

Antitumorigenic effect of interferon- β by inhibition of undifferentiated glioblastoma cells

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Abstract. Glioma stem-like cells (GSCs) are undifferentiated cells that are considered to be an origin of glioblastomas. Furthermore, they may contribute to treatment resistance and recurrence in glioblastomas. GSCs differentiate into differentiated glioma cells (non-glioma stem-like cells: non-GSCs), and interconversion might occur between GSCs and non-GSCs. We investigated whether interferon-beta (IFN- β) could exert any efficacy towards GSCs or such interconversion processes. The neural stem cell marker CD133 and pluripotency marker Nanog in GSCs were analyzed to evaluate their differentiation levels. GSCs were considered to undergo differentiation into non-GSCs upon serum exposure, since the expression of CD133 and *Nanog* in the GSCs was negatively affected. Furthermore, the cells regained their undifferentiated features upon removal of the serum. However, we verified that IFN- β reduced cell proliferation and tumor sphere formation in GSCs, and induced suppression of the restoration of such undifferentiated features. In addition, we also confirmed that IFN- β suppressed the acquisition process of undifferentiated features in human malignant glioma cell lines. Our data thus suggest that IFN- β could be an effective agent not only through its cell growth inhibitory effect on GSCs but also as a means of targeting the interconversion between GSCs and non-GSCs, indicating the possibility of IFN- β being used to prevent treatment resistance and recurrence in glioblastomas, via the inhibition of undifferentiated features.

Introduction

Malignant gliomas, especially glioblastomas, are the most frequently occurring primary tumors of the central nervous system, and represent one of the most lethal malignancies. Glioblastomas have been reported to be heterogeneous bulk tumors comprising differentiated and undifferentiated cells with a self-renewal ability, pluripotency, and tumorigenicity (1). Such heterogeneity may contribute to tumor expansion, invasion, metastasis, and drug resistance. The undifferentiated cells, a distinct subpopulation, within the tumors may derive from a limited source of glioblastoma cells and are termed glioblastoma stem-like cells (GSCs) (2-4). These cells are considered to be capable of aberrantly differentiating into diverse cell types, differentiated glioma cells (non-glioma stem-like cells: non-GSCs), in response to their microenvironment (5-7). Furthermore, there may be interconversion between GSCs and non-GSCs (7).

The efficacy of postoperative radiotherapy with concomitant and adjuvant temozolomide (TMZ) as the first-line treatment for glioblastomas was reported to be 9.8% in terms of the 5-year survival rate versus 1.9% with radiotherapy alone in a recent EORTC/NCIC randomized phase III trial (8,9). Concomitant radiotherapy with TMZ followed by adjuvant TMZ chemotherapy has thus become a current standard post-operative treatment for glioblastomas. Among the factors that may contribute to TMZ resistance, O⁶-methylguanine-DNA methyltransferase (MGMT, a protein that removes drug-induced alkylguanine adducts from DNA created by TMZ) is thought to be involved in its crucial mechanisms (9,10).

Human interferon-beta (IFN- β), a type I interferon, was first discovered on the basis of its antiviral activities. Subsequently, it was found to exhibit pleiotropic biological activities including immunomodulatory activity, anti-angiogenic activity and direct antitumor effects: e.g., growth inhibition, and apoptosis (11-13). Recently, a synergistic antitumor effect between TMZ and IFN- β was reported in malignant glioma cells *in vitro* (14,15). Natsume *et al*

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suggested that a sensitizing effect between IFN- β and TMZ in TMZ-resistant glioma cells was possibly due to attenuation of MGMT expression via induction of the protein p53 (14). More recently, the INTEGRA clinical study (integrated Japanese multicenter clinical trial: a phase II study on IFN- β and TMZ for glioma in combination with radiotherapy) was undertaken to evaluate the clinical effectiveness in glioblastomas (16,17).

Concerning the treatment of glioblastomas, it is important to elucidate the detailed features of GSCs as well as the underlying mechanisms of interconversion between GSCs and non-GSCs. To this end, we examined whether IFN- β could exert some effect on the interconversion between GSCs and non-GSCs, especially the conversion process of non-GSCs into GSCs.

Materials and methods

Cell culture. As GSCs, we employed 0222-GSC provided by Nagoya University School of Medicine (Nagoya, Japan) (7,8). The 0222-GSC satisfied the following criteria: i) the cell lines could be maintained in serum-free-media for 3 months (minimum) and ii) 10^3 cells formed tumors in the brain of nonobese diabetic mice with severe combined immunodeficiency disease (18). 0222-GSC culture was undertaken in serum-free neurobasal (NBE) media (Invitrogen, Carlsbad, CA, USA) comprising N2 and B27 supplements (Invitrogen), human recombinant basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA), and epidermal growth factor (EGF; R&D Systems).

Human malignant glioma cell lines A-172, AM-38, T98G, U-251MG, YH-13 (purchased from Health Science Research Resources Bank, Sennan, Osaka, Japan), U-87MG, and U-138MG (purchased from American Type Culture Collection, Manassas, VA, USA) were also used in the present study. These human malignant glioma cell lines were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY, USA) (18,19).

Populations of serum-induced brain tumor cells (S-BTC) were established by culturing 0222-GSC in serum medium for 3 weeks. Moreover, populations of revertant-glioma stem-like cells (Rev-GSC) were established by additional culturing of S-BTC in serum-free medium for 2 weeks. On the other hand, populations of S-BTC+IFN were established by culturing 0222-GSC in serum medium with 10 IU/ml IFN- β (Toray Industries, Tokyo, Japan) twice a week for 3 weeks (the total number of administrations was 6). Populations of Rev-GSC+IFN were then established by additional culturing of S-BTC+IFN in serum-free medium for 2 weeks (Fig. 1). Additionally, populations of GSC+IFN were established by culturing 0222-GSC in serum-free medium with 10 IU/ml IFN- β for one week.

Rev-A-172, Rev-AM-38, Rev-T98G, Rev-U-87MG, Rev-U-138MG, Rev-U-251MG, and Rev-YH-13 were established by culturing the respective cells in serum-free medium for 2 weeks. Moreover, Rev-A-172+IFN, Rev-AM-38+IFN, Rev-T98G+IFN, Rev-U-87MG+IFN, Rev-U-138MG+IFN, Rev-U-251MG+IFN, and Rev-YH-13+IFN were established by culturing the respective cells in serum-free medium for

2 weeks after culture in serum supplemented medium with 10 IU/ml IFN- β twice a week for 3 weeks (the total number of administrations was 6) (Fig. 2).

Flow cytometric analysis. The neural stem cell marker CD133 was employed as a marker of GSCs. Furthermore, glial fibrillary acidic protein (GFAP) was used as a marker of astrocytes, and galactocerebroside C (GalC) was used as a marker of oligodendrocytes (2,18,20-22). We employed the following fluorescence conjugated monoclonal antibodies: anti-CD133 (CD133-PE, 130-080-801; Miltenyi Biotec, Auburn, CA, USA), anti-GFAP (anti-GFAP-Alexa Fluor 488, 561449; Becton-Dickinson, NJ, USA), and anti-GalC (antiGalC-Alexa Fluor 488, MAB342A4; Millipore, Temecula, CA, USA).

The expressions of CD133, GFAP and GalC in 0222-GSC, S-BTC, Rev-GSC, Rev-GSC+IFN, and the 7 human malignant glioma cell lines were analyzed with a fluorescence-activated cell sorter (FACS). The fluorescence was measured using FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA), and the DNA histograms were analyzed with Flowjo software (BioLegend, San Diego, CA, USA).

The FACS analyses were repeated at least 3 times in each experiment, and we confirmed that similar tendencies were obtained.

mRNA expressions of Nanog. We analyzed the mRNA expression of pluripotency markers, Nanog, in 0222-GSC, S-BTC, Rev-GSC, Rev-GSC+IFN, Rev-U-87MG, and Rev-U-87MG+IFN by the real-time polymerase chain reaction (real-time PCR) (23,24). An RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) was employed for the extraction of mRNA. A SepOne Real-time PCR System (Applied Biosystems, Foster City, CA, USA) was used for the RT-PCR reaction. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was employed for the control. The following primers, synthesized by Opero (Tokyo, Japan), were used in the real-time PCR as described previously (7): *Nanog* (forward, 5'-GTC CCG GTC AAG AAA CAG AA; reverse, 5'-TGC GTC ACA CCA TTG CTA TT) and *GAPDH* (forward, 5'-TCG GTG CGT GCC CAG TTG AAC C; reverse: 5'-ATG CGG CTG ACT GTC GAA CAG GAG). The real-time PCR was carried out with a final volume of 50 μ l containing 10 pmol of each sense and antisense primer, 2.5 μ l of 50 mM Mn(OAc)₂, 25 μ l of RNA-direct™ SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan), 2 μ g of extracted mRNA, and RNA-free water. Amplification was carried out by initial denaturing at 90°C for 30 sec, reverse transcription at 61°C for 20 min, second denaturing at 95°C for 1 min, followed by 40 cycles of extension at 95°C for 15 sec, 55°C for 15 sec, and 74°C for 45 sec. The expression levels were calculated using the following equations by comparing the threshold cycles (CT): Δ CT = CT of Nanog - CT of GAPDH, $\Delta\Delta$ CT = Δ CT (target cell line) - Δ CT (reference cell line), and ratio = $2^{-\Delta\Delta$ CT} (25).

Growth inhibitory effect of IFN- β on 0222-GSC. The growth inhibitory effect of IFN- β was evaluated by counting the number of cells using a Coulter Counter (Coulter Counter ZI, Beckman coulter, Fullerton, CA, USA). Briefly, cells were plated at 2×10^4 cells per well in 24-well, flat-bottomed plates

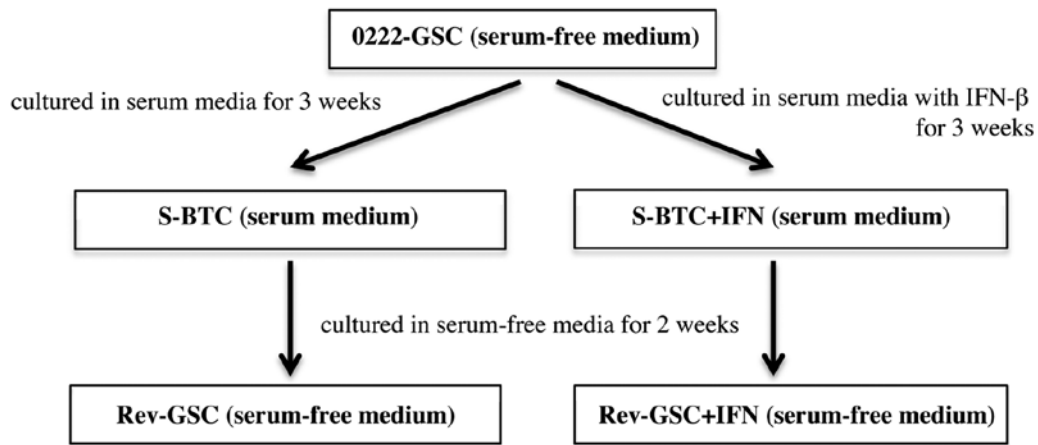


Figure 1. Flowchart of experiments on GSC. 0222-GSC, a glioma stem-like cell (GSC) line was cultured in serum-free medium. S-BTC was established by culturing 0222-GSC in serum media for 3 weeks. S-BTC+IFN was established by culturing 0222-GSC in serum media for 3 weeks, with administration of IFN- β . Rev-GSC and Rev-GSC+IFN were established by additional culturing of S-BTC and S-BTC+IFN in serum-free medium for 2 weeks, respectively.

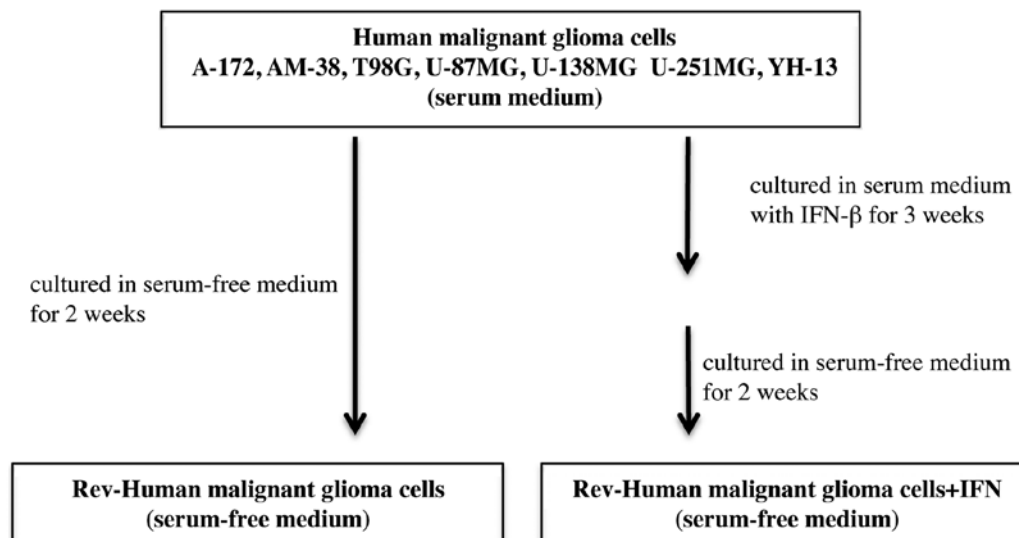


Figure 2. Flowchart of experiments on 7 human glioma cell lines. Rev-Human malignant glioma cells were established by culturing the respective cells in serum-free medium for 2 weeks. Rev-human malignant glioma cells+IFN were established by culturing the respective cells in serum-free medium for 2 weeks after culture in serum medium with IFN- β for 3 weeks.

(Iwaki, Chiba, Japan) and incubated in the medium with or without 1.0-100 IU/ml of IFN- β . After 5 days of exposure to various concentrations of IFN- β , the cells were counted with the cell counter.

Sphere formation assay. 0222-GSC cells were placed into 96-well plates (50 cells/well) in serum-free medium. IFN- β (10 IU/ml) was administered on one side (48-wells). At day 7 after seeding, the spheres containing >10 cells were counted.

Statistical evaluations. Statistical analyses were performed using the unpaired, Mann-Whitney U test. If the samples comprised more than three groups, the significance of the overall samples was evaluated by the Kruskal-Wallis test before evaluating the significant differences between pairs of groups by the Mann-Whitney U test. All quantitative data are

presented as the means \pm SE from at least six samples per data point. Statistical software IBM SPSS Statistics version 21.0 (International Business Machines Corp., Armonk, NY, USA) was employed for the data analysis.

Results

Characteristics of GSCs and effects of IFN- β . 0222-GSC formed tumor spheres in serum-free medium. However, the cells did not show tumor spheres in response to a change to serum medium for 3 weeks (S-BTC). In addition, S-BTC reformed tumor spheres again after culturing in serum-free medium for 2 weeks (Rev-GSC) (Fig. 3A).

In the FACS analysis, 0222-GSC expressed a positive reaction for CD133, but the expressions became negatively converted in S-BTC. Further, the expression of CD133 again

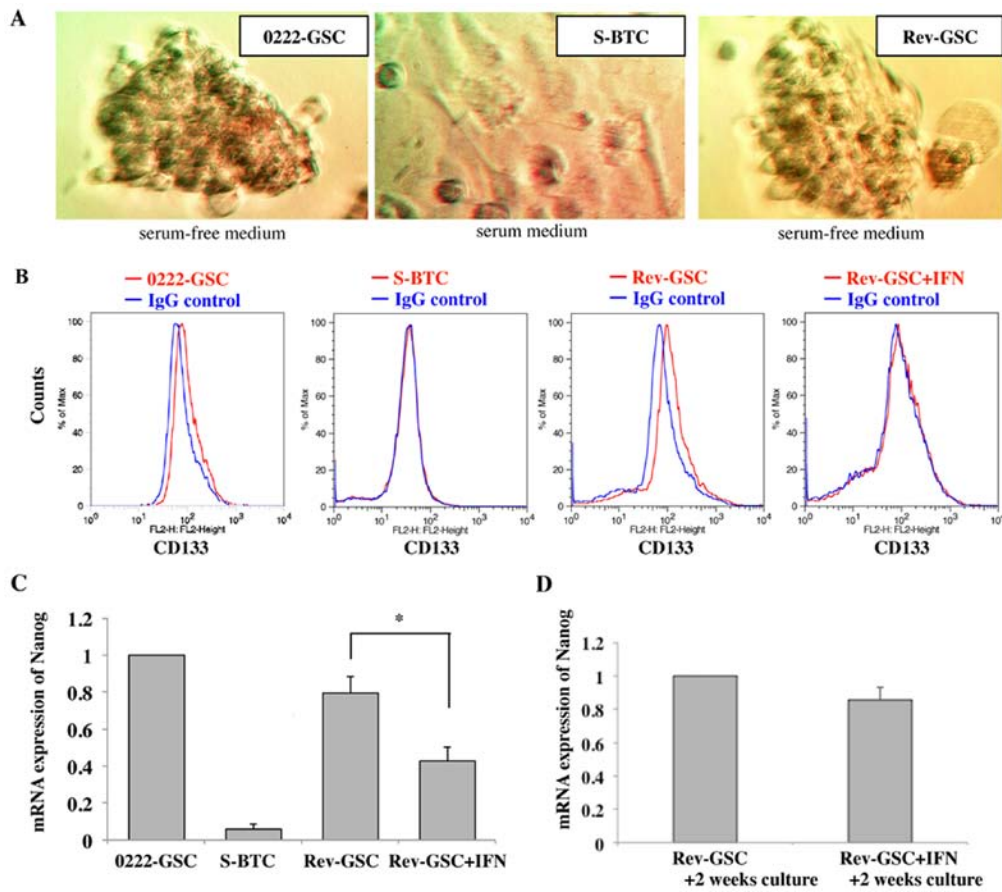


Figure 3. IFN- β suppresses interconversion between GSCs and non-GSCs. (A) Phase contrast images of 0222-GSC, S-BTC and Rev-GSC observed under a microscope (x100). (B) FACS analyses of the CD133 expression in 0222-GSC, S-BTC, Rev-GSC and Rev-GSC+IFN. (C) RT-PCR analyses of the mRNA expression of *Nanog* in 0222-GSC, S-BTC, Rev-GSC and Rev-GSC+IFN. The values are expressed relative to the expression of mRNA *Nanog* in 0222-GSC (means \pm SE). * p <0.05. (D) Expression of mRNA *Nanog* (means \pm SE) in Rev-GSC and Rev-GSC+IFN after 2 weeks of continuous culture in serum-free medium.

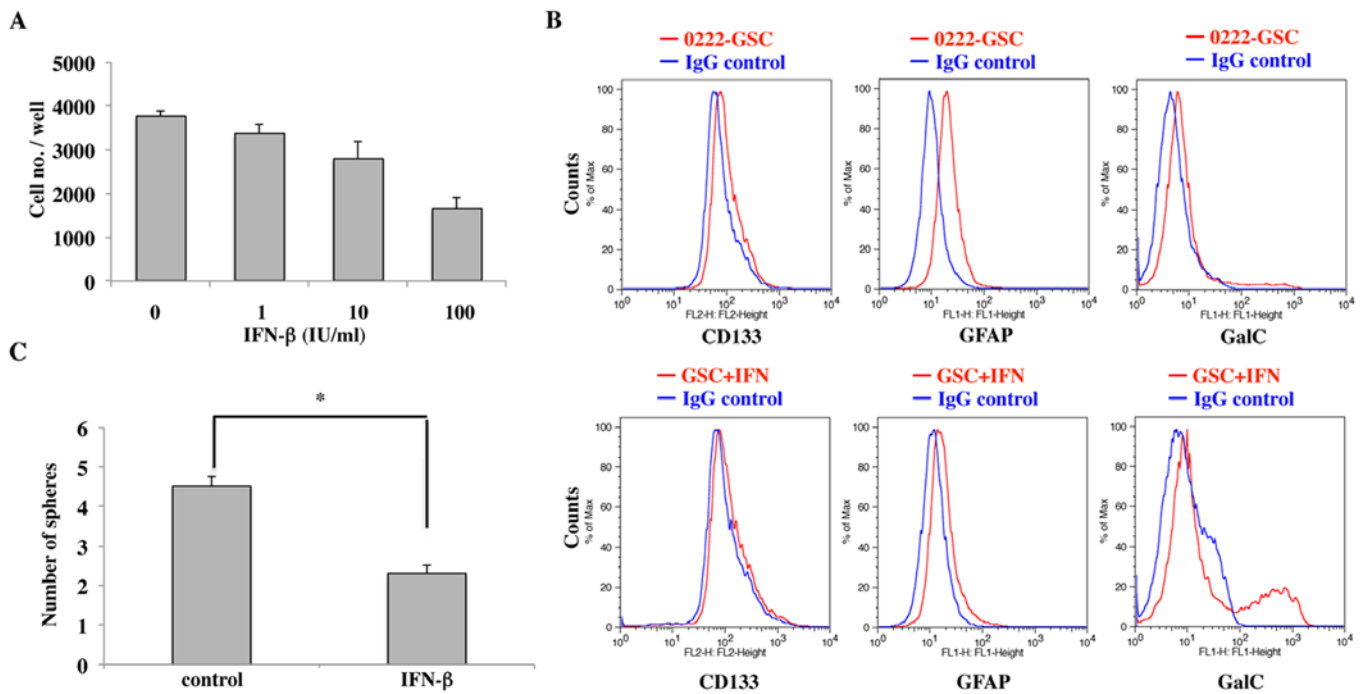


Figure 4. IFN- β reduced GSC proliferation and tumor sphere formation, and influenced cell differentiation of GSCs. (A) Proliferation assays of 0222-GSC with IFN- β (0-100 IU/ml). Cells were counted 5 days after seeding. There were significant differences between the 4 groups by the Kruskal-Wallis test. Means \pm SE. (B) FACS analysis of the expression of CD133, GFAP, and GalC in 0222-GSC and GSC+IFN (0222-GSC cultured for one week after IFN- β administration). (C) Tumor sphere formation assay of 0222-GSC with IFN- β . The number of spheres was counted 7 days after seeding. Means \pm SE, ** p <0.01.

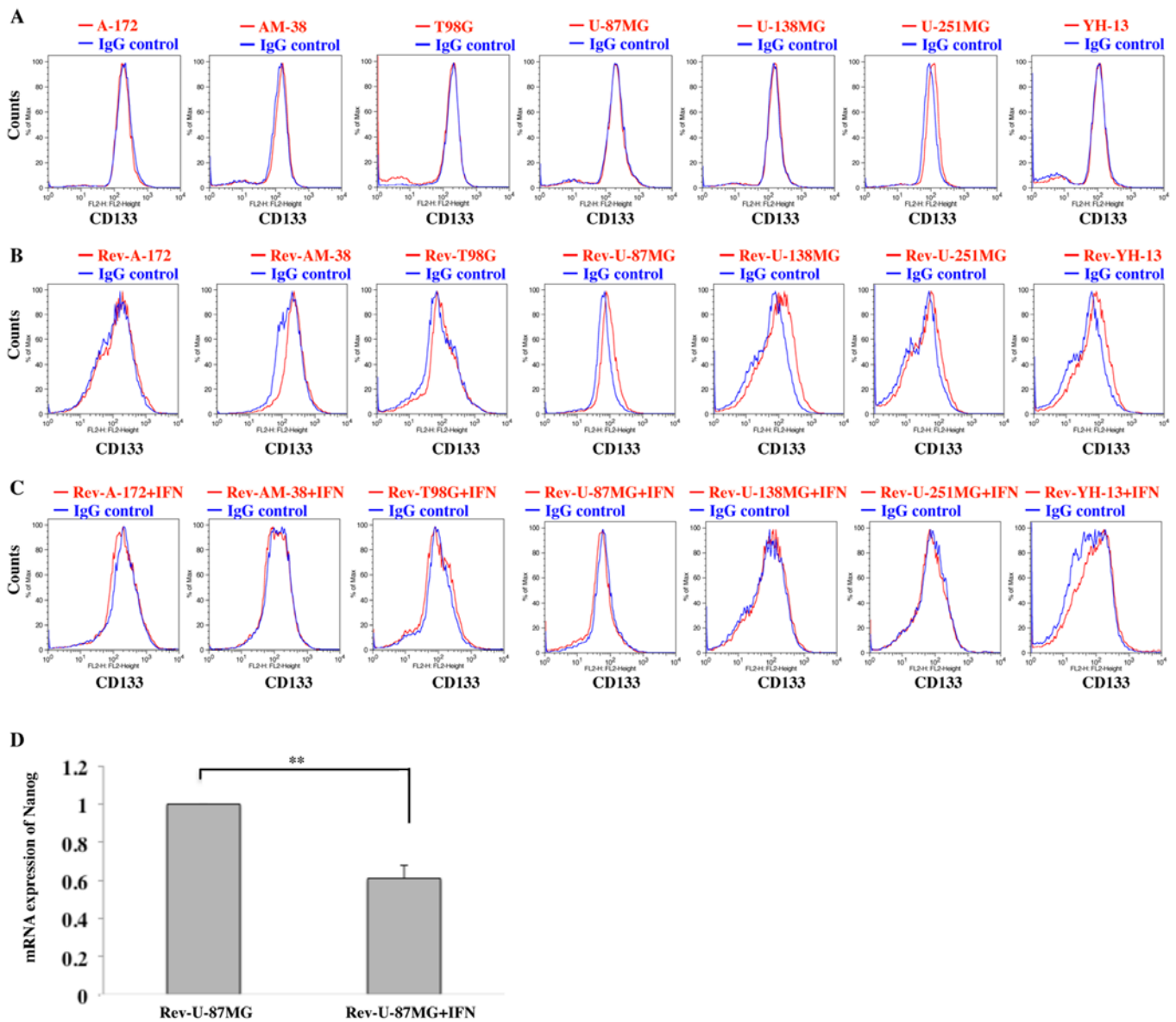


Figure 5. Effects of IFN- β on human malignant glioma cell lines. (A-C) FACS analyses of the CD133 expression in human malignant glioma cell lines: original state (A), culture of the respective cell lines in serum-free medium for 2 weeks (B), and culture of the respective cell lines in serum-free medium for 2 weeks, after administration of IFN- β (C). (D) RT-PCR analyses of the expression of mRNA *Nanog* in Rev-U-87MG and Rev-U-87MG+IFN. The values are expressed relative to the expression of mRNA *Nanog* in Rev-U-87MG (means \pm SE). ** $p < 0.01$.

positively converted in Rev-GSC (expression was not observed when S-BTC were cultured in serum-free medium for only a week; data not shown). On the other hand, the expression of CD133 was suppressed in Rev-GSC+IFN (Fig. 3B).

The mRNA expression of *Nanog* in 0222-GSC, S-BTC, Rev-GSC, and Rev-GSC+IFN were analyzed by the real-time PCR. The expression in S-BTC was reduced to 0.06 ± 0.03 -fold as compared to the expressions in 0222-GSC. However, the expression in Rev-GSC increased again to 0.79 ± 0.09 -fold as compared to the expressions in 0222-GSC. On the other hand, the expression of mRNA *Nanog* in Rev-GSC+IFN was also increased to 0.42 ± 0.07 -fold as compared to the expressions in 0222-GSC, although the level was significantly lower than that for Rev-GSC ($p < 0.01$; Fig. 3C).

Furthermore, Rev-GSC and Rev-GSC+IFN were cultured continuously for an additional 2 weeks in serum-free media

(total of 4 weeks culture in serum-free media). Although the expression of mRNA *Nanog* in Rev-GSC+IFN was reduced to 0.86 ± 0.08 -fold as compared to those in Rev-GSC, there was no significant difference between Rev-GSC+IFN and Rev-GSC ($p = 0.09$; Fig. 3D).

Effect of IFN- β on GSC. As shown in Fig. 4A, cell growth inhibitory effects of IFN- β on 0222-GSC were observed in a dose-dependent manner.

We next examined the expression of CD133, GFAP and GalC in 0222-GSC and GSC+IFN by FACS analysis. The expression of CD133 and GFAP was suppressed, but the expression of GalC was enhanced in GSC+IFN as compared to those in 0222-GSC (Fig. 4B). The data obtained indicated IFN- β induced oligodendrogenesis in 0222-GSCs, as reported previously (18).

The numbers of tumor spheres were counted after 7 days of culture in serum-free medium and compared between IFN- β treatment, but not in 0222-GSC. As shown in Fig. 4C, the number of tumor spheres in the IFN- β -treated cells were significantly lower than the number of tumor spheres in the non-treated cells ($p < 0.01$). We found that the sphere formation ability was attenuated by IFN- β treatment, although both groups could form tumor spheres.

Effect of IFN- β on malignant glioma cell lines. The human malignant glioma cell lines did not form tumor spheres. On FACS analysis, U-251MG expressed CD133, even though the other 6 cell lines did not (Fig. 5A). Subsequently, each of the cell lines was able to form tumor spheres when cultured in serum-free medium for 2 weeks (*viz.*, Rev-A-172, Rev-AM-38, Rev-T98G, Rev-U-87MG, Rev-U-138MG, Rev-U-251MG and Rev-YH-13). Expressions of CD133 was newly observed in U-87MG, U-138MG and YH-13 on FACS analysis (Fig. 5B). Each of the cell lines, which had been administered IFN- β previously under serum medium, also formed tumor spheres when cultured in serum-free medium for 2 weeks (*viz.*, Rev-A-172+IFN, Rev-AM-38+IFN, Rev-T98G+IFN, Rev-U-87MG+IFN, Rev-U-138MG+IFN, Rev-U-251MG+IFN and Rev-YH-13+IFN), but none of these cell lines expressed CD133 (Fig. 5C).

Moreover, we analyzed the mRNA expressions of *Nanog* in Rev-U-87MG and Rev-U-87MG+IFN by the real-time PCR. The expressions in Rev-U-87MG+IFN was significantly reduced to 0.61 ± 0.07 -fold as compared to the expression in Rev-U-87MG ($p < 0.01$; Fig. 5D).

Discussion

GSCs share many properties with normal stem cells including self-renewal and pluripotency, and exhibit tumorigenic ability. Furthermore, GSCs may contribute to tumor development, invasion, recurrence and chemo/radiation resistance in glioblastomas (7,18,26,27). It is important therefore to take GSCs fully into account when deciding treatment strategies for glioblastomas.

The undifferentiated state of GSCs displays characteristics such as tumor sphere formation, CD133 expression, and mRNA *Nanog* expression (7,18,20-22). The GSC cell line, 0222-GSC, demonstrated tumor sphere formation, CD133 expression, and a high expression of mRNA *Nanog*. However, it lost such characteristics on exposure to serum medium (S-BTC). This may indicate that GSCs can change/differentiate to non-GSCs in response to signals from their microenvironment (7,18). On the other hand, S-BTC re-exhibited the characteristics of tumor sphere formation, CD133 expression, and a high expression of mRNA *Nanog* upon removal of the serum from the medium. Such changes suggested that the non-GSCs had regained an undifferentiated state in response to signals from their microenvironment. These findings are in keeping with those described in previous reports (7).

Human malignant glioma cell lines cultured in conventional medium did not form tumor spheres, nor did they express CD133 (except for U-251MG) in the present study. All cell lines formed tumor spheres when cultured in serum-free medium, and furthermore U-87MG, U-138MG, and YH-13

expressed CD133. Qiang *et al*, investigated the percentage of CD133-positive cells in A-172, U-87MG, and U-251MG by FACS analysis, and found that the existence of CD133-positive cells was common in U-251MG (28). They also reported that human malignant glioma cell lines formed tumor spheres and showed increased expression of CD133 when cultured in serum-free medium (28). We obtained similar results, and confirmed that human malignant glioma cells (non-GSCs) could acquire an undifferentiated state (return to GSCs) in response to signals from their microenvironment.

It might be possible to enhance the effects of radiotherapy/chemotherapy for glioblastomas, if we could promote the differentiation of GSCs and/or suppress the return process of non-GSCs to GSCs. Not only GSCs themselves but also the interconversion between GSCs and non-GSCs could be new targets in glioblastoma treatment. In the present study, IFN- β revealed a cell growth inhibitory effect and a cell differentiation effect with suppression of tumor sphere formation and CD133 expression in 0222-GSC. In addition, IFN- β suppressed the acquisition process of undifferentiated features in S-BTC and some of the human malignant glioma cell lines investigated. Thus, IFN- β might represent an effective agent not only through its cell growth inhibitory effect on GSCs but also as a means of targeting the interconversion between GSCs and non-GSCs.

0222-GSC cells were induced to differentiate into oligodendrogenetic cells in the present study, as reported previously (8). The oligodendrogliomas display a better prognosis than the astrocytomas, through their high sensitivity to adjuvant therapy including radiotherapy and chemotherapy (29). Such a differentiation effect is therefore considered to offer a new treatment strategy for glioblastomas, and IFN- β may represent an effective drug for the treatment of glioblastomas, not only when used alone but also in combination with TMZ. Further, in the present study, IFN- β also induced suppression of acquisition processes involved in the undifferentiation of non-GSCs at 2 weeks, although the suppression of mRNA *Nanog* was not significant at 4 weeks after IFN- β treatment. The interval of IFN- β administration employed during maintenance therapy in the INTEGRA study was once every 4 weeks (17), so that a higher effect could be expected if the intervals were shorter.

Finally, the effects of IFN- β on the tumor cells were considered as not only temporary, but also genetic or epigenetic changes, since the effects of IFN- β lasted >2 weeks. DNA methylation, and histone modification (acetylation, methylation, and phosphorylation) are known to represent epigenetic changes of the cells. Among them, histone methylation has been reported to play an important role in the control of normal stem cell differentiation. In particular, histone H3 lysine 27 trimethylation (H3K27me3) in the promoter region of the differentiation-associated genes is known to inhibit the transcription of these genes (30,31). H3K27me3 is catalyzed by enhancer of zeste homolog 2 (EZH2; histone methyltransferase). EZH2 has been reported to contribute to undifferentiated features in normal stem cells (32-36). Enhanced gene expressions of *EZH2* have been observed in various cancers (32,37-39), and inhibitors of EZH2 have been found to exhibit antitumor effects (40,41). While *EZH2* has been described as an oncogene, it has also been reported as

a tumor-suppressor gene since mutations of *EZH2* have been observed extinguished in several cancers (42). Although the detailed relationships between *EZH2* and cancer remain to be elucidated, there is a report suggesting that *EZH2* may contribute to the interconversion between GSCs and non-GSCs (7). Further research is clearly necessary, but an association could exist between *EZH2* and the effects of IFN- β .

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