and p-Tyr705), STAT3 (Cell Signaling), and β-actin (AC-15; Sigma-Aldrich).

Intracranial Glioma Xenograft and IFN-β Treatment

Animal experiments were done according to the principles described in the Guide for the Care and Use of Laboratory Animals prepared by the Office of the Prime Minister of Japan. We stereotactically injected 0316-GICs (1×10^5 cells) resuspended in 5 μL of PBS into 5- to 6-wk-old nonobese diabetic/severe combined immunodeficient female mice (SLC, Shizuoka, Japan) as described previously (40). IFN-β (2×10^5 IU/animal) was given i.p. at 4 wk after tumor inoculation. The control mice received PBS. Treatments were repeated at 24-h intervals for a total of five doses.

In situ *Hybridization*

Tissues were fixed with Tissue Fixative (Genostaff), embedded in paraffin, and sectioned at 6 μm. For performing *in situ* hybridization, the tissue sections were deparaffinized with xylene, and rehydrated using a series of ethanol washes and PBS. The sections were fixed with 4% paraformaldehyde in PBS for 15 min and then washed with PBS. They were then treated with 10 μg/mL of Proteinase K in PBS for 30 min at 37°C, washed with PBS, refixed with 4% paraformaldehyde in PBS, washed again with PBS, and placed in 0.2 mol/L of HCl for 10 min. After washing with PBS, the sections were acetylated by incubation in 0.1 mol/L of triethanolamine-HCl (pH 8.0) and 0.25% acetic anhydride for 10 min. After washing with PBS, the sections were

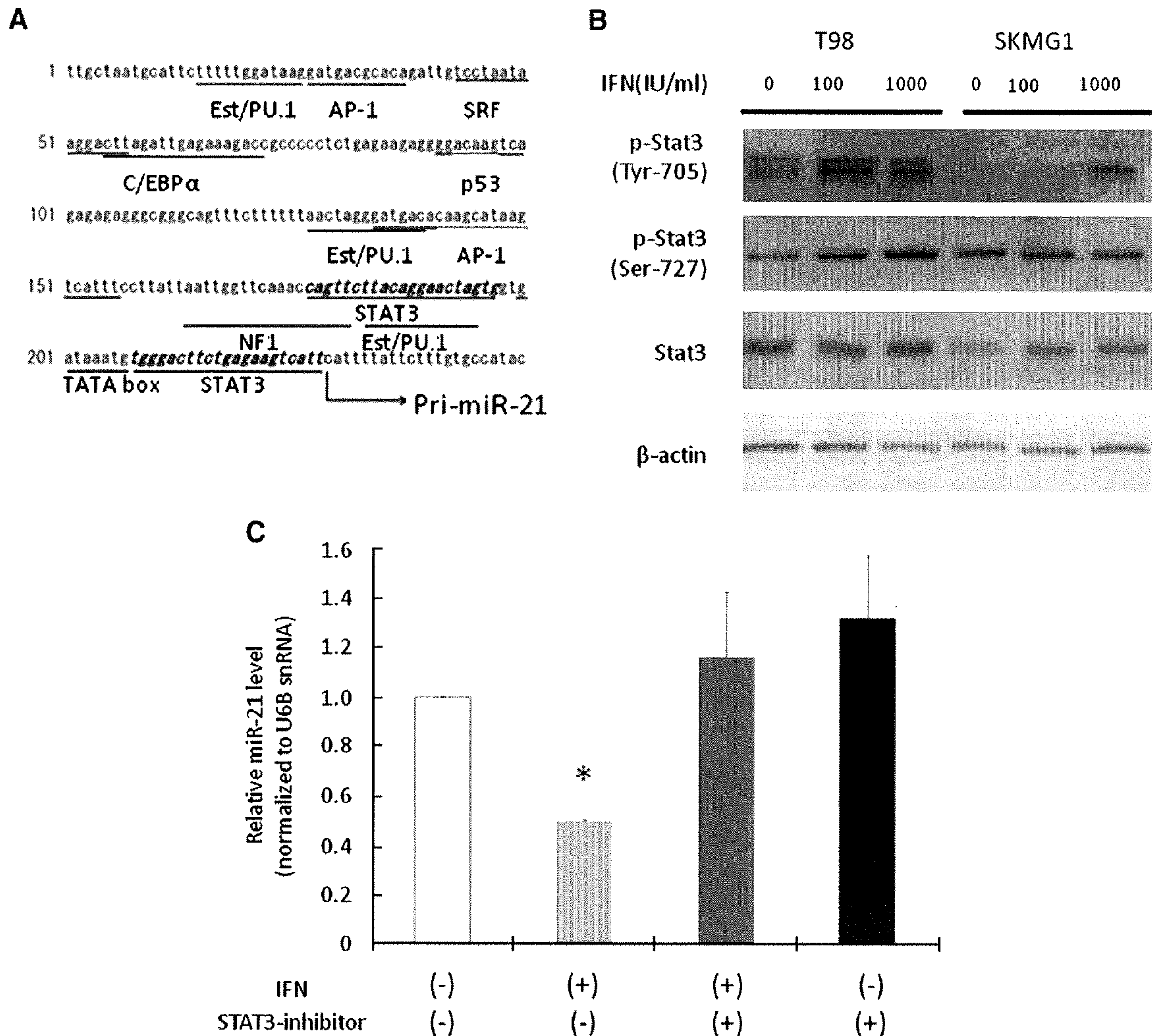

FIGURE 4. STAT3 negatively regulates miR-21 transcription. **A.** Consensus sequence upstream of the transcription start site of the *pri-miR-21* obtained using TRANSFAC matrices (http://www.gene-regulation.com/pub/databases.html). **B.** IFN-β phosphorylates tyrosine and, in part, serine residues of STAT3 in both T98 and SKMG1 glioma cells. **C.** Treatment with a STAT3-specific inhibitory peptide increased the level of miR-21 expression and inhibited IFN-β–mediated suppression of miR-21 (*, $P < 0.05$).

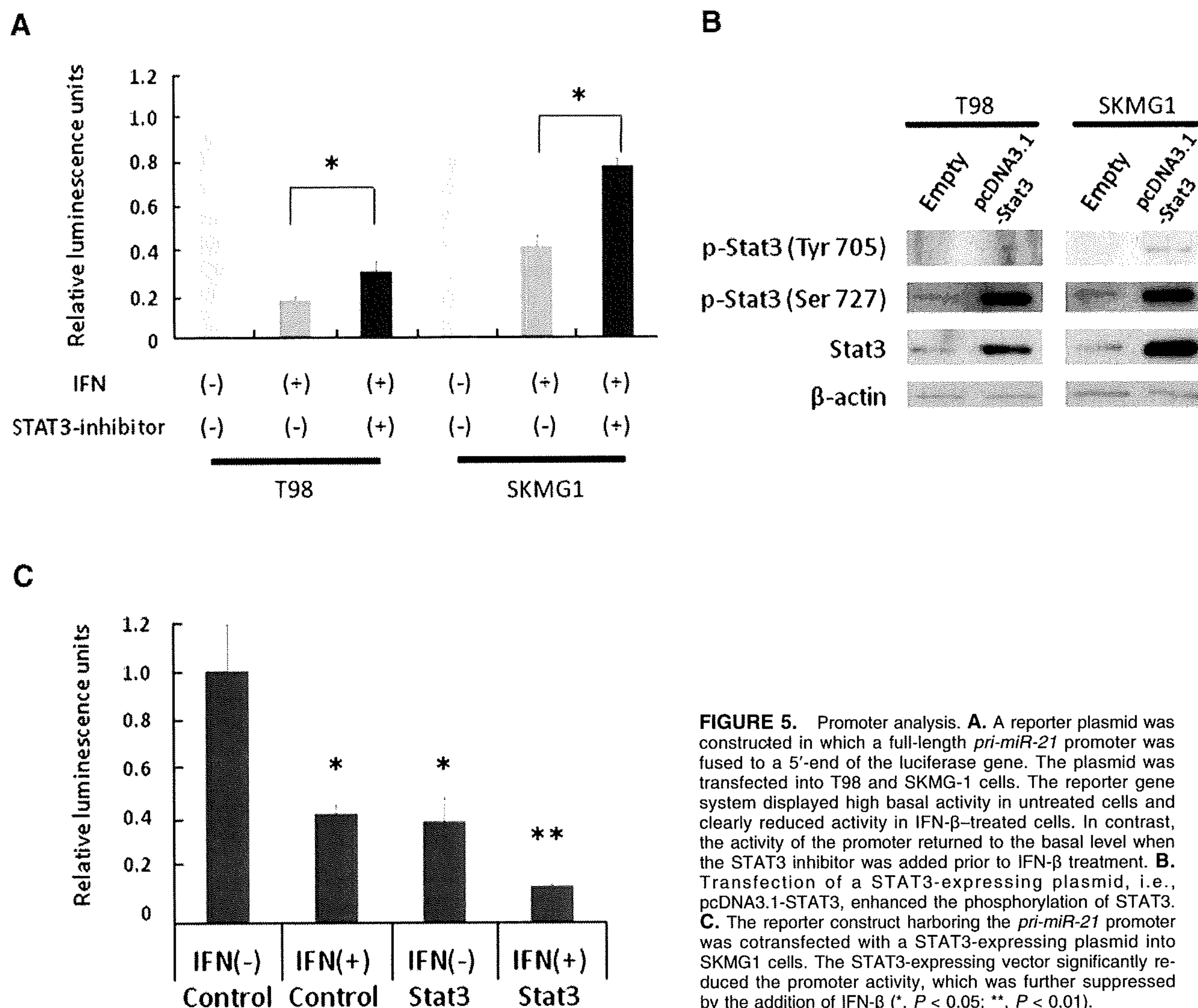

FIGURE 5. Promoter analysis. **A.** A reporter plasmid was constructed in which a full-length *pri-miR-21* promoter was fused to a 5′-end of the luciferase gene. The plasmid was transfected into T98 and SKMG-1 cells. The reporter gene system displayed high basal activity in untreated cells and clearly reduced activity in IFN-β–treated cells. In contrast, the activity of the promoter returned to the basal level when the STAT3 inhibitor was added prior to IFN-β treatment. **B.** Transfection of a STAT3-expressing plasmid, i.e., pcDNA3.1-STAT3, enhanced the phosphorylation of STAT3. **C.** The reporter construct harboring the *pri-miR-21* promoter was cotransfected with a STAT3-expressing plasmid into SKMG1 cells. The STAT3-expressing vector significantly reduced the promoter activity, which was further suppressed by the addition of IFN-β (*, $P < 0.05$; **, $P < 0.01$).

dehydrated using a series of ethanol washes. Hybridization was done with either the locked nucleic acid–enhanced miR-21 probe (5′-TCAACATCAGTCTGAATAAGCTA-3′; Exiqon) or a scramble probe (5′-GTGTAACACGTCTATACGCCCA-3′) at concentrations of 18 nmol/L in the Probe Diluent (Genostaff) at 50°C for 16 h. After hybridization, the sections were washed in 5× HybriWash (Genostaff; equivalent to 5× SSC) at 50°C for 20 min and then in 50% formamide, 2× HybriWash at 50°C for 20 min. Subsequently, the sections were washed thrice with 2× HybriWash at 50°C for 20 min, and once with TBS Tween 20 (TBST; 0.1% Tween 20 in TBS). After treatment with 0.5% blocking reagent (Roche) in TBST for 30 min, the sections were incubated with anti–DIG-AP conjugate (Roche) diluted 1:1,000 with TBST for 2 h. The sections were washed twice with TBST and then incubated in 100 mmol/L of NaCl, 50 mmol/L of $MgCl_2$, 0.1% Tween 20, and 100 mmol/L of Tris-HCl (pH 9.5). Coloring reactions were done using nitroblue tetrazolium-5-bromo-4-choloro-3-indolylphosphate, an alkaline phosphate color substrate. The sections were counterstained with Kernechtrot stain solution (Muto, Tokyo, Japan), dehydrated, and then mounted with Malinol (Muto).

Luciferase Reporter Assay

The miR-21 promoter/enhancer region was amplified by PCR from human genomic DNA using the primers 5′-TTTGGTACCTTGCTAATGCATTCT-3′ and 5′-TTTAGATCTAGTTCA GCTATGGTAAGAGC-3′ and inserted into the *Kpn*I and *Bgl*II sites of pGL3-Enhancer vector (Promega) immediately downstream of the luciferase gene to form a pGL-miR-21 promoter/enhancer construct. The overexpression of STAT3 in the cells was achieved by transfection with pcDNA3.1-STAT3 that was provided by Dr. Takeshi Senga (Department of Tumor Biology, Nagoya University School of Medicine, Nagoya, Japan). The SKMG1 and T98 cell lines were seeded in 24-well plates for 24 h and then transfected with 1 μg of pGL-miR-21 promoter/enhancer construct with or without pcDNA 3.1-STAT3 for 48 h.

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 $100\,\mu g\,ml^{-1}$ 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 $3\,\mu g\,ml^{-1}$ 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 ($2.5\,\mu g\,ml^{-1}$) and gentamicin ($20\,\mu g\,ml^{-1}$).

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 $500\,\mu g\,ml^{-1}$, 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 $500\,\mu g\,ml^{-1}$ 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

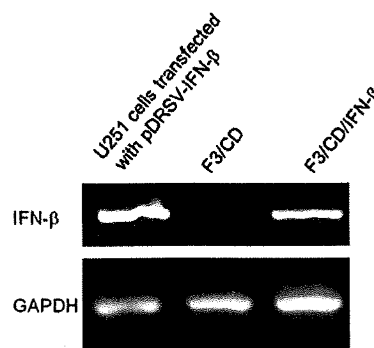


Figure 1 The expression of human interferon-β (IFN-β) in the F3.CD/IFN-β cell line. The IFN-β transcript was expressed only in F3.CD.IFN-β human neural stem cells.

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

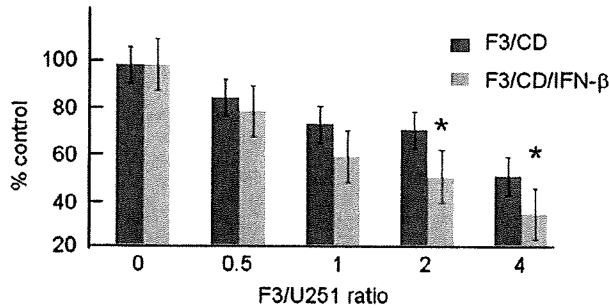


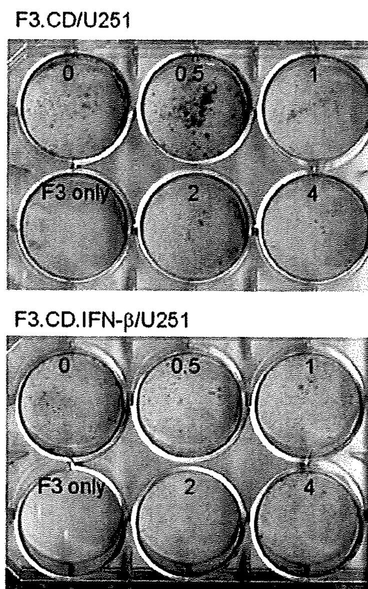
Figure 2 A bystander killing effect. The U251 cells were seeded in a 24-well plate (2×10^4 cells per well) and cocultured with either F3.CD or F3.CD.IFN-β cells at various U251/F3 ratios ranging from 1:0 to 1:4. After the day 1 of culture, 5-fluorocytosine (5-FC) was added to the medium at a final concentration of $500\,\mu g\,ml^{-1}$, 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.



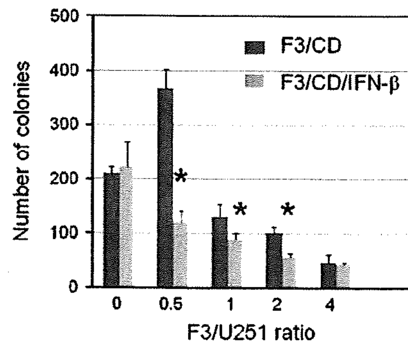


Figure 3 Clonogenic assay. U251 cells were seeded in a 24-well plate (2×10^4 cells per well) and cocultured with either F3.CD or F3.CD.IFN-β cells at various U251/F3 ratios ranging from 1:0 to 1:4. After 24 h culture, 5-fluorocytosine (5-FC) was added to the medium at a final concentration of $500\,\mu g\,ml^{-1}$, 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.

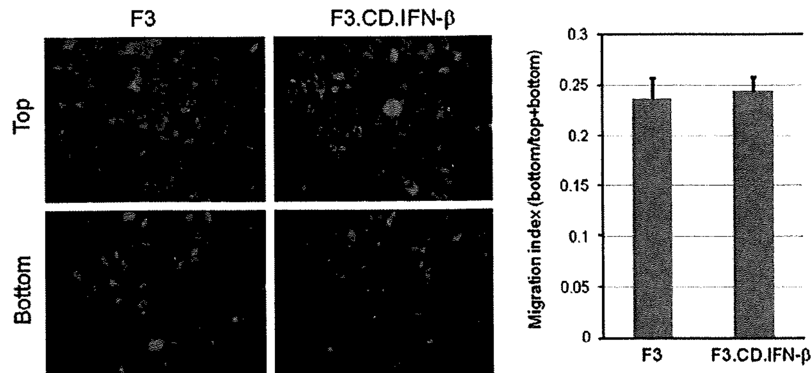


Figure 4 Migration assay *in vitro*. U251 cells (1 × 10^5) were plated on the 24-well plate and cultured for 48 h. CM-DiI-labeled F3 or F3.CD.IFN-β cells (2 × 10^4) were seeded into the upper wells of the FluoroBlok inserts. After a 24-h incubation, migrated (bottom) and non-migrated cells (top) were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

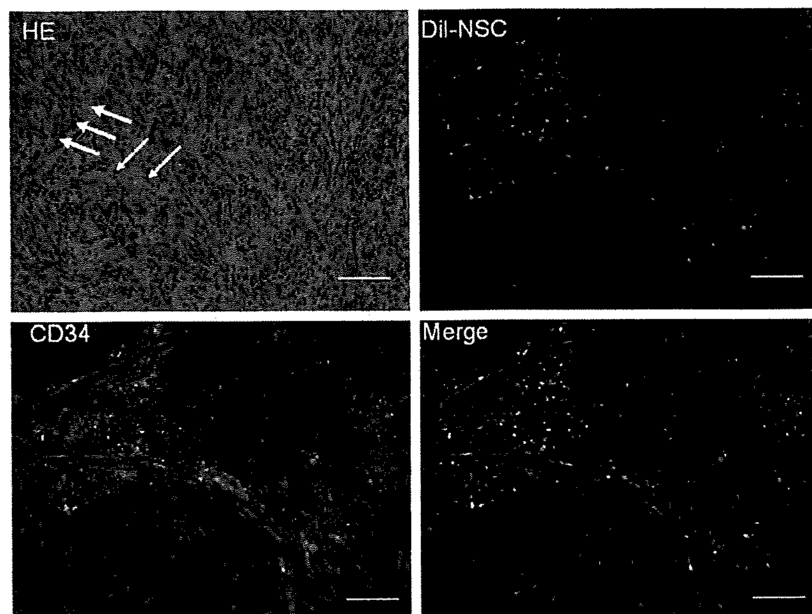


Figure 5 Migration of F3.CD.IFN-β cells into the tumor parenchyma. To ascertain the migratory capability of the neural stem cells (NSCs) from the vessels to the tumor parenchyma, the DiI-labeled F3.CD.IFN-β cells (red) were injected into the tail vein, and the newly formed tumor vessels were immunostained 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

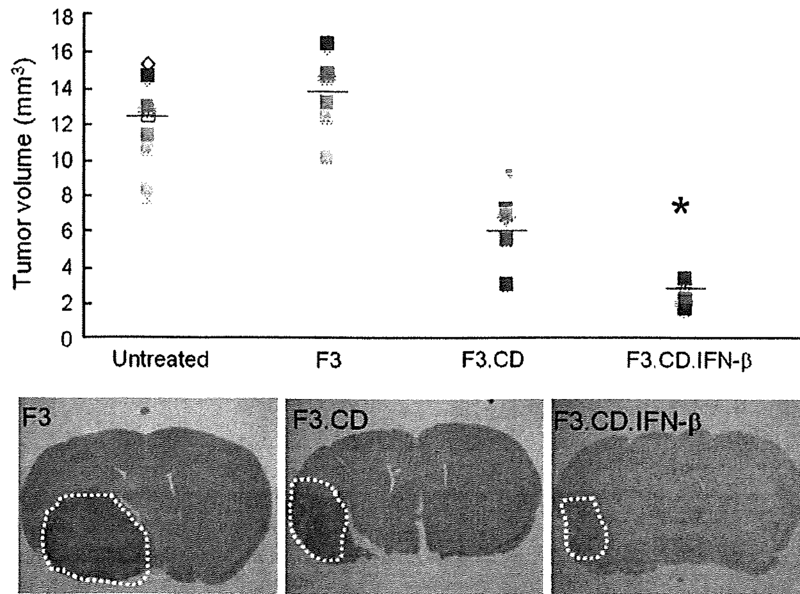


Figure 6 The growth inhibitory effect of genetically engineered neural stem cells (NSCs) on the glioma cells *in vivo*. The mice with the U251-derived intracerebral tumor were injected with F3 cells, and then i.p. injections of 5-fluorocytosine (5-FC) were administered for the next 10 days. The volume 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.

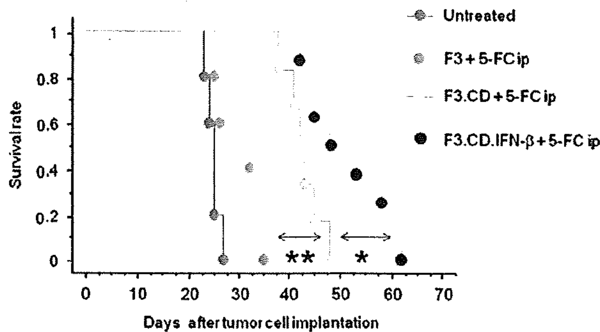


Figure 7 The survival time of experimental animals. Mice were inoculated with U251 intracranially, and subsequently with each type of neural stem cells (NSCs) (F3.CD.IFN-β ($n=8$), F3.CD ($n=6$) and F3 ($n=5$)), followed by intraperitoneal injection of prodrug 5-fluorocytosine (5-FC) and compared with 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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Clinical Trial Note

A Multicenter Phase I Trial of Interferon-β and Temozolomide Combination Therapy for High-grade Gliomas (INTEGRA Study)

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A multicenter phase I clinical trial, namely, Integrated Japanese Multicenter Clinical Trial: A Phase I Study of Interferon-β and Temozolomide for Glioma in Combination with Radiotherapy (INTEGRA Study), is being conducted for patients with high-grade glioma in order to evaluate the safety, feasibility and preliminary clinical effectiveness of the combination of interferon-β and temozolomide. The primary endpoint is incidence of adverse events. The secondary endpoints are progression-free survival time and overall survival time. In addition, objective tumor response will be evaluated in a subpopulation of patients with the measurable disease. The reduction rate of tumor will be calculated according to Response Evaluation Criteria In Solid Tumors for measurable tumors as determined by magnetic resonance imaging. Subsequently, the overall response will be evaluated based on the results of measurable and non-measurable tumors. Ten newly diagnosed and 10 recurrent patients will be enrolled in this study.

Key words: chemo-phase I-II-III – clinical trials – CNS

INTRODUCTION

Gliomas account for ~40% of all brain tumors and are thus the most common primary tumors of the central nervous system. Primary brain tumors are classified according to their cell type and histological grade into categories defined by the World Health Organization (WHO) (1). High-grade (WHO grades III and IV) gliomas, which include anaplastic astrocytoma (AA), anaplastic oligodendroglioma (AO), anaplastic oligoastrocytoma (AOA) and glioblastoma multiforme (GBM), are often resistant to treatment; GBM, the most common glioma in adults, kills patients within a median time span of a year after diagnosis despite treatment with aggressive surgical resection, nitrosourea-based chemotherapy and radiotherapy (2–4). A number of studies by large cooperative groups have shown the benefits of radiation therapy in doses up to 60 Gy after surgery for improving overall survival and time to progression (5). In Japan, nitrosourea agents such as 1-(4-amino-2-methyl-5-pyridiminyl)methyl-3-(2-chloroethyl)-3-nitrosourea and methyl-6-[3-(2-chloroethyl)-3-nitrosoureido]-6-deoxy-alpha-D-glucopyranoside have been used to treat malignant gliomas for a long time; however, this treatment offered few clinical benefits. Temozolomide (TMZ), an oral alkylating agent, has been demonstrated to possess antitumor activity against malignant gliomas, with minimal additional toxicity; furthermore, in a previous study of concomitant radiation therapy and chemotherapy with TMZ followed by adjuvant TMZ, survival duration substantially improved (6). In 2006, TMZ

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was certified as the treatment agent for malignant gliomas by the National Ministry of Health and Welfare of Japan, and a combination of radiotherapy and chemotherapy with TMZ is now used as the first-line therapy. However, its clinical outcomes depend on the *O*-(6)-methylguanine-DNA methyltransferase (MGMT) status, and MGMT modification is one of the key factors to obtain greater clinical benefits in the future.

Interferon-β (IFN-β) exhibits pleiotropic biological effects and has been widely used either alone or in combination with other antitumor agents in the treatment of malignant gliomas and melanomas (7). In the treatment of malignant gliomas, IFN-β can act as a drug sensitizer, enhancing toxicity against various neoplasms when administered in combination with nitrosourea. IFN-β and nitrosourea combination therapy has been particularly used for the treatment of gliomas in Japan (8). Previously, we demonstrated that IFN-β markedly enhanced chemosensitivity to TMZ in an *in vitro* study of human glioma cells (9); this finding suggested that one of the major mechanisms by which IFN-β enhances chemosensitivity is the downregulation of MGMT transcription via *p53* induction. This effect was also observed in an experimental animal model (10). These two studies suggested that chemotherapy with IFN-β and TMZ plus radiation might further improve the clinical outcome in malignant gliomas when compared with TMZ plus radiation therapy. Here, in order to evaluate the safety, feasibility and preliminary clinical effectiveness of the combination of IFN-β and TMZ, we are conducting a clinical study, namely, Integrated Japanese Multicenter Clinical Trial: A Phase I Study of Interferon-β and Temozolomide for Glioma in Combination with Radiotherapy (INTEGRA study). This study involves eight medical institutions, covering the entire regional population of Japan.

PROTOCOL DIGEST OF THE STUDY

Purpose

The main aim of this study is to evaluate the safety, feasibility and preliminary clinical effectiveness of IFN-β and TMZ for the treatment of malignant gliomas.

Study Setting and Protocol Review

This is a multicenter clinical trial involving eight neurosurgical institutions: Yamagata, Saitama Medical, Nippon Medical, Nagoya, Osaka, Kyoto, and Hiroshima Universities and Kitano Hospital. The protocol has been reviewed and approved by institutional review boards of each of these institutions.

Registration and Monitoring

Participating investigators are instructed to send an eligibility criteria report to the Data Center at Nagoya University, which is a third party different from the study director. Ten newly diagnosed and 10 recurrent patients are registered for a period of 6 months from December 2007. Data, including those of magnetic resonance imaging (MRI), blood tests, and pathology, will be collected at the data center. The quality of data will be checked and verified at the data center. If required, the data center would provide feedback to the institutions. The data center will send high-quality data to the study director. Committees of safety and efficacy (Dr Kazuo Tabuchi, Koyanagi Memorial Hospital, Saga), radiotherapy (Dr Shinji Naganawa, Department of Radiology, Nagoya University School of Medicine), pathological review (Dr Youichi Nagasato, Department of Pathology, Gunma University School of Medicine) and statistics (Dr Kunihiko Hayashi, Gunma University School of Health Science) will send their reports to the head office.

Endpoints

The primary endpoint is incidence of adverse events. The secondary endpoints are progression-free survival time and overall survival time. In addition, objective tumor response will be evaluated in a subpopulation of patients with measurable disease. The reduction rate of tumor will be calculated according to Response Evaluation Criteria In Solid Tumors for measurable tumors as determined by MRI. Non-measurable tumors are classified into four grades: complete remission, partial response, progression and not evaluable. Subsequently, the overall response will be evaluated based on the results of measurable and non-measurable tumors.

Eligibility Criteria

The eligibility criteria are as follows:

(i) Histologically confirmed diagnosis of newly diagnosed or recurrent high-grade glioma (AA, AO, AOA or GBM). More than 50% volume of tumor is located in the supratentorial region.
(ii) No tumor recognized in the optic nerve, olfactory nerve and pituitary gland on pretreatment MRI.
(iii) No dissemination detected by MRI. Age between 18 and 75 years at the time of registration.
(iv) Performance status is 0–2, 3 only due to neurological deficits.
(v) Sufficient organ function before chemotherapy according to the following laboratory data: WBC $\geq$ 3000/mm^3 or neutrophils $\geq$1500/mm^3, platelets $\geq$100 000/mm^3, hemoglobin $\geq$8.0 g/dl, bilirubin $\leq$1.5 mg/dl, serum glutamic oxaloacetic transaminase $\leq$ 100 IU, serum glutamic pyruvic transaminase $\leq$ 100 IU, creatinine $\leq$1.5 mg/dl, creatinine clearance $\geq$50 ml/min and electrocardiogram showing no serious arrhythmia and no serious ischemic heart disease.
(vi) No prior chemoradiotherapy for newly diagnosed patients.

(vii) The interval from the end of prior anti-tumor therapy (e.g. chemotherapy, radiotherapy, immunotherapy) must be at least 4 weeks for recurrent patients, regardless of the regimen.
(viii) Written informed consent.

Exclusion Criteria

The exclusion criteria are as follows:

(i) synchronous double cancer or metachronous double cancer in last 5 years; carcinoma *in situ* accepted;
(ii) meningitis or pneumonia;
(iii) pregnant, possibly pregnant, or nursing women;
(iv) mental disorder;
(v) uncontrolled diabetes mellitus (DM) or under treatment with insulin for DM;
(vi) myocardial infarction in last 3 months;
(vii) history of pulmonary fibrosis or interstitial pneumonia.

Treatment Methods

For newly diagnosed patients:

Radiotherapy 60 Gy/30 fr, 2 Gy ×5 days/week;
IFN-β 3 MIU/body, administered intravenously on alternate days during radiotherapy;
TMZ 75 mg/(m^2 day), daily from the first day to the last day of radiotherapy.

After completing this induction period, all patients will have 4 weeks of washout period, and they will be then shifted to adjuvant period.

IFN-β 3 MIU/body, administered on the first day morning every 4 weeks;
TMZ 150 mg/(m^2 day) (days 1–5: first cycle);
200 mg/(m^2 day) (days 1–5: second to sixth cycle).

In the absence of hematologic toxicity, the dose is increased to 200 mg/(m^2 day), beginning with the second cycle to the sixth cycle.

This cycle is repeated six times every 28 days in the absence of tumor progression, serious adverse events such as grade 4 hematological toxicity, refusal of therapy and deviation from the protocol.

For recurrent patients:

IFN-β 3 MIU/body, administered the first day morning every 4 weeks (day 1);
TMZ 150 mg/(m^2 day) (days 1–5: first cycle);
200 mg/(m^2 day) (days 1–5: second to sixth cycle).

In the absence of hematologic toxicity, the dose is increased to 200 mg/(m^2 day), beginning with the second cycle to the sixth cycle.

This cycle is repeated six times every 28 days.

This regimen has been considered to be the most promising based on previous clinical studies (8,11–14). Thus, dose-limiting toxicity was not evaluated in this study.

Follow-up and Statistical Methods

Disease progression and occurrence of new disease will be examined by MRI performed at baseline and at least after every 4–5 weeks during treatment. Blood tests and symptom checks will be carried out before treatment and at least after every 2 weeks during treatment. Follow-up will continue for 3 months from the end of treatment. In cases wherein therapy is discontinued due to toxicity, clinicians would follow-up patients until they recover from toxicity. In addition, overall survival, progression-free survival and treatment success curves are constructed as time-to-event plots by the Kaplan–Meier method.

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Conflict of interest statement

None declared.

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