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Boron neuron capture therapy using epithermal neutrons for recurrent cancer in the oral cavity and cervical lymph node metastasis

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Received March 23, 2007; Accepted June 18, 2007

Abstract. The purpose of this clinical trial was to evaluate the utility of boron neutron capture therapy (BNCT) using epithermal neutrons for cases of recurrent cancer in the oral cavity, which are not indicated for a conventional treatment modality. We enrolled four patients with local recurrence or metastasis to the regional lymph nodes after completion of initial treatments, including surgery, chemotherapy and radiotherapy. Before receiving BNCT, patients underwent ^{18}F-p-bononophenylalanine (BPA) positron emission tomography (PET) examinations to assess the BPA accumulation ratios in tumors and normal tissues. All patients showed at least a tentative partial response, while a marked improvement in quality of life was seen in one patient. Before BNCT, that patient could not be discharged from the hospital because of eating difficulties and malaise; after treatment, he was comfortably discharged. Mild malaise, oral mucositis and alopecia were seen as mild adverse effects; however, no life-threatening systemic symptoms were observed in any of the cases. Our results suggested that BNCT is a useful treatment modality for recurrent or regionally metastasized oral cancer.

Introduction

Treatment of advanced and/or recurrent oral cancer is a major concern, because the related tumors are known to be resistant to conventional treatment modalities. We primarily treat patients with advanced squamous cell carcinoma using systemic chemotherapy consisting of Docetaxel and Nedaplatin (1,2). However, because chemotherapy generally induces severe systemic side effects including leukocytopenia, it is difficult to treat patients who have a poor performance status (PS).

Boron neutron capture therapy (BNCT) is a type of tumor-cell targeted radiotherapy that has a significantly increased therapeutic ratio in comparison to conventional radiotherapy, without severe systemic side effects. BNCT is based on the nuclear reaction that occurs when boron-10 is irradiated with low-energy thermal neutrons to yield high-linear-energy transfer α particles and recoiling lithium-7 nuclei (3). Clinical interest in BNCT has focused primarily on the treatment of brain tumors and either cutaneous primary or cerebral metastasis from a melanoma; more recently, it has focused on head and neck malignancies (4,5). In the present study, we assessed the usefulness of BNCT for cases of recurrent cancer in the oral cavity and neck metastasis following conventional treatment.

Patients and methods

Patients. The subjects were 4 patients with recurrent oral cancer and/or cervical neck lymph node metastasis following conventional treatment, including surgery, chemotherapy and radiotherapy (Table I). Each patient signed an informed-consent statement approved by the medical and ethics committees of Kyoto University Research Reactor Institute (KURI) and Osaka Medical College (OMC) prior to enrollment.

Diagnoses of local recurrence and/or metastasis in regional lymph nodes was determined from the results of histopathological and/or cytological examinations, as well as imaging modalities including computed tomography (CT), magnetic resonance images (MRI), and ultrasound (US). After confirmation of recurrence and/or metastasis, the indications for

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Key words: boron neutron capture therapy, epithermal neutron, oral cancer, boronophenylalanine

Table I. Characteristics of patients.

Case no.	Age/Sex	Location	Histopathological diagnosis	Previous treatment	BNCT fraction
1	41/M	Cervical lymph node[a]	SqCC	Surg, Chem, Rad (39 Gy)	2 (2 months)
2	57/M	Maxilla	SqCC	Surg, Chem, Rad (60 Gy)	2 (1 month)
3	67/F	Maxilla	MC	Surg, Chem, Rad (60 Gy)	1
4	69/F	Maxilla	AC	Surg, Chem, Rad (60 Gy), Therm	1

[a]Original tumor site, tongue. M, male; F, female; SqCC, squamous cell carcinoma; MC, mucoepidermoid carcinoma; AC, adenocarcinoma; Surg, surgery; Chem, chemotherapy; Rad, conventional radiotherapy (total doses); Therm, thermotherapy.

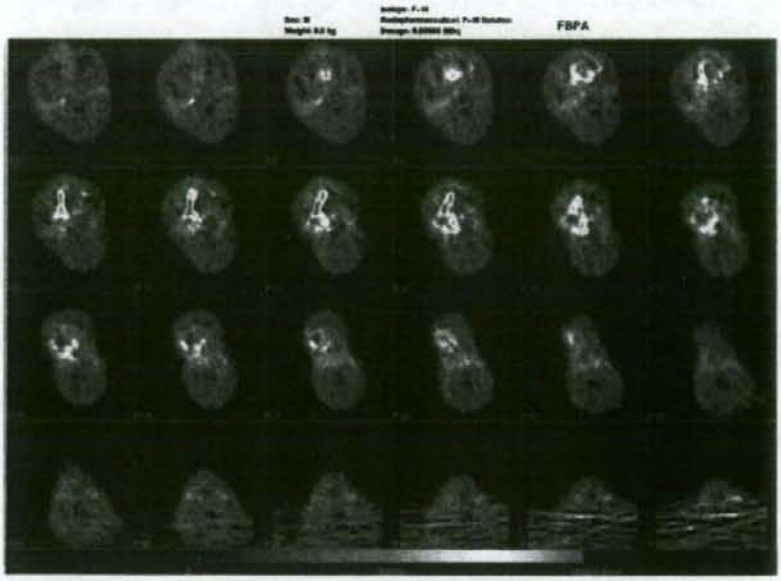

Figure 1. ^{18}F-BPA PET study (case 1). Ratios of ^{18}F-BPA accumulation in the area that responsible to tumor and normal tissues were assessed, and the T/N ratio was calculated.

other conventional treatments were assessed. Patients not indicated for another treatment modality were enrolled in the present study and treated with BNCT.

Methods. Prior to BNCT, all patients underwent a fluoride-18-labelled p-boronophenylalanine positron emission tomography (^{18}F-BPA PET) study to assess the distribution of BPA and estimate the boron concentration in the tumor (Fig. 1) (6,7). The tumor/normal (T/N) tissue ratio of BPA uptake was estimated from those results and dose planning was made according to the T/N ratio. Neutron flux was determined using a computer work station equipped with SERA dose planning software (Idaho National Engineering and Environmental Laboratory, Idaho Falls, ID) before the radiation was delivered. The total dose of BPA was 500 mg/kg body weight. Two hours before neutron irradiation, an intravenous administration of 200 mg/kg body weight/h of BPA was started. Patients were positioned for neutron radiotherapy in the reactor room and irradiation started after changing the flow rate of BPA to 100 mg/kg body weight/h (8). All patients were placed in a sitting position during irradiation. To monitor the boron concentration in the blood, venous blood samples were obtained every 30 min to 1 h after BPA was administered until neutron irradiation was completed. ^{10}B concentrations in the blood were measured by prompt γ-ray analysis and time vs. ^{10}B concentration curves were plotted. The boron concentrations from BPA in the tumor and normal tissues were estimated by the T/N ratio of ^{18}F-BPA on PET. Based on the boron concentration, the neutron fluence rate simulated by the SERA work-station, and factors related to the relative biological effectiveness of the neutron beam and BPA, the total dose delivered to the tumor and normal tissues could be estimated. The duration of irradiation was set to deliver up to 10-15 Gy-Eq to the oral mucosa and as much as possible to the tumor. Here, Gy-Eq (Gy:Gray) means the biologically equivalent X-ray dose that would produce effects equivalent to that of the total BNCT radiation.

Treatment results were assessed by examining clinical features and the results of imaging modalities, including CT and MR imaging. The criteria for responses were defined as follows: complete response (CR), complete disappearance of all clinically evident tumors; partial response (PR), >50%

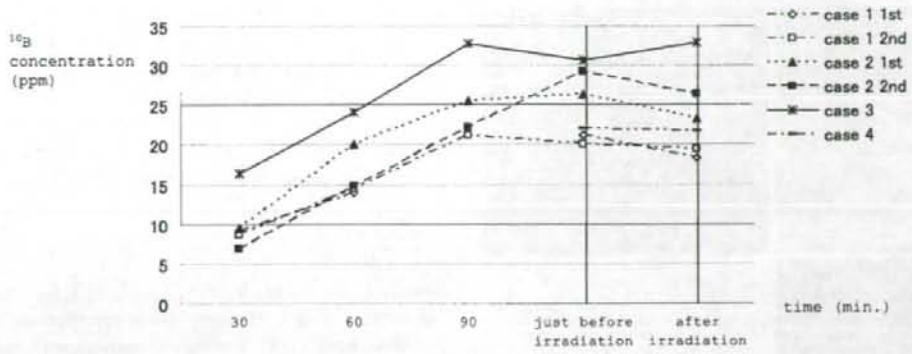

Figure 2. ^{10}B concentrations in blood. Boron concentrations in blood were monitored using venous blood samples taken before and after irradiation.

Table II. Parameters of BNCT.

Case	T/N ratio of ^{18}F-BPA	Irradiation time (min)	Dose (Gy-Eg) Skin surface	Oral mucosa	Tumor peak (depth from skin surface, cm)	Minimum tumor dose (depth from skin surface, cm)
1	4.0	90	4.9	14.4	39.1 (1.8)	15.0 (7.0)
	-	75	2.9	9.5	25.5 (2.5)	10.3 (7.0)
2	3.4	41	3.2	9.1	21.6 (2.0)	9.1 (6.0)
	-	60	4.6	14.4	20.1 (2.5)	8.9 (6.0)
3	2.2	36	3.7	15.3	24.8 (2.5)	22.0 (4.0)
4	2.4	26	7.2	15.0	38.3	17.1

reduction in the sum of the two largest perpendicular diameters of each measurable disease site, with no appearance of new lesions or progression of any existing lesions; progressive disease (PD), at least a 25% increase in tumor area or the appearance of new lesions; no change (NC), all other outcomes. Adverse effects were assessed using the National Cancer Institute Common Toxicity Criteria (NCI-CTC ver.2.0).

Results

BNCT parameters. The T/N ratios of ^{18}F-BPA, which were assessed in the PET examinations, ranged from 2.2 to 4.0. The fraction of irradiation was 2 in two cases (cases 1 and 2) and 1 in two cases (cases 3 and 4). The duration of neutron irradiation was from 26 to 90 min. The concentration of ^{10}B just before and just after irradiation ranged from 30.7 to 20.0 ppm and 32.9 to 18.3 ppm, respectively (Fig. 2). The minimum tumor doses (usually at the deepest part of the tumor) ranged from 22.0 to 8.9 Gy-Eq. The maximum doses for the oral mucosa and skin surface ranged from 15.3 to 9.1 Gy-Eq and 7.2 to 2.9 Gy-Eq, respectively (Table II).

Clinical response, adverse effects, PS and outcome. Clinical response was determined to be PR in 3 cases and PD in 1

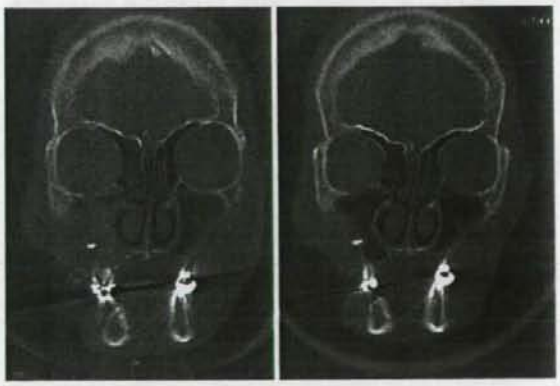

Figure 3. Case presentation (case 3). Left, prior to irradiation; right, 5 months after irradiation. A CT examination revealed a marked decrease in tumor size. Before irradiation (left), the right antrum was filled with a dense and soft tissue mass. Following BNCT (right), the tumor mass was decreased in size and the right antrum appeared as a pneumatic space.

(Figs. 3 and 4). As for systemic adverse effects, mild fatigue was detected in all cases, though apparent hematological toxicities were not seen. Locally, mucositis appearing within

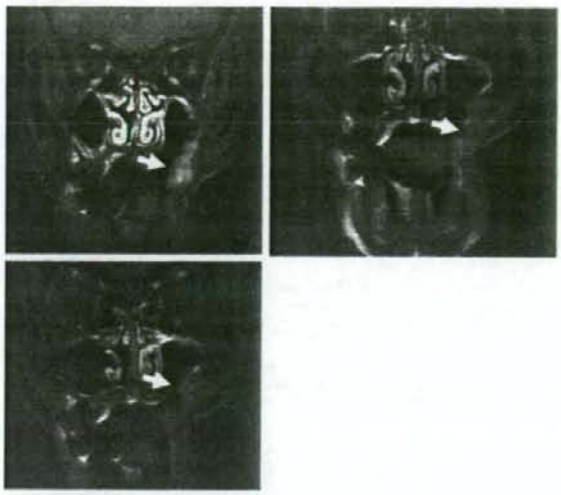

Figure 4. Case presentation (case 2). Top left, prior to irradiation; top right, just prior to second irradiation; bottom, 3 months after completion of BNCT. An enhanced mass lesion was seen in the left buccal area in all three phases (arrows). Although no apparent decrease in tumor size was demonstrated just before the second irradiation, marked tumor downsizing was noted 3 months after the completion of BNCT.

1 week after irradiation and continuing for 3-5 weeks was the most severe adverse effect. No skin defects that had a direct relation to BNCT were seen in the irradiated fields, except in the case 1 patient, whose skin already showed tumor invasion before BNCT. PS was improved in 3 of 4 cases, while the remaining patient, who did not complain of any symptoms including severe pain before irradiation, showed no change (Table III). The most apparent improvement in PS was seen in the case 1 patient, who had been hospitalized because of severe pain and difficulties in eating before BNCT. His neck mass was markedly downsized and pain was relieved, and he was tentatively discharged from the hospital after BNCT. Two of 4 patients (cases 3 and 4) were alive with evidence of disease during follow-up periods ranging from 6 to 12 months. One patient (case 1) died from tumor re-growth followed by aspiration pneumonia 2 months after completion of BNCT, and the remaining patient (case 2) died from local tumor re-growth 12 months after completion of BNCT.

Discussion

Results of the first clinical trial of BNCT for head and neck cancer including oral cancer were reported in 2004 (5). In 2005, we began BNCT at our institution, mainly for patients with advanced non-resectable and recurrent oral cancer. In general, such patients suffer from severe pain, difficulty with eating that induces malnutrition, difficulty with speaking, and poor PS. For recurrent cancer patients, it is controversial which kind of treatments should be chosen, radical or palliative. To resolve this issue, both the clinical response, including prognosis, and improvement of PS should be discussed when a treatment modality for these patients is decided.

BNCT is a novel treatment modality for these patients, because it offers the possibility of inducing radical rather than palliative treatment outcomes. Prior to administering BNCT to oral cancer patients, it is important to discuss the protocol, as conventional BNCT for a brain tumor has a number of problems, such as lack of neutron penetration, especially in patients with deep-seated tumors, an insufficient contrast in boron concentration between tumors and normal tissues, an absolute lack of boron in tumor tissues, and an uncertain estimation of neutron flux captured by the ^{10}B atoms in tumor cells (9). In the present study, we assessed macroscopic treatment effects and investigated clinical problems associated with BNCT administered for recurrent oral cancer and neck metastasis following conventional treatment.

BNCT indications were assessed using ^{18}F-BPA PET, which provides an accurate assessment of BPA accumulation

Table III. Clinical results.

Case	Clinical response	Adverse effects	Improved PS	Outcome (duration of BNCT and time of outcome determination)
1	PD	Fa (1) Mu (2)	2+	Died (4 months)
2	PR	Fa (1) Mu (1)	+-	Died (13 months)
3	PR	Fa (1) Al (1)	1+	AD (13 months)
4	PR	Fa (1) Mu (2)	1+	AD (7 months)

Clinical response: PD, progressive disease; PR, partial response. Adverse effects: Fa, Fatigue; Mu, mucositis; Al, alopecia. Improved PS: 2+, improved by 2 grades or more; 1+, improvement of 1 grade; +-, no change; -, exacerbation. Outcome: AD, alive with disease.

and distribution before irradiation. Kato *et al* (5) reported that a head and neck tumor with a T/N ratio of <2.5 or a tumor in a location deep under the skin surface should not be recommended for BNCT. In the present 4 cases, the T/N ratios were 4.0, 3.4, 2.2 and 2.4, respectively. The latter 2 (cases 3 and 4) showed low T/N values according to the criteria of Kato *et al*; however, relatively good responses were obtained. The tumors in those patients were superficially located near the skin surface and their positions allowed for relatively easy access during irradiation. Conversely, cases that showed higher T/N values (cases 1 and 2) had unfortunate outcomes within a 12-month follow-up period. The clinical effects seen in the present study suggest that tumor location and patient performance are important, in addition to the T/N ratio obtained by ^{18}F-BPA PET. Patient positioning during irradiation has a strong influence on the effects of BNCT. Notably, patients with a neck dissection, such as case 1, find it difficult to stretch their neck, while the shoulder interferes with accurate positioning. To improve the effects of BNCT, it is necessary to modify patient positioning for each case.

Kato *et al* (5) indicated that BNCT represents a new and promising treatment approach, even for large or far advanced head and neck malignancies, because it can induce a remarkable reduction of huge tumors, improve QOL, and has very mild side effects. In the present study, 3 of 4 patients were categorized as PR and the remaining patient as PD. We considered that the overall clinical effects were excellent for our patients, who had recurrent disease and had already undergone other conventional treatment modalities including surgery, chemotherapy and radiotherapy. In the PD case (case 1), a tentative tumor decrease was seen; however, tumor re-growth occurred and the patient died 4 months after the first course of BNCT. That tentative decrease in tumor mass led to an improvement in PS, namely, pain relief, and as a result the patient was discharged temporarily from the hospital. In both of the fatal-outcome cases (cases 1 and 2), tumor re-growth occurred in the part deep from the skin surface. These results suggest that the minimum tumor dose was not enough to destroy the tumor cells at the areas of recurrence. Between the tumor re-growth cases (cases 1 and 2) and no-re-growth cases, there was no essential difference in estimated minimum tumor dose. Thereafter, the recurrence might be ascribed to heterogeneous distribution of boron compounds in the re-growth cases (strategies for overcoming this problem are discussed later). Recurrence in these cases occurred at the deepest part of the tumor, where the absorbed dose in tumor tissue was the lowest.

Kato *et al* (5) stated that one of the merits of BNCT is its indication for recurrent or metastatic cases that have already received a full dose of radiotherapy. All of the present patients had experienced conventional radiotherapy before undergoing BNCT. Three of the 4 had already received 60 Gy of radiation in the same field that received BNCT, and the remaining case (case 1) had received 39 Gy. As a result, following BNCT, there was a concern regarding perforation of the covering skin, osteoradionecrosis or rupture of vital vessels like the carotid artery. At present, no such severe complication has been seen in the patients. In one patient who died (case 1), the tumor had already invaded the skin, and skin perforation was evident before performing BNCT. Skin perforation was also seen in case 2. In this case, although the tumor showed good response to BNCT, aggressive tumor re-growth was seen eight months after irradiation, and tumor invasion to the skin followed by perforation occurred. The irradiated doses to the skin were not significantly different between cases 1 and 2 (fatal outcomes) and 3 and 4 (good outcomes). These results suggest that previous irradiation should not prohibit the administration of BNCT. On the other hand, because the follow-up period is limited, we can not predict whether osteoradionecrosis of the maxilla and adjacent bones will occur in the case 3 and 4. As a result, we can make no definite conclusion as to whether previous irradiation should prohibit the administration of BNCT or restrict its dose. To resolve these issues, long-term follow-up of a large number of cases is required.

From a clinical point of view, it is important to assess adverse effects as well as clinical response. Regarding brain tumors, one of the authors of this paper (Miyatake *et al*) (9) reported that all of their patients exhibited alopecia, and no acute brain swelling or consciousness disturbance occurred. As for head and neck tumors, Kato *et al* (5) noted that there were few side effects such as transient mucositis and alopecia, and all side effects were less than grade-2 by NCI-CTC. In the present patients, no severe systemic adverse effects including malaise, anemia or leukocytopenia were seen. On the other hand, mucositis, which can cause difficulties with eating and degrade QOL, occurred and continued for 3 to 4 weeks in all patients. In addition, in the patient with maxillary cancer and the case of a metastatic neck lymph node, localized alopecia was seen. Those systemic and localized adverse effects suggest that, except for the possibility of mucositis, BNCT has more benefits for patients than conventional treatments, including chemotherapy and radiotherapy.

Selection of the boron delivery agents is also an important factor for BNCT (4). Previous clinical trials (5,9) used a combination of BPA and sodium borocaptate (BSH). Ono *et al* (8) reported that accumulation of BPA occurred in cycling tumor cells but not in quiescent cells of solid tumors, and combination with BSH was one of the solutions for the problem of heterogeneous micro-distribution of BPA (10,11). Further, Ono *et al* (8) considered that the new BPA injection and neutron irradiation protocol was able to overcome the difficulties of BPA. In the present study, we used the same new BPA injection and neutron irradiation protocol, and good results were obtained. However, the number of cases was quite limited; thus, further investigation is required to determine the utility of this new BPA protocol for oral cancer. Other boron compound infusion methods for treatment of brain tumors including long-term infusion of BPA (12) and intracarotid injection of compounds (13) have been reported. Nevertheless, the indications for those methods in regard to oral cancer including metastatic neck nodes should also be investigated.

Acknowledgements

This study was supported in part by a Grant-in-Aid for Scientific Research (C) (No.18592224) to M.S. from Japan Society for the Promotion of Science and by a grant from the Takeda Science Foundation to Osaka Medical College.

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VASCULAR ENDOTHELIAL GROWTH FACTOR GENE-TRANSFERRED BONE MARROW STROMAL CELLS ENGINEERED WITH A HERPES SIMPLEX VIRUS TYPE 1 VECTOR CAN IMPROVE NEUROLOGICAL DEFICITS AND REDUCE INFARCTION VOLUME IN RAT BRAIN ISCHEMIA

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Received, October 3, 2006.
Accepted, April 14, 2007.

OBJECTIVE: Several reports recently suggested that vascular endothelial growth factor (VEGF) may have a therapeutic benefit against experimental cerebral infarction animal models. In addition, bone marrow stromal cells (BMSCs) are known to have therapeutic potency in improving neurological deficits after occlusive cerebrovascular diseases. In the present study, we evaluated the hypothesis that intracerebral transplantation of VEGF gene-transferred BMSCs could provide a greater therapeutic effect than intracerebral transplantation of native (non-gene-transformed) BMSCs by using a transient middle cerebral artery occlusion (MCAO) rat model.

METHODS: Adult Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) were anesthetized. VEGF gene-transferred BMSCs engineered with a replication-deficient herpes simplex virus type 1 1764/4-/pR19-hVEGF165 vector, native BMSCs, or phosphate-buffered saline were administered intracerebrally 24 hours after transient MCAO. All animals underwent behavioral testing for 28 days, and the infarction volume was determined 14 days after MCAO. The brain water contents in the ipsilateral and contralateral hemispheres of the MCAO were measured 2 and 7 days after the MCAO. Fourteen days after MCAO, immunohistochemical staining for VEGF was performed.

RESULTS: The group receiving VEGF-modified BMSCs demonstrated significant functional recovery compared with those receiving native BMSCs. Fourteen days after the MCAO, there was a significantly lower infarct volume without aggravating cerebral edema in the group treated with VEGF gene-modified BMSCs compared with the control groups. The transplanted VEGF gene-modified BMSCs strongly expressed VEGF protein for at least 14 days.

CONCLUSION: Our data suggest that the intracerebral transplantation of VEGF gene-transferred BMSCs may provide a more potent autologous cell transplantation therapy for stroke than the transplantation of native BMSCs alone.

KEY WORDS: Bone marrow stromal cells, Cerebral infarct, Replication-deficient herpes simplex virus type 1 vector, Vascular endothelial growth factor

Neurosurgery 61:586–595, 2007 DOI: 10.1227/01.NEU.0000280010.56943.37 www.neurosurgery-online.com

Many previous studies showed that intracerebral or intravenous transplantation of bone marrow stromal cells (BMSCs), which have the ability to produce many kinds of important growth factors and cytokines, including fibroblast growth factor (FGF)-2, nerve growth factor, hepatocyte growth factor (HGF), and brain-derived neurotrophic factor (7), can improve neurological function after ischemic stroke (1, 4–6, 11, 16, 17, 19, 30, 34, 35). Therefore, BMSCs, including the primitive pluripotent mesenchymal stem cells, are thought to be a source for autoplastic therapy. If we could enhance the therapeutic

potential of BMSCs by ex vivo gene transfer of specific neurotrophic growth factors, such modified BMSCs might become even more promising tools for stroke treatment. In fact, the autologous transplantation of BMSCs modified with specific growth factor genes that have potential neuroprotective effects could help to improve the neurological function after ischemic cerebral stroke, as previously reported (12, 36).

It has been suggested that specific kinds of growth factors can ameliorate the neurological deficits caused by various neurological disorders (13, 25, 26, 29). Vascular endothelial growth factor (VEGF) is a strong angiogenic peptide with potent neurotrophic function in cerebrovascular diseases (14, 18) that also has a potential vascular permeability function (9, 10). Currently, the action of VEGF against acute ischemic disorders is considered a double-edged sword (15, 31, 35). Despite its neurotrophic function, application of this cytokine in cases of acute ischemic stroke is still controversial because of its vascular permeability-increasing effects, which can aggravate peri-infarct edema. In this study, we investigated whether or not the ex vivo gene modification of BMSCs with the VEGF gene could help to achieve greater therapeutic effects against acute cerebral infarction.

MATERIALS AND METHODS

Replication-deficient Herpes Simplex Virus Type 1 Vector Expressing the Human VEGF165 Gene

We prepared a replication-deficient herpes simplex virus type 1 (HSV-1) vector expressing human VEGF165 (HSV-1 1764/4-/pR19/hVEGF165), which originated from HSV-1 strain 17 and was made replication-incompetent by the deletion of the ICP4 and ICP34.5 genes and the inactivation of the VP16 gene (8, 20, 24). This vector contained the pR19 expression cassette driving a gene of interest under the control of fusion promoters, latency-associated transcript promoters, cytomegalovirus enhancers, and woodchuck posttranscriptional regulatory elements, as shown in *Figure 1A*.

Donor BMSC Preparation

We harvested donor BMSCs from 8-week-old Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) as described previously (6, 12, 36). The bilateral femurs and tibias were sterilely dissected, and their bone marrow was extruded into a flask with the culture medium (Minimum Essential Medium with 10% fetal bovine serum, 1% antibiotic-antimycotic [GIBCO Invitrogen, Carlsbad, CA], and 2 mmol/L L-glutamine), and cultured at 37°C, 98% humidity, and 5% CO_2. After 48 hours, the nonadherent cells were removed by replacing the medium. When the cells had grown to confluence, they were lifted by 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid in phosphate-buffered saline (PBS). The cells were passed five to seven times and were used in all procedures.

In Vitro Study

Human VEGF165 Secretion into the Culture Supernatant from VEGF Gene-enhanced BMSCs

The following eight experimental groups (n = 4 for each group) were used: Group 1, only culture medium (without BMSCs); Group 2, BMSCs without HSV-1 vector infection incubated in culture medium; and Groups 3, 4, 5, 6, 7, and 8: BMSCs infected with HSV-1 1764/4-/pR19/VEGF at multiplicities of infection of 0.1, 1, 2, 5, 10, and 20, respectively, in PBS-1% glucose for 1 hour and then incubated in the culture medium. Twenty-four hours after HSV-1 vector infection, we determined the VEGF concentration in the culture supernatant with an enzyme-linked immunosorbent assay (Endogen Human VEGF ELISA Kit; Pierce Biotechnology, Inc., Rockford, IL). Absorbency was measured with a microplate reader (450 nm; NALGEN-NUNC Immunoreader NJ-2001; Nalgen-Nunc, Rochester, NY) as previously described (12, 36).

In Vivo Study

Transient Middle Cerebral Artery Occlusion Model

All procedures and virus inoculates were approved by the Committees of Recombinant Deoyribonucleic Acid and Animal Experiments, Osaka Medical College. Eight-week-old male Wistar rats weighing 250 to 280 g (Japan SLC, Inc.) were anesthetized initially with 3.5% halothane and maintained with 1.0 to 2.0% halothane in 70% N2O and 30% O_2 using a face mask. Rectal temperature was maintained at 37°C throughout the surgical procedure. We induced transient middle cerebral artery occlusion (MCAO) using a previously described method of intraluminal vascular occlusion with minor modifications (12, 21, 32, 36). Two hours after MCAO, the animals were reanesthetized using the same procedure as for MCAO, and reperfusion was performed by withdrawing the suture; then, the end of the external carotid artery was tied. After recovery from anesthesia, the animals were allowed free access to food and water.

Experimental Groups: Intracerebral Transplantation of Donors

Twenty-four hours after MCAO, the animals were reanesthetized with halothane and maintained with intraperitoneal pentobarbital (10 μg/g body weight) administration, and then fixed with a stereotactic head holder (Model 900; David Kopf Instruments, Tujunga, CA). Donor cells were stereotactically transplanted by inserting a 26-gauge needle with a Hamilton syringe into the ipsilateral striatum of the MCAO, as previously described (12, 36). There were 1 x 106 cells in the total 10-μL fluid volume that was transplanted into each animal over a 10-minute period. The experimental groups included Group 1 (sham-operated group), consisting of rats given MCAO without cell administration but with 10 μL of PBS; Group 2 (native BMSC-treated group), consisting of rats given BMSCs without gene transfer (1 x 106 cells); and Group 3 (VEGF-modified, BMSC-treated group), consisting of rats given BMSCs (1 x 106 cells) into which the VEGF gene had been transferred by HSV-1 1764/4-/pR19/hVEGF165.

Ex Vivo Gene Transfer to BMSCs

BMSCs were prepared and passaged five to seven times. Twenty-four hours before the stereotactic transplantation, the VEGF gene was transferred to the BMSCs with HSV-1 1764/4-/pR19/hVEGF165 at a multiplicity of infection of five. Untreated BMSCs and VEGF-modified BMSCs were harvested just before the transplantation and diluted to 1 x 106 cells/10 μL of PBS, as previously described (12, 36).

Behavioral Testing

All animals (n = 5 for each group) underwent behavioral tests before MCAO and 1, 3, 7, 14, 21, and 28 days after MCAO. We used the modified neurological severity score (*Table 1*), which was described by Schallert et al. (27) to grade the aspects of neurological function. The

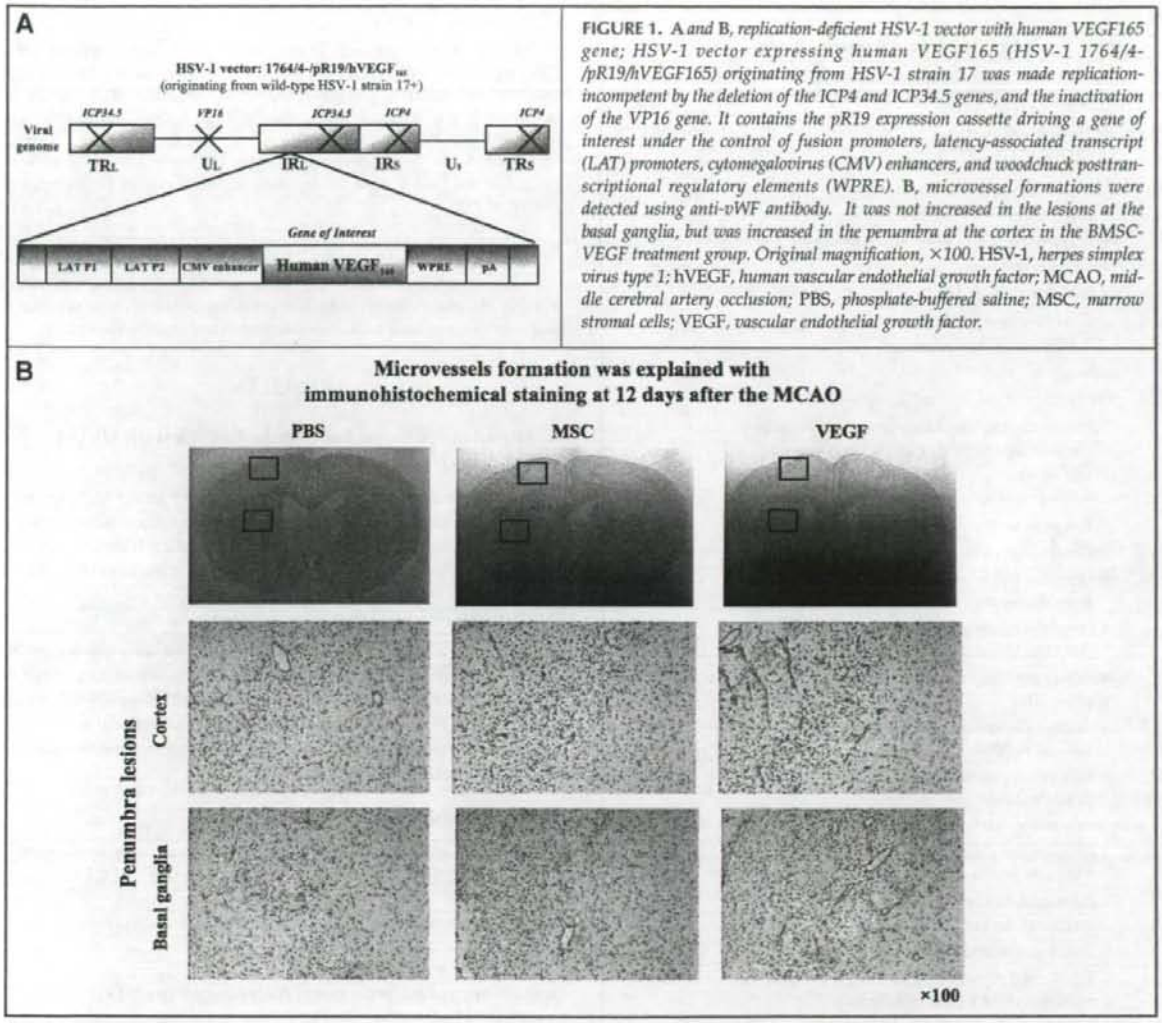

FIGURE 1. *A and B, replication-deficient HSV-1 vector with human VEGF165 gene; HSV-1 vector expressing human VEGF165 (HSV-1 1764/4-/pR19/hVEGF165) originating from HSV-1 strain 17 was made replication-incompetent by the deletion of the ICP4 and ICP34.5 genes, and the inactivation of the VP16 gene. It contains the pR19 expression cassette driving a gene of interest under the control of fusion promoters, latency-associated transcript (LAT) promoters, cytomegalovirus (CMV) enhancers, and woodchuck posttranscriptional regulatory elements (WPRE). B, microvessel formations were detected using anti-vWF antibody. It was not increased in the lesions at the basal ganglia, but was increased in the penumbra at the cortex in the BMSC-VEGF treatment group. Original magnification, ×100.* HSV-1, *herpes simplex virus type 1;* hVEGF, *human vascular endothelial growth factor;* MCAO, *middle cerebral artery occlusion;* PBS, *phosphate-buffered saline;* MSC, *marrow stromal cells;* VEGF, *vascular endothelial growth factor.*

tests used to determine the modified neurological severity score included motor (muscle status, abnormal movement), sensory (visual, tactile, and proprioceptive), and reflex tests. The higher the score, the more severe the neurological deficit.

Infarction Volume

Fourteen days after the MCAO, rats of all groups (n = 5 for each group) were deeply anesthetized with halothane. Then, transcardiac perfusion was performed with saline. The brain was immediately removed and sliced into seven equally spaced (2 mm) coronal sections using a rodent brain matrix. These sections were soaked in a 2% solution of 2-3-5-triphenyltetrazolium (Wako Pure Chemical Industries, Osaka, Japan) for 30 minutes at 37°C. The infarct area was defined as the area not stained with 2-3-5-triphenyltetrazolium, as described previously (2, 16). The lesion volume was calculated as the ratio of the volume of the lesion to the volume of the contralateral hemisphere.

Identification of the Transplanted BMSCs and Immunohistochemical Assessment for hVEGF165

Before transplantation, the BMSCs were incubated with culture medium containing 1 μg/mL bisbenzimide (Hoechst 33258; Polysciences, Eppelheim, Germany) over 24 hours to label the nuclei fluorescently. Twenty-four hours after MCAO, the rats were treated with PBS or fluorescence-marked native or VEGF-modified BMSCs as described above. Fourteen days after MCAO, the rats were deeply anesthetized with halothane and transcardially perfused with normal saline, followed by 4% paraformaldehyde. Then, the brains were removed and incubated overnight in 20% sucrose at 4°C. The samples were immediately frozen by liquid nitrogen, and 6-μm sections were

TABLE 1. Modified neurological severity score[a]

Motor tests	No. of points
Raising the rat by the tail	3
1 Flexion of forelimb	
1 Flexion of hindlimb	
1 Head moved more than 10 degrees to the vertical axis within 30 s	
Walking on the floor (normal, 0; maximum, 3)	3
0 Normal walk	
1 Inability to walk straight	
2 Circling toward the paretic side	
3 Fall down to the paretic side	
Sensory tests	2
1 Placing test (visual and tactile test)	
2 Proprioceptive test (deep sensation, pushing the paw against the table edge to stimulate limb muscles)	
Beam balance tests (normal, 0; maximum, 6)	6
0 Balances with steady posture	
1 Grasps side of beam	
2 Hugs the beam and one limb falls down from the beam	
3 Hugs the beam and two limbs fall down from the beam or spins on beam (<60 s)	
4 Attempts to balance on the beam but falls off (<40 s)	
5 Attempts to balance on the beam but falls off (<20 s)	
6 Falls off, no attempt to balance of hang onto the beam	
Reflexes absent and abnormal movements	4
1 Pinna reflex (head shake when touching the auditory meatus)	
1 Corneal reflex (eye blink when lightly touching the cornea with cotton)	
1 Startle reflex (motor response to a brief noise from snapping a clipboard and paper)	
1 Seizures, myoclonus, myodystony	
Maximum no. of points	18

[a] One point was awarded for the inability to perform the tasks or for the lack of a tested reflex. 1 to 6 indicates mild injury, 7 to 12 indicates moderate injury, and 13 to 18 denotes severe injury.

prepared with a cryostat for immunohistochemical staining. After permeabilization with 0.02% Triton X and blocking in 3% bovine serum albumin, these sections were treated with the mouse monoclonal antibody against hVEGF165 (Chemicon International, Inc., Temecula, CA) for 120 minutes at 4°C. Subsequently, the sections were washed with PBS, and fluorescein rhodamine-conjugated donkey secondary antibody against mouse immunoglobulin-G (Cosmo Bio, Ltd., Tokyo, Japan) was added, followed by incubation for 20 minutes at 4°C. The prepared sections were observed with a fluorescent microscope (Olympus AX70; Olympus Corp., Tokyo, Japan).

Brain Water Content

The rats (n = 4 for each group) were deeply anesthetized with halothane 2 and 7 days after MCAO, and the brains were removed and dissected immediately. The whole brain was separated into an ischemic hemisphere and a nonischemic hemisphere. Subsequently, the wet weight of each side was rapidly measured, and the brain was dried at 95°C for 24 hours to obtain the dry weight. The brain water content was calculated as (wet weight − dry weight)/dry weight, as previously described (11).

Statistical Analysis

Data are expressed as the means ± standard deviation. One-way analysis of variance followed by Bonferroni's post hoc analysis was used, and P values less than 0.05 were considered statistically significant.

RESULTS

In Vitro Study: Gene Transfer to BMSCs with HSV-1 Vector and hVEGF165 Secretion

The culture supernatant of hVEGF165 gene-transferred BMSCs was measured by enzyme-linked immunosorbent assay. hVEGF165 production by hVEGF165-modified BMSCs proportionately increased with the multiplicity of infection (*Fig. 2A*).

Neurological Outcome

Between 14 and 28 days after MCAO, the rats treated with native BMSCs or VEGF-modified BMSCs showed significant improvements in neurological function compared with PBS-treated rats ($P < 0.05$). In addition, the VEGF-modified BMSC group displayed more remarkable amelioration than the native BMSC group ($P < 0.05$) (*Fig. 3A*).

Measurement of Infarction Volume

Fourteen days after MCAO, the infarction volume was significantly less in the rats receiving BMSCs with the hVEGF165 gene (28.42 ± 2.65%) than in either the PBS group (38.95 ± 3.47%) or the native BMSC group (35.14 ± 2.66%; $P < 0.05$) (*Fig. 4*).

Identification of the Transplanted BMSCs and Immunohistochemical Assessment for VEGF

Fourteen days after MCAO, some Hoechst-positive cells were observed around the transplantation track, but no Hoechst-positive cells had migrated into the remote site from transplantation points such as the contralateral brain or ipsilateral cortex. VEGF expression by transplanted donor cells was detected with immunohistochemical staining at 14 days after MCAO at the region around the injection point. Some Hoechst-marked cells stained weakly for VEGF in the native BMSC-treated rats, whereas the Hoechst-marked cells stained strongly for VEGF in the VEGF-modified, BMSC-treated rats (*Fig. 5*).

Brain Water Content

Brain water content was measured in the three treatment groups 2 and 7 days after MCAO. With respect to the mean level of water content in the ischemic cerebral hemisphere,

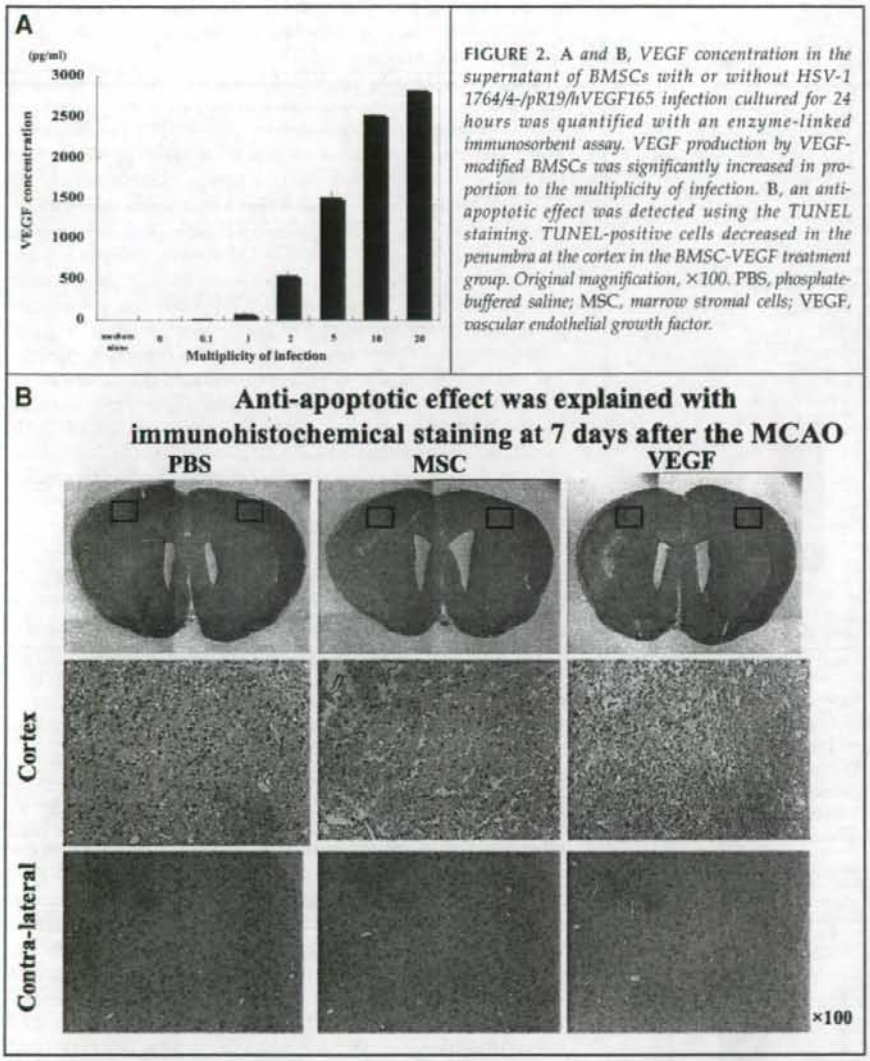

FIGURE 2. A *and* B, *VEGF concentration in the supernatant of BMSCs with or without HSV-1 1764/4-/pR19/hVEGF165 infection cultured for 24 hours was quantified with an enzyme-linked immunosorbent assay. VEGF production by VEGF-modified BMSCs was significantly increased in proportion to the multiplicity of infection.* B, *an anti-apoptotic effect was detected using the TUNEL staining. TUNEL-positive cells decreased in the penumbra at the cortex in the BMSC-VEGF treatment group. Original magnification,* ×*100.* PBS, *phosphate-buffered saline;* MSC, *marrow stromal cells;* VEGF, *vascular endothelial growth factor.*

there was no significant difference among the three groups at either analysis point (*Fig. 6*).

DISCUSSION

Recent animal studies have strongly suggested that cell transplantation therapy can be a promising therapeutic option for patients with ischemic stroke. Of the several kinds of cell candidates, including adult neural stem cells (NSCs), embryonic stem cells, and embryonic neural cells, BMSCs are by far the most accessible. Furthermore, their use is not associated with ethical or immunological problems. In fact, because many reports have insisted that only native BMSC transplantation could improve neurological function after cerebral ischemic stroke (1, 4–6, 16, 19), BMSCs have become one of the major cellular candidates for such treatments. Bone marrow mesenchymal stem cells have the potential to differentiate into neural cells (22, 33), and BMSCs can produce many kinds of important growth factors and cytokines, including FGF-2, nerve growth factor, HGF, brain-derived neurotrophic factor, and VEGF (7). Thus, BMSCs may provide important cues for neuron survival in the damaged brain, with or without direct commitment to brain tissue repair. With regard to the possible mechanisms for their therapeutic effect, it has been reported that BMSCs have the ability to differentiate into other cell types (22, 33) and have a potent ability to secrete multiple cytokines, including VEGF, in ischemic circumstances (7). Therefore, we believe that BMSCs may be useful vehicles for autotransplantation in both cell and gene therapy for ischemic cerebral stroke. In addition, many researchers have tried to deliver genes of interest directly into the brain. For example, Kurozumi et al. (16) reported the efficacy of direct gene therapy for a rat MCAO model using a glial cell line-derived neutrophilic factor-expressing fiber-mutant adenovirus vector.

In the present study, with the intent of enhancing the therapeutic potential of BMSCs, we prepared gene-modified BMSCs that strongly expressed VEGF using replication-deficient HSV-1 vectors. In a preliminary study, we confirmed that BMSCs could be efficiently gene-transferred by this HSV-1 vector with little cytotoxicity; their intracerebral transplantation did not result in greater immune reactions than transplantation of native BMSCs (data not shown). Just as we had intended, both BMSCs transferred with the HGF gene and those transferred with the FGF-2 gene using this vector seem to have achieved impressive thera-

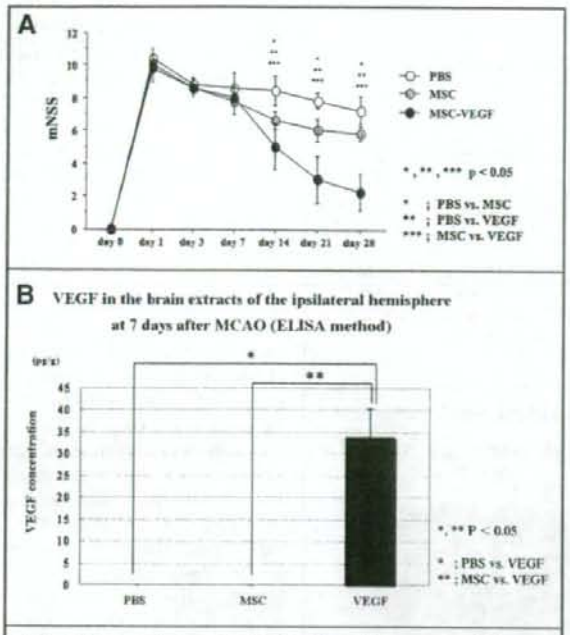

FIGURE 3. A *and* B, *neurological function of the experimental groups after MCAO determined by the modified neurological severity score. Significant neurological functional recovery was observed at 14, 21, and 28 days after MCAO in the rats treated with VEGF-modified BMSCs compared with the rats treated with native BMSCs alone ($P < 0.05$).* B, *VEGF concentration acquired from brain tissue at Day 7 was quantified with an enzyme-linked immunosorbent assay. VEGF concentration of the ipsilateral hemispheres in the BMSC-VEGF treatment group was significantly higher than that in the other groups ($P < 0.05$).*

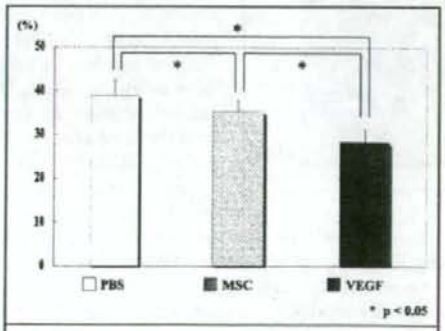

FIGURE 4. *Bar graph showing the ratio of the infarction volume of the lesion to the volume of the contralateral hemisphere detected with 2-3-5-triphenyltetrazolium staining at 14 days after the MCAO. There was a significantly lower infarction volume in the VEGF-modified, BMSC-treated group than in the other groups* (Asterisk, $P < 0.05$).

peutic effects in a rat stroke model. Therefore, our novel therapeutic strategy may be more beneficial than either direct gene therapy or cell therapy alone (12, 36).

It is widely known that VEGF is induced in hypoxic brains that have had an ischemic stroke and may play important roles in the remodeling of the damaged tissue. VEGF has two important biological properties. One is a strong angiogenic peptide (14, 18), and the other is a vascular permeability factor (9, 10). Many researchers have reported that VEGF has a strong therapeutic potency for ischemic stroke (11, 30, 34, 35). However, there is concern that VEGF increases blood-brain barrier leakage and might, as a consequence, induce a vasogenic brain edema in the acute stage of ischemic stroke (35). Indeed, some reports insist that the inhibition of VEGF in the super-acute stage of stroke attenuates brain edema and reduces infarction volume. For instance, Kimura et al. (15) demonstrated that inhibition of VEGF immediately after venous infarction attenuates vascular permeability and reduces cerebral venous infarction. Their results suggested that VEGF has a malignant influence on infarcts, especially in the super-acute stage. On the other hand, Sun et al. (30) indicated that VEGF reduced infarct size, improved neurological performance, enhanced the delayed survival of newborn neurons in the dentate gyrus and subventricular zone, and stimulated angiogenesis in the striatal ischemic penumbra. Shen et al. (28) also showed the efficacy of VEGF gene therapy in a rat cerebral ischemia model by using the adeno-associated virus vector with a hypoxia-responsive element controlling VEGF gene expression. The controlled overexpression of VEGF achieved a significant reduction of neuronal apoptosis and infarct volume. In the present study, we also observed an immunohistochemical increase in the formation of microvessels and an antiapoptotic effect in the cortex penumbra, especially in the VEGF-modified, BMSC-treated group (*Figs 1B and 2B*). We speculate that these beneficial effects may have been related to the surprising reduction of infarct volumes. On the other hand, some researchers revealed that although VEGF administration produced angiogenic effects, it did not increase cerebral blood flow (11, 34). Therefore, the therapeutic contribution of VEGF-induced angiogenesis to acute stroke remains obscure. The above discrepancy might arise from the fact that VEGF plays different roles in different stages of ischemic stroke. That is to say, the action of VEGF in patients with stroke is considered a double-edged sword, with the cytokine demonstrating both desirable and unfavorable activity.

Zhang et al. (35) demonstrated that administration of VEGF to ischemic rats in the late stage (48 hr after ischemic stroke) enhanced angiogenesis in the ischemic penumbra and significantly improved neurological recovery. On the other hand, administration of VEGF to ischemic rats in the early post ischemic stage (1 hr after ischemic stroke) significantly increased blood-brain barrier leakage, hemorrhagic transformation, and ischemic lesions. These data indicated that VEGF can markedly enhance angiogenesis in the ischemic brain and reduce neurological deficits during stroke recovery, and that inhibition of VEGF at the super-acute stage of stroke may reduce blood-brain barrier permeability and the risk of

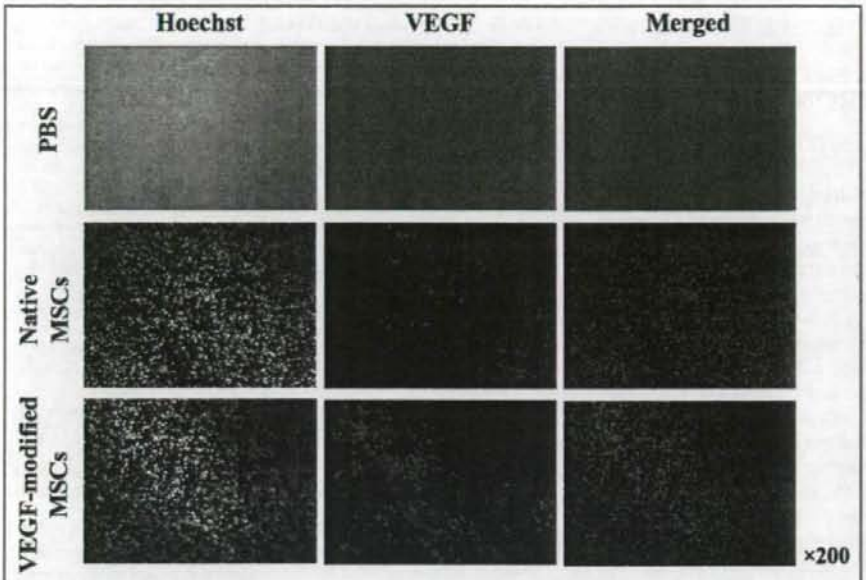

FIGURE 5. *VEGF production by transplanted BMSCs was detected by immunohistochemical staining 14 days after the MCAO. Some Hoechst 33258-marked transplanted BMSCs expressed weak VEGF production in the native BMSC-treated rat brains. On the other hand, VEGF-modified BMSCs expressed VEGF more abundantly. Original magnification, ×200.* VEGF, *vascular endothelial growth factor;* PBS, *phosphate-buffered saline;* MSC, *marrow stromal cells.*

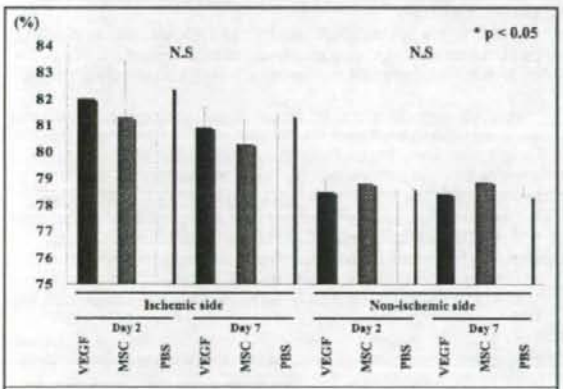

FIGURE 6. *Bar graph showing the brain water content in the three treatment groups 2 and 7 days after MCAO. There was not a significant difference among the three groups at either analysis point ($P < 0.05$).*

hemorrhagic events after focal cerebral ischemia. Thus, if we can determine the optimal timing, artificial enhancement of VEGF in the hypoxia-damaged brain may achieve desirable advantages without inducing adverse effects.

In our study, we performed therapeutic transplantation 24 hours after MCAO, and the VEGF gene-modified BMSC-treated group showed significantly higher therapeutic gain without any aggravation of brain edema in comparison with the control groups. We have no definitive explanation for why brain edema deterioration did not occur under our study conditions. One possibility is the participation of other cytokines. Cao et al. (3) showed that FGF-2 and VEGF induced significant angiogenic effects and that although VEGF-induced capillaries demonstrated numerous fenestrations, no fenestrations were detected in capillaries induced by FGF-2. Murohara et al. (23) demonstrated that VEGF, but not FGF-2 or HGF, significantly increased vascular permeability. BMSCs can also secrete FGF-2 and HGF, and it is probable that the combination of these cytokines can modify the VEGF function against the vessel permeability. Zhu et al. (37) showed that the direct transplantation of VEGF gene-transfected NSCs could confer sufficient neuroprotection against transient focal cerebral ischemia in rats. Their results demonstrated that the transplantation of nontransfected NSCs into the ischemic rat brain also improved the neurological symptoms in comparison with PBS-injected and sham-operated groups. The improvement of neurological symptoms occurred earlier in the VEGF gene-transfected NSC group than in the nontransfected NSC group. Their data suggested the possibility that overexpression of VEGF in the ischemic brain from gene-modified cells could accelerate the neurological recovery after stroke.

The therapeutic efficacy of BMSC therapy against brain ischemia has been clarified by several studies that used different methods of implantation (i.e., intracerebral, intra-arterial, or intravenous). We believe that intravenous administration of BMSCs is a less invasive method than direct transplantation. In our preliminary study, however, only approximately 3% of BMSCs successfully migrated into the ischemia-lesioned brain after intravenous injection. Most of them were trapped by other organs, including the liver, spleen, and lungs (data not shown). Under such conditions, the serum VEGF level would also increase and systemic edema might occur, as in Crow-Fukase syndrome. Thus, in the present study, we chose direct grafting of BMSCs to an ischemic lesioned brain because the purpose of this study was to clarify the impact of VEGF gene modification on BMSC therapy. Of course, if the delivery of BMSCs to the brain could be improved by some technical advance in the

future, the present method could be applied to the clinical treatment of patients with stroke.

CONCLUSION

In view of the accumulated data over the past several years, it is clear that the therapeutic benefit of BMSC transplantation against stroke may derive from multiple mechanisms, including providing focal growth factors with neuroprotective effects, enhancing angiogenesis, and providing for new neurons, or "exogenous neurogenesis," from marrow stromal stem cells. Although clinical trials for acute ischemic stroke with allograft bone marrow mesenchymal stem cells have already been initiated in Japan, the development of methods for improving cell grafting, intensifying its therapeutic potential, or evaluating subpopulations with specific properties, as well as clinical trials, will be required in the future to assess and improve the efficacy of this treatment. We think that the therapeutic modification of BMSCs using a gene-engineering method might be one way to realize the above requirements. In the present study, we proposed a gene-engineering strategy with replication-incompetent HSV-VEGF to treat acute stroke. In fact, 24 hours after the rat MCAO, the intracerebral transplantation of hVEGF165 gene-enhanced BMSCs contributed to remarkable functional recovery and infarct reduction without the exacerbation of acute brain edema compared with that of native BMSCs or administration of PBS. Although it is thought that VEGF is a double-edged sword against the ischemic brain, further examination of the mechanisms of this autoplastic gene-engineered cell therapy is suggested.

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Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research (B) (14370448) and (C) (12671353), and by a Grant-in-Aid for Exploratory Research (14657350) from the Japanese Ministry of Education, Science and Culture to Shin-Ichi Miyatake, M.D., Ph.D. Additional support was provided in the form of a grant from the Promotion and Mutual Aid Corporation for Private Schools of Japan and the Science Research Promotion Fund to Shin-Ichi Miyatake, M.D., Ph.D., and by the High-Tech Research Program of Osaka Medical College. This work was also supported in part by a Grant-in-Aid 17790989 from the Ministry of Education, Science and Culture, Japan, to Naosuke Nonoguchi, M.D.

We thank Daisuke Furutama, M.D., Ph.D., First Department of Internal Medicine, Osaka Medical College, for provision of hVEGF165 complementary deoxyribonucleic acid; Mari Dezawa, M.D., Ph.D., Department of Anatomy and Neurobiology, Kyoto University Graduate School of Medicine, for careful guidance in BMSC issues; and Ming-Zhu Zhao, M.D., Ph.D., Department of Neurosurgery, Osaka Medical College, for constant support.

COMMENTS

This report represents another study showing the value of cellular repair for ischemic stroke. In this experiment, the authors hypothesized that vascular endothelial growth factor (VEGF) producing cells would have better results than nonproducing marrow stromal cells (MSCs). The study was well designed but is limited by a rather simplistic method to measure infarct volume (magnetic resonance imaging was not used) and to assess animal function. Approximately 10% of transplanted cells appeared to survive in the authors' early analysis, which is in line with several other reports using different cell lines.

Certainly, animal models can be of value, but many questions remain to be addressed. These could include a study of the timing of cell delivery after stroke, cell number, use of additional compounds to promote graft viability, delivery method, or location. Other questions will require human clinical trials. I applaud the authors on their fine work.

Douglas Kondziolka
Pittsburgh, Pennsylvania

There has been increasing experimental evidence that cellular transplantation therapy may play a role in the treatment of ischemic stroke. Recently, bone MSCs have shown particular promise in animal models of ischemic strokes. The mechanism by which the beneficial therapeutic effect is exerted is still unclear, with secretion of various cytokines and differentiation potential being important. In previous work, the authors established that MSCs can be efficiently engineered to secrete cytokines at elevated levels by gene-transfer with herpes simplex virus type 1 vector. The engineered MSCs, when transplanted in a rat transient ischemia model, can secrete human growth factor and fibroblast growth factor-2 at elevated levels with beneficial effect. In this work, the authors extend their consideration to VEGF in a similar model. As the authors point out, VEGF has a potential downside in treating ischemic stroke due to its role in increasing vascular permeability and, thus, cerebral edema. This work demonstrates that MSCs can be engineered to secrete VEGF at increased levels, and that cellular transplantation therapy in the rat transient ischemia model improves outcome as determined by behavioral testing. Importantly, there was no significant increase in cerebral edema between the experimental animals and control animals receiving native MSCs. It is unclear why this is so. As the authors suggest, it may be the timing of the "treatment" at 24 hours that makes the difference. It would be interesting to see if this bears out in future experiments. It may also be that the other cytokines secreted by the MSCs ameliorates vascular permeability effect of VEGF. Although not statistically significant, it does appear that the animals treated with native MSC have less cerebral edema compared with those control animals receiving the phosphate-buffered saline injections. As with other work showing beneficial effects of cellular transplantation therapy, there remain many questions about the mechanism of action of the therapeutic intervention. Nevertheless, this work provides further optimism that this approach may ultimately be useful in the treatment of ischemic stroke. The fact that MSCs can be harvested easily with little ethical debate lends further enthusiasm.

Charles Y. Liu
Los Angeles, California

Decades of promising laboratory research has translated into failed clinical trials of neuroprotective or neuroregenerative therapy for acute ischemic stroke. It is hoped that stem cell therapy will succeed where other approaches have not. Previous studies have suggested that the important effect of stem cell transplantation is in VEGF expression as penumbral salvage of infarcted tissue has been demonstrated despite the failure of graft survival. The authors' experiment specifically addresses whether or not additional infarct survival results with enhancement of VEGF expression. They achieved a significant improvement in functional neurological recovery and infarct volume that seemed to be a graded response with VEGF-enhanced MSCs outperforming unenhanced MSCs.

As MSC therapy is already making its way into human clinical trials, it is critical that we have a better understanding of the potential mechanism of action and optimum timing of such therapy. To make the translational leap, laboratory experiments should parallel the typical

clinical scenario of MCA occlusion and major hemispheric stroke and be supported by serial neuroimaging, particularly with diffusion-weighted magnetic resonance imaging scans. Although the authors' conclusions are perhaps more hopeful than the data supports, their work provides another essential piece of the puzzle.

Cathy Sila
Cleveland, Ohio

Miki et al. have examined a VEGF expressing bone MSC-based transplant therapy after ischemic stroke in rats. They have utilized the transient MCA occlusion model in rats to test their hypothesis that VEGF expressing bone MSCs are more effective in limiting stroke volume and clinical deterioration after middle cerebral artery ischemia. This group has engineered a VEGF expressing MSC line that they have validated in previous publications as well as this one. These cells are directly transplanted into ischemic brain. Nuclear labeling is used to track cells and confirm that engraftment has occurred. The behavioral assessment of rats is done with a previously validated scale. The results are impressive for the VEGF expressing MSCs versus the MSCs in terms of behavioral improvement post infarct and reduction in stroke volume. There is a marked and significant improvement for the VEGF expressing MSC-treated group beginning at 7 days after infarct versus MSC alone or control. The stroke volume is also significantly reduced. Colabeling demonstrates that the engrafted cells express VEGF. Histology reveals neoangiogenesis in the VEGF-treated group, whereas little to no angiogenesis is demonstrated in the control and non-VEGF groups. The main complication of VEGF administration has been the exacerbation of cerebral edema. The authors demonstrate that this is not an issue in this model by performing water content measures from each group and demonstrating no significant difference. It is not clear why this is the case in this model versus direct VEGF administration. This study uses validated techniques and demonstrates the promising effects of VEGF expressing BMSCs as a tissue engineering approach to stroke protection and recovery.

Douglas J. Cook
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Toronto, Canada

Miki et al. evaluated the hypothesis that intracerebral transplantation of VEGF gene-transferred MSCs could provide therapeutic benefit in a middle cerebral artery occlusion rat model. Results demonstrated that stroke animals receiving VEGF-modified MSCs had significant functional recovery compared with those receiving native MSCs. Moreover, there was a significantly lower infarct volume in the group treated with VEGF gene-modified MSCs compared with the control groups. Proof of principle was confirmed as the transplanted VEGF gene-modified MSCs strongly expressed VEGF protein for at least 14 days, leading the authors to conclude that intracerebral transplantation of VEGF gene-transferred MSCs may be a beneficial avenue for future stroke therapy.

Although only in the early stages, stem cell therapy holds much promise for the development of stroke treatment protocols. By reducing ischemic brain injury in animal models after safe administration at delayed time points, stem cells offer greater utility than currently available agents. Moving forward, caution should be exercised in future studies and eventual clinical trials, as previous failed attempts at translating stroke therapies to the bedside have shown that application of under-elucidated therapies leads to wasted resources and the potential for poor patient outcomes.

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"Oil Factory" (1954), woodblock and paper, copied from a 1773 picture scroll, Taiyo Fishing Company. (Courtesy of New Bedford Whaling Museum). *From:* Matthews, LH: *The Whale*. New York, Simon & Schuster, 1968.

FLUORESCENCE OF NON-NEOPLASTIC, MAGNETIC RESONANCE IMAGING-ENHANCING TISSUE BY 5-AMINOLEVULINIC ACID: CASE REPORT

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Received, January 27, 2007.
Accepted, June 7, 2007.

OBJECTIVE: It has been established that fluorescence-guided resection using 5-aminolevulinic acid is useful in glioma surgery. In this study, we describe three cases in which even perinecrotic tissue could be recognized as fluorescence positive.

METHODS: Three cases of central nervous system disease, showing gadolinium enhancement on magnetic resonance imaging scans, were operated on with the aid of fluorescence derived from 5-aminolevulinic acid. Two of these were diagnosed as radiation necrosis and the other as a neurodegenerative demyelinating disease.

RESULTS: In all cases, at least some parts of the gadolinium-enhanced area could be labeled as fluorescence positive, whereas centers of necrotic tissue were negative for fluorescence. Histologically, cell infiltration was marked in each case that showed fluorescence activity.

CONCLUSION: Both malignant tumors and the perinecrotic area in radiation necrosis or neurodegenerative disease can be labeled as fluorescence positive using 5-aminolevulinic acid.

KEY WORDS: 5- aminolevulinic acid, Neurodegenerative disease, Protoporphyrin IX, Radiation necrosis

Neurosurgery 61:E1101–E1104, 2007 DOI: 10.1227/01.NEU.0000280106.90528.DD www.neurosurgery-online.com

Recent advancements in fluorescence diagnosis technology allow tumors to be easily visualized by a fluorescence guide, especially with the administration of 5-aminolevulinic acid (5-ALA) in esophageal, bronchial, urothelical, epithelial, and bladder tumors (1, 6, 8, 10). 5-ALA has also been used clinically in the removal of malignant gliomas because of its high tumor specificity and safety (13–16). It actively accumulates in the neoplasm and is converted to the fluorescent substance, protoporphyrin IX (PpIX) (12, 15). It is reported in the literature that with the systemic administration of 5-ALA in animal models, this compound is delivered from the blood to the cerebrospinal fluid via the choroid plexus (2). PpIX accumulates only in limited areas in the normal brain, including the choroid plexus (11). With rare exceptions such as those mentioned in this study, PpIX fluorescence has been thought to be restricted to tumor tissues, with the fluorescence intensity correlating to tumor cell density (13). For the last couple years, we have routinely applied fluorescence-guided surgery in the removal of malignant gliomas. We experienced that even nontumor tissue showed vague and sometimes strong fluorescence of PpIX with the administration of 5-ALA. We present three cases, two of radiation necrosis and one of a neurodegenerative disease, in which tissues showed fluorescence activity with 5-ALA administration and in which no tumor cells were identified in a surgical specimen.

Illustrative Cases

Patient 1

A 67-year old woman had a left parietal glioblastoma (*Fig. 1A*). During the operation for this tumor, we used 5-ALA and the Karl Storz D-light system (Karl Storz, Tuttlingen, Germany) for excitation to identify the tumor (*Fig. 2C*). The tumor could be labeled very clearly by strong red fluorescence. The tumor was removed completely, and the patient received boron neutron capture therapy (BNCT) (5, 9). An additional 20 Gy was applied by fractionated external x-ray irradiation for a shortage in the dose absorbed by the tumor tissue, especially in the deep part of the mass. No tumor recurrence was observed on magnetic resonance imaging (MRI) scans and no

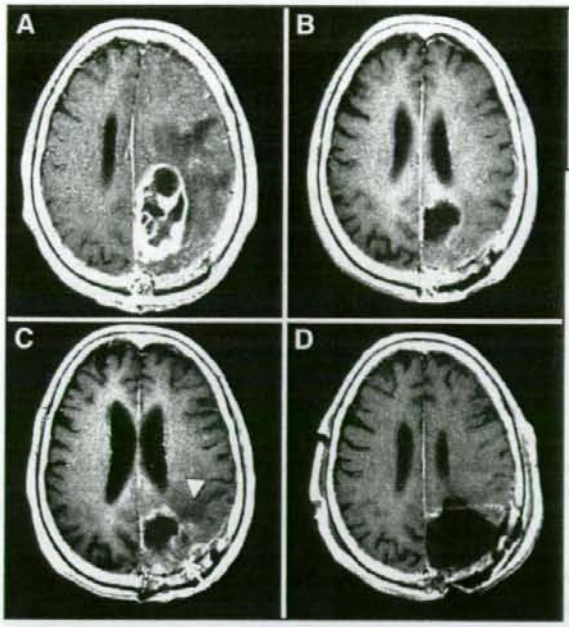

FIGURE 1. *Serial Gd-enhanced T1-weighted MRI scan in Patient 1.* **A,** *just before the second operation.* **B,** *20 months after BNCT. No enhanced lesion and no perilesional edema were observed.* **C,** *14 months after BNCT.* White arrow *shows the new Gd-enhanced lesion. A* white arrowhead *indicates the perilesional edema, which was not observed in* **B.** **D,** *just after the third operation. The Gd-enhanced lesion was completely resected by the third operation.*

neurological deficits were seen for 13 months after BNCT (*Fig. 1B*). Fourteen months after BNCT, the patient experienced right hemiparesis. An MRI scan revealed a gadolinium (Gd)-enhanced lesion (*Fig. 1C*). Radiation necrosis was suspected, but we could not neglect the possibility of a recurrence of the glioblastoma. We then performed a craniotomy during which the mass was removed *en bloc*. Histological examination of the center of the mass showed complete necrosis (*Fig. 2D*). Bright- and dark-field operative photographs are shown in *Figure 2, A* and *B*. In this operation, OPMI Pentero (Carl Zeiss, Oberkocken, Germany) was used as the operation microscope; a Blue 400 system (Carl Zeiss) equipped on this microscope was used for excitation and detection of the fluorescence of PpIX. Red fluorescence by Blue 400 is relatively weak in comparison with Carl-Stortz D-light or the high power violet laser system described below. Vague fluorescence activity was observed in the resected plane of the mass. A surgical specimen from this vague fluorescence area showed some cell infiltration (*Fig. 2E*). With anti-glial fibrillary acidic protein (GFAP) antibody, some cells were positive for immunoreactivity (data not shown). The pathological diagnosis was radiation necrosis; no apparent tumor cells were found.

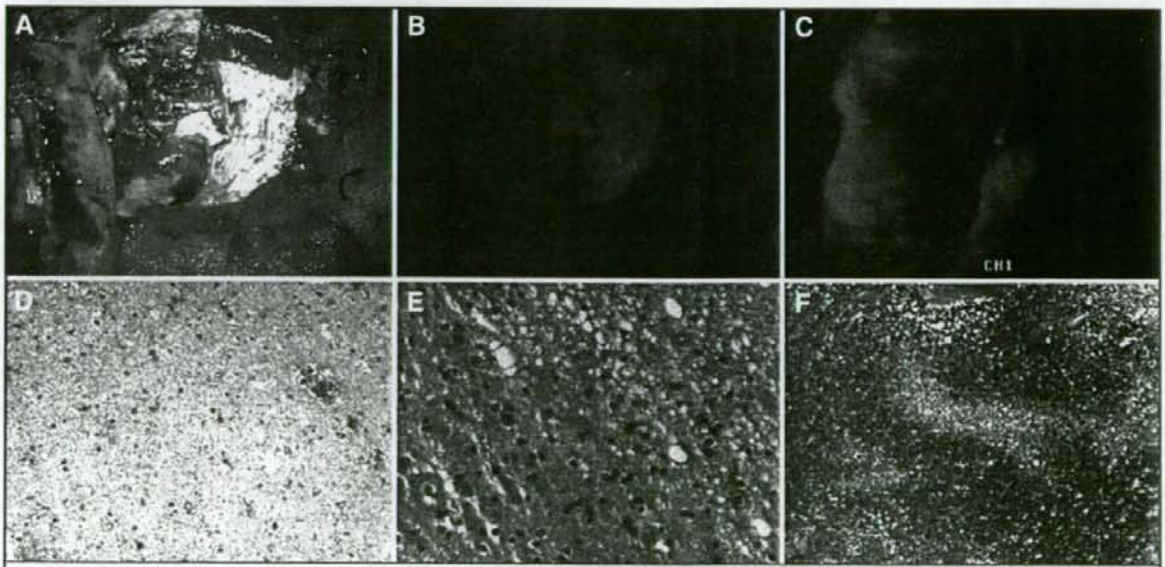

FIGURE 2. *Intraoperative photographs and histopathological findings of Patient 1.* **A,** *a bright-field photograph of the third operation.* **B,** *a dark-field photograph with the Blue 400 system that is in the same field as that shown in* **A.** *Vague but definitive fluorescence activity could be observed in the resection plane.* **C,** *a dark-field photograph with the excitation of the Carl-Stortz D-light system in the second operation. Strong fluorescence activity could be observed on the surface of the tumor.* **D,** *hematoxylin and eosin (H & E) staining of the core lesion that was negative for fluorescence in the third operation (original magnification, ×5).* **E,** *H & E staining with vague fluorescence in the third operation (original magnification, ×20). Some cells (nontumorous) infiltrated into the brain.* **F,** *H & E staining of the lesion that was strongly positive for fluorescence in the second operation (original magnification, ×5). The histological diagnosis was glioblastoma in the second operation.*

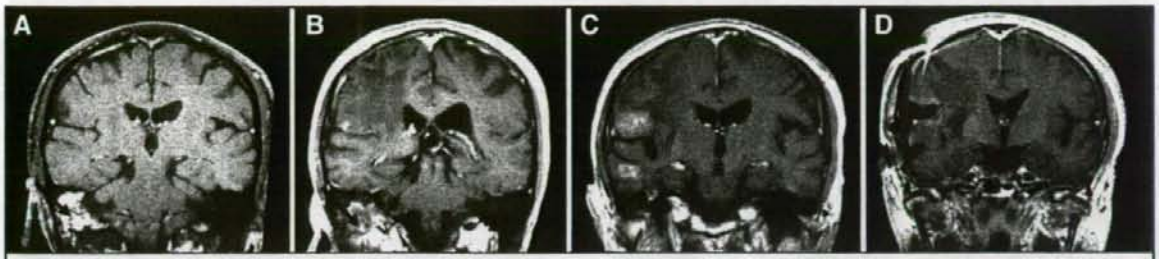

FIGURE 3. *Serial Gd-enhanced T1-weighted coronal MRI scans of Patient 2.* **A**, *MRI scan obtained just before BNCT. The* black arrow *shows the Gd-enhanced, growing tumor.* **B**, *MRI scan obtained 2 years after BNCT. The* black arrow *shows a decrease in the intensity of the Gd-enhanced area. Note that low intensity lesions are recognized in the parietal and temporal regions.* **C**, *MRI scan obtained at the same time as* **B**. *Note the Gd-enhanced lesions that can be identified in the frontal and temporal regions.* **D**, *MRI scan obtained 24 hours after the operation. No apparent enhanced lesion was observed.*

Patient 2

A 79-year-old man underwent operation for a salivary duct carcinoma, followed by 60 Gy x-ray irradiation. A recurrence of the mass was recognized 11 years after the treatment (*Fig. 3A*). He received BNCT for his subtemporal lesion twice within a 1-month interval. Unfortunately, in the second BNCT, the patient lowered his head during the neutron irradiation while in the sitting position. The BNCT was able to control the tumor efficiently during the follow-up period (*Fig. 3B*). Twenty months after the second BNCT, the patient experienced a generalized convulsive seizure with mild left hemiparesis. Gd-enhanced lesions were recognized in the left frontoparietal operculum and the left temporal region independently with perifocal edema (*Fig. 3C*). We diagnosed these enhanced lesions as radiation necrosis, and the patient was treated with the administration of steroids for 1 month. However, no improvement of his hemiparesis resulted, and a slight aggravation of the enhanced lesions was recognized on follow-up images, even after the steroid administration. The patient could not walk alone, and he wanted the lesions removed by craniotomy to improve his hemiparesis. Both lesions were removed via a craniotomy (*Fig. 3D*). In this operation, the Blue 400 system equipped with OPMI Pentero was used for the detection of the fluorescence of PpIX. In the operation for the frontoparietal operculum lesion, we removed the necrotic mass and left the fluorescence-positive area so as not to aggravate the hemiparesis. In removing the temporal lesion, we removed even the faint fluorescence area shown in *Figure 4A* because there was no fear of aggravating the symptoms by this procedure. *Figure 4B* shows the border of the total necrosis and the surrounding tissue where the cell infiltration was marked. Some infiltrative cells were positive for GFAP, whereas others were positive for macrophage marker (KP-1) (*Fig. 4, C and D*). By GFAP immunohistochemistry, a gliotic plane was prominent between the completely necrotic area and normal white matter. A frontoparietal operculum lesion also showed total necrosis with no malignancy (data not shown). After the surgery, the patient's hemiparesis

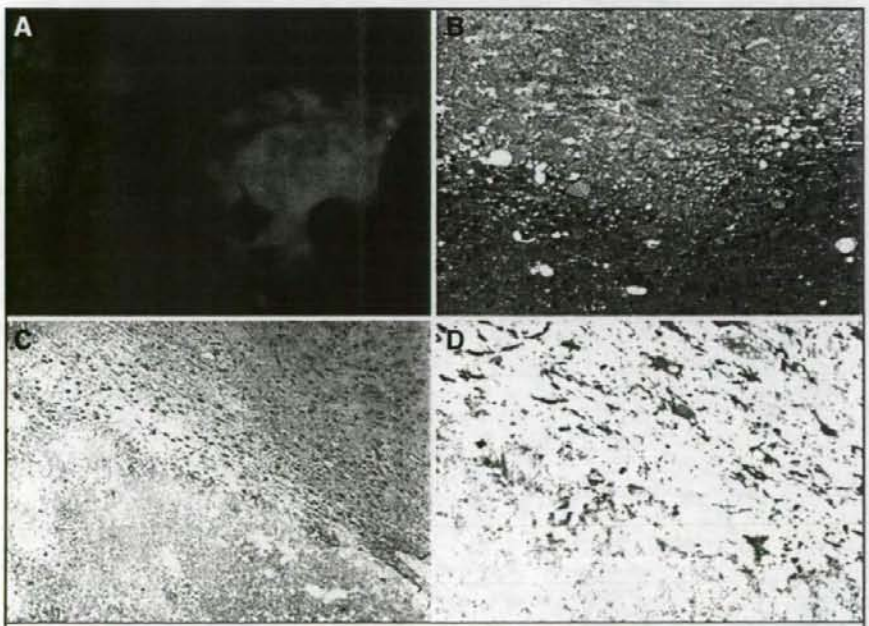

FIGURE 4. *Intraoperative photograph and histopathological findings of Patient 2.* **A**, *a dark-field intraoperative photograph with the Blue 400 system taken during the removal of the temporal lesion. Vague fluorescence activity was identified at the bottom of the enhanced mass. No apparent fluorescence was observed in the core of this lesion.* **B**, *H & E staining of a vague fluorescence-positive area (original magnification, ×10). Total necrosis and cell infiltration in the brain were seen with a relatively distinct border.* **C** *and* **D**, *immunohistochemistry for GFAP (***C***) and KP-1 (***D***) of the same specimen as that shown in* **B** *(original magnification, ×5 [***C***] and ×10 [***D***]).*