alpha v beta 3 plays a key role in endothelial cell survival and migration during angiogenesis;[21] and CD105 is considered an appropriate marker of tumor-related angiogenesis and neovascularization.[22] Therefore, different from single-target therapies, such as "VEGF-targeted therapy" or peptide-based immunotherapy, HUVEC vaccine therapy is a multi-targeted immunotherapy. Compared with single-targeted therapies, multi-targeted immunotherapy has the advantage of reducing the risk of resistance to therapy, as well as the *in vivo* selection of the ideal antigen to be exposed to the immune system by antigen-presenting cells. The most important limiting factor of single peptide-based immunotherapy is the use of peptides that are effectively presented only by specific human leukocyte antigen (HLA) subtypes, eliciting HLA-restricted CTL responses. Thus, it cannot be applied to every patient, but to a limited population with a specific HLA subtype. In comparison, the HUVEC vaccine, which consists of whole cells expressing various kinds of angiogenic antigens (multi-target), may allow the different antigen presenting cells expressing different HLA to "select" the best antigenic determinant from the antigen "repertoire" to be presented and to generate the anti-angiogenic CTL. As evidence to support this theory, for at least 6 months after enrollment, the HUVEC

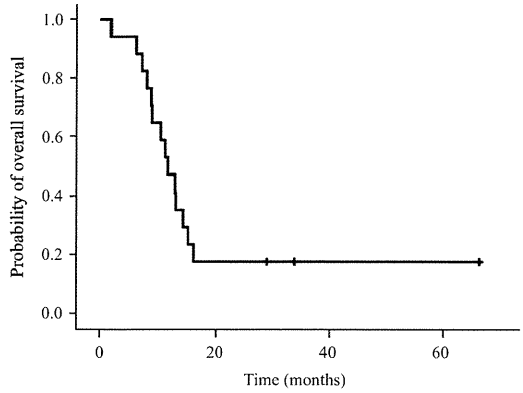

Fig. 4. Kaplan–Meier overall survival.

vaccine therapy resulted in significant elongation of the tumor doubling time and delay of tumor progression in patients selected without consideration of their HLA subtypes (Fig. 2). Specific antibodies and cellular immunity reactive with HUVEC's membrane antigens were detected in monthly samples of the patients. At 1 month after enrollment, specific antibodies such as CD31, CD51, CD105 and CD146 were also detected. IFN-γ secretion by patients' peripheral blood mononuclear cells was measured in the presence of HUVEC by enzyme-linked immunospot.[12] The evident reduction of the tumor growth rate, however, could be observed from the third month after the start of the HUVEC vaccine therapy.

In this study, hematological adverse reactions of grades 2–3 were recognized, but they were associated with the administration of chemotherapy. Although we continued the vaccination protocol for long periods of time with an expectation of inducing a long-lasting immune response, no adverse effect caused by the HUVEC vaccine therapy was observed, except for a DTH-like skin reaction at the injection site. In contrast, patients with recurrent GBM receiving BEV alone or in combination with irinotecan experienced grade 3 adverse events in 46.4 and 65.8% of cases, respectively, including hypertension, proteinuria, convulsion and neutropenia. Intracranial hemorrhage was also reported in 2 patients (2.4%) of a BEV-alone group (grade 1) and in three patients (3.8%) of a BEV plus irinotecan group (grades 1, 2 and 4, respectively).[6] In addition, BEV has been linked to increased invasiveness.[11] Thus, the HUVEC vaccine seemed to be superior to BEV in terms of adverse reactions. The main difference between BEV and the HUVEC vaccine is that BEV targets one angiogenic factor, namely VEGF, whereas the HUVEC vaccine targets the angiogenic vascular endothelium itself, inducing humoral and cellular immune responses against it.

Cancer cells may acquire the ability to produce angiogenic factors other than VEGF, such as acidic or basic fibroblast growth factor, interleukin-8, and epidermal growth factor, among others, overcoming the effect of BEV. Different from cancer cells, the HUVEC or angiogenic endothelial cells are normal cells, which rarely develop mutations. Because the HUVEC provide several targets, such as CD31 and CD105, different from the BEV, in which the only target is the VEGF, the HUVEC vaccine therapy might not increase the degree of invasiveness.

Once the immunity against tumor angiogenesis is stimulated, theoretically, it can be permanently reactivated by periodic vaccinations, and might be useful for the control of not only brain tumors, but also other solid tumors that also depend on angiogenesis for their growth and metastasis. Unfortunately, in the case of fast-growing and large tumors, the vaccine is not successful in controlling the growth of tumor mass (Fig. 1). The HUVEC vaccine therapy against recurrent GBM, therefore, should be used in combination with other treatment modalities. In a pilot study including metastatic colorectal cancer as well as other malignant brain tumors, such as recurrent anaplastic oligodendroglioma and recurrent pineoblastoma, specific antibodies and cellular effectors against HUVEC membrane antigens were detected in all the patients.[12] However, patients with malignant brain tumors had better clinical response than those with metastatic colorectal cancer, which might be mainly because of smaller lesions targeted. Different from patients with GBM, colorectal cancer patients were enrolled in the HUVEC vaccine protocol only after the relapse of all available treatment modalities. Thus, a similar trial could not be conducted for colorectal cancer. Patients with other tumor types have also been included, but only a few cases have been evaluated, without conclusive findings. Because the safety of this treatment modality could be proved, similar trials should be conducted to analyze the other solid tumor types that might benefit from its application.

In conclusion, the HUVEC vaccine therapy is a promising new treatment modality, without important adverse effects. Acquisition of resistance to multi-target immunotherapy, namely the HUVEC vaccine therapy, seems to be less frequent. As a matter of course, a large-scale prospective study will help confirm our present results.

Table 2. Survival according to clinical variables

Variable	Number of patients	Overall survival			*P*
		Median (95% CI) months	6 months (95% CI) %	12 months (95% CI) %	
Age					
<50 years	5	7.8 (5.4–10.2)	75.0 (37.7–93.7)	25.0	0.012
>50 years	12	14.2 (9.8–18.6)	100	(6.3–62.3) 55.6 (25.1–82.3)	
Location					
Frontal or temporal	11	12.7 (10.3–15.1)	100	58.3 (30.7–81.5)	0.040
Parietal or occipital	6	6.9 (4.8–9.0)	60.0 (20.0–90.0)	20.0 (2.7–69.1)	
Disease progression					
<6 months pre-enrollment	6	8.7 (4.3–13.0)	100	33.3 (8.3–73.2)	0.8
>6 months pre-enrollment	11	12.7 (9.8–15.6)	81.8 (49.3–95.4)	54.5 (26.8–79.7)	
Corticosteroid use					
Yes	4	8.7 (0.0–20.9)	75.0 (23.8–96.6)	50.0 (12.3–87.7)	0.8
No	13	11.4 (8.3–14.5)	92.3 (60.9–98.9)	46.2 (22.4–71.8)	

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Disclosure Statement

The authors have no conflict of interest to declare.

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ORIGINAL ARTICLE

Randomized trial of chemoradiotherapy and adjuvant chemotherapy with nimustine (ACNU) versus nimustine plus procarbazine for newly diagnosed anaplastic astrocytoma and glioblastoma (JCOG0305)

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Abstract
Purpose Glioblastoma (GBM) is one of the worst cancers in terms of prognosis. Standard therapy consists of resection with concomitant chemoradiotherapy. Resistance to nimustine hydrochloride (ACNU), an alkylating agent, has been linked to methylguanine DNA methyltransferase (MGMT). Daily administration of procarbazine (PCZ) has been reported to decrease MGMT activity. This study investigated the efficacy of ACNU + PCZ compared to ACNU alone for GBM and anaplastic astrocytoma (AA).

Methods Patients (20–69 years) who had newly diagnosed AA and GBM were randomly assigned to receive radiotherapy with ACNU alone or with ACNU + PCZ. The primary endpoint was overall survival (OS). This was designed as a phase II/III trial with a total sample size of 310 patients and was registered as UMIN-CTR C000000108.
Results After 111 patients from 19 centers in Japan were enrolled, this study was terminated early because temozolomide was newly approved in Japan. The median OS and median progression-free survival (PFS) with ACNU alone ($n = 55$) or ACNU + PCZ ($n = 56$) in the

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intention-to-treat population were 27.4 and 22.4 months ($p = 0.75$), and 8.6 and 6.9 months, respectively. The median OS and median PFS of the GBM subgroup treated with ACNU alone ($n = 40$) or ACNU + PCZ ($n = 41$) were 19.0 and 19.5 months, and 6.2 and 6.3 months, respectively. Grade 3/4 hematologic adverse events occurred in more than 40 % of patients in both arms, and 27 % of patients discontinued treatment because of adverse events.

Conclusions The addition of PCZ to ACNU was not beneficial, in comparison with ACNU alone, for patients with newly diagnosed AA and GBM.

Keywords Glioblastoma · Anaplastic astrocytoma · Nimustine · ACNU · Procarbazine · MGMT

Abbreviations

GBM	Glioblastoma
AA	Anaplastic astrocytoma
ACNU	Nimustine hydrochloride
BCNU	Carmustine
TMZ	Temozolomide
MGMT	Methylguanine DNA methyltransferase
WHO	World Health Organization
PFS	Progression-free survival
OS	Overall survival
RT	Radiotherapy
HR	Hazard ratio
AE	Adverse event
ND	Not determined
CR	Complete response
PR	Partial response
SD	Stable disease
PD	Progressive disease
WBC	White blood cell
3D-CRT	Three-dimensional conformal radiotherapy
CT	Computed tomography
IMRT	Intensity-modulated radiation therapy
BEV	Beam's eye views
DVH	Dose–volume histograms
GTV	Gross tumor volume
CTV	Clinical target volume
PTV	Planning target volume
ICRU	International Commission on Radiation Units
FLAIR	Fluid-attenuated inversion recovery
OAR	Organ-at-risk

Introduction

Glioblastoma (GBM) is one of the worst cancers in terms of prognosis, with almost all patients experiencing progression without cure. According to the report of the Brain Tumor Registry of Japan, the %5-year survival of World Health Organization (WHO) grade IV GBM is 6.9 % and that of WHO grade III anaplastic astrocytoma (AA) is 33.9 % [1].

Standard therapy against GBM consists of the maximal resection that is safely possible, with concomitant chemo-radiotherapy. Currently, temozolomide (TMZ) is the

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standard agent used in the treatment of GBM. However, before the TMZ era, nitrosourea had been widely used for GBM and AA. The Glioma Meta-analysis Trialists Group described that chemotherapy including nitrosourea showed significant prolongation of survival, with a hazard ratio of 0.85 ($p < 0.0001$) [2].

Nimustine hydrochloride (ACNU) was developed in Japan, and for more than 20 years since 1980, it has been the standard chemotherapeutic agent against gliomas [3]. Wolff et al. [4] analyzed 364 studies, including a total of 24,193 patients with high-grade glioma, and reported that the survival gain in the 15 ACNU-treated cohorts was 8.9 months, compared to those who received different drugs or no chemotherapy. Takakura et al. [5] reported that the overall survival (OS) of AA and GBM treated by radiotherapy (RT) and concomitant ACNU were 36 and 12 months, respectively. Furthermore, the response rate of a more than 50 % reduction in tumor size was 46.2 % in both AA and GBM. Alkylating agents, including ACNU and procarbazine (PCZ), confer cytotoxic effects on glioma cells by alkylation at the O^6-position of guanine in DNA. This results in the formation of DNA cross-links [6]. Methylguanine DNA methyltransferase (MGMT) removes methylation damage induced by nitrosourea from the O^6-position of DNA guanines before cell injury, and this enzyme was detectable in 76 % of glioma tissues [7]. MGMT in glioma cells is a primary defense against nitrosourea, but the cellular methyltransferase activity of MGMT is exhausted after MGMT takes effect. Daily administration of PCZ for 10 days was reported to cause the accumulation of O^6-methylguanine; it also decreased MGMT activity in rat liver [8] and lymphocytes in lymphoma patients [9]. Inhibition of MGMT by O^6-benzylguanine increased the cytotoxicity of TMZ and carmustine (BCNU) to tumor cells [10]. From these results, it can be predicted that daily administration of PCZ, by depleting MGMT activity, will increase the efficacy of ACNU against AA and GBM.

To prove this hypothesis and establish a more potent standard therapy for AA and GBM, the Brain Tumor Study Group of the Japan Clinical Oncology Group (JCOG) conducted this clinical trial. The study was terminated at the end of the phase II part. The current report describes the final outcome of the study.

Subjects and methods

Patient eligibility criteria

Patients aged 20 to less than 70 years of age who had newly diagnosed and histologically proven supratentorial GBM or AA were eligible for this study. Patients were enrolled between 3 and 14 days after their operation. To be eligible, a patient's preoperative MRI had to show that more than 50 % of the tumor was located in supratentorial areas, except the optic nerve, olfactory nerve, or pituitary gland. Eligible patients had Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0–2 or 3 (only in cases with neurologic symptoms caused by a tumor) and adequate hematologic, pulmonary, renal, and hepatic function, defined as follows: white blood cell (WBC) count $\geq 3.0 \times 10^3$/mL, hemoglobin level ≥ 8.0 g/dL, platelets count $\geq 1.0 \times 10^6$/mL, aspartate transaminase (AST) level ≤ 100 IU/L, alanine transaminase (ALT) level ≤ 100 IU/L, serum creatinine level ≤ 1.0 mg/dL. Additionally, written informed consent was obtained from all the participating patients. We excluded patients with multiple or disseminated tumors or large tumors in which the planned target volume for irradiation exceeded 1/3 of the whole-brain volume. Additionally, we also classified as ineligible any patient who was pregnant, had meningitis, pneumonia, diabetes mellitus with insulin injection, myocardial infarction, or unstable angina pectoris within the last 3 months, mental disorders, a history of pulmonary fibrosis or interstitial pneumonia, or other forms of active cancer occurring within 5 years of treatment. The study protocol was approved by JCOG Protocol Review Committee and institutional review board at each center.

Treatment

After the confirmation of the eligibility criteria, registration was made by telephone or fax to the JCOG Data Center. Patients were randomized within 14 days of surgery to either ACNU with RT (the control arm, A) or to ACNU + PCZ with RT (the experimental arm, B) (Fig. 1a) by a minimization method with adjustment factors consisting of histology (GBM vs. AA), age (younger than 60 vs. 60 years or older), residual tumor (presence vs. absence), and institution. Residual tumor was assessed using a gadolinium-enhanced MRI obtained within 72 h of the surgery.

Radiotherapy with concomitant chemotherapy was started within 3 weeks after the surgery. Patient positioning and immobilization with an individual head mask and computed tomography (CT)-based planning were required. Treatment was delivered using linear accelerators with nominal energies ≥ 4 MV. Intensity-modulated radiation therapy (IMRT) technique was not permitted. All fields were to be treated every day. Three-dimensional conformal radiotherapy (3D-CRT) planning including the use of beam's eye views (BEV) and dose–volume histograms (DVH) were recommended for volumetric dose evaluation. Quality assurance reviews were done at the Radiotherapy Support Centre in Tokyo, Japan, with feedback sent to each

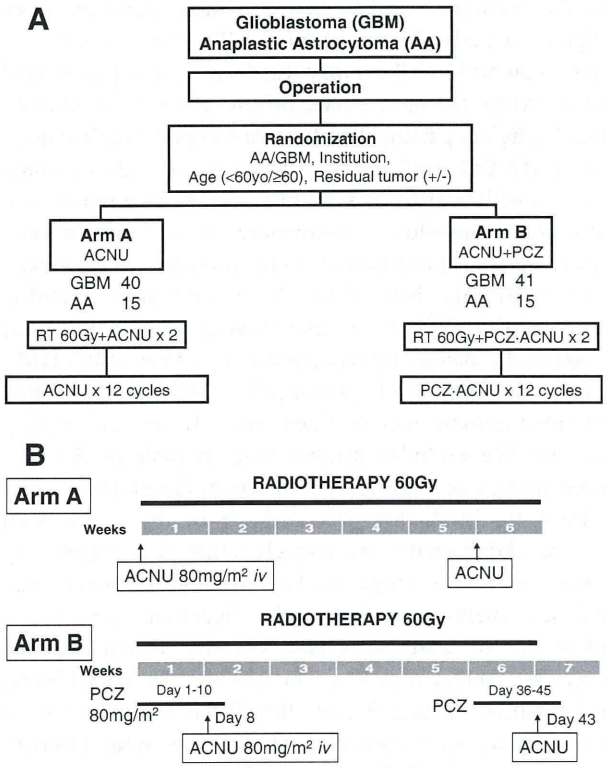

Fig. 1 **a** Study design of JCOG 0305: RT + ACNU versus RT + ACNU + PCZ; 40 patients with GBM and 15 patients with AA were assigned to *arm A*, and 41 patients with GBM and 15 patients with AA were assigned to *arm B*. **b** Treatment schedule of RT + ACNU (*Arm A*) and RT + ACNU + PCZ (*Arm B*)

institution by the radiotherapy study coordinator (Minako Sumi). The minimum and maximum dose to the PTV should be comprised between 95 and 107 % of the International Commission on Radiation Units (ICRU) reference point dose. The gross tumor volume (GTV) was defined as the primary tumor with or without enhancement on CT or magnetic resonance imaging (MRI). The clinical target volume 1(CTV1) included GTV, the resection cavity and surrounding edema (high-intensity area on T2-weighted or fluid-attenuated inversion recovery (FLAIR) image) plus a 1.5-cm margin. The CTV2 included GTV and the resection cavity plus a 1.5-cm margin. Planning target volume (PTV) was defined as CTV plus a margin of 0.5 cm or more. The doses for PTV1 and PTV2 were 50 and 10 Gy, respectively. The protocol required contouring organ-at-risk (OAR), including optic chiasm, brain stem, and retina. Cumulative doses to the optic chiasm and brainstem were limited to a maximum dose of 50 and 45 Gy for the retina.

In the control arm A, 80 mg/m^2 of ACNU was administered intravenously on days 1 and 36 during RT (Fig. 1b). In the experimental arm B, 80 mg/m^2 of oral PCZ was administered daily from days 1 to 10 and days 36 to 45, and given together with intravenous ACNU (80 mg/m^2) on days 8 and 43. Adjuvant therapy consisting of 80 mg/m^2 of ACNU alone in arm A or ACNU plus PCZ (PCZ: 80 mg/m^2 orally on days 1–10, ACNU: 80 mg/m^2 intravenously on day 8) in arm B started 56 days from the final administration of ACNU and was given every 8 weeks, for up to 12 cycles. Doses of ACNU and PCZ were calculated using actual body surface area, reduced for toxicity, and were not escalated.

Evaluations and follow-up

Baseline and follow-up examinations included vital signs, subjective symptoms, neurologic examination, MRI scan, and blood and serum laboratory examinations. For each patient, these examinations were performed weekly, with the exception of MRI scans, which were performed between the end of the initial chemoradiotherapy and the beginning of adjuvant therapy. All examinations were performed before each cycle of adjuvant chemotherapy, at a frequency of nearly every 2 months. After completion of the treatment protocol, patients were assessed every 3 months until progression. Toxicity was graded using the National Cancer Institute Common Toxicity Criteria (version 2). Findings of radiation necrosis were also assessed on MRI. Each patient was required to undergo a follow-up examination for at least 2 years from the date of randomization.

Tumor progression on MRI was defined according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.0 [11]. Progression of disease was defined as a 20 % increase in tumor size, as shown by contrast-enhanced imaging, or the development of new lesions, neurologic deterioration, or death by any cause. Further treatment at recurrence or progression was discretionary, but recorded.

A central pathology review by 3 independent pathologists (Yoichi Nakazato, a member of the Working Group for WHO 2007 classification; Nobuaki Funata; and Toru Iwaki) was performed and determinations given. A central review of radiological response was also performed.

Statistical analysis

When we planned this study, TMZ had been widely approved and was used worldwide. However, TMZ was not available in Japan. ACNU remained the standard therapy in Japan, but there was no sufficient data regarding this treatment. We planned a phase II/III clinical trial, with the phase II part designed to confirm the feasibility of ACNU and ACNU + PCZ.

The primary and secondary endpoints for the phase II part were %6-month survival and adverse events (AEs) in ACNU + PCZ arm. The primary endpoint of the phase III part was OS, while the secondary endpoints were PFS, response rate, complete response rate, and AEs.