Statistical Analysis

The statistical significance of the differences observed was determined by ANOVA (StatView; SAS Institute), and Bonferroni's correction was applied for multiple comparisons.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

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ORIGINAL ARTICLE

Human neural stem cells transduced with IFN-β and cytosine deaminase genes intensify bystander effect in experimental glioma

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Previously, we have shown that the genetically modified human neural stem cells (NSCs) show remarkable migratory and tumor-tropic capability to track down brain tumor cells and deliver therapeutic agents with significant therapeutic benefit. Human NSCs that were retrovirally transduced with cytosine deaminase (CD) gene showed remarkable 'bystander killer effect' on the glioma cells after application of the prodrug, 5-fluorocytosine (5-FC). Interferon-β (IFN-β) is known for its antiproliferative effects in a variety of cancers. In our pilot clinical trial in glioma, the IFN-β gene has shown potent antitumor activity in patients with malignant glioma. In the present study, we sought to examine whether human NSCs genetically modified to express both CD and IFN-β genes intensified antitumor effect on experimental glioma. *In vitro* studies showed that CD/IFN-β-expressing NSCs exerted a remarkable bystander effect on human glioma cells after the application of 5-FC, as compared with parental NSCs and CD-expressing NSCs. In animal models with human glioma orthotopic xenograft, intravenously infused CD/IFN-β-expressing NSCs produced striking antitumor effect after administration of the prodrug 5-FC. Furthermore, the same gene therapy regimen prolonged survival periods significantly in the experimental animals. The results of the present study indicate that the multimodal NSC-based treatment strategy might have therapeutic potential against gliomas.
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Keywords: giloma; human neural stem cells; retrovirus; cytosine deaminase; interferon-β

Introduction

Malignant glioma represents about 20% of all intracranial tumors. Despite advances in radiation therapy and chemotherapy administered after the surgical resection, the prognosis of malignant glioma remains poor with a median survival of <10 months.[1] The infiltrative nature of malignant gliomas and the limited penetration of chemotherapeutic agents through the tight blood–brain barrier are obstacles in the treatment of these formidable tumors.

Our group and others have previously shown that because of their remarkable migratory and tumor-tropic properties, neural stem cells (NSCs) represent a potentially powerful tool for the treatment of brain tumors by delivering therapeutic drugs into the intracranial glioma across the blood–brain barrier.[2–5] Genetically modified NSCs selectively migrate toward brain tumor cells and deliver therapeutic agents with significant beneficial effects. In particular, NSCs that are retrovirally transduced with suicide genes such as the cytosine deaminase (CD) gene show a remarkable 'bystander killer effect' on 5-fluorocytosine (5-FC)-treated glioma cells.[3,5–8]

Interferon-β (IFN-β) is known for its ability to interfere with viral replication and also for its antiproliferative effects on a variety of cancer cells. However, the efficacy of IFN-β is limited because of its extremely short half-life after intravenous administration as well as the systemic toxicity when this protein is administered at doses required to achieve the desired antitumor effect. In our pilot clinical trial, liposome-mediated *IFN-β* gene therapy has shown potent antitumor activity in patients with malignant glioma.[9,10] The clinical trial suggested that the *IFN-β* gene delivery permitted locally sustained IFN-β production at levels sufficient to yield antitumor efficacy with minimal systemic adverse effects.

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In the present study, we sought to examine whether intravenously administered human NSCs expressing CD and IFN-β migrate into the intracranial tumor bed through the blood vessels, and exhibit antitumor effect by the combined delivery of a suicide gene and a cytotoxic cytokine gene onto the experimental glioma.

Materials and methods

Human glioma cells and NSCs

A human glioma cell line, U251, was obtained from the American Tissue Culture Collection (ATCC, Manassas, VA) and grown in Eagle's minimal essential medium (Nissui, Tokyo, Japan) containing 10% fetal bovine serum, 5 mM of L-glutamine, 2 mM nonessential amino acids and antibiotics (100 U ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2. HB1.F3 (F3) human NSC line was generated from the human fetal telencephalon, and was immortalized by transfection with a retroviral vector encoding the *v-myc* oncogene, as described previously.[11] It has been confirmed that this human NSC line is capable of self renewal and is multipotent, that is, these NSCs can differentiate into cells of the neuronal and glial lineages, both *in vivo* and *in vitro*.[11] In this study, the clonal F3.CD.IFN-β line was derived from the parental F3.CD cells.[3] An expression plasmid was constructed using the pBabePuro retroviral vector (Cell Biolabs, San Diego, CA) as the backbone to include the human IFN-β cDNA transcribed from the long terminal repeat ends of the *IFN-β* gene.[9,10] The IFN-β.puro plasmid and the MV12 envelope-coding plasmid (provided by Dr KS Aboody[3]) were cotransduced into pA317 cells (ATCC). The supernatant containing the IFN-β-expressing retroviral vector was used for multiple infections of the F3.CD cells. The transduced F3.CD.IFN-β cells were selected by culturing them for 4 weeks in a medium containing 3 μg ml^{-1} of puromycin. Successful establishment of the F3.CD.IFN-β cells was confirmed by reverse transcription PCR. The *IFN-β* transcript in these cells was amplified by touchdown PCR using the following primers: sense, 5′-GCCG CATTGACCATCTATGAGA-3′; antisense, 5′-GAGATCT TCAGTTTCGGAGGTAAC-3′. Glyceraldehyde 3-phosphate dehydrogenase was used as a control to confirm equal RNA loading. U251 cells transfected with liposomes containing the human *IFN-β* gene (pDRSV-IFN-β) were used as positive controls.[10] Parental F3, F3.CD and F3.CD.IFN-β cells were cultured in Dulbecco's modified Eagle's medium with high glucose (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, fungizone (2.5 μg ml^{-1}) and gentamicin (20 μg ml^{-1}).

DiI labeling of NSCs

F3 cells were prelabeled by incubating for 20 min in culture medium containing cell tracker CM-DiI (Invitrogen). CM-DiI has maximum fluorescence emission at 570 nm.

Boyden chamber migration assay

Filters were coated with a fibronectin (Sigma-Aldrich, St Louis, MO; dilution, 1:40 in phosphate-buffered saline (PBS)) solution and air-dried before use. Migration assays were performed in a modified Boyden chamber by using a 24-well HTS FluoroBlok insert system (Falcon Becton Dickinson, Heidelberg, Germany). The inserts contained a polyethylene membrane with a pore size of 8.0 μm, which blocks 99% of the light transmitted at wavelengths 490–700 nm. U251 cells (1×10^5 cells) were incubated on a 24-well plate. After 48 h, 1×10^4 single cells were placed on the top of an insert and incubated for 24 h in Dulbecco's modified Eagle's medium. The cells were fixed using 4% paraformaldehyde in 0.1 M PBS. Then the membrane was cut out of the insert and covered in 4′,6-diamidino-2-phenyl-indole as a mounting medium (absorption at 360 nm and emission at 460 nm; Vectashield, Vector Laboratories, Burlingame, CA) between two thin coverslips. The total number of migrated cells at the bottom of the membrane and the number of non-migrated cells at the top were counted ($n = 5$). The migration index was calculated using the following formula: number of migrated cells at the bottom/number of both migrated and non-migrated cells.

In vitro *quantitative assay for bystander killing effect*

The bystander killing effect of F3.CD and F3.CD.IFN-β cells on the U251 cells after 5-FC (Sigma-Aldrich) treatment was quantified. U251 cells were seeded in a 24-well plate (2×10^4 cells per well) and cocultured with either F3.CD or F3.CD.IFN-β cells at various U251/F3 ratios ranging from 1:0 to 1:4. After 24 h culture, 5-FC was added to the medium at a final concentration of 500 μg ml^{-1}, and the culture was maintained for 3 more days. Each experiment was performed in triplicate. After the day 3, the cells were rinsed twice with PBS, and the adherent cells were detached using trypsin/EDTA. The number of viable cells was counted by the trypan blue method and expressed as the percentage of untreated U251 cells.

Clonogenic assay

The U251 cells were seeded in a 24-well plate (2×10^4 cells per well) and cocultured with either F3.CD or F3.CD.IFN-β cells at various U251/F3 ratios ranging from 1:0 to 1:4. After 24 h culture, 5-FC was added to the medium at a final concentration of 500 μg ml^{-1} and the culture was maintained for 3 more days. The surviving cells were detached from the plates and re-seeded in a six-well plate at a cell density of 500 cells per well (duplicate). The cells were cultured for 9 days and then stained with 0.25% 1,9-dimethylmethylene blue (Sigma-Aldrich) in 50% ethanol. The number of colonies was counted by two independent observers.

Intracerebral glioma model

The experiments were performed in accordance with the Guidelines for Animal Experiments of the Nagoya University Graduate School of Medicine. BALB/c female nude mice (SLC, Shizuoka, Japan) were anesthetized by

administering an intraperitoneal (i.p.) injection of pentobarbital (60–70 mg per kg body weight). The animals were injected with 1×10^6 U251 cells suspended in 5 µl of PBS using a Hamilton syringe under stereotactic guidance; the injections were administered into the forebrain (2 mm into the lateral side and 1 mm into the anterior side of the bregma; at a depth of 4 mm from the dural surface) for over 5 min. This intracerebral model was reproducible and exhibited a survival of ~25–40 days.

Histological study of intravenously injected F3 cells into intracerebral glioma

At 14 days after the establishment of the intracerebral glioma model, the animals ($n = 3$) were injected intravenously through the tail vein with 2×10^6 Dil-labeled F3, F3.CD or F3.CD.IFN-β cells diluted in 100 µl of PBS. The control animals ($n = 3$) were injected with PBS alone. After 7 days, the animals were killed and transcardially perfused with 10% buffered formalin. Paraffin-embedded coronal sections were immunostained with rat anti-mouse-CD34 antibody (MEC 14.7; HyCult Biotechnology, Uden, Netherlands) followed by anti-rat Alexa Fluor 488 (Molecular Probes, Eugene, OR), and nuclei were counterstained with Hoechst 33342. CD34 has been most commonly used in studies of tumor angiogenesis, and the detection of CD34 in endothelial cells can be interpreted as indicative of angiogenesis. The adjacent sections also were processed for hematoxylin and eosin staining.

Intravenous transplantation of F3.CD or F3.CD.IFN-β cells followed by 5-FC treatment

At 3 days after the implantation of U251 glioma cells into the brain, the animals were randomly divided, and five mice each were intravenously injected through the tail vein with PBS, F3, F3.CD or F3.CD.IFN-β cells (2×10^6 cells in 100 µl of PBS). After 2 days, 5-FC was injected i.p. at a dose of 900 mg per kg body weight daily for 10 consecutive days. At 28 days after implantation of glioma cells, animals were killed and brain sections were processed for hematoxylin and eosin staining. The antitumor effect of the NSCs was evaluated by measuring the long (a) and the short (b) axes of the coronal sections with maximal tumor area. The approximate volume of the tumor (V) was calculated according to the formula, $V\ (\text{mm}^3) = a \times b^2/2$. The overall survival time from implantation of glioma cells was assessed in another set of mice treated in the same manner.

Statistical analyses

The statistical significance of the observed difference was determined by analysis of variance (StatView software, SAS Institute, Cary, NC), and subsequently, Bonferroni's correction for multiple comparisons was applied. Survival curves were generated using the Kaplan–Meier method. The log-rank statistic (StatView) was used to compare the distribution of the survival times. All reported P-values were two-sided; a value <0.05 was considered to be statistically significant.

Results

F3.CD.IFN-β human NSCs produce human IFN-β

The expression of the human IFN-β in F3.CD.IFN-β cells was confirmed by reverse transcription PCR. The human IFN-β transcript was found to be expressed in both the clonal cell lines, namely, the F3.CD.IFN-β cell line and the positive control IFN-β-expressing U251 cell line, but not in the parental F3.CD cell line (Figure 1).

F3.CD.IFN-β cells show higher bystander killing effect on glioma cells in vitro

To quantify the bystander effect of F3.CD.IFN-β cells on the U251 glioma cells, both types of cells were cocultured at various ratios and subsequently treated with 5-FC. The number of viable cells was assessed after 3 days, and this number was then compared with the number of viable 5-FC-treated U251 cells cultured alone. We confirmed that the F3.CD and F3.CD.IFN-β cells did not survive after treatment with 500 µg ml^{-1} 5-FC (data not shown). As shown in Figure 2, the number of viable cells decreased with an increase in the F3/U251 ratio in both groups, that is, F3.CD + U251 and F3.CD.IFN-β + U251. Notably, the bystander killing effect exhibited by the F3.CD.IFN-β cells was more significant than that of the F3.CD cells, at the F3/U251 ratio of 2:1 and 4:1 ($P<0.05$). Consistent with this result, the clonogenic potential of the U251 cells surviving after coculture with F3.CD.IFN-β and 5-FC treatment was significantly lower than the clonogenic potential for similar U251 cells that were cocultured with F3.CD cells (Figure 3).

F3 cells migrate to intracranial glioma in mice

The *in vitro* migration assay revealed that F3.CD.IFN-β cells had the same migratory pattern as that of the parental F3 cells (Figure 4). Next, to ascertain the migratory capability of the NSCs from the vessels to the tumor mass, the Dil-labeled F3.CD.IFN-β cells were injected into the tail vein, and the newly formed tumor vessels were immunostained by using an anti-CD34 antibody. The Dil-labeled NSCs were found to be present

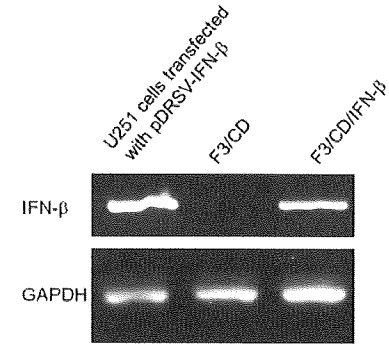

Figure 1 The expression of human interferon-β (IFN-β) in the F3.CD/IFN-β cell line. The IFN-β transcript was expressed only in F3.CD.IFN-β human neural stem cells.

in both, tumor stroma and tumor parenchyma. Thus, NSCs appeared to migrate into the tumor parenchyma extending from tumor vessels (Figure 5).

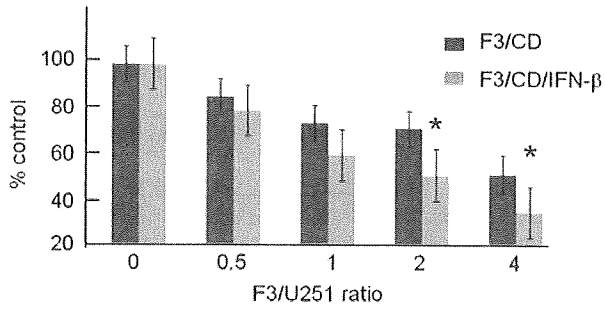

Figure 2 A bystander killing effect. The U251 cells were seeded in a 24-well plate (2×10^4 cells per well) and cocultured with either F3.CD or F3.CD.IFN-β cells at various U251/F3 ratios ranging from 1:0 to 1:4. After the day 1 of culture, 5-fluorocytosine (5-FC) was added to the medium at a final concentration of 500 μg ml^{-1}, and the culture was maintained for 3 more days. Each experiment was performed in triplicate. The number of viable cells was counted by the trypan blue method, and expressed as the percentage of untreated U251 cells. The number of viable cells decreased with an increase in the F3/U251 ratio in both the cocultures, that is, in F3.CD + U251 and F3.CD.IFN-β + U251. Notably, the bystander killing effect exhibited by the F3.CD.IFN-β cells was more significant than that of the F3.CD cells, at the F3/U251 ratio of 2:1 and 4:1 (*$P<0.05$). IFN-β, interferon-β.

F3.CD.IFN-β cells reduce tumor burden in experimental glioma in mice

We studied the growth inhibitory effect of genetically engineered NSCs (that is, F3.CD.IFN-β and F3.CD cells) on glioma cells *in vivo*. The mice with the U251-derived intracerebral tumor were injected with F3 cells, and the i.p. injections of 5-FC were administered for the next 10 days. The volume of the tumor was assessed on day 28 after FC treatment. As shown in Figure 6, the residual tumor mass obtained in the group treated with F3.CD.IFN-β was much smaller than that obtained in the group treated with F3.CD cells ($P<0.05$).

F3.CD.IFN-β cells increase the survival periods in experimental animals

To determine whether F3.CD.IFN-β implantation followed by i.p. injections of 5-FC can produce therapeutic benefits *in vivo*, the implantation was performed 3 days after intracranial inoculation of U251 cells. We measured the survival periods of mice first inoculated with U251 intracranially, and subsequently with each type of NSCs (F3.CD.IFN-β, F3.CD and F3) and i.p. injected with 5-FC. The survival of mice treated with F3.CD.IFN-β was significantly longer than that of mice treated with F3.CD or F3 cells (Figure 7). These results suggest that the antitumor effect of F3.CD.IFN-β cells on glioma cells is greater than the antitumor effect of the F3.CD cells.

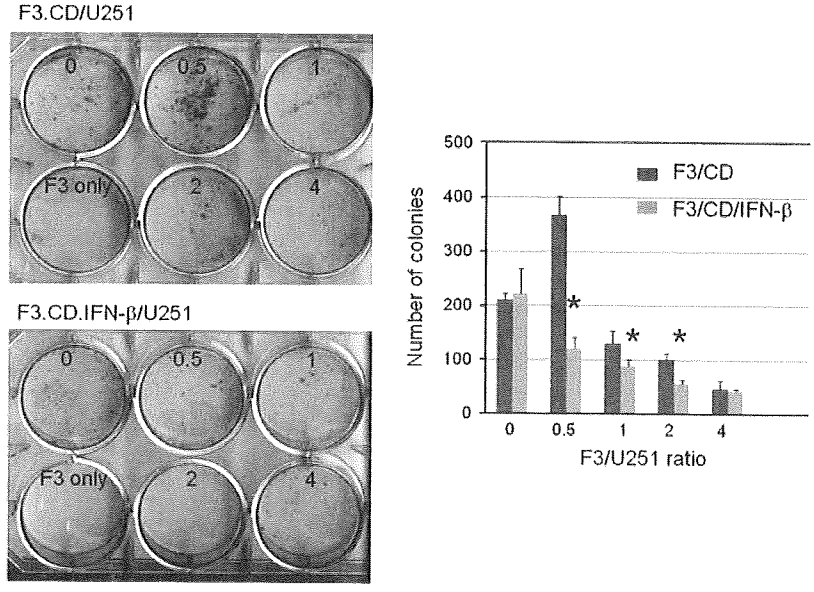

Figure 3 Clonogenic assay. U251 cells were seeded in a 24-well plate (2×10^4 cells per well) and cocultured with either F3.CD or F3.CD.IFN-β cells at various U251/F3 ratios ranging from 1:0 to 1:4. After 24 h culture, 5-fluorocytosine (5-FC) was added to the medium at a final concentration of 500 μg ml^{-1}, and the culture was maintained for 3 more days. The surviving cells were detached from the plates and re-seeded in a six-well plate at a cell density of 500 cells per well (duplicate). The cells were cultured for 9 days and then stained with 0.25% 1,9-dimethylmethylene blue (Sigma-Aldrich) in 50% Ethanol. The number of colonies was counted by two independent observers. The clonogenic potential of surviving U251 cells after coculture with F3.CD.IFN-β after 5-FC treatment was significantly reduced compared with coculture with F3.CD (*$P<0.05$). IFN-β, interferon-β.

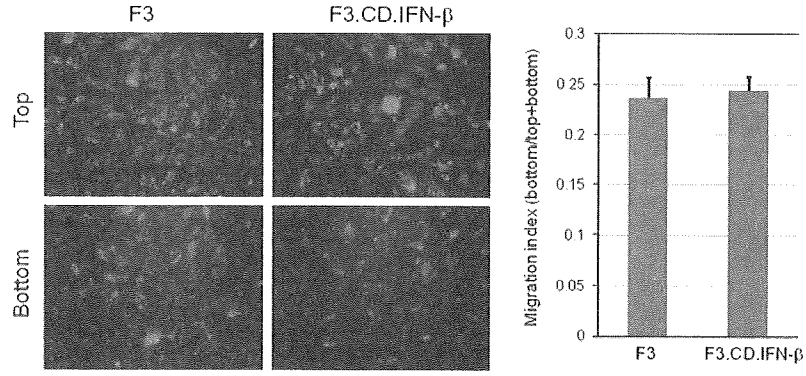

Figure 4 Migration assay *in vitro*. U251 cells (1×10^5) were plated on the 24-well plate and cultured for 48 h. CM-DiI-labeled F3 or F3.CD.IFN-β cells (2×10^4) were seeded into the upper wells of the FluoroBlok inserts. After a 24-h incubation, migrated (bottom) and non-migrated cells (top) were counted. There was no significant difference between the migration indices of these two cell lines. IFN-β, interferon-β.

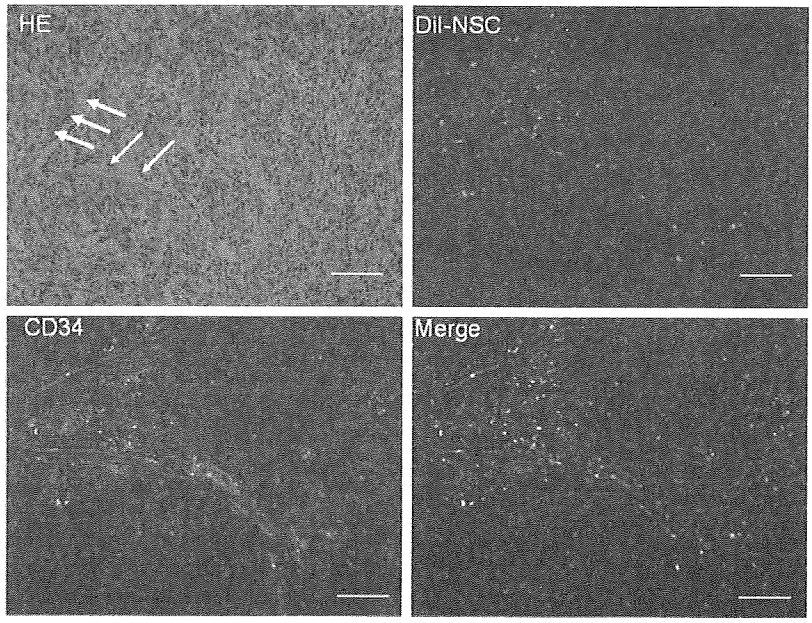

Figure 5 Migration of F3.CD.IFN-β cells into the tumor parenchyma. To ascertain the migratory capability of the neural stem cells (NSCs) from the vessels to the tumor parenchyma, the DiI-labeled F3.CD.IFN-β cells (red) were injected into the tail vein, and the newly formed tumor vessels were immunostained by using an anti-CD34 antibody (green). The DiI-labeled NSCs were found to be present in both, tumor stroma and tumor parenchyma. An adjacent section was also stained with hematoxylin and eosin, and the field relevant to the fluorescence images is displayed. The arrows indicate tumor vessels. Scale bar, 100 μm. IFN-β, interferon-β.

Discussion

The principal finding of the present study is that the genetically engineered human NSCs expressing CD and IFN-β appear to exert an additive effect in destroying intracerebral gliomas. The number of viable tumor cells present in a coculture of 5-FC-treated F3.CD.IFN-β + U251 cells was ~60% of that in a coculture of 5-FC-treated F3.CD + U251 cells at a F3/U251 ratio of 2:1 or 4:1 (Figure 2). Further, compared with the mice injected with F3.CD, the mice intravenously injected with F3.CD.IFN-β showed a significantly higher reduction in the tumor volume as well as a longer survival period (Figures 6 and 7).

Interferon-β is a type I IFN that exerts pleiotropic biological effects.[12] We have previously undertaken preclinical and experimental studies to investigate the application of cationic liposomes for delivering the *IFN*-β gene in glioma patients. *In vitro* experiments showed that the cationic liposome-mediated human *IFN*-β gene transfer into the cultured human glioma cells induced a cytocidal but not a cytostatic response even in

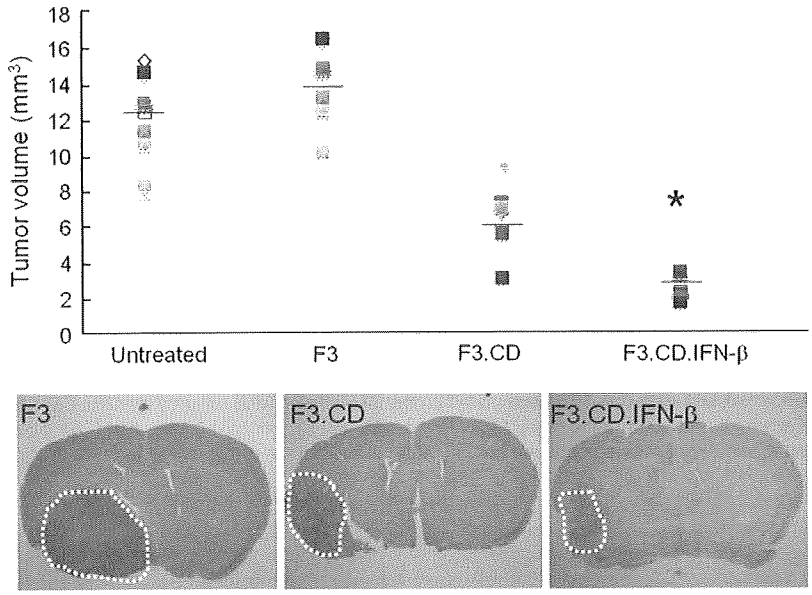

Figure 6 The growth inhibitory effect of genetically engineered neural stem cells (NSCs) on the glioma cells *in vivo*. The mice with the U251-derived intracerebral tumor were injected with F3 cells, and then i.p. injections of 5-fluorocytosine (5-FC) were administered for the next 10 days. The volume of the tumor was assessed on day 28 after FC treatment. The residual tumor mass obtained in the group treated with F3.CD.IFN-β cells was much smaller than that obtained in the group treated with F3.CD cells (*$P<0.05$). IFN-β, interferon-β.

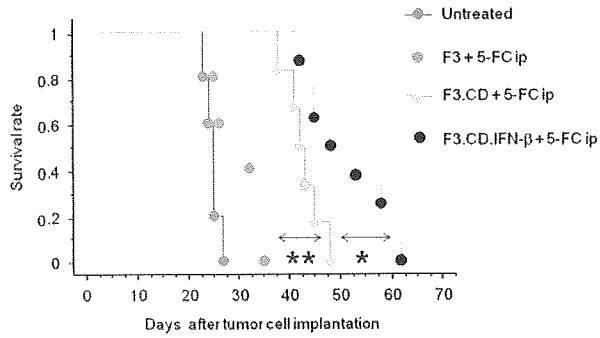

Figure 7 The survival time of experimental animals. Mice were inoculated with U251 intracranially, and subsequently with each type of neural stem cells (NSCs) (F3.CD.IFN-β ($n=8$), F3.CD ($n=6$) and F3 ($n=5$)), followed by intraperitoneal injection of prodrug 5-fluorocytosine (5-FC) and compared with untreated animals ($n=5$). We measured the survival time from U251 cell inoculation. The rates of survival of mice treated with F3.CD.IFN-β cells were significantly higher than those of mice treated with F3.CD (*$P<0.05$); mice that received F3.CD therapy had significantly higher survival rates than those that received only F3 cells (**$P<0.005$). CD, cytosine deaminase, IFN-β, interferon-β.

IFN-resistant human glioma cell lines, probably by inducing apoptosis.[12] Cationic liposome-mediated *IFN-β* gene transfer exhibited a much stronger inhibition of glioma cell growth than exogenous IFN-β. A 40-fold increase in the concentration of IFN-β was required to obtain an inhibitory effect similar to that observed with exogenous transfer of the *IFN-β* gene. Presumably, a sustained higher expression of IFN-β in the microenvironment may induce a direct apoptotic effect on the surrounding tumor cells. *In vivo* experiments using mice implanted with human glioma cells revealed that local administration of cationic liposomes containing the human *IFN-β* gene induced an apparent reduction in the tumor growth and prolonged the survival.[13–16] On the basis of these observations, a phase I clinical trial of *IFN-β* gene therapy was performed on five patients with recurrent malignant glioma.[9] At 10 weeks after treatment initiation, two patients showed more than 50% tumor reduction, whereas others did not show any significant improvement. The median survival was longer in the treated subjects than in the matched historical controls from our institution. After gene therapy, significant changes were observed in the histology and gene expression related to immune response, apoptosis and neovascularization.[17] A recent study has reported the findings of a phase I clinical trial, in which stereotactic injections of IFN-β-expressing adenoviral vectors were administered to 11 patients with malignant glioma and resulted in modest clinical outcome.[18] However, local administration of therapeutic IFN-β vectors used in these clinical trials could not address the issues of selective targeting of infiltrative satellite tumors.

To overcome this limitation, the inherently migratory, tumor-tropic NSCs can serve as a potentially powerful therapeutic tool. NSCs display remarkable tropism and migratory capacity to sites of malignant growth.[19,20] In this study, we chose the human F3 NSC cell line, as it is a well-characterized and a well-established human NSC line.[3,5–7,11] No signs of local or systemic toxicity were

observed in case of animals injected with F3 cells alone. Importantly, these cells can be modified to stably express a therapeutic transgene. In NSC-based gene therapy strategies targeting brain tumors, NSCs were mostly used to transport the CD/5-FC prodrug system to the tumor cells.[3–8] Recently, Dickson *et al.*[21] showed that in mice, F3 human NSCs transiently expressing human IFN-β displayed tropism for sites of disseminated neuroblastoma, resulting in significant tumor growth. The sustained expression of IFN-β at disseminated sites of microscopic disease represents a novel therapeutic approach. In the present study, we investigated the additive efficacy of NSCs for delivering CD as well as IFN-β to the tumor site. Further studies are required to elucidate the mechanisms by which IFN-β intensifies the bystander effect of CD against glioma cells. Moreover, the application of NSCs in clinical settings raises some concerns. A recent study reported an NSC-derived brain tumor in a patient with ataxia telangiectasia who had been administered intracerebellar and intrathecal injections of human fetal NSCs.[22] In this study, we systematically delivered a human NSC cell line immortalized by v-*myc*. In our previous publication, we have reported that intravenously injected NSCs tend to be trapped in the spleen, kidney and liver.[23] Systemic administration of immortalized NSCs might cause neoplasm formation. These issues need to be addressed before clinical application. Nevertheless, our study indicates that the toxic effect against glioma cells exerted by a combination of two treatments is more effective than that exerted by CD-based suicide strategy. These findings support the possible application of a one–two-punch combination therapy for the treatment of malignant gliomas.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Induction of oligodendrogenesis in glioblastoma-initiating cells by IFN-mediated activation of STAT3 signaling

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ABSTRACT

The response of cancer patients to interferon (IFN) treatment is long-lasting, indicating that IFN may act on small cancer stem cell populations. Glioma-initiating cells (GICs) can self-renew and induce the formation of heterogeneously differentiated tumor cells and are resistant to chemotherapeutic agents like temozolomide. In this study, we showed that via STAT3 signaling, IFN-β suppressed the proliferation, self-renewal, and tumorigenesis of GICs, induced their terminal differentiation to mature oligodendroglia-like cells, and exhibited synergistic cytotoxicity with temozolomide. Therefore, IFN may be a potential therapeutic agent for inducing the terminal differentiation of GICs.

1. Introduction

Glioblastoma multiforme (GBM) is the most lethal form of primary glioma with less than 12 months of median survival time despite the availability of various therapies, including surgical resection, radiotherapy, and chemotherapy [1]. For many years, gliomas, including GBM, were considered to be heterogeneous bulk tumors comprising differentiated and undifferentiated cells with self-renewal and partial differentiation capabilities [2]. Therefore, the failure of treatments against gliomas may be attributed to certain undifferentiated tumor cells responsible for regrowth.

Cancers have been reported to harbor small cell populations possessing growth sustaining and tumorigenesis abilities. These cells—termed cancer stem cells (CSCs) or cancer initiating cells—have been identified in the cases of leukemia, multiple myeloma, breast cancer, and glioma. In solid tumors, CSCs share many properties, including self-renewal and multi-potency, with normal stem cells; moreover, they can initiate tumor growth. Glioma-initiating cells (GICs) also share some properties of CSCs. GICs express genes characteristic of neural stem cells and differentiate into phenotypically diverse populations, including neuronal, astrocytic, and oligodendroglial cells [3]. Moreover, GICs have been reported to contribute to the radioresistance and chemoresistance of tumors by activating preferential checkpoint responses and overexpressing DNA repair genes [4,5]. Since GICs play an important role in tumorigenesis and tumor recurrence, effective cancer treatments should be targeted at them. Lee et al. demonstrated that bone morphogenetic protein (BMP) mediated the differentiation of astroglial cells from GBM-initiating cells via the Janus-activated kinase (JAK)/signal transducer and activator of transcription (STAT) pathways [6]. Their study demonstrated

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the potential for therapeutic approaches that can induce terminal differentiation of GICs.

Type I interferons (IFNs), including IFN-α and IFN-β, are cytokines exhibiting immunomodulatory, cell differentiative, antiangiogenic, and antiproliferative effects against various neoplasms, particularly glial tumors, by activating the JAK/STAT pathways [7,8]. By utilizing several GIC lines and animal models, we demonstrated that IFN-β elicits a remarkable antiproliferative effect on GICs and induces their terminal differentiation into oligodendroglial cells. Further, we speculated that STAT3 activation is a possible action mechanism that promotes oligodendroglial differentiation.

2. Materials and methods

2.1. Primary tumor sphere cultures

After obtaining written informed consent, we collected tumor samples from three patients (# 1228, 0316, and 0222) who were newly diagnosed with GBM and undergoing surgical treatment at the Nagoya University hospital, Japan. One hour after surgical resection, the tumors were washed and enzymatically dissociated into single cells. Red blood cells were removed by Ficoll gradient centrifugation. Tumor cells were cultured either in neurobasal (NBE) media comprising neurobasal media with N2 and B27 supplements (Invitrogen), human recombinant basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) (20 ng/ml each; R&D Systems) (NBE cells), or in DMEM media (Invitrogen) containing 10% fetal bovine serum (FBS) (serum cells). We established three sets of cell lines that satisfied the following criteria: (a) NBE cells could be maintained in the NBE media for 3 months (minimum) and (b) 10^3 NBE cells could form tumors in the brains of nonobese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) (as described below), but 10^5 serum cells could not. The NBE cells were subcultured monthly (minimum) by dissociating the spheres with NeuroCult (StemCell Technologies).

2.2. Human neural stem cells

A human neural stem cell (NSC) line, namely, HB1/F3, was generated from the human fetal telencephalon as described previously [9]. It has been confirmed that this human NSC line is capable of self renewal and is multipotent, i.e., the NSCs can differentiate into cells of the neuronal and glial lineages, both in vivo and in vitro [9].

2.3. Reagents

Human IFN-β and temozolomide (TMZ) were obtained from Toray, Co., Ltd., and the Schering-Plough Research Institute, respectively. Stat3 inhibitor (PpYLKTK-mts) was purchased from Calbiochem.

2.4. Intracranial tumor cell injection

Cells cultured either under the NBE or the serum condition were resuspended in 5-μl phosphate-buffered saline (PBS) and injected stereotactically into 5–6-wk-old NOD/SCID female mice, as described previously [10].

2.5. Cell proliferation assay

Cell proliferation was assessed by WST-8 (Dojindo), 5′-bromodeoxyunidine (BrdU) incorporation, and colony formation assays. The former two assays were performed according to manufacturers' protocols. In the colony-formation assay, dissociated NBE cells were seeded in 6-well dishes in a medium containing 10% FBS and cultured for 5 days. Cell colonies were fixed and stained with 0.04% crystal violet. The number of colonies per dish was counted by three observers blinded to the treatment conditions.

2.6. Immunocytochemistry

After fixation in 4% paraformaldehyde, the cells were permeabilized on ice. The following primary antibodies were used: anti-β-tubulin (Tuj-1; Chemicon), anti-GFAP (Z0334; DAKO), anti-galactocerebroside C (GalC) (MAB342; Chemicon), and anti-nestin (MAB1259; R&D). The primary antibodies were visualized using anti-mouse Alexa Fluor 546 or anti-rabbit Alexa Fluor 488 antibodies (Molecular Probes). Nuclei were counterstained using 4′,6-diamino-2-phenylindole (DAPI).

2.7. Flow cytometric analysis

Following fixation and permeabilization, cells were treated with anti-GalC and anti-mouse Alexa Fluor 488 (Molecular Probes) antibodies. For cell cycle analysis, cells were resuspended in PBS containing propidium iodide and RNaseA. Stained cells were analyzed using fluorescence-activated cell sorter (FACS) Calibur flow cytometer (Becton Dickinson).

2.8. Western blotting

Cell lysis and immunoblotting were performed as previously described [11]. Antibodies against the following proteins were used: phospho-STAT3 (p-Ser727 and p-Tyr705) and STAT3 (Cell Signaling), O^6-methylguanine methyltransferase (MGMT) (MT3.1; Neomarkers), Myelin-basic protein (MBP) (MAB382; Chemicon), CD133 (ab19898; Abcam), or β-actin (AC-15; Sigma–Aldrich).

2.9. Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed using Superscript II RT Kit (Invitrogen) according to the manufacturer's protocol. Supplementary Table 1 lists the PCR primers employed. Amplification cycle numbers varied for different genes at a range of 25–35 cycles. Band intensities were densitometrically semi-quantified by NIH-image.

2.10. Statistical analysis

The statistical significance of the differences observed was determined by analysis of variance (ANOVA) (Stat-

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View; SAS Institute, Cary, NC), and Bonferroni's correction was employed for multiple comparisons.

3. Results

3.1. Characterization of GICs from human glioblastomas

Dissociated tumor cells formed neurosphere-like aggregates within 7 days of culture. Spheres from these GIC lines continuously proliferated in the presence of serum-free NBE media containing bFGF and EGF; further, all lines (1228-NBE, 0316-NBE, and 0222-NBE) could be maintained for 3 months (minimum). Cells cultured in the medium containing 10% FBS were found to attach to the culture dish (1228-serum, 0316-serum, and 0222-serum). As previously reported [3], cells in spheres were strongly immunoreactive to the neural progenitor marker nestin. Further, they expressed the stem cell surface marker CD133; however, the expression of both nestin and CD133 was weak in cells cultured under serum conditions (Fig. 1A). In order to identify cells on the basis of their differentiation stage, the expression of several stem cell markers was examined by RT-PCR; these included fucosyltransferase 4 (FUT4; catalyzes sialyl Lewis X determinant (SSEA) synthesis during human embryogenesis) [12], Oct-4 and Sox-2 (undifferentiated pluripotent cell markers), and nestin. Sox-2 and nestin expression levels were downregulated in the serum cells compared to their expression in the NBE cells; however, early differentiation stage stem cell markers—

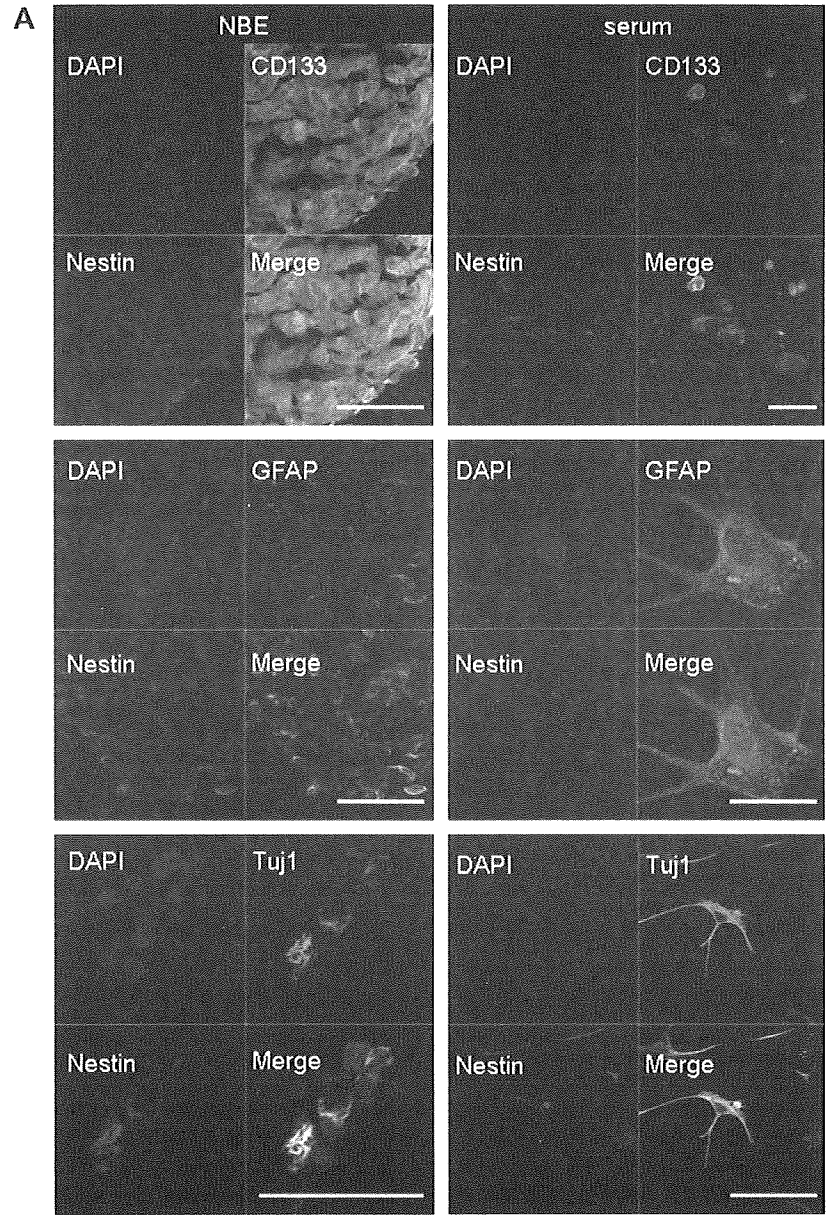

Fig. 1. Characterization of GICs from glioblastomas. A, Immunocytochemical analysis of cells grown in serum-free NBE media (NBE cells) and serum-containing media (serum cells). Scale bar: 50 μm. B, RT-PCR analysis of stem cell markers. Internal control: GAPDH. The level of gene expression was quantified by NIH-image. The values are expressed relative to the intensity of GAPDH (mean ± SD of three independent PCRs in the three cell lines). C, Microphotographs of parental tumor and NBE-cell-derived intracranial xenograft tumor (hematoxylin-eosin, 200×).

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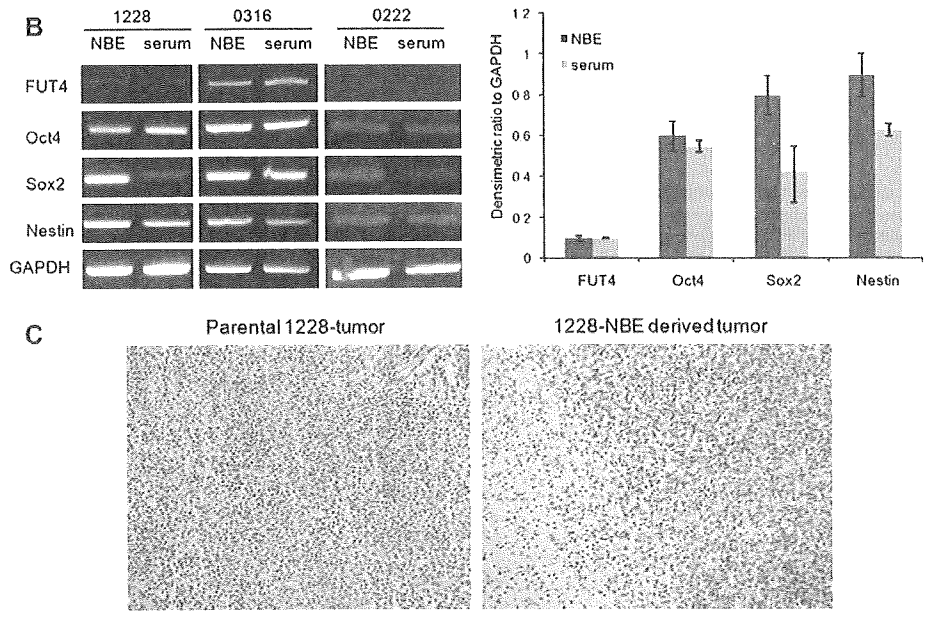

Fig. 1 *(continued)*

FUT4 and Oct4—did not display such difference in expression between the NBE and serum cells (Fig. 1B). The expression of glial (GFAP) and neuronal (Tuj1) phenotype markers was stronger in the serum cells than that in the NBE cells (Fig 1A). These findings were reproducible in other NBE cells (data not shown). To test whether tumor spheres established in culture retained the intracranial tumor formation ability, we injected 1×10^5 cells dissociated from tumor spheres into the brains of NOD/SCID mice. Transplantation of these cells led to the formation of tumors mimicking the phenotypic features of the parental human GBM tumors (e.g., infiltrative and angiogenic) (Fig. 1C). In addition, only 1000 dissociated NBE cells were sufficient for induction of tumors, while even 1×10^5 serum cells were unable to induce tumor formation in the brains of NOD/SCID mice.

3.2. IFN-β reduces GIC proliferation

We seeded 1000 dissociated NBE or serum cells in a 96-well plate and treated each culture with IFN-β (1000 IU/ml), TMZ (200 μM), or a combination of both. The relative cell number was determined after 48 h of treatment. Compared to the untreated cells, the NBE cells treated with IFN-β showed significantly slow growth; however, IFN-β treatment did not affect the growth of the serum cells ($p < 0.01$), and the number of NBE cells treated with IFN-β reached a plateau within 4 days (Fig. 2A). In contrast, TMZ did not exert an antiproliferative effect on either NBE or serum cells. Treatment with the IFN-β and TMZ combination induced remarkable synergistic growth suppression in 0316- and 0222-NBE cells and in all the serum cells ($p < 0.01$); however, no synergistic effect on growth suppression was observed in the 1228-NBE cells. This finding was consistent with our previous finding that the combination treatment of IFN-β and TMZ exerts a synergistic effect on glioma cells [11]. Colony formation was suppressed by approximately 50% in the NBE cells after treatment with 1000-IU/ml IFN-β (Fig. 2B). This was confirmed by the concomitant increase in the number of NBE cells in the G0/G1 phase, decrease in the number of cells in the S-G2 M phase (Fig. 2C), and a significant decrease (20%) in the number of BrdU-positive cells in response to IFN-β treatment compared to that in the untreated cells (Fig. 2D, $p < 0.05$).

3.3. IFN-β mediates oligodendrogenesis in GICs via the STAT3 signaling pathway

Since IFN-β delayed the growth of GICs, we hypothesized that this might lead to the activation of the differentiation process in GICs. STAT3 activation is crucial for stem cell function, differentiation, and survival [13]. Recent studies demonstrated that BMP and leukemia inhibitory factor/ciliary 35 neurotrophic factor (LIF/CTNF) induced STAT3-mediated differentiation of neural [14] and glioma stem cells [6]. Our study showed that STAT3 expression was strongly upregulated in the NBE cells, and that IFN-β phosphorylates STAT3 tyrosine (Fig. 3A). Furthermore, consistent with other reports [5], we observed that the expression of MGMT—a key molecule responsible for acquiring resistance to alkylating chemotherapeutic agents—was remarkably higher in the NBE cells than in the serum cells; however, IFN-β led to the reduction of its expression. This may partly explain the synergistic cytotoxic effect induced by the TMZ and IFN-β combination (Fig. 2A), which is quite similar to the findings of our previous study [11]. In analyses of RT-PCR, Western blotting and FACS, we also observed that IFN-β treatment enhanced the expression of the oligodendroglial marker, oligodendrocyte-specific protein (OSP), GalC, and MBP in the NBE cells; however, their expression was inhibited by a STAT3-specific inhibitory peptide (Fig. 3B–D). Further, IFN-β treatment did not induce the expression of OSP, GalC, and MBP in human neural stem cells (Fig. 3E). In contrast, while the expression of other neural lineage markers (i.e., glial fibrillary acidic protein (GFAP) and neuron-specific class III beta-tubulin (Tuj1)) was unaffected (data not shown), the expression of nestin and CD133 was reduced in the NBE cells after IFN-β treatment (Fig. 3E and F).

3.4. IFN-β treatment reduces the tumorigenic GIC pool

The above mentioned results suggest that GICs acquire an oligidendroglial cell-like fate after treatment with IFN-β and hence there is depletion in the tumorigenic GIC pool. Dissociated 0316-NBE cells were treated with 1000 IU/ml of IFN-β for 48 h. The viability of cells treated ex vivo with IFN-β was confirmed by the trypan-blue dye exclusion method before the unilateral intrastrial injection of 0316-NBE cells (1×10^5 cells) into NOD/SCID mice. IFN-β-treated cells were unable to form tumors in four of the five mice injected with NBE cells. If tumors developed, they were in the form of small, delimited lesions surrounding the injection site (Fig. 4AB). In contrast, all animals receiving untreated 0316-NBE cells developed large tumor masses showing characteristic GBM features and died 40–55 days post-injection. Subsequently, we examined whether systemic IFN-β delivery could reduce tumor growth. IFN-β (2×10^5 IU) was administered intraperitoneally every 2 days for 14 days (six pulses) from day 7 after injection with untreated 0316-NBE cells. Mice treated with IFN-β in this manner displayed small, confined lesions and survived for a significantly longer duration ($p < 0.01$). Although the effect of systemically administered IFN-β on

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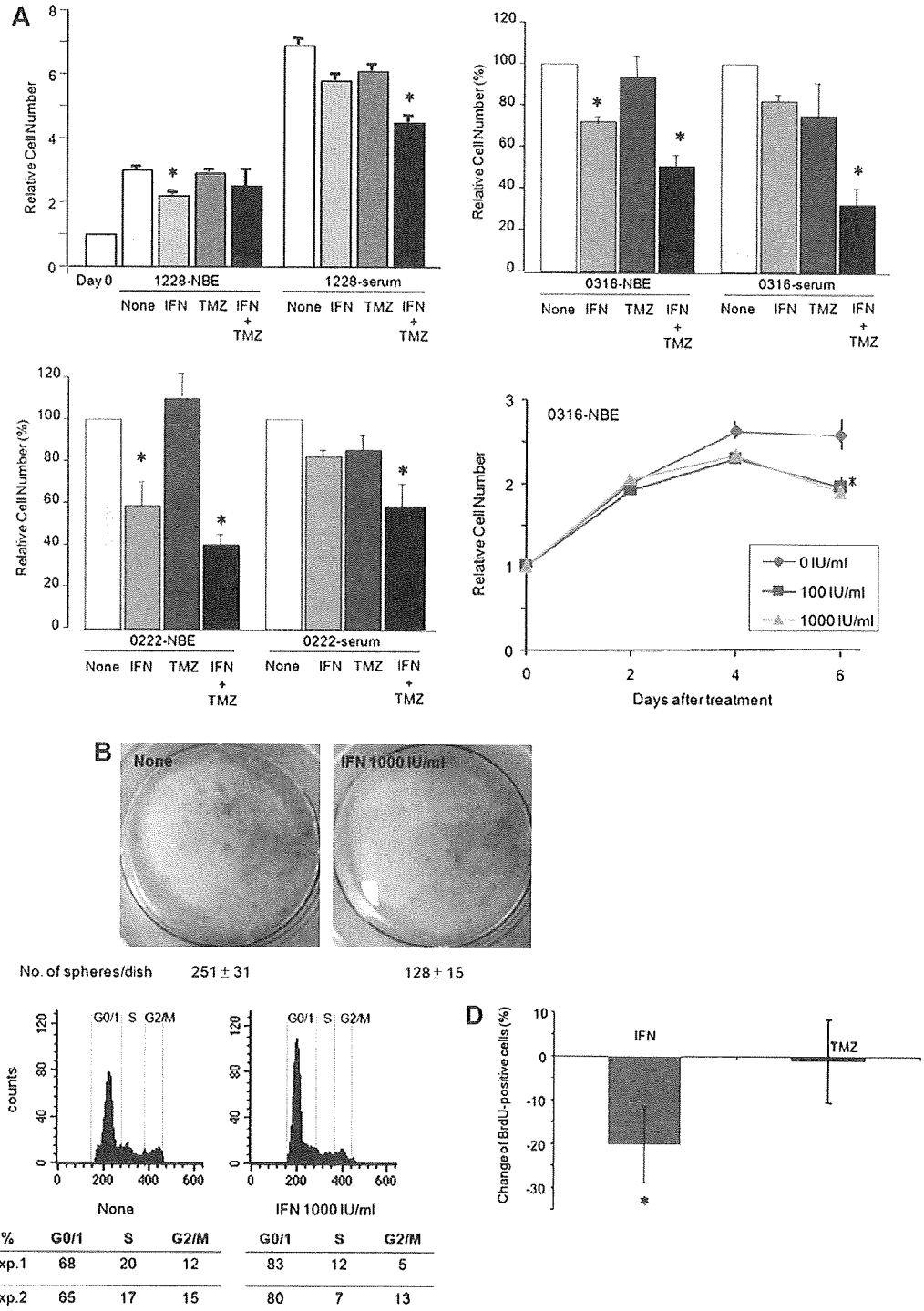

%	G0/1	S	G2/M	G0/1	S	G2/M
Exp.1	68	20	12	83	12	5
Exp.2	65	17	15	80	7	13

Fig. 2. IFN-β reduces GIC proliferation. A, Proliferation assays of NBE and serum cells treated with IFN-β (1000 IU/ml) and TMZ (200 μM). Cells were counted using the WST-8 reagent, and relative changes were determined by comparing the viability of cells with those on day 1 (1228 cells) after treatment or with untreated cells (0316 and 0222 cells). Compared to untreated cell, the growth of all NBE cells but not of the serum cells was significantly delayed by IFN-β treatment. In the time course study, the IFN-β-treated NBE cell number reached a plateau within 4 days. In contrast, TMZ did not exert antiproliferative effect on either NBE or serum cells. IFN-β and TMZ combination induced remarkable synergistic growth suppression in 0316- and 0222-NBE cells and in all serum cells. Mean ± SD, $*p < 0.01$. B, IFN-β inhibits colony formation. The number of colonies per culture dish was counted by three observers blinded to the treatment conditions. C, Cell cycle analysis of NBE cells cultured with IFN-β. Percentage of cells in each stage of cell cycle obtained in two independent experiments is shown in the table. D, BrdU incorporation assay. Relative changes were determined by comparing the number of BrdU-positive cells in treated groups with those in the untreated group. Mean ± SD, $*p < 0.05$.

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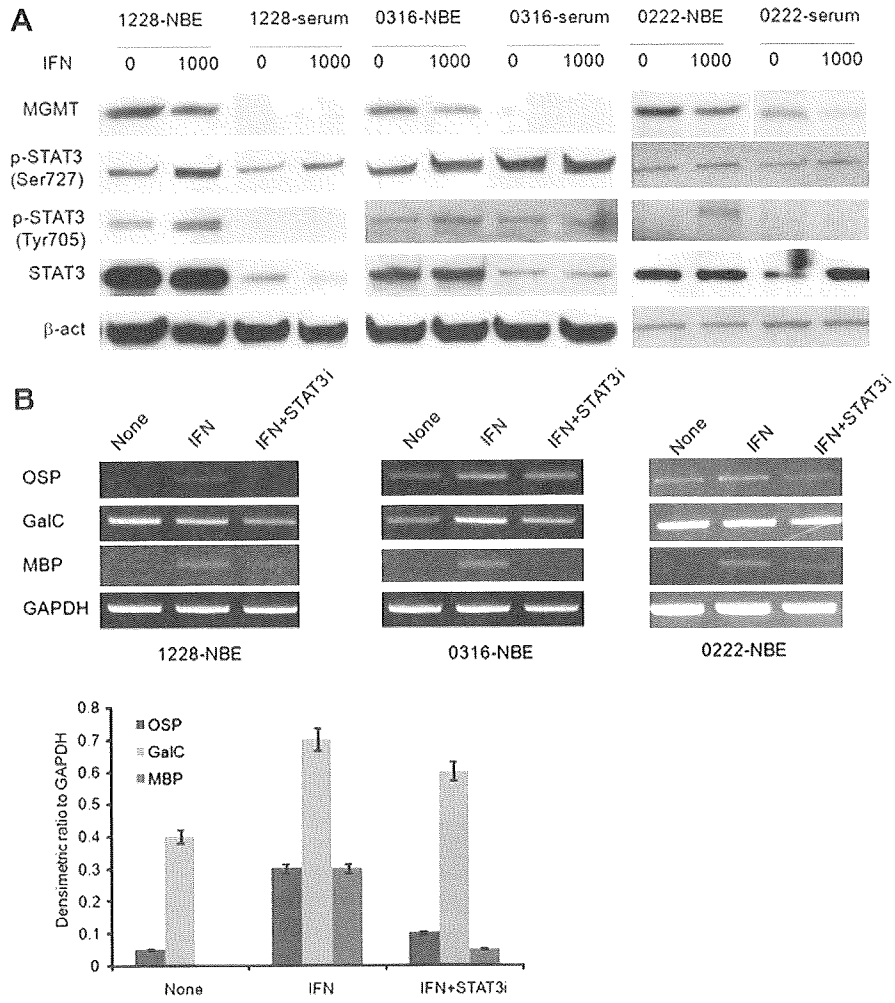

Fig. 3. IFN-β mediates oligodendrogenesis by the activation of STAT3 signaling. A, Western blot analysis of STAT3 and MGMT in IFN-β-treated NBE and serum cells. B, RT-PCR for detecting oligodendroglial markers in NBE cells treated with IFN-β with or without a STAT3 inhibitor. The level of expression was quantified by NIH-image. The values are expressed relative to the intensity of GAPDH (mean ± SD of three independent PCRs in the three cell lines). C, Western blot analysis of MBP and STAT3 in IFN-β-treated NBE cells with or without a STAT3 inhibitor. Cells treated with a STAT3 inhibitor for 30 min were cultured with IFN-β for 2 days. D, Immunocytochemical and FACS analyses of GalC expression in NBE cells cultured with IFN-β. E, RT-PCR for the detection of nestin and oligodendroglial markers in human neural stem cells and 022-NBE cells treated with IFN-β. F, Western blot for the detection of CD133 in 0316- and 0222-NBE cells treated with IFN-β.

the established tumor masses was limited probably due to its short half life, these findings suggest that IFN-β reduced the tumorigenesis ability of GICs.

4. Discussion

Response–survival paradox in cancer therapy has long been suggested [15]. Although most cancer patients respond to therapy, few are cured. Objective clinical response to treatment might not generally be helpful in predicting the overall survival improvement. For example, the clinical response patterns toward imatinib and IFN treatment in chronic myeloid leukemia (CML) patients are remarkably different. The rapid imatinib-induced responses are probably a consequence of its efficient activity against differentiated cells constituting the bulk of CML. The rapid, but short-lasting response to imatinib could be attributed to the resistance of CML stem cells. In contrast, IFN-induced responses are slow and gradual, frequently requiring years to establish their effect; however, they could be long-lasting. This is consistent with the data suggesting that IFN activity is principally directed at small CML stem cell population. The response–survival paradox also applies to other malignancies.

In ovarian cancer, Moserle et al. demonstrated that IFN exerted marked antiproliferative effect on side population cells with higher proliferation rates [16]. However, its action mechanism remains unclear. These studies prompted the elucidation of how IFN could target cancer stem cells.

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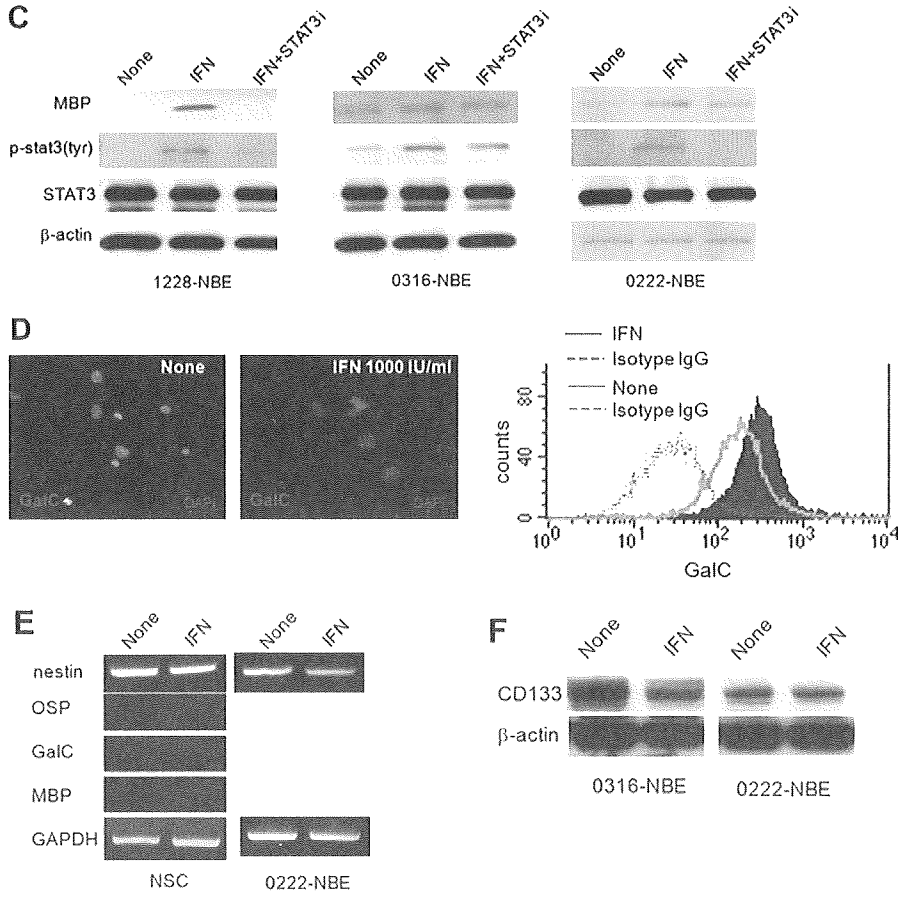

Fig. 3 *(continued)*

In this study, we demonstrated that IFN-β elicited significant antiproliferative effects on GICs although such effects were not elicited by TMZ. STAT3 activation signaling pathway appears to be essential for antiproliferation in glioblastoma [17]; however, the role of STAT3 activation is debatable because its overactivation has been reported to be oncogenic in various tumor types [18]. Our data suggest that IFN-β-mediated STAT3 phosphorylation is necessary for the differentiation of GICs into oligodendroglial cells. Thus, STAT3 activation seems to be cytostatic.

An unresolved question pertains to what regulates oligodendrogenesis of GICs. Oligodendroglial lineage arises from undifferentiated progenitor cells, mainly found in the subventricular zone, that mature into myelinating oligodendrocytes due to the interactions between BMP, sonic hedgehog, and Notch signaling pathways [19]. IFN-mediated activation of BMP/Smad pathway and Notch signaling was not observed in our study (data not shown). There is strong evidence suggesting that thyroid hormones (THs) directly regulate the differentiation and maturation of oligodendroglial cells [19]. Triiodothyronine (T3) is an active hormone that regulates gene expression by binding to specific intracellular TH receptors. Our group previously demonstrated that 95% of glioma cells expressed a member of the thyroid/steroid hormone receptor superfamily—peroxisome proliferative-activated receptor gamma (PPARγ), which activates the transcription of target genes after forming heterodimers with retinoid × receptors (RXR) [20]. PPARs are known to be responsible for the acquisition of specific NSC fate. It has been reported that PPARs regulate the proliferation, migration, and differentiation of NSCs via STAT3 activation [21]. Although further confirmative studies are warranted in this regard, we were able to show that treatment of GICs with IFN-β resulted in T3 release, which was inhibited by the STAT3 inhibitor. Treatment with exogenous T3 resulted in the formation of MBP-positive mature oligodendrocytes displaying a multibranched morphology (Supplementary Fig. S1). This suggests that GICs may possess neuroendocrinal properties and differentiate into mature oligodendrocytes.

In summary, we demonstrated that IFN-β inhibits the self-renewal ability of GICs and induces their terminal differentiation to mature oligodendroglia-like cells. This IFN activity may be directed only toward GICs.

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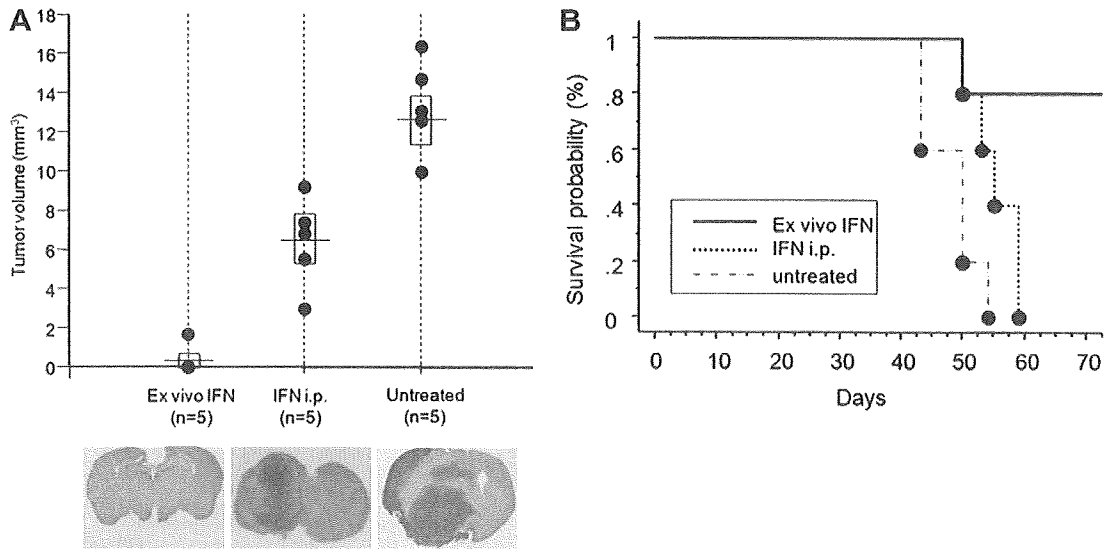

Fig. 4. IFN-β treatment reduces the pool of tumorigenic GICs. Ex vivo IFN: NBE cells treated with IFN for 2 days were implanted into the brains of NOD/SCID mice. IFN i.p.: IFN-β (2×10^5 IU) was administered intraperitoneally every 2 days for 14 days. A, Tumor length (a) and width (b) were measured in the largest coronal section 30 days after implantation, and tumor volumes were calculated as $V\,(mm^3) = a \times b^2/2$. Mean (bar) ± SD (box). B, Survival curve of mice injected with NBE cells.

Conflict of interest

None to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2009.04.020.

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Pediatr Blood Cancer 2009;53:37–41

Interferon-Beta, MCNU, and Conventional Radiotherapy for Pediatric Patients With Brainstem Glioma

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Background. Most children with brainstem glioma die within 2 years of diagnosis, and the median survival time for patients with this condition is less than 1 year. The role of chemotherapy in the treatment of children with brainstem glioma is not well defined. The primary aim of this study is to evaluate the effects of treatment with interferon-β (IFN-β), ranimustine (MCNU), and radiotherapy (IMR therapy) administered to brainstem glioma patients treated at our institution. We also determined patient response to IMR therapy by evaluating O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation in serum DNA. ***Procedures.*** We retrospectively reviewed 15 patients who were newly diagnosed to have brainstem tumors and were administered IFN-β (1–2 MIU/day, days 1–7; 0.5–1 MIU/day, days 8–14) and MCNU (80 mg/m^2 on day 2) concurrently with conventional radiotherapy. Responses were assessed by MRI scan, and data on clinical course and toxicity were obtained from the medical records. The MGMT promoter methylation in serum DNA of five patients was assayed by methylation-specific PCR. ***Results.*** Of the 15 patients, partial response, stable disease, and progressive disease were noted in 5 patients each. The median overall survival time and the median progression-free survival time were 14.7 and 4.6 months, respectively. The protocol was not terminated in any of the patients because of hematological toxicity, nephrotoxicity, or neurotoxicity. The MGMT promoter methylation status in the serum appeared to correlate with a positive response to IMR therapy. ***Conclusions.*** The IMR combination therapy is well tolerated and may be a promising treatment for brainstem glioma. Pediatr Blood Cancer 2009;53:37–41.

Key words: brainstem glioma; chemotherapy; interferon-beta; MCNU; methylation; MGMT

INTRODUCTION

Brain tumors account for 20% of all pediatric neoplasms, which comprise the largest group of solid tumors that occur during childhood [1]. These tumors are both anatomically and histologically diverse. One of the most complex families of such tumors is the brainstem tumor family that accounts for 15–20% of all central nervous system tumors affecting children. This range corresponds to 50 annual cases of this tumor in Japan. Despite collaborative efforts between pediatric neurosurgery, oncology, and hematology, there has been no improvement in the survival of brainstem tumor patients over the past 3 decades. Overall, greater than 90% of children die within 2 years of diagnosis with a median survival time of less than 1 year. Most prospective studies that focused on the treatment of brainstem gliomas have investigated several radiotherapy options such as conventional and hyperfractionated radiotherapy. Studies have concluded that standard conventional radiotherapy is as efficient as alternative radiation techniques for tumor treatment; thus, the standard treatment for this tumor currently involves conventional focal radiotherapy. However, outcomes for patients remains poor. Conventional radiotherapy often results in a transient improvement in neurological signs and symptoms; however, it does not aid in the improvement of event-free and overall survival rates. Theoretically, randomized control clinical trials (RCTs) should be designed to detect the benefits of a new strategy over the existing ones and to prove new hypotheses. Since the standard treatment arm of RCTs provides no guarantee of success, the rationale underlying the designing of RCTs based on their comparisons with a standard treatment group is questionable from the point of view of treatment efficacy. Furthermore, such a design is very difficult to establish, particularly in children. The best method for the clinical study of brainstem glioma patients remains to be defined. For these reasons, a few phase III studies to assess the role of chemotherapy have been conducted; further, chemotherapy has not shown any significant effectiveness in improving outcomes in patients with brainstem glioma. Although it has become standard practice to perform cooperative multicenter studies in clinical research in pediatric neurooncology, the differences in the characteristics of patients between institutions is a matter of concern because it precludes appropriate comparisons. The need for performing a central radiology review is critical, even when diagnostic criteria have been validated. Consequently, this retrospective study in a single institution adopted a standardized approach by focusing on uniform diagnostic criteria and treatment plans, which allowed for a more simple and reproducible outcome. The primary aim of this study is to evaluate the effects of treatment with IFN-β and a water-soluble nitrosourea derivative, namely, ranimustine (MCNU), and radiotherapy (IMR therapy) performed in brainstem glioma patients treated at our institution.

Free DNA in the serum of cancer patients can be detected. It has been proven that hypermethylation of a normally unmethylated CpG island observed in the promoter region of certain tumor suppressor genes such as *p16*, *RB*, and *death-associated protein kinase* (*DAPK*) can be detected in the serum of the majority of cancer patients by using methylation-specific polymerase chain reaction (PCR), which is a sensitive and specific technique for methylation analysis [2–6]. Previously, we demonstrated that *p16* hypermethylation was detected in the serum of brainstem glioma patients; further, it can aid in the differentiation of brainstem glioma from other types of histologies [7]. Current diagnosis of brainstem tumors is largely performed by graphical examinations such as magnetic resonance imaging (MRI) and positron emission tomography (PET). Although biopsy provides a

Additional Supporting Information may be found in the online version of this article.

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definitive histological diagnosis, it cannot be used in all cases because of its invasive nature. This limitation of biopsy prompted us to attempt the development of non-invasive biomarkers for brainstem tumors. In this study, we also performed a pilot study to evaluate the O^6-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation levels in the serum of five brainstem glioma patients who were treated with IMR therapy and evaluated the response of these patients to this treatment with respect to the MGMT promoter methylation in their serum.

METHODS

Patients

We retrospectively reviewed the cases of 15 patients aged less than 17 years who were newly diagnosed to have brainstem tumors and who received chemoradiotherapy consisting of IFN-β and MCNU (IMR therapy) at the Nagoya University School of Medicine from 1995 to 2006. The diagnosis was based on the review of the MRI scans of the patients; in four patients, histopathology was verified by biopsy. Table I presents the clinical characteristics of each patient. The tumor radiological findings were classified according to the classification system of Choux et al. [8]

Treatment Regimen: Pre-Radiation, Concomitant, and Adjuvant IFN-β Plus MCNU

Figure 1 summarizes the treatment regimen. Patients 1–10 received IFN-β at a daily dose of 1 million international units (MIU) on days 1–7, a reduced dose of 0.5 MIU on days 8–14, and MCNU at a dose of 80 mg/m^2 on day 2. Patients 11–15 received IFN-β at a daily dose of 2 MIU on days 1–7 and a reduced dose of 1 MIU on days 8–14. From day 3, radiotherapy was initiated to deliver radiation to the tumor bed with a conventional fractionation of 1.5–2 Gy. Radiation was delivered once daily for 5 days per week to deliver a total dose of 40–60 Gy. The adjuvant administration of IFN-β and MCNU at the same dose was repeated every 6 weeks in cases with absence of tumor progression, with serious adverse events such as grade 4 hematological toxicity, and with refusal of therapy.

Response Criteria

The response to treatment was evaluated by detailed neurological examination and serial postcontrast CT and MRI at intervals of 3–6 weeks prior to and following completion of IMR therapy. The efficacy of IMR therapy was determined by assessing the reduction in tumor size, deterioration of symptoms, and survival rates. Tumor size was defined as the area of the high-intensity region revealed on T2-weighted MR images.

The neuroradiological criteria employed to determine the response or relapse were as follows: (1) complete response (CR), the disappearance of all known diseases for a minimum of 4 weeks; (2) partial response (PR), reduction of at least 50% in the sum of the products of the largest diameters of measurable lesions for at least 4 weeks; (3) stable disease (ST), decrease of <25% or no increase in the sum of the products of the largest diameters of measurable lesions; and (4) progressive disease (PD), any exacerbation in known disease conditions or the appearance of new lesions.

Toxicity

All patients were evaluated for toxicity; the toxicity was graded according to the National Institutes of Health Common Toxicity Criteria version 3.

MGMT Promoter Hypermethylation in Serum

The molecular genetic analysis performed in the study was approved by the Institutional Ethics Committee of Nagoya University; further, all patients who registered for this study provided written informed consent. We analyzed serum samples

TABLE I. Clinical, Radiological, Histological, and Serum MGMT Methylation Data of Patients With Brainstem Glioma

Pt. no.	Age (years)	Sex	Classification[a]	Histology[b]	RT (Gy)	MGMT met	Radiological response	Second. Tx	PFS (months)	OS (months)
1	8	F	Instric, focal	—	59	—	PD	None	0	4.8
2	5	F	Diffuse	—	60	—	PD	CBDCA+VP16	2.4	14.7
3	7	M	Diffuse	—	60	—	ST	None	7.2	19.3
4	9	F	Diffuse	Astro-G2	50	M	ST	CBDCA+VP16	3.1	36.2
5	9	F	Diffuse	—	60	M	PR	CBDCA+VP16	7.2	19.1
6	5	M	Diffuse	Astro-G2	60	M	PR	None	2.6	5.7
7	4	M	Instric, focal	Astro-G3	60	—	ST	None	7.3	18.4
8	9	M	Diffuse	Astro-G2	59	U	PD	CBDCA+VP16	0	7.1
9	5	M	Diffuse	—	60	—	PR	CBDCA+VP-16 and ICE	5.5	12.3
10	5	M	Diffuse	—	60	—	PD	None	3.4	10.4
11	8	F	Diffuse	—	54	—	ST	TMZ	4.6	8.4
12	7	F	Diffuse	—	54	—	PR	None	14.4	17
13	9	M	Diffuse	—	54	—	PR	None	33.5	33.5
14	12	F	Diffuse	—	45	—	ST	TMZ	4	10.2
15	17	M	Diffuse	—	60	U	PD	None	6.6	22.4

Pt, patient; M, male; F, female; Astro-G, astrocytoma grade. RT, radiotherapy; met, methylation; M, methylated; U, unmethylated; PD, progressive disease; ST, stable disease; PR, partial response; Second. Tx, Second-line treatment; CBDCA, carboplatin; TMZ, temozolomide; ICE, ifosmide + cisplatin + etoposide; PFS, progression-free survival; OS, overall survival. [a]The diagnosis was based on MRI scan; the tumor radiological findings were classified with classification system by Choux et al.; [b]Histopathology was verified by biopsy in Patients #4, 6–8.