TABLE II – HUMAN CDCA1-DERIVED PEPTIDES PREDICTED TO BE BOUND TO HLA-A2 AND USED FOR VACCINATION OF MICE[1]

No.	Position	Subsequence residue listing	Binding score
1	65	YMMPVNSEV	855
2	120	FLSGIINFI	607
3	222	RLNELKLLV	285
4	351	KLATAQFKI	211
5	182	QLSDGIQEL	201
6	141	FLWQYKSSA	190
7	3	TLSFPRYNV	69.6
8	285	CLPSCQLEV	69.6
9	228	LLVVSLKEI	40.8
10	386	AVYERVTTI	27.5
11	372	TVIEDCNKV	25.0
12	243	KIVDSPEKL	20.7
13	257	KMKDTVQKL	17.8
14	88	LVTHLDSFL	17.5
15	447	KIDEKTAEL	16.9
16	358	KINKKHEDV	16.4
17	416	KLKSQEIFL	14.4
18	82	FLPFSNLVT	14.1
19	344	LMIVKKEKL	12.9
20	44	VLHMIYMRA	12.7
21	227	KLLVVSLKEI	311
22	222	RLNEIKLLVV	269
23	294	QLYQKKIQDL	157
24	87	NLVTHLDSFL	117
25	181	KQLSDGIQEL	64.5
26	47	MIYMRALQIV	49.1
27	402	KLGIQQLKDA	40.0
28	343	RLMIVKKEKL	38.7
29	309	KLASILKESL	36.6
30	22	ILTGADGKNL	36.3
31	193	SLNQDFHQKT	28.3
32	52	ALQIVYGIRL	21.4
33	44	VLHMIYMRAL	16.7
34	35	DLYPNPKPEV	16.7
35	165	KLERIDSVPV	15.6
36	65	YMMPVNSEVM	12.3
37	154	QLNAAHQEAL	10.5
38	60	RLEHFYMMPV	10.2
39	344	LMIVKKEKLA	6.1
40	453	AELKRKMFKM	4.8

[1]To identify HLA-A2-restricted CTL epitopes of human CDCA1 by using HLA-A2.1 (HHD) transgenic mouse (Tgm), we selected 40 kinds of peptides having amino acid sequences with high predicted binding scores to HLA-A2 (A*0201).

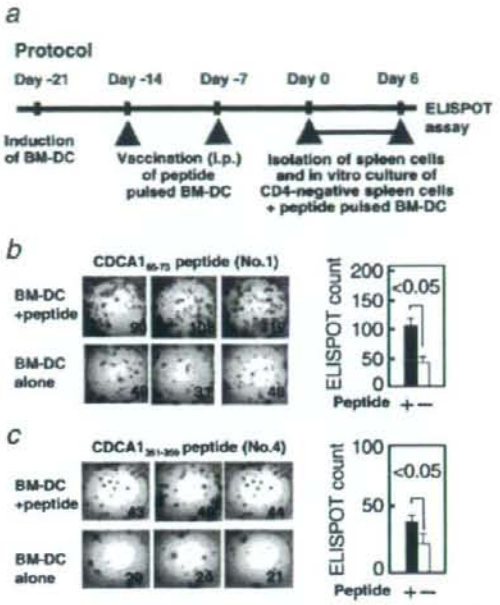

FIGURE 4 – The identification of HLA-A2-restricted mouse CTL epitopes of human CDCA1 by using HLA-A2.1 (HHD) Tgm and ELISPOT assay. (*a*) Protocol for identification of human CDCA1-derived and HLA-A2-restricted CTL epitopes. We immunized the HLA-A2.1 (HHD) Tgm with BM-DCs (5×10^5/well) pulsed with the mixture of CDCA1-derived peptides carrying HLA-A2 (A*0201) binding motif into the peritoneal cavity once a week for 2 weeks. Seven days after the last DC vaccination, spleen cells (5×10^6/well) were stimulated with syngenic BM-DCs (2×10^5/well) pulsed with each peptide *in vitro* for 6 days. We used these cultured CD4-negative spleen cells as responder cells in ELISPOT assay to evaluate CDCA1-specific response of CTLs. (*b* and *c*) Two candidate peptides were selected from 40 kinds of CDCA1 peptides by using IFN-γ ELISPOT assay. (*b*) CD4-negative spleen cells showed 102 ± 10.1 spot counts/well, in response to the BM-DC pulsed with the $CDCA1_{65-73}$ peptide (upper), whereas they showed 42.0 ± 9.64 spot counts/well in the presence of BM-DC without peptide loading (lower) ($p < 0.05$). (*c*) CD4-negative spleen cells showed 42.3 ± 4.02 spot counts/well, in response to the BM-DC pulsed with the CDCA1 351–359 peptide (upper), whereas they showed 24.6 ± 7.19 spot counts/well in the presence of BM-DC without peptide loading (lower) ($p < 0.05$).

risk of immune escape of cancer cells attributable to deletion, mutation or downregulation of TAAs, as a consequence of therapeutically driven immune selection.[12] One of the function of CDCA1 is reportedly to couple kinetochores to spindle microtubules and it is critical for retaining the cell cycles.[14] Furthermore, CDCA1 is one of the component of nuclear division cycle complex, which is an essential kinetochore component, highly conserved across species with a crucial role in proper chromosome segregation during mitosis.[38] CDCA1 is required for stable kinetochore localization of centromere-associated protein E (CENP-E) in Hela cells, and depletion by RNAi of CDCA1 caused aberrant chromosome segregation resulting in a prolonged mitotic blockade followed by cell death.[15] This aberrant exit from mitosis has characteristics of both apoptosis and catastrophe.[14] Consequently, CDCA1 is essential for normal cellular function, and it also plays an important role in proliferation and survival of cancer cells.

In addition, CDCA1 and kinetochore associated 2 (KNTC2) have been reported to be members of the evolutionarily conserved centromere protein complex.[13] Their elevated expressions were associated with poorer prognosis of NSCLC patients by using immunohistochemical analysis, and the growth of NSCLC was inhibited by the dominant negative peptides of CDCA1.[13] Therefore, the expression levels of CDCA1 in NSCLC tissue may be a useful marker for the prediction of the prognoses of the patients after surgical treatment. Furthermore, the results suggest a possible involvement of CDCA1 in the progression of NSCLC. Thus, immunotherapy targeting at CDCA1 may be effective for such NSCLC patients with a poor prognosis.

In this study, we identified 2 HLA-A2-restricted CDCA1 epitope peptides that can stimulate generation of HLA-A2-restricted mouse CTL by vaccination in HLA-A2.1 (HHD) Tgm without expression of endogenous mouse $H\text{-}2^b$-encoded class I molecules. In addition, we found that the CDCA1-reactive human CTLs could be generated from PBMCs stimulated with these peptides in healthy donors and a cancer patient. We demonstrated that these CTL lines specific to peptides derived from CDCA1 killed tumor cells expressing CDCA1 in a HLA-A2-restricted manner (Fig. 4). HLA-A2.1(HHD) Tgm has been reported to be a versatile animal model for the preclinical evaluation of peptide-based immunotherapy.[18,19,32] We could also identify its usefulness for the identification of HLA-A2-restricted CTL epitopes in this study.

In this study, we selected CDCA1-derived peptides which were predicted to have high binding affinity to HLA-A0201-encoded

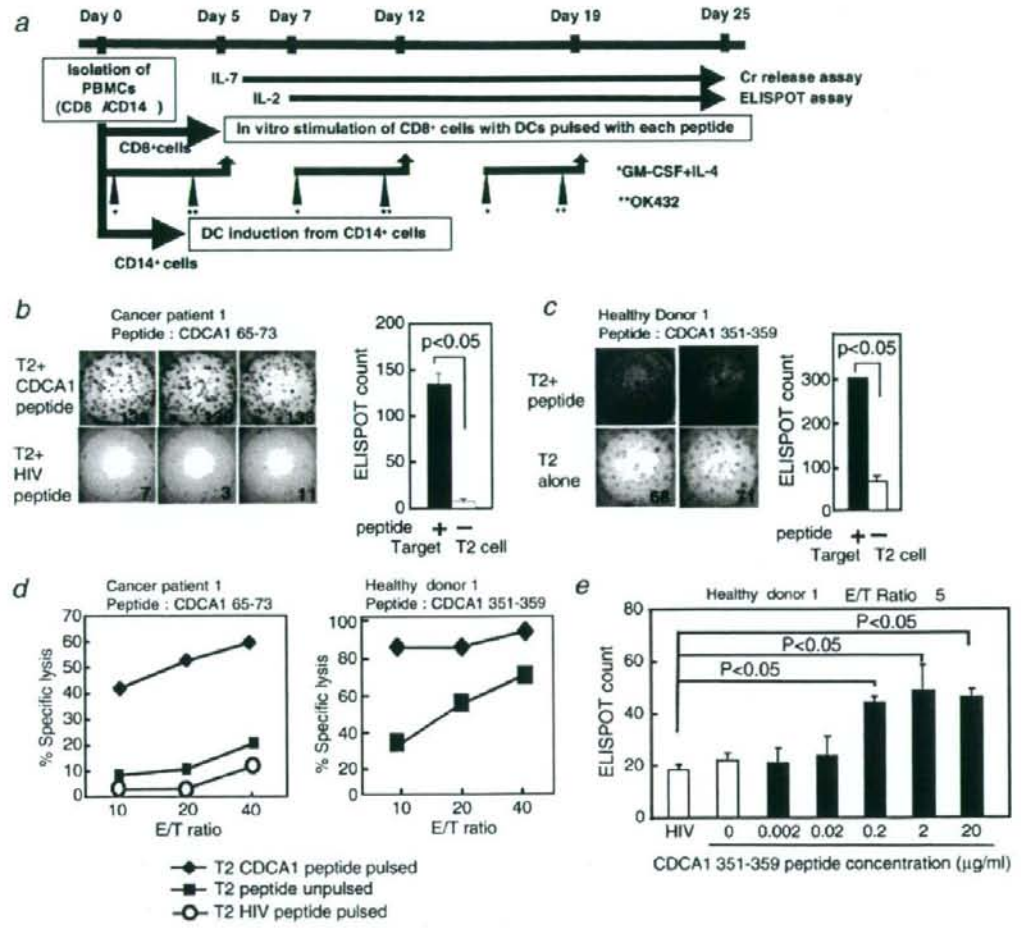

FIGURE 5 – CDCA1-specific immune response of CTLs induced from a healthy donor and a NSCLC patient. (*a*) Protocol for induction of CDCA1-specific CTLs from PBMC. We isolated PBMCs from donors and $CD8^+$ T cells and $CD14^+$ cells were isolated using each microbeads from the PBMC of the same donors. Thereafter, peptide-reactive $CD8^+$ CTLs were generated. We generated DC from $CD14^+$ cells, by culturing in the presence of GM-CSF and IL-4 for 5 days. DC were pulsed with HLA-A2-binding peptides in the presence of β2-microglobulin for 4 hr at 37°C. These peptide-pulsed DC were then irradiated and mixed at 1:20 ratio with autologous $CD8^+$ T cells. Cells were cultured with IL-7 in AIM-V with 2% auto serum. After 3 days, these cultures were supplemented with IL-2. On days 12 and 19, the T cells were further restimulated with the autologous peptide-pulsed DC. The DCs were prepared each time in the same way as described earlier. IFNγ ELISPOT assay and Cr release assay were performed after 5 or 6 days from the third round of peptide stimulation. CTL induced from a donor was cocultured with target cells and ELISPOT assay was done by using CDCA1 65–73 peptide (No. 1) (*b*) or CDCA1 351–359 peptide (No. 4) (*c*). The IFNγ production stimulated with peptide-pulsed T2 cells was significantly greater than that stimulated with nonpulsed T2 cells or HIV peptide-pulsed T2 cells. (*d*) The CTLs induced from PBMCs of the cancer patient 1 and the healthy donor 1 also showed cytotoxic activity to T2 cells pulsed with the CDCA1 peptide. (*e*) Peptide dose-dependent response of CDCA1 351–359 peptide-induced CTLs was investigated in the healthy donor 1. CTLs produced a significant amount of IFNγ in response to the T2 cells pulsed with more than 0.2 μg/ml of the peptide at E/T ratio 5.

molecules by the BIMAS software program; however, some of their amino acid sequences are not conserved between human and mouse CDCA1. There are 2 amino acid replacements between human and mouse $CDCA1_{65-73}$ peptide (human YMMPVNSEV/mouse YMMPMNIEV) and 1 amino acid replacement in $CDCA1_{351-359}$ peptide (human KLATAQFKI/mouse KLATARFKI). Hence, we worried that the mouse CTLs induced by stimulation with these nonself human CDCA1 peptides would stimulate strong responses in Tgm, but not in human, whereas we could induce CTLs responding to these epitope peptides from healthy donors and a cancer patient. Because it may be possible that human CTLs can recognize CDCA1-derived peptide in the context of HLA-A2, but that mouse CTL cannot recognize those peptides, we are planning to evaluate antigenicity of 38 other CDCA1 peptides by stimulating human PBMCs in a future study.

We found that it is possible to induce CDCA1-reactive CTLs by stimulation of PBMCs from healthy donors and a cancer patient with the CDCA1 peptides *in vitro*. The CTLs induced by the peptide-

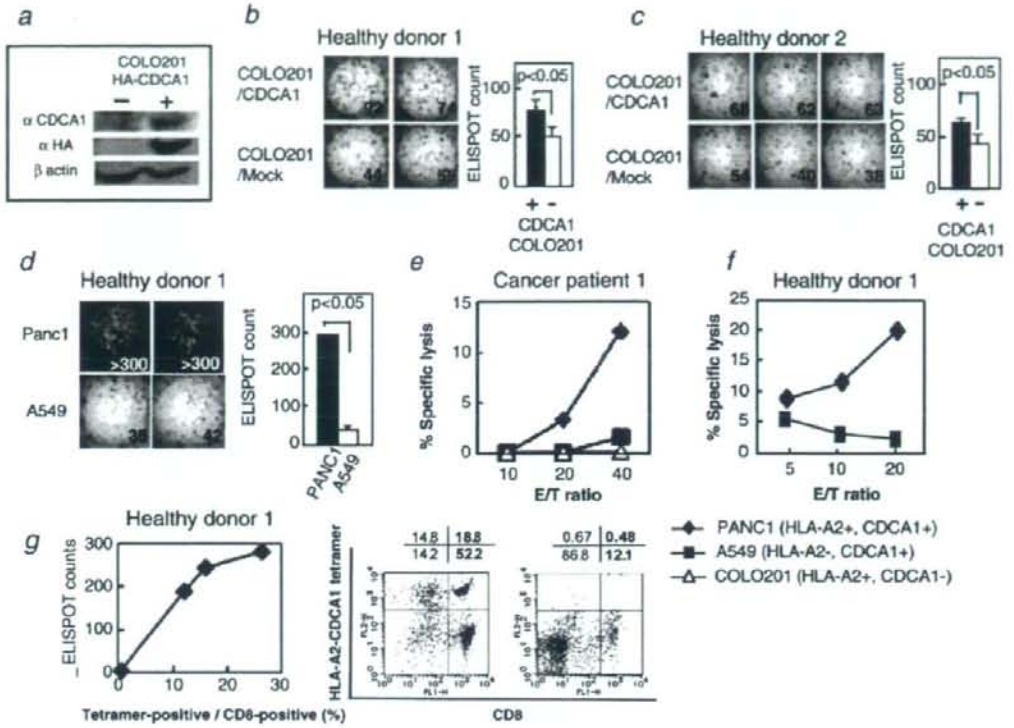

FIGURE 6 – The CDCA1-specific and CDCA1-positive tumor cell-directed cytotoxic activity of CTLs induced from healthy donors and a NSCLC patient. (*a*) The introduction and expression of CDCA1-gene expression vector in COLO201 cells. Lenti-viruses harboring EF-1a promoter and CMV promoter-driven CDCA1-HA expression vector were infected 3 times to cancer cell line, COLO201, which expresses HLA-A2 but not CDCA1. The whole cell lysate was subjected to the Western blot analyses using anti-HA antibody (middle) or anti-CDCA1 antibody (upper). (*b–d*) The IFNγ production by CTLs stimulated with COLO201-CDCA1 was significantly larger than that stimulated with mock transfected tumor cell line, COLO201. Furthermore, the IFNγ production by CTLs stimulated with PANC1 which endogenously expresses both CDCA1 and HLA-A2 was significantly greater than that stimulated with A549 which endogenously expresses CDCA1 but not HLA-A2. (*e, f*) The CTLs were cocultured with target cells and ^{51}Cr release assay was done. These CTLs exhibited cytotoxic activity to PANC1 (CDCA1$^+$, HLA-A2$^+$) but not to A549 (CDCA1$^+$, HLA-A2$^-$) nor COLO201 (CDCA1$^-$, HLA-A2$^+$). (*g*) Correlation between the frequency of CDCA1 peptide-reactive CTLs and the frequency of HLA-A2-CDCA1 tetramer-positive CTLs in CD8-positive T cells. Left: In ELISPOT assay, the target cells were peptide-pulsed T2 cells and E/T ratio was 5. Right: In FACS analyses, cells analyzed were CTLs generated from the healthy donor 1 by stimulation of PBMCs 3 times with peptide-pulsed DC (left) and freshly isolated naive CD8-positive cells separated from PBMC of the donor (right).

loaded DC exerted a significant cytotoxic activity against CDCA1-expressing cancer cells in a HLA-A2-restricted manner. Propagation of the CDCA1 peptide-specific and HLA-A2 (*A0201)-restricted CTLs from the donor's PBMCs was also confirmed by the specific HLA-peptide tetramer assay. Although the background CTL responses were high and/or the antigen-specific CTL responses were small in several experiments, these responses were highly reproducible in experiments repeated several times. Therefore, we are convinced that these CTL responses were specific to the CDCA1 peptides. These relatively weak CTL responses to PANC1 tumor cell line may be due to poor processing of the CTL epitope from CDCA1 protein in PANC1 cells or relatively CTL-resistant nature of PANC1 cells used as the target cells. Whereas, these CTLs killed very well the T2 cells pulsed with the CDCA1 peptide. These problems await solving in our future experiment.

The induction of CDCA1-specific CTLs from healthy donors and a cancer patient has important implications for ongoing efforts to search additional TAAs. In addition, we are now also trying to induce the CDCA1-reactive CTLs from PBMCs isolated from patients with NSCLC, SCLC, cholangiocellular cancer, bladder cancer and renal cell cancer. There are several methods for cell-mediated cancer immunotherapy, including the vaccination of peptide or protein,[39] immunization with dendritic cells pulsed with a peptide, protein or tumor lysate,[40,41] the immunization with dendritic cell/tumor cell hybrids[42] and adaptive transfer of tumor-specific CTL lines propagated *ex vivo*.[43] Our CDCA1 peptides may well be applicable in some of these immunotherapeutic approaches.

In summary, we have found a novel cancer testis antigen, CDCA1, expressed in NSCLC, SCLC, cholangiocellular cancer, bladder cancer and renal cell cancer. We can induce tumor-reactive CTLs from PBMCs, which were stimulated with the specific peptide. The CDCA1 epitope peptides identified in this study may well provide a new cancer immune therapy for NSCLC.

Acknowledgements

The authors thank Dr. Hideyuki Saya (Keio University, Tokyo, Japan), the Cell Resource Center for Biomedical Research Insti-

tute of Development, Aging and Cancer, Tohoku University and Health Science Research Resources Bank for providing the cell lines, Tatsuko Kubo (Department of Molecular Pathology, Kumamoto, Japan) for technical assistance of immunohistochemical analyses and Dr. Hiroyuki Miyoshi (Riken BioResource Center) for providing a lentiviral vector.

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J Neurosurg 108:963–971, 2008

Phase II clinical trial of Wilms tumor 1 peptide vaccination for patients with recurrent glioblastoma multiforme

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Object. The object of this study was to investigate the safety and clinical responses of immunotherapy targeting the *WT1* (Wilms tumor 1) gene product in patients with recurrent glioblastoma multiforme (GBM).

Methods. Twenty-one patients with WT1/HLA-A*2402–positive recurrent GBM were included in a Phase II clinical study of WT1 vaccine therapy. In all patients, the tumors were resistant to standard therapy. Patients received intradermal injections of an HLA-A*2402–restricted, modified 9-mer WT1 peptide every week for 12 weeks. Tumor size, which was obtained by measuring the contrast-enhanced area on magnetic resonance images, was determined every 4 weeks. The responses were analyzed according to Response Evaluation Criteria in Solid Tumors (RECIST) 12 weeks after the initial vaccination. Patients who achieved an effective response continued to be vaccinated until tumor progression occurred. Progression-free survival and overall survival after initial WT1 treatment were estimated.

Results. The protocol was well tolerated; only local erythema occurred at the WT1 vaccine injection site. The clinical responses were as follows: partial response in 2 patients, stable disease in 10 patients, and progressive disease in 9 patients. No patient had a complete response. The overall response rate (cases with complete or partial response) was 9.5%, and the disease control rate (cases with complete or partial response as well as those in which disease was stable) was 57.1%. The median progression-free survival (PFS) period was 20.0 weeks, and the 6-month (26-week) PFS rate was 33.3%.

Conclusions. Although a small uncontrolled nonrandomized trial, this study showed that WT1 vaccine therapy for patients with WT1/HLA-A*2402–positive recurrent GBM was safe and produced a clinical response. Based on these results, further clinical studies of WT1 vaccine therapy in patients with malignant glioma are warranted.
(DOI: 10.3171/JNS/2008/108/5/0963)

Key Words • **cancer vaccine** • **glioblastoma multiforme** • **glioma** • **immunotherapy** • **Wilms tumor 1**

Currently, the standard treatment for gliomas is surgery, followed by external radiation and chemotherapy. In patients with newly diagnosed GBM, however, concurrent irradiation and temozolomide therapy, followed by adjuvant temozolomide therapy for at least 6 months, offered a modest benefit, with a median survival of 14.6 months and a 2-year survival rate of 26.5%.[22] To date, therapeutic options for patients with malignant glioma have been limited, and extensive research is ongoing.

Immune therapy against malignant gliomas includes several therapeutic approaches that involve dendritic cell–based immunotherapy and antibody-mediated immunotherapy.[31] Cancer vaccination is another novel form of therapy.[30] Recent advances in molecular biology and tumor immunology have resulted in the identification of a large number of tumor-associated antigens that could be used for cancer vaccination, since their epitopes associated with HLA Class I molecules were recognized by CTLs. One of the identified tumor-associated antigens was the product of the Wilms tumor gene, *WT1*.[17]

Abbreviations used in this paper: CTL = cytotoxic T-lymphocyte; DSMC = Data Safety Monitoring Committee; ECOG = Eastern Cooperative Oncology Group; FDG = fluorodeoxyglucose; GBM = glioblastoma multiforme; HLA = human leukocyte antigen; MR = magnetic resonance; PBMC = peripheral blood mononuclear cell; PET = positron emission tomography; PFS = progression-free survival; RECIST = Response Evaluation Criteria in Solid Tumors; SPECT = single-photon emission computed tomography; WHO = World Health Organization; WT1 = Wilms tumor 1.

The *WT1* gene was isolated as a gene responsible for Wilms tumor. It encodes a zinc finger transcription factor, which is involved in cell proliferation and differentiation, apoptosis, and organ development. Although the *WT1* gene was first categorized as a tumor suppressor gene, it was later proposed that the wild-type *WT1* gene functions as an oncogene rather than as a tumor-suppressor gene. In response to granulocyte colony–stimulation factor, growth promotion and differentiation inhibition were identified in wild-type *WT1* gene–transfected myeloid progenitor cells.[25] In many reports, the wild-type *WT1* gene was shown to be overexpressed in various types of solid tumors. The WT1 protein was found to be an attractive target antigen for immunotherapy against these malignancies.[20]

Recently, we performed a Phase I clinical trial to examine the safety of a WT1-based vaccine, as well as the clinical and immunological responses of patients with a variety of cancer types, including leukemia, lung cancer, and breast cancer.[19] The authors demonstrated that WT1 peptide vaccine emulsified with Montanide ISA51 adjuvant and administered at a dosage of 0.3, 1.0, or 3.0 mg at 2-week intervals was safe for patients other than those with myelodysplastic syndromes. Furthermore, the vaccination induced peptide-specific CTLs and was associated with clinical response. Very recently, it was confirmed that the potential toxicities of the weekly WT1 vaccination treatment schedule (3 mg per week) with the same adjuvant were also acceptable.[15]

An increasing number of central nervous system studies have reported that systemic immunotherapy is capable of inducing an antitumor response within the immunologically privileged brain.[29–31] These advances suggest the possibility of the development of a new peptide-based cancer immunotherapy. The blood–brain barrier, which was thought to be one of the hurdles hindering the development of therapeutically effective immunotherapy for gliomas, does not always function effectively in cases involving recurrent gliomas.[29]

Like many other solid tumors, gliomas have been found to express WT1 protein.[8] A definite correlation has been observed between WT1 expression and cellular proliferation activity, as indicated by WHO grade.[8] In the present study, we investigated the clinical responses to peptide-based immunotherapy targeting the *WT1* gene product in patients with recurrent GBMs. We also evaluated the correlation between the clinical response and the WT1 expression level in tumor tissues using immunohistochemical staining, as well as WT1-specific CTL frequencies (tetramer assay) in patients' PBMCs.

Clinical Materials and Methods

The WT1 Peptide

The immunization consisted of an HLA-A*2402–restricted, modified 9-mer WT1 peptide (amino acids 235–243 CYTWNQMNL), in which Y was substituted for M at amino acid position 2 (the anchor position) of the natural WT1 peptide. About 60% of Japanese have HLA-A*2402 which is the most common HLA Class I type in the Japanese population. The modified 9-mer WT1 peptide was shown to induce much stronger CTL activity against WT1-expressing tumor cells than the natural peptide.[26] The WT1 peptide (GMP grade) was purchased from Multiple Peptide Systems as the lyophilized peptide.

Patient Population

Twenty-one patients were enrolled in this study. All patients seen in our unit who had recurrent or progressive GBM were eligible to be enrolled if their disease was resistant to conventional chemotherapy and radiotherapy. Patients who had refused chemotherapy but wanted to receive WT1 vaccine therapy under the auspices of this clinical trial were also eligible. In patients who received stereotactic radiosurgery as part of their initial therapy, true recurrence or progression was distinguished from radiation necrosis by metabolic imaging or histological confirmation.

Additional inclusion criteria were: 1) age between 16 and 80 years, 2) expression of WT1 in the glioma cells determined by immunohistochemical analysis, 3) HLA-A*2402–positivity, 4) estimated survival of more than 3 months, 5) ECOG Performance Status Grade 0–2, 6) no severe organ function impairment, and 7) the written informed consent of the patient. All enrolled patients had histologically proven GBM (Grade 4) based on the WHO criteria. After initial resection of the tumor, patients underwent a course of external radiation therapy and chemotherapy. Magnetic resonance imaging was used to monitor patients for recurrence or progression of their tumor during initial therapy and during maintenance therapy. No patient was treated with chemotherapy or radiotherapy during the 4 weeks prior to WT1 immunotherapy. Registered patients had methionine-PET, FDG-PET, thallium-SPECT, and MR imaging to confirm recurrence or progression and to exclude radiation necrosis. All patients underwent electrocardiography, and blood samples were obtained to confirm that there were no abnormalities.

After informed consent was obtained, it took at least 2 weeks for the immunohistochemical analysis, HLA-typing analysis, image analysis, and other tests to be completed. Therefore, the presence of tumor recurrence or progression was again assessed $>$ 2 weeks after registration for WT1 treatment. The DSMC independently reviewed the eligibility of each enrolled patient. Protocol compliance, safety, and on-schedule study progress were also monitored by the DSMC. The WT1 peptide–based Phase II study was approved by the ethical review boards of the Osaka University Faculty of Medicine.

Vaccine Preparation and Vaccination

Patients received intradermal injections of 3.0 mg of HLA-A*2402–restricted modified 9-mer WT1 peptide emulsified with Montanide ISA51 adjuvant. The WT1 vaccinations were scheduled to be given weekly for 12 consecutive weeks. Twelve weeks after the initial vaccination, the response was evaluated on MR imaging. If an effect was observed after the 12 vaccinations, WT1 vaccination was continued at 1-week intervals (with the patients' informed consent) until tumor progression was again noted.

Immunohistochemical Analysis

Immunohistochemical analysis was performed to confirm WT1 protein expression in malignant glioma tissue using a procedure that has been previously described.[8] Brief-

ly, formalin-fixed tissue sections were prepared from the resected tumors. Sections were microwaved in citrate buffer for antigen retrieval and incubated with anti–human WT1 mouse monoclonal antibody 6F-H2 (DAKO; diluted 1:50). The WT1 reaction was visualized with the Vectastain ABC kit (Vector Laboratories) and diaminobenzidine (WAKO). The sections were then counterstained with hematoxylin. Control positive staining was evaluated with Wilms tumor, and control negative staining was evaluated with normal brain. Expression of WT1 seen in the sections was classified on a scale from 0 to 4 based on the staining density and the pattern of the glioma cells according to the following criteria: 0, negative staining; 1, slightly increased staining in some tumor cells compared with that in normal glial cells; 2, staining at intermediate intensity in some tumor cells; 3, strong staining in some tumor cells and intermediate staining in almost all tumor cells; and 4, greatly increased staining in almost all tumor cells compared with that in normal glial cells. Three investigators scored every sample independently; scores agreed upon by at least 2 investigators were accepted.

For MIB-1 immunostaining, antibody against the Ki 67 antigen (DAKO) was diluted 1:50 and used as previously described.[11] In each case, MIB-1 immunostaining was performed on the same serial sections used for WT1 immunohistochemical evaluation. The staining index reflecting each tumor's proliferation activity was determined by calculating the percentage of positively stained tumor cell nuclei out of 1000 evaluated tumor cell nuclei. All assessments were made in areas with the greatest degree of immunostaining.

*The WT1 peptide/HLA-A*2402 Tetramer Assay of WT1-Specific CTLs*

The WT1 (a natural, HLA-A*2402–restricted, 9-mer WT1 peptide)/HLA-A*2402 tetramer was kindly provided by M. Gotoh of Sumitomo Pharmaceuticals. This tetramer stained > 90% of the TAK-1 cells, which were WT1-specific CTLs that could recognize the complex of the natural 9-mer WT1 peptide and HLA-A*2402 molecules. The PBMCs from HLA-A*2402–positive patients were double-stained with PerCP-CD8 antibody (BD Pharmingen) and phycoerythrin tetramer. The cells were analyzed by fluorescence-activated cell sorting. A double-positive fraction was considered to represent WT1-specific CD8-positive CTLs.

Evaluation of Toxicity

Blood samples were evaluated every week. Toxicities were evaluated according to the US National Cancer Institute Common Toxicity Criteria and independently reviewed by the DSMC.

Evaluation of MR Images

Magnetic resonance imaging was performed every 4 weeks. After the WT1 vaccine was administered 12 times, the antitumor effect of the treatment was assessed by determining the response of the target lesions on MR images. The tumor size, corresponding to the contrast-enhanced area on T1-weighted MR images, was measured and analyzed according to RECIST,[23] with results reported as complete response, partial response, stable disease, and progressive disease. The response rate was calculated as the percentage of the number of cases in which there was a complete or partial response divided by the total number of cases. The effective rate was calculated as the percentage of the number of cases in which there was a complete or partial response or stable disease divided by the total number of cases.

Additional Vaccinations and Calculation of the Survival Period

If an effect was observed after 12 vaccinations, further WT1 vaccination at 1-week intervals was given only with the patients' informed consent. The PFS period was calculated from the day of the first WT1 vaccination to the day of the last image prior to the detection of disease progression; this was used as the principal end point. The overall survival period after WT1 vaccination was also calculated, as was the overall survival period after tumor recurrence for WT1-vaccinated patients.

Statistical Analysis

Our main objectives were to evaluate the duration of PFS, the 6-month PFS rate, the overall response rate, the disease control rate, and toxicity based on the WHO criteria. The objective assessments of tumor response were reported using RECIST and were based on major changes in tumor size seen on Gd-enhanced MR images in comparison with the baseline images. Hematological and nonhematological toxicities were assessed using the US National Cancer Institute Common Toxicity Criteria, and the safety and tolerability of the treatment were estimated. Statistical evaluation was performed using Stat View version 4.5 (Abacus Concepts, Inc.). Probability values < 0.05 were considered statistically significant. The Kaplan–Meier method was used to analyze overall survival and PFS. The log-rank test was used to assess the strength of the association between survival time and single variables corresponding to factors that were considered prognostic for survival.

The required sample size for this Phase II trial was estimated to be 20 at 5% Type I and 20% Type II errors, under the assumption of 10 and 30% 6-month PFS rates for the null and alternative hypotheses, respectively. Allowing for the possibility that we might not be able to obtain complete data in all cases, the sample size was set at 21.

Results

Patient Characteristics

During the trial period, 37 patients were assessed for inclusion in the trial. All 37 had WT1-positive GBM, as determined by immunohistochemical analysis. Because we use HLA-A*2402–restricted WT1 peptide, 16 patients with HLA-A*2402–negative type were excluded, and 21 patients (7 women and 14 men) with HLA-A*2402–positive type were enrolled in the study (Table 1). In all the cases involving HLA-A*2402–negative excluded patients, the survival time from recurrence or progression to death was investigated. The median survival time after tumor recurrence in the HLA-A*2402–negative patients was 21 weeks, which was almost the same as that of the historical

TABLE 1

*Characteristics of and clinical results in all enrolled patients**

Case No.	Age (yrs), Sex	RT Dosage (Gy)	Chemo	Add'l Tx	Steroid Tx	KPS Score	Response	PFS (wks)	OS (wks)	WT1 Score
1	63, M	60	CE × 3	IFN	yes	50	SD	28.1	36.1	4
2	33, M	60	—	—	yes	60	PR	23.4	32.4	4
3	45, M	60	CE × 3	IFN	yes	70	PD	5.1	32.6	1
4	29, F	60	CE × 3, ACNU × 2	IFN	—	90	SD	16.0	30.1	2
5	69, M	60	—	IFN	—	80	PD	8.0	36.7	3
6	69, M	60	CE × 3	IFN	—	80	SD	24.4	106.1	3
7	42, M	50	—	—	—	60	SD	32.0	87.1	4
8	46, F	56	—	SRS	yes	60	SD	>96.0	>96.0	3
9	63, M	60	ACNU × 3	SRS	yes	80	PD	0	>87.3	4
10	67, M	60	ACNU × 3	IFN	—	90	PD	4.0	15.0	3
11	40, F	60	ACNU × 3	—	—	80	SD	51.3	69.4	3
12	76, M	60	ACNU × 3	IFN	yes	70	SD	21.1	>79.4	1
13	54, M	50	CE × 3	IFN	yes	50	PD	4.0	18.4	2
14	55, M	60	CE × 3	IFN	—	90	PD	2.0	28.4	2
15	58, F	60	CE × 3	IFN	—	90	SD	42.4	61.7	3
16	20, F	60	ACNU × 2	—	—	90	PR	20.0	29.3	4
17	42, M	60	—	—	—	90	PD	4.3	35.6	3
18	41, M	60	CE × 3, ACNU × 2	SRS	yes	100	SD	>43.6	>43.6	3
19	54, M	60	ACNU × 3	IFN	yes	90	PD	8.0	>41.6	2
20	58, F	50	—	SRS	—	50	SD	>32.1	>32.1	4
21	55, F	60	—	—	yes	100	PD	0	>31.4	4

* ACNU = nimustine hydrochloride; Add'l = additional; CE = carboplatin and etoposide; Chemo = chemotherapy; IFN = β-interferon; KPS = Karnofsky Performance Scale; OS = overall survival; PD = progressive disease; PR = partial response; RT = radiotherapy; SD = stable disease; SRS = stereotactic radiosurgery; Tx = therapy; — = not administered.

control patients at Osaka University Hospital (20 weeks, data not shown). The mean age of the 21 enrolled HLA-A*2402–positive patients was 51.4 years (range 20–76 years). Of the 21 patients, 15 had recurrent disease and 6 had disease progression after initial therapy. All patients had radiotherapy with or without chemotherapy or interferon treatment. All enrolled patients had an ECOG performance status of 0–2 (Karnofsky Performance Scale score > 50), and 10 of them were receiving a maintenance dose (1–4 mg/day betamethasone) of glucocorticoid therapy at the time of vaccination due to local symptoms or symptoms of increased intracranial pressure caused by edema in the area around the tumor. Eight patients underwent surgery after recurrence for mass reduction and confirmed recurrence, and methionine-PET, thallium-SPECT, and FDG-PET were performed in all cases to confirm tumor recurrence.

Clinical Response to Vaccination

All treated patients had a local inflammatory response with erythema at the WT1 vaccine injection site. No Grade 3 or 4 toxicities were observed. Liver dysfunction was detected in Case 9, but improved after the patient's anticonvulsant therapy was changed. This event was considered by the DSMC and was judged to have had no relationship to the WT1 treatment.

A summary of patient responses to WT1 immunotherapy is shown in Table 1. Clinical responses included partial response in 2 patients; stable disease in 10 patients; and progressive disease in 9 patients, including 2 who dropped out of the trial due to tumor progression and poor general condition (Cases 10 and 13). Patients who had an effective response continued to receive vaccinations until tumor progression was demonstrated. All responses were assessed by the DSMC.

The overall response rate was 9.5%. The disease control rate, calculated from the number of patients with complete response, partial response, or stable disease in the initial 3 months (the clinical trial period) was 57.1%. The Kaplan–Meier survival probability curves are shown in Fig. 1. Median PFS in the 21 patients with GBM who were included in the study was 20.0 weeks, and the PFS rate at 6 months (26 weeks) was 33.3%. Median overall survival after initial vaccination was 36.7 weeks. Median overall survival after tumor recurrence in WT1-vaccinated patients was 46 weeks.

Two patients (Case 2 and Case 16) experienced partial response. In both cases, immunohistochemical analysis of the tumor specimens showed high WT1 expression levels, but neither patient survived for a long period (PFS of 23.4 weeks in Case 2 and 20.0 weeks in Case 16). Both patients had disease progression after the 12-week trial period, with leptomeningeal dissemination of the glioma cells and formation of a mass at a different site.

In contrast, in the stable disease group 4 patients (Cases 8, 11, 15, and 18) experienced gradual tumor stabilization; that is, they had a response during the late period of the 3-month WT1 vaccination course. These patients survived for a long time without progression (PFS > 96.0 weeks in Case 8, 51.3 weeks in Case 11, 42.4 weeks in Case 15, and > 43.6 weeks in Case 18).

Relationship Between PFS and WT1-Immunostaining Intensity

In all 21 patients, immunostaining was positive for WT1. The WT1 expression score was 4 in 7 cases, 3 in 8 cases, 2

in 4 cases, and 1 in 2 cases (Table 1). Figure 2 shows representative photomicrographs of Score 2 (Fig. 2A), Score 3 (Fig. 2B), and Score 4 (Fig. 2C) WT1 immunostaining, and Fig. 2D shows MIB-1-immunostaining of a section from the same lesion as Fig. 2C. Both of the patients who had a partial response to vaccination had Score 4 immunostaining. The patients were grouped according to WT1 expression scores, and PFS curves were estimated for each group and then compared. The patients with Score 3 immunostaining tended to have the longest PFS time. The patients with Score 3 or 4 had a statistically longer PFS time than the patients with Score 1 or 2 ($p = 0.0020$, Fig. 3 *right*). Among the patients with high WT1-immunostaining scores (3 and 4), the patients with Score 4 had a shorter PFS time than those with Score 3, although partial response was achieved in 2 patients with Score 4. This might reflect the fact that the patients with Score 4 had high proliferation activity of the GBM cells that was recognized by the high MIB-1 staining index, although they also had the highest amount of target WT1 protein recognized by the induced WT1-specific CTLs.

Relationship Between PFS and MIB-1 Staining Index

The MIB-1 staining index, which reflects each tumor's proliferation activity, was determined by calculating the percentage of positively stained tumor cell nuclei. No statistical difference in PFS was observed between the 2 groups (Fig. 3 *left*). The proliferation activity was found not to directly affect PFS after WT1 vaccination.

Evaluation of WT1-Specific CTL Frequencies in PBMCs

The frequencies of WT1-specific CTLs before WT1 vaccination were significantly higher in patients with GBM than in healthy controls ($p = 0.0019$, Fig. 4). These results indicate that the immune system in patients with WT1-expressing GBM cells responded to the WT1 protein derived from the tumor cells and elicited WT1-specific CTLs that were present before WT1 vaccination; this suggests that the WT1 protein in GBM cells is naturally immunogenic. The existence of the high frequencies of WT1-specific CTLs before WT1 vaccination may have contributed to the favorable clinical responses in patients with GBM. There was no correlation between the induction of a clinical response and WT1-specific CTL frequencies in the PBMCs of the patients prior to vaccination (Fig. 4). Furthermore, the CTL frequencies did not increase after vaccination, even in the patients who responded.

Discussion

The WT1 gene is physiologically expressed in some organs, such as the kidneys, bone marrow, and pleura. Experimental evidence shows that WT1-specific CTLs kill WT1-expressing tumor cells without killing normal cells.[24] Consistent with these data, in the present study, patients with a clinical response had adverse effects of the WT1 vaccination that were limited to local erythema at the injection sites of the WT1 vaccine.

The primary end points of this study were PFS and the PFS rate at 6 months. The objective response rate and the disease control rate with WT1 vaccination, as well as its safety and tolerability, were also estimated.

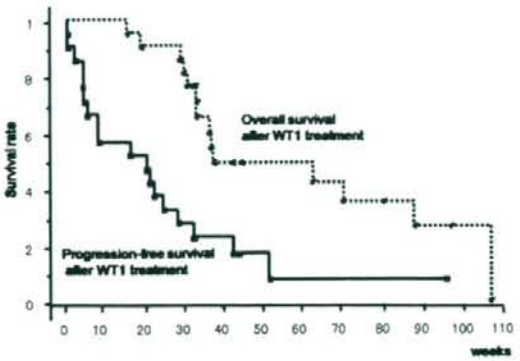

Fig. 1. Kaplan–Meier curves for PFS *(solid line)* and overall survival *(dotted line)* after initial WT1 vaccination for patients with recurrent GBM.

A review of the literature suggested that an agent demonstrating a 6-month progression-free survival rate ≥ 10% would be considered active.[9] A retrospective analysis of 8 Phase II chemotherapy trials conducted from 1986 to 1995 and involving a total of 225 patients with GBM was performed at the M. D. Anderson Cancer Center; a median PFS of 9 weeks and a 6-month PFS rate of 15% were reported.[28] Temozolomide, the most recent drug to be introduced for the treatment of GBM, has been shown to produce results that were not very different from those achieved with carmustine (BCNU). A study that included a series of 112 patients with GBM demonstrated a response rate of 6% with a 6-month PFS rate of 21%;[32] another study, which included a series of 138 patients with GBM, demonstrated a response rate of 8% and a 6-month PFS rate of 18%.[3] The use of BCNU chemotherapy in recurrent GBM was also recently studied; the median time to progression was 13.3 weeks, and the 6-month PFS rate was 17.5%.[4] Following these reports, 6-month PFS rates for the null and alternative hypotheses were assumed to be 10 and 30%, respectively, in this trial, and the sample size was set at 21.

In our study, the median duration of PFS was 20.0 weeks, and the PFS rate at 6 months was 33.3%. The response rate was 9.5%, whereas the disease control rate was 57.1%. The 6-month PFS rate was 33.3% in our patients with GBM—which was higher than the 10% that was set as indicating an active level—and, moreover, was higher than the 30% that was set as the alternative hypothesis before the study was started. Thus, this result suggested that WT1 vaccination was active. The median PFS and median overall survival after WT1 vaccination were 20.0 weeks and 36.7 weeks, respectively; these results are comparable to those reported in the literature for various combination regimens of chemotherapy and/or radiotherapy.

All the treated patients had an inflammatory response with erythema at the WT1 vaccine injection site, but no systemic toxicities were observed. Taken together, these findings allow one to conclude that WT1 vaccination had an anti-GBM effect, it was safe, and the patients tolerated it well.

Although the response rate in our study (9.5%) was not

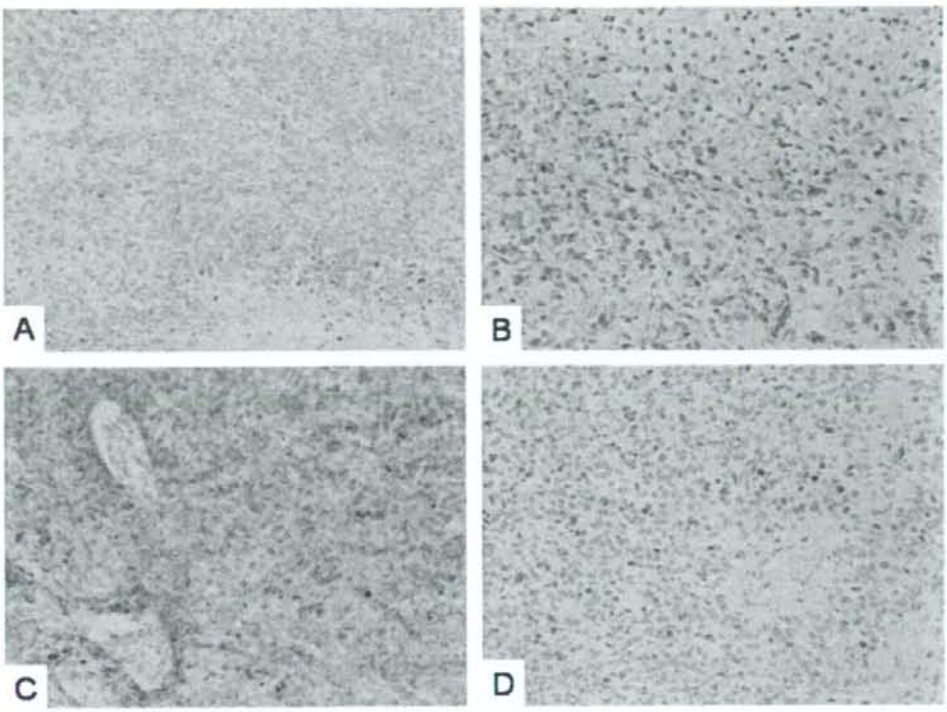

FIG. 2. Photomicrographs showing WT1 (A, B, and C) and MIB-1 (D) immunostaining. Representative WT1 immunostaining with scores of 2, 3, and 4 are shown in A, B, and C, respectively; MIB-1 immunostaining (D) in a specimen from the same case as panel C. Specimens were stained with antibody against WT1 protein (A, B, and C) or antibody against Ki 67 antigen (MIB-1) (D). Original magnification × 200.

very high compared with findings reported in chemotherapy studies, the disease control rate of 57.1% was favorable. The ability of WT1 vaccination to stabilize tumor growth might explain a good PFS of the patients treated with the vaccine. It should be emphasized that WT1 immunotherapy is less toxic than all of the chemotherapy treatments reported. Taken together, the patients in our study had a median PFS, 6-month PFS rate, and disease control rate that were comparable to those achieved using other chemotherapy regimens but with much less toxicity. These findings indicate that WT1 vaccination may be useful for the treatment of GBM.

In our study, WT1-specific CTL frequencies were higher in the PBMCs of patients with GBM than in those of healthy controls; this same phenomenon has been seen in other solid cancers.[19] The results, including good PFS and 6-month PFS rate and high stable disease rate, might be at least partly due to the high frequency of WT1-specific CTLs in the PBMCs of the patients prior to vaccination. Even in the responders, however, the CTL frequencies did not increase after vaccination. In our recent report,[19] we found a correlation between the clinical response and an increase in WT1-specific CTL frequencies in the PBMCs of cancer patients after vaccination. The correlation was clear in patients with leukemia, but it was not that clear in those with solid tumors (lung and breast cancer; unpublished data). Several cancer immunotherapy trials[2,13,14,21] have shown a poor correlation between clinical response and an increase in antigen-specific CTL frequencies. Germeau et al.[7] reported that high frequencies of the antigen-specific CTL were observed before vaccination and did not correlate the clinical response in solid cancers. They suggest that a spontaneous antitumor T-cell response that has become ineffective can be awakened by vaccination and contribute to tumor rejection. After the vaccination, CTLs in the responders might change qualitatively, but not quantitatively. The successfully activated CTLs could have more migratory ability, which would lead to the accumulation of CTLs in the brain.[12] These issues should be addressed by an intense analysis of the CTLs in WT1 vaccine-treated patients with GBM.

Immunohistochemical analysis showed that the patients with a high expression of WT1 protein in tumor specimens tended to respond well to WT1 vaccination. This finding suggests that the presence of high target antigen levels in the tumor cells plays an important role in the clinical responses. Taken together, both a high frequency of WT1-specific CTLs and a high WT1 protein expression level in tumor tissues may be needed for good clinical response to WT1 vaccination.

Under normal conditions, no lymphocytes are present in the brain parenchyma. However, tumor-infiltrating lym-

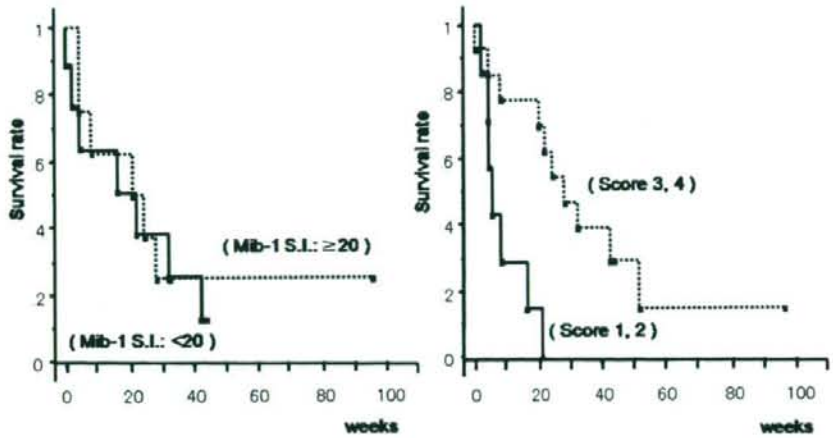

FIG. 3. *Left:* Kaplan–Meier curves for PFS after initial WT1 vaccination for patients with recurrent GBM classified by MIB-1 staining index (S.I.) determined by means of immunohistochemical analysis. The *solid line* indicates cases with an MIB-1 staining index of < 20%, and the *dotted line* indicates cases with an MIB-1 staining index of ≥ 20%. No statistical difference in PFS was observed between the 2 groups. *Right:* Kaplan–Meier curves for PFS after initial WT1 vaccination of patients with recurrent GBM classified by WT1 expression level. The *solid line* indicates cases with low WT1 expression on tumor cells (Score 1 or 2), and the *dotted line* indicates cases with high WT1 expression on tumor cells (Score 3 or 4). Cases with scores of 3 or 4 were associated with better PFS than cases with scores of 1 or 2 ($p = 0.002$).

phocytes are found in and around the tumors in 35–80% of patients with malignant glioma;[5] this may indicate that tumor-specific CTLs would be available to attack the tumor. It has also been reported that immunosuppressive mechanisms, such as the existence of regulatory T cells,[6] hamper CTL function. Thus, the combination of a cancer vaccine with other modalities to inhibit immunosuppressive mechanisms may be useful for improving the efficacy of the vaccine.

It is probable that some cancer patients treated with cancer vaccines can survive long-term without remarkable tumor regression. On the other hand, their tumors could be stabilized or could regress following a temporary increase in size after vaccination since, in general, immunotherapy does not act as quickly as chemotherapy. In fact, some patients in the stable disease group in this study survived for a long time without the treatments achieving partial response. In Case 8, a decrease in tumor size, although it did not reach the partial response level, was observed 7 months after the initial WT1 vaccination. Furthermore, in some of the patients whose clinical response was classified as progressive disease (Cases 3 and 9), tumor stabilization was induced by WT1 vaccination at a later time during the trial. Therefore, one has to consider whether RECIST, which is the gold standard for evaluating the response of solid tumors to cancer chemotherapy, is suitable for evaluating the clinical response to cancer vaccine treatment.[18]

The mechanisms of tumor escape from immune recognition/destruction are thought to be multifactorial. They include: downregulation of major histocompatibility complex Class I molecules, loss of tumor antigens, defective death receptor signaling, lack of costimulation, and the production of immunosuppressive cytokines and suppressive cells.[1]

Given the many different potential mechanisms, combination therapy strategies that use several treatment modalities could include sequential chemotherapy, radiotherapy, and immunotherapy protocols; these will need to be considered.[27]

Conclusions

In HLA-A*2402–positive patients with GBM, immu-

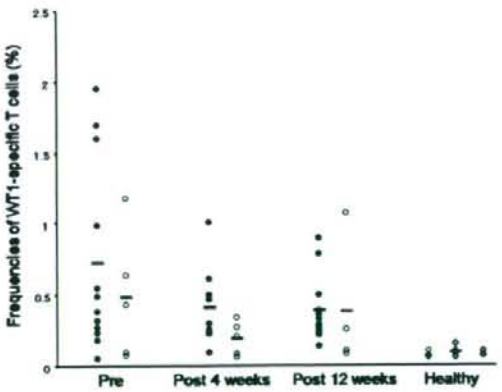

FIG. 4. Graph showing the frequencies of WT1-specific CTLs before WT1 vaccination, 4 and 12 weeks after WT1 vaccination, and in healthy controls. Patients with controlled disease (partial response or stable disease, *closed circles*) as well as those with uncontrolled disease (progressive disease, *open circles*) had a higher frequency of WT1-specific CTLs during the entire evaluation period than healthy controls *(diamonds)*. The *horizontal bars* indicate mean frequencies.

notherapy with HLA-A*2402–restricted, modified 9-mer WT1 peptide vaccination had disease-stabilizing, as well as disease progression–inhibiting, effects that were equal or superior to those of chemotherapy, with systemic toxicity that was much less than that of chemotherapy and thus allowed the vaccinations to be given for a long time. The WT1 protein is considered to be one of the most promising tumor antigens, since injection of a single WT1 peptide type can induce a clinical response. This is another advantage of the vaccine—one does not need to choose a suitable combination of peptides in the laboratory before vaccination. Compared with dendritic cell therapy, WT1 vaccination is simple. The use of a more suitable adjuvant, such as *Mycobacterium bovis* bacillus Calmette–Guérin cell wall skeleton (BCG-CWS),[16] or combination therapy involving vaccination[10] and other modalities may further enhance the clinical usefulness of this treatment for patients with GBM.

Disclaimer

The authors have no conflicts of interest related to this paper.

Acknowledgments

We thank T. Umeda, Y. Watatani, R. Fujita, and M. Kakinoki for technical assistance and coordination of clinical research.

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Manuscript submitted April 26, 2007.
Accepted August 16, 2007.
This work was supported by grants-in-aid from the Ministry of Health, Labour and Welfare, Japan (H16-TRANS-003), to Dr. Sugiyama, and from the Japanese Foundation for Multidisciplinary Treatment of Cancer to Dr. Izumoto.

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A Novel Antiangiogenic Effect for Telomerase-Specific Virotherapy through Host Immune System[1]

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Soluble factors in the tumor microenvironment may influence the process of angiogenesis; a process essential for the growth and progression of malignant tumors. In this study, we describe a novel antiangiogenic effect of conditional replication-selective adenovirus through the stimulation of host immune reaction. An attenuated adenovirus (OBP-301, Telomelysin), in which the human telomerase reverse transcriptase promoter element drives expression of E1 genes, could replicate in and cause selective lysis of cancer cells. Mixed lymphocyte-tumor cell culture demonstrated that OBP-301-infected cancer cells stimulated PBMC to produce IFN-γ into the supernatants. When the supernatants were subjected to the assay of in vitro angiogenesis, the tube formation of HUVECs was inhibited more efficiently than recombinant IFN-γ. Moreover, in vivo angiogenic assay using a membrane-diffusion chamber system s.c. transplanted in *nu/nu* mice showed that tumor cell-induced neovascularization was markedly reduced when the chambers contained the mixed lymphocyte-tumor cell culture supernatants. The growth of s.c. murine colon tumors in syngenic mice was significantly inhibited due to the reduced vascularity by intratumoral injection of OBP-301. The antitumor as well as antiangiogenic effects, however, were less apparent in SCID mice due to the lack of host immune responses. Our data suggest that OBP-301 seems to have antiangiogenic properties through the stimulation of host immune cells to produce endogenous antiangiogenic factors such as IFN-γ. *The Journal of Immunology,* 2009, 182: 1763–1769.

Angiogenesis is the development of new capillaries from preexisting capillary blood vessels and is necessary for the growth of solid tumors beyond 1–2 mm in diameter (1). Targeting the angiogenic process is therefore regarded as a promising strategy in cancer therapy. Angiogenesis consists of dissolution of the basement membrane, migration and proliferation of endothelial cells, canalization, branching and formation of vascular loops, and formation of a basement membrane (2). These steps might be regulated by the local balance between the amount of angiogenic stimulators and inhibitors (3–5). As cells undergo malignant transformation, angiogenic mitogens such as vascular endothelial growth factor (VEGF),[3] basic fibroblast growth factor, platelet-derived epithelial cell growth factor, and TGF become dominant, causing the aberrant angiogenesis. In contrast, many endogenous angiogenic inhibitors such as platelet factor 4, thrombospondin 1, angiostatin, endostatin, various antiangiogenic peptides, hormone metabolites, and cytokines constitutively suppress angiogenesis in normal tissues (6). These scenarios suggest the possibility that endogenous angiogenic inhibitors that outweigh the stimulators could turn off the angiogenic switch.

Recent studies have demonstrated that the tumor microenvironment, which orchestrates with the host immune system, is a critical component of both tumor progression and tumor suppression (7). Indeed, the production of cytokines at tumor sites can either stimulate or inhibit tumor growth and progression (8). These findings provide a unique therapeutic opportunity based on selective and locoregional production of endogenous antitumor mediators such as angiogenic inhibitors. We reported previously that telomerase-specific replication-competent adenovirus (Telomelysin, OBP-301), in which the human telomerase reverse transcriptase promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosomal entry sequence, induced selective E1 expression and efficiently killed human cancer cells, but not normal human fibroblasts (9–12). Although the precise molecular mechanism of OBP-301-induced cell death is still unclear, the process of oncolysis is morphologically distinct from apoptosis and necrosis. We found that tumor cells killed by OBP-301 infection could stimulate host immune cells more efficiently compared with chemotherapeutic drug-induced apoptotic cells and necrotic cells by freeze/thaw, thus enhancing the antitumor immune response (13). These results suggest that oncolytic virus is effective not only as a direct cytotoxic drug but also as an immunostimulatory agent that could modify the tumor microenvironment.

In the present article, we explored whether OBP-301-infected oncolytic cells can activate host immune cells and influence tumor

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Received for publication July 22, 2008. Accepted for publication November 26, 2008.

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[1] This work was supported by Grants-in-Aid from the Ministry of Education, Science, and Culture, Japan (to T.F.) and grants from the Ministry of Health and Welfare, Japan (to T.F.).

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[3] Abbreviations used in this paper: VEGF, vascular endothelial growth factor; MLTC, mixed lymphocyte-tumor cell culture; MOI, multiplicity of infection.

cell-mediated angiogenesis in vitro and in vivo. Antineoplastic effect of intratumoral administration of OBP-301 on s.c. murine colon tumors transplanted was compared in syngenic immunocompetent mice and SCID mice. Finally, we examined the effect of neutralizing anti-IFN-γ Ab on OBP-301-mediated antiangiogenic potential in vivo.

Materials and Methods

Cell lines and reagents

The human colorectal carcinoma cell lines SW620 (HLA-A02/A24) and the murine colon adenocarcinoma cell line Colon-26 were maintained in vitro in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Recombinant human IFN-γ was purchased from Peprotech.

Mice

Female BALB/c (BALB/cAnNCrlCrlj), BALB/c *nu/nu* (CAnN.Cg-*Foxn1*^{nu}/CrlCrlj), and SCID (CB17/Icr-*Prkdc*^{scid}/CrlCrlj) mice, 5–6 wk of age, were purchased from Charles River Japan Breeding Laboratories. Animals were housed under specific pathogen-free conditions in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Adenovirus

The recombinant replication-selective, tumor-specific adenovirus vector OBP-301 (Telomelysin), in which the human telomerase reverse transcriptase promoter element drives the expression of *E1A* and *E1B* genes linked with an internal ribosomal entry sequence, was constructed and previously characterized (9–12). The virus was purified by $CsCl_2$ step gradient ultracentrifugation followed by $CsCl_2$ linear gradient ultracentrifugation.

Cell viability assay

XTT assay was performed to measure cell viability. Briefly, cells were plated on 96-well plates at 5×10^3 per well 24 h before treatment and then infected with OBP-301. Cell viability was determined at the times indicated by using a Cell Proliferation kit II (Roche Molecular Biochemicals) according to the protocol provided by the manufacturer.

Mixed lymphocyte-tumor cell culture (MLTC) and cytokine production assay

For MLTC, SW620 tumor cells were infected with OBP-301 at a multiplicity of infection (MOI) of 10, washed three times in PBS 72 h after infection, and then cocultured with PBMC at a ratio of 1:40. The supernatant was collected at the indicated times and stored at −80°C until assay. The concentration of IFN-γ was measured with ELISA kits (BioSource International).

In vitro angiogenesis assay

In vitro angiogenesis was assessed based on the formation of capillary-like structures by HUVECs cocultured with human diploid fibroblasts according to the instructions provided with the angiogenesis kit (Kurabo). In brief, the HUVECs were incubated in a medium containing the diluted supernatants of MLTC or recombinant IFN-γ in the presence or absence of VEGF (10 ng/ml). The medium was replaced at days 4, 7, and 9. At day 11, the HUVECs were fixed and stained by using an anti-human CD31 Ab (Kurabo) according to the instructions provided. The formation of the capillary network was observed with a microscope at a magnification of ×40.

In vivo assay for tumor angiogenesis

In vivo angiogenesis was determined using the dorsal air-sac method (14). Briefly, 2×10^6 SW620 cells were suspended in PBS containing the diluted supernatants of MLTC or control medium, and placed into round-shaped chambers that consisted of a ring covered with cellulose ester filters (pore size, 0.45 μm; Millipore) on both sides. These chambers were implanted into a dorsal air sac produced in female BALB/c *nu/nu* mice by the injection of 10 ml of air. Five mice in each group were sacrificed on day 5, and the formation of a dense capillary network in s.c. regions was examined under a dissecting microscope. The neovascularization was assessed semiquantitatively by counting the number of cork screw vessels. For each slide, a total of three fields at a magnification of ×4 were selected at random, and the scores were averaged.

In vivo tumor growth and determination of microvessels

Female BALB/c and SCID mice were s.c. implanted with 2×10^6 Colon-26 cells. When tumors grew to ~5–6 mm in diameter, the mice were randomly assigned into three groups and a 100 μl of solution containing 1×10^8 PFU of dl312 or OBP-301, or PBS was injected into the tumor on days 1, 3, and 5. Tumors were measured for perpendicular diameters every 3 or 4 days, and tumor volume (in cubic millimeters) was calculated using the following formula: $a \times b^2 \times 0.5$, where a is the longest diameter, b is the shortest diameter, and 0.5 is a constant to calculate the volume of an ellipsoid. For histological analysis, 2 wk after treatment, the tumors were harvested, embedded in Tissue Tek (Sakura), cut into 5 μm-thick sections, and assessed by a standard H&E and immunohistochemical staining using a rat anti-mouse mAb against CD31 (BD Pharmingen). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences.

In vivo inhibition of IFN-γ with neutralizing Abs

For neutralizing IFN-γ, mice were i.p. administered 200 μg of rat anti-mouse IFN-γ mAb (XMG1.2; BD Pharmingen) 1 day before the first injection of OBP-301 and on days 1 and 3 after the first injection. Control mice received i.p. administration of isotype-matched rat IgG1 (BD Pharmingen).

Statistical analysis

Determination of significant differences among groups was assessed by calculating the value of Student's t test using the original data analysis. Statistical significance was defined at $p < 0.01$.

Results

Effect of OBP-301-infected human colorectal cancer cells on PBMC in vitro

First, we examined whether OBP-301 infection affects the viability of human colorectal cancer cells using the XTT assay. SW620 cells were either mock-infected with culture medium or infected with OBP-301 at an MOI of 1 or 10. As shown in Fig. 1*A*, OBP-301 infection induced death of SW620 cells in a dose-dependent manner. Next, we examined the ability of OBP-301-infected oncolytic cells to stimulate PBMC in MLTC. For this purpose, SW620 cells (HLA-A02/A24) treated with 10 MOI of OBP-301 for 72 h were cocultured with HLA-matched PBMC obtained from HLA-A24$^+$ healthy volunteers at a ratio of 1:40. The production of IFN-γ in the supernatants was then explored by ELISA analysis at the indicated time points. PBMC incubated with OBP-301-infected oncolytic SW620 cells secreted large amounts of IFN-γ as early as 24 h after MLTC, whereas PBMC alone induced little IFN-γ secretion (Fig. 1*B*). The maximum level of IFN-γ was ~250 pg/ml. We previously confirmed that addition of OBP-301 alone without target tumor cells did not affect the cytokine secretion from PBMC into the supernatant, indicating that infection of OBP-301 itself had no apparent effect on PBMC (13). These results suggest that PBMC stimulated with oncolytic tumor cells preferentially secrete high-level IFN-γ.

Inhibition of in vitro and in vivo angiogenesis by MLTC supernatants with OBP-301-infected human tumor cells

In the next step, we investigated the effects of MLTC supernatants with oncolytic SW620 tumor cells and HLA-matched PBMC on VEGF-induced angiogenesis in vitro. The addition of VEGF enhanced the formation of vascular-like structures of HUVECs, although tubule formation was almost absent without VEGF. This VEGF-induced angiogenesis was completely impaired by the addition of MLTC supernatants even at 1/4 dilution (Fig. 2). In contrast, although MLTC supernatants were confirmed to contain ~250 pg/ml IFN-γ, 10-fold more concentration of recombinant IFN-γ was needed to attenuate the tubule formation close to basal levels. The supernatants of PBMC

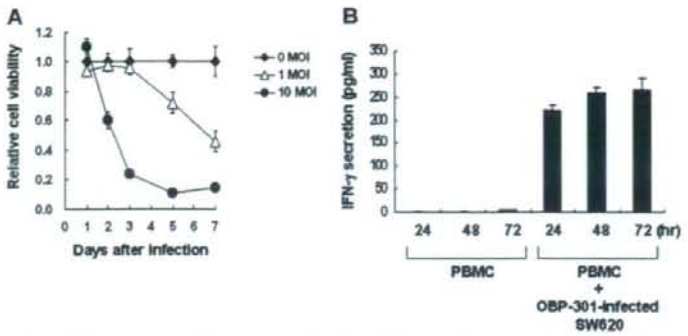

FIGURE 1. In vitro cytopathic effects of OBP-301 and IFN-γ secretion by oncolytic cell-stimulated PBMC. *A*, SW620 human colorectal cancer cells were infected with OBP-301 at indicated MOI values, and surviving cells were quantitated over 7 days by XTT assay. The cell viability of mock-treated cells on day 1 was considered 1.0, and the relative cell viability was calculated. Data are mean ± SD of triplicate experiments. *B*, IFN-γ concentrations in the supernatants of MLTC analyzed by ELISA. SW620 cells were treated with 10 MOI of OBP-301 for 72 h, and then cocultured with PBMCs obtained from HLA-A24$^+$ healthy volunteers for the indicated time periods in MLTC. The culture supernatants were harvested and tested by ELISA for IFN-γ concentrations. As a control, the supernatants of PBMC alone were also examined. Data are mean ± SD of triplicate experiments.

alone had no effect on in vitro angiogenesis. These results suggest that MLTC supernatants may contain more antiangiogenic factors in addition to IFN-γ.

We also assessed whether MLTC supernatants inhibited in vivo angiogenesis induced by human cancer cells. SW620 cells in PBS containing supernatants of OBP-301-infected SW620 cells, PBMC, or both, which were packed into membrane chambers, were implanted into a dorsal air sac produced in *nu/nu* mice. The chambers consisted of membranes that allowed the passage of macromolecules such as IFN-γ, but not cells. Five days after implantation, neovascularization, as demonstrated by the development of capillary networks and curled microvessels in addition to the preexisting vessels, occurred in the dorsal subcutis touched by the chamber, which contained SW620 cells alone. The addition of MLTC supernatants, however, reduced the size and tortuosity of the preexisting vessels, and significantly reduced the development of curled microvessels (Fig. 3). Although the preexisting vessels became thinner by supernatants of OBP-301-infected SW620 cells or PBMC, the number of curled microvessels, which is characteristic of tumor neovasculature, was consistent in these two groups with that in the group compared with SW620 cells alone. Thus, MLTC supernatants exhibited a profound antiangiogenic activity in vivo.

Involvement of host immune activity on antiangiogenic effect of OBP-301

The finding that OBP-301-infected tumor cells stimulated PBMC to produce antiangiogenic factors prompted us to study whether immunodeficiency of host animals could affect the antitumor effect of OBP-301 in vivo. When 2×10^6 Colon-26 murine colon adenocarcinoma cells were inoculated s.c. into BALB/c and SCID mice, palpable tumors appeared in 100% of the mice within 2 wk after tumor injection. Fourteen days after tumor inoculation, animals bearing Colon-26 tumors with a diameter of 5–6 mm were treated with the direct intratumoral injection of 10^8 PFU OBP-301 every 2 days for three cycles. As shown in Fig. 4, treatment with OBP-301 resulted in a significant growth suppression compared with tumors injected with PBS at least for 12 days starting on day 4 after last virus injection ($p < 0.01$) in BALB/c mice; however, OBP-301-mediated antitumor effect was partially impaired in SCID mice, as significant inhibition was observed only for 6 days starting on day 10. Intratumoral injection of replication-deficient dl312 adenovirus had no effect on the tumor growth in BALB/c or SCID mice (data not shown). These results indicate the partial involvement of the host immune system in the OBP-301-mediated antitumor effect.

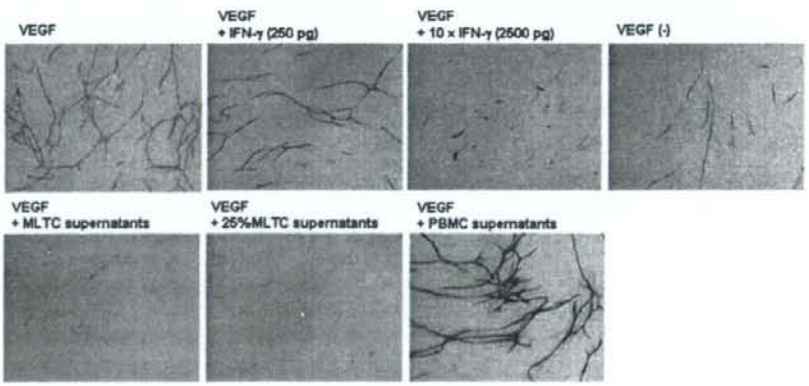

FIGURE 2. Inhibition of in vitro angiogenesis by the supernatants of OBP-301-infected oncolytic cells and PBMC. HUVECs were incubated in a medium containing the supernatants of MLTC obtained 72 h after coculture with OBP-301-infected oncolytic cells and PBMC or recombinant IFN-γ in the presence or absence of VEGF (10 ng/ml). The formation of the capillary network was confirmed by staining with anti-human CD31 Ab on day 11. Representative images depicting formation of capillary-like tube structures by HUVECs are shown. Original magnification is at ×40.

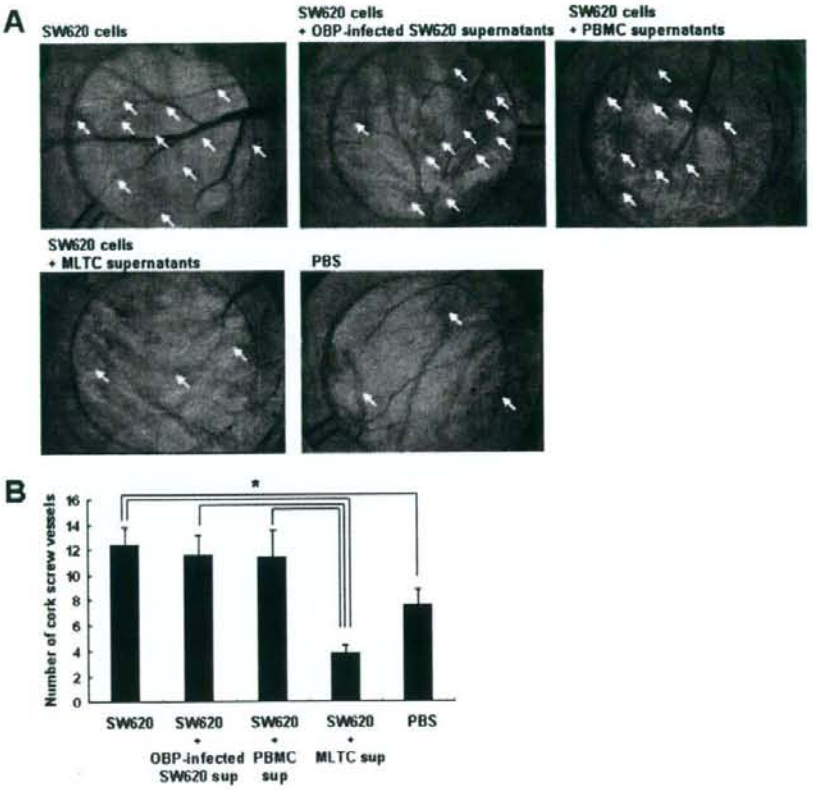

FIGURE 3. Inhibition of tumor cell-mediated in vivo angiogenesis by the supernatants of OBP-301-infected oncolytic cells and PBMC. *A*, SW620 human colorectal tumor cells at a density of 2×10^6 were placed in a diffusion chamber in PBS containing the diluted supernatants of MLTC obtained 72 h after coculture with OBP-301-infected oncolytic cells and PBMC or control mediums, and it was implanted into a dorsal air space produced in BALB/c *nu/nu* mice on day 0. Mice were sacrificed on day 5, and the chamber was removed from the s.c. tissue. A new ring without filters was placed on the same site to mark the position of the chamber. The capillary networks developed inside the rings were photographed to determine the effect of treatments. Representative images of treatment groups are shown. Curled microvessels are shown (arrow). *B*, The number of cork screw vessels was semiquantitatively counted to assess the neovascularization. Data are mean ± SD. *, $p < 0.01$. Similar results were observed in two independent experiments conducted in triplicate.

Antiangiogenic effect of OBP-301 on syngenic and immunodeficient murine tumor models

When Colon-26 s.c. tumors implanted in BALB/c mice were injected with PBS, replication-deficient dl312 adenovirus, or OBP-301. Macroscopically, tumors treated with OBP-301 were consistently smaller than those of the other two cohorts of mice 14 days after last virus injection (Fig. 5*A*). Furthermore, a reddish area was noted on the tumor surface on two of six mice treated with OBP-301, indicating virus-induced intratumoral necrosis of tumor cells in vivo.

To better understand the mechanisms underlying the induction of necrosis following OBP-301 treatment, histologic and immunohistochemical analyses were performed on Colon-26 tumors harvested 14 days after last injection. A standard H&E staining demonstrated the presence of many vessels in tumors injected with PBS or dl312. However, OBP-301-treated tumors showed few vessels. In addition, massive tumor cell death and cellular infiltrates at the central portions of the tumors were observed where OBP-301 was injected (Fig. 5*B*). Immunohistochemical staining of tumor sections with the Ab for CD31 Ag, an endothelial cell marker, also revealed that Colon-26 tumors injected with OBP-301 displayed very few and extremely small blood vessels (Fig. 5*C*). In contrast, OBP-301 injection could not apparently reduce the vessel numbers on Colon-26 tumors implanted in SCID mice (Fig. 5*D*). These in vivo studies demonstrated that inhibition of angiogenesis due to the stimulation of host immune system might be an important mechanism of OBP-301-mediated in vivo antitumor effect.

Contribution of in vivo IFN-γ production to the OBP-301-mediated antiangiogenic effects

Finally, to determine whether IFN-γ is involved in OBP-301-mediated antiangiogenic effects, in vivo neutralizing experiments were performed by using anti-IFN-γ mAb or isotype-matched control mAb. Angiogenesis was reduced by intratumoral injection of

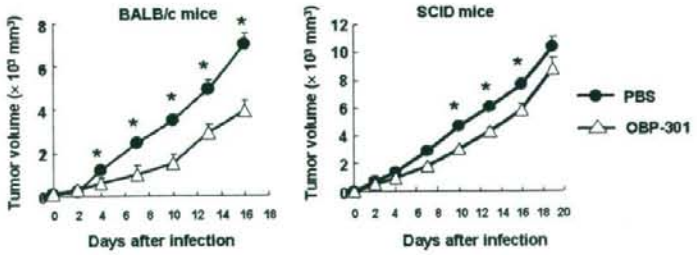

FIGURE 4. Antitumor effects of intratumorally injected OBP-301 against Colon-26 murine colon adenocarcinoma tumors in syngenic immunocompetent BALB/c and immunodeficient SCID mice. Colon-26 cells (2×10^6 cells/each) were injected s.c. into the right flank of mice. OBP-301 (1×10^8 PFU/body) was administered intratumorally for three cycles every 2 days. PBS was used as a control. Six mice were used in each group. Tumor growth was expressed by tumor mean volume ± SD. *, $p < 0.01$.

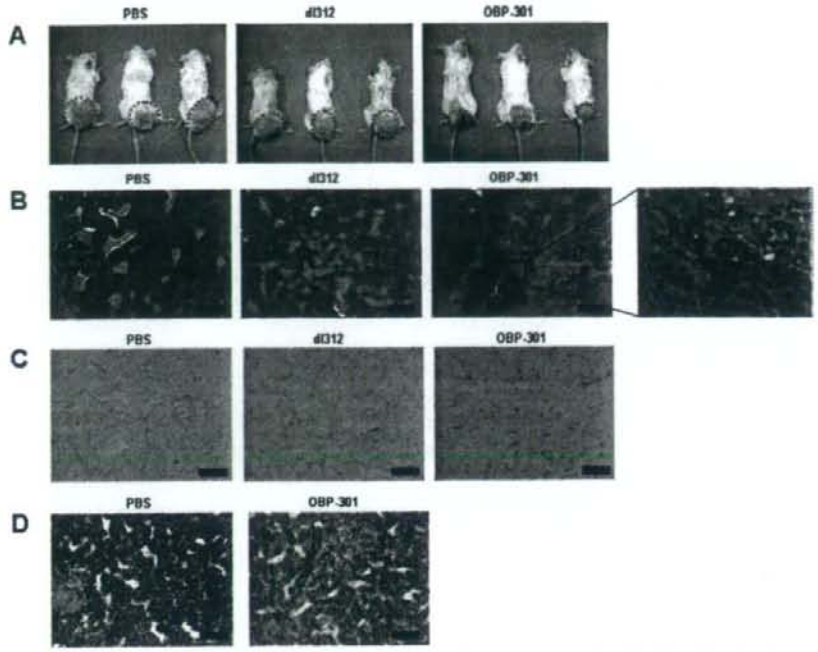

FIGURE 5. Macroscopic and histopathological analysis of Colon-26 tumors treated intratumorally with OBP-301. Colon-26 cells (2×10^6 cells/each) were injected s.c. into the right flank of syngenic BALB/c mice and SCID mice and OBP-301 (1×10^8 PFU/body) was administered intratumorally for three cycles every 2 days as described in Fig. 4. *A*, Macroscopic appearance of Colon-26 tumors on BALB/c mice 14 days after treatment. Note the reddish area on the tumor surface in two mice treated with OBP-301. *B*, Tumor sections were obtained from BALB/c mice 14 days after final administration of OBP-301. Frozen sections of tumors were stained with H&E. Scale bar represents 100 μm, and magnification is $\times$100. Magnified view of the boxed region in *B* is shown. The area with cellular infiltrates is indicated with the green dotted line. *C*, Blood vessel formation in Colon-26 tumors injected with OBP-301. Frozen sections of the tumors were also probed with an Ab against CD31. Scale bar represents 50 μm, and magnification is a $\times$200. *D*, Tumor sections were obtained from SCID mice 14 days after final administration of OBP-301. Frozen sections of tumors were stained with H&E. Scale bar represents 100 μm, and magnification is at $\times$100 magnification.

OBP-301 on Colon-26 tumors; this antiangiogenic effect, however, could be partially inhibited in the presence of anti-IFN-γ mAb (Fig. 6). Treatment with control IgG1 had no effect on the antiangiogenic effects of OBP-301. These results suggest that IFN-γ may be one of the important factors for OBP-301 to inhibit angiogenesis in vivo.

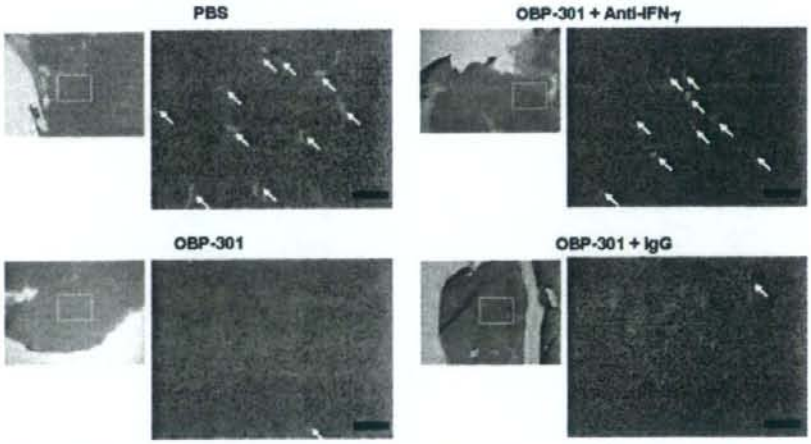

FIGURE 6. Effects of anti-IFN-γ Abs on angiogenesis in Colon-26 tumors. Colon-26 cells (2×10^6 cells/each) were injected s.c. into the right flank of syngenic BALB/c mice and OBP-301 (1×10^8 PFU/body) was administered intratumorally for three cycles every 2 days as described in Fig. 4. Mice were administered 200 μg of anti-IFN-γ mAb (XMG1.2) i.p. to neutralize IFN-γ 1 day before the first injection of OBP-301 and on days 1 and 3 after the first injection. Control mice received i.p. administration of isotype-matched rat IgG1 or PBS. Frozen sections of tumors obtained 14 days after final administration of OBP-301 were stained with H&E. Magnified view (*right*) of the boxed region (*left*). Microvessels are shown (arrow). Scale bar represents 50 μm, and magnification is at $\times$200.

Discussion

The tumor vasculature provides a new and attractive target for cancer therapy because of the reliance of most tumor cells on an adequate vascular supply for their growth and survival. Although the beneficial effects of novel antiangiogenic agents such as bevacizumab have been recently shown (15), regulation of endogenous antiangiogenic mediators may be another approach to inhibit angiogenesis. In the present study, we showed that OBP-301 infection and replication induced cytolysis of tumor cells with subsequent stimulation of host immune cells, which in turn inhibited tumor angiogenesis in vivo. Treatment of established murine colon tumors with intratumoral injection of OBP-301 resulted in a significant antitumor response characterized by extensive necrosis and reduced vascularity.

We reported previously that wild-type *p53* tumor suppressor gene transfer by a replication-deficient adenovirus vector (Advexin) could have antiangiogenic effects. The effects could be through down-regulation of angiogenic factor VEGF and up-regulation of antiangiogenic factor BAI1 because tumor p53 protein is a potent transcriptional factor (16, 17). In contrast, OBP-301 contains no therapeutic genes such as *p53* and, therefore, its infection may not directly influence the angiogenic property of infected tumor cells. However, because viral infection is known to trigger innate and adaptive immune responses presumably through the release of proinflammatory cytokines (18–20), local administration of OBP-301 might affect the tumor microenvironment, thus explaining the potential therapeutic benefit on tumor angiogenesis. In fact, dying tumor cells infected with OBP-301 promoted the production of Th1 cytokines by PBMC such as IFN-γ, which is one of the most potent antiangiogenic factors (21, 22) (Fig. 1). Viral infection itself has been reported to activate dendritic cells to secrete pro- or anti-inflammatory cytokines (23); our preliminary experiments, however, demonstrated that OBP-301 alone had no effect on cytokine production by PBMC (13), indicating that OBP-301 itself may be less infective or stimulatory to PBMC. The result is consistent with our previous finding that OBP-301 attenuated replication as well as cytotoxicity of human normal cells (9, 10). Moreover, OBP-301-infected tumor cells, but not untreated tumor cells, enhanced IFN-γ-inducible proteasome activator PA28 expression in the presence of PBMC (13), indicating that only dying tumor cells could trigger IFN-γ production by PBMC.

IFN-γ has been also known to inhibit tumor angiogenesis through the subsequent stimulation of secondary mediators, including monokine induced by IFN-γ and IFN-inducible protein 10 (24). Indeed, the observation that the supernatants of PBMC cocultured with OBP-301-infected human colorectal cancer cells exhibited a more profound antiangiogenic effect than recombinant IFN-γ (Fig. 2) suggests that other factors in addition to IFN-γ, which may not be related to IFN-γ, play important roles in inhibition of tumor cell-mediated angiogenesis. For example, we also found that oncolytic cells stimulated PBMC to secrete IL-12, which is an inducer of IFN-γ as well as an antiangiogenic factor, into the culture supernatants (13). The supernatants of neither virus-infected tumor cells alone nor PBMC alone were more antiangiogenic compared with those of MLTC in vivo (Fig. 3). Therefore, the interaction of oncolytic cells and PBMC is required to produce antiangiogenic mediators and to inhibit in vivo angiogenesis following OBP-301 treatment. The question what kind of cells produce mediators for antiangiogenic effects is of interest. We reported previously that OBP-301 replication produced the endogenous danger signaling molecule, uric acid, in infected human tumor cells, which in turn stimulated dendritic cells to produce IFN-γ as well as IL-12 into the supernatants (13). The amount of IFN-γ produced by dendritic cells was ~40 pg/ml, although 250 pg/ml IFN-γ was detected in the MLTC supernatants (Fig. 1*B*), indicating that other cell types may contribute to IFN-γ production. Lymphocytes that promote innate immunity (i.e., NK cells) as well as classical $CD4^+$ and $CD8^+$ T cells are also know to produce IFN-γ (25). Thus, dendritic cells represent one of the sources of IFN-γ; however, IL-12 secreted from dendritic cells activated with OBP-301-infected tumor cells might trigger these cells to produce IFN-γ.

To more directly evaluate the antiangiogenic effect of OBP-301, we used a syngenic BALB/c model established by s.c. inoculation of Colon-26 murine colon adenocarcinoma cells. OBP-301 is reported to have high infectivity and the potential to induce cell death in a variety of human cancer cells (9–12), whereas murine cells are relatively refractory to adenovirus infection due to the low expression of the coxsackievirus and adenovirus receptor. We have confirmed previously that telomerase-specific oncolytic adenovirus could infect and replicate in Colon-26 cells (12). Intratumoral administration of OBP-301 significantly inhibited the growth of Colon-26 tumors in syngenic immunocompetent BALB/c mice, although the magnitude of suppression was much less when compared with that in human tumor xenografts (9, 10). The finding that tumor growth suppression by OBP-301 was partially inhibited in immunodeficient SCID mice (Fig. 4) indicates that the host immune system could be partially responsible for the antitumor effect of OBP-301. Histopathologic analysis revealed that the presence of the immune cell infiltrates and the massive necrosis in Colon-26 tumors are exclusively due to the tumor-specific viral replication because dl312-injected tumors showed neither cellular infiltrates nor tissue damages (Fig. 5*B*). In view of the fact that a cellular infiltration could be still observed as late as 14 days after the last OBP-301 injection, immune responses are likely to be induced by oncolytic tumor cells. Furthermore, as expected, tumors injected with OBP-301 formed less blood vessels than mock- or dl312-treated tumors (Fig. 5, *B* and *C*), suggesting that inhibition of angiogenesis by infiltrating cell-secreted mediators partially elicits the antitumor activity of OBP-301. In contrast, antiangiogenic effect of OBP-301 was impaired in SCID mice (Fig. 5*D*), indicating that host immune cells are necessary for this function of OBP-301. Moreover, IFN-γ is considered to be partially responsible for the antiangiogenic effects of OBP-301 because in vivo neutralization of IFN-γ by anti-IFN-γ mAb increased angiogenesis on Colon-26 tumors (Fig. 6).

It remains to be studied whether OBP-301-infected oncolytic cells are capable of inhibiting the growth of distant tumors. Circulating inhibitors of angiogenesis such as angiostatin and endostatin can suppress the growth of remote metastases (26). The observation that none of mice treated with OBP-301 showed signs of viral distress (ruffled fur, weight loss, lethargy, or agitation) as well as histopathologic changes in any organs at autopsy (data not shown) suggests that the cytokine secretion by oncolytic cell-stimulated immune cells might be local rather than systemic. Thus, it is unlikely that locally produced antiangiogenic factors interfere with the distant tumor growth, although the circulating virus itself can infect and replicate in metastatic tumors. This question is being currently investigated in our laboratory.

In conclusion, we provide for the first time evidence that oncolytic virotherapy induces novel antiangiogenic effect by stimulating host immune cells to produce antiangiogenic mediators such as IFN-γ. Our data suggest that the antitumor effect of OBP-301 might be both direct and indirect.