Differentiation therapy targeting indoleamine 2,3-dioxygenase 1 in glioblastoma cells *in vitro*

KAORU NISHIDE, SHUN YAMAMURO, YUMI AIDA, YOSHINARI OZAWA, EMIKO SANO and ATSUO YOSHINO

Department of Neurological Surgery, Nihon University School of Medicine, Tokyo 173-8610, Japan

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Abstract. Glioblastoma cells strongly express the tryptophan-metabolizing enzyme indoleamine 2,3-dioxygenase 1 (IDO1), which is important in enabling immune evasion. Notably, glioma stem-like cells (GSCs) express higher levels of IDO1 than differentiated glioblastoma cells (DGCs); therefore, it was hypothesized that IDO1 inhibition in GSCs may induce differentiation. The present study used the glioblastoma cell line U-87MG, and newly established GSC lines 0125-GSC and 0222-GSC. A model GSC line, Rev-U-87MG, was created by culturing U-87MG in serum-free medium. Rev-U-87MG-IDO(-) was generated by continuous treatment with an IDO1 inhibitor. The DGC lines 0125-DGC and 0222-DGC were derived by culturing 0125-GSC and 0222-GSC in serum-containing medium. IDO1-inhibited GSC lines 0125-GSC-IDO(-) and 0222-GSC-IDO(-) were generated by continuous IDO1 inhibitor exposure. The expression levels of IDO1 and stem cell markers (Nestin, Nanog and Sox2) were measured by western blotting, and temozolomide sensitivity was evaluated by cell counting. Notably, U-87MG, 0125-DGC and 0222-DGC adhered without sphere formation. By contrast, Rev-U-87MG, 0125-GSC and 0222-GSC formed spheres, whereas IDO1-inhibited cells showed partial adhesion with reduced sphere formation. IDO1 and stem cell marker expression were reduced in all IDO1-inhibited cell lines. Rev-U-87MG exhibited decreased temozolomide sensitivity compared with U-87MG, whereas Rev-U-87MG-IDO(-) showed increased sensitivity. Similarly, 0125-DGC and 0222-DGC were more sensitive than their parent GSC lines, and 0125-GSC-IDO(-) and 0222-GSC-IDO(-) also exhibited enhanced drug sensitivity compared with their parent GSC lines at multiple concentrations. These findings suggest that IDO1 inhibition may induce the differentiation of GSCs and reduce their resistance to temozolomide.

Correspondence to: Dr Shun Yamamuro, Department of Neurological Surgery, Nihon University School of Medicine, 30-1 Oyaguchi Kami-cho, Itabashi, Tokyo 173-8610, Japan
E-mail: yamamuro.shun@nihon-u.ac.jp

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Introduction

The prognosis for patients with glioblastoma remains extremely poor, as the median survival time is less than 20 months even with postoperative chemoradiation therapy using temozolomide (1-3). Recent studies have revealed that glioma stem-like cells (GSCs), a type of cancer stem cell, are crucial in the malignancy of glioblastomas (4,5). GSCs can differentiate into glioma cells, including glioblastoma cells, in response to the microenvironment. Interestingly, these differentiated glioblastoma cells (DGCs) have been reported to dedifferentiate back into GSCs, indicating bidirectional interconversion between GSCs and DGCs (6,7). Our group has previously conducted extensive research into the biological characteristics of GSCs (7-9).

Indoleamine 2,3-dioxygenase 1 (IDO1), a tryptophan-metabolizing enzyme, promotes the catabolic pathway from tryptophan to kynurenine, which is known to suppress cytotoxic T cells and activate regulatory T cells (10-12). IDO1 is highly expressed in various malignant tumors, including glioblastomas, and has been reported to suppress tumor immunity (10,13). Furthermore, IDO1 expression has been shown to correlate with poor prognosis in patients with glioblastomas and to increase with higher tumor grade of gliomas (14-16).

We previously demonstrated that IDO1 is more strongly expressed in GSCs than in DGCs, suggesting that GSCs suppress antitumor immunity via IDO1 expression (8). Based on this finding, we hypothesized that inhibition of IDO1 in GSCs could promote differentiation and attenuate resistance to temozolomide. The present study tried to develop a novel differentiation-inducing therapy for glioblastomas.

Materials and methods

Cell lines and cell culture. The present experiments used the widely available human glioblastoma cell line U-87MG (glioblastoma of unknown origin; cat. no. HTB 14; lot no. 2497162; purchased from the American Type Culture Collection), and GSC lines 0125-GSC and 0222-GSC, newly established from surgical specimens. The primary cell lines 0125-GSC and

0222-GSC were originally established at Nagoya University (Nagoya, Japan) from glioblastoma specimens obtained after written informed consent from the patients, as previously reported (17), and have been used in multiple studies (6-9,18). The use of these cell lines in the present study was approved by the Institutional Review Board of Nihon University School of Medicine (approval no. 2026-09; Tokyo, Japan). Additionally, a GSC model line Rev-U-87MG, derived by culturing U-87MG in serum-free medium for more than 2 weeks, was used (8). DGC model lines 0125-DGC and 0222-DGC were generated by culturing 0125-GSC and 0222-GSC in serum-containing medium for more than 2 weeks (8). Furthermore, Rev-U-87MG, 0125-GSC, and 0222-GSC were cultured in the continuous presence of the IDO1 inhibitor for over 2 weeks to generate IDO1-suppressed cell lines Rev-U-87MG-IDO(-), 0125-GSC-IDO(-), and 0222-GSC-IDO(-).

Cell culture used serum-containing medium consisting of Dulbecco's modified Eagle's medium supplemented (Nissui Pharmaceutical) with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.), and serum-free medium consisted of Neurobasal Medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with L-glutamine, epidermal growth factor, recombinant human basic fibroblast growth factor (R&D Systems), N2, and B27 (Invitrogen; Thermo Fisher Scientific, Inc.). Serum-containing medium was used for culturing DGCs, whereas serum-free medium was used for culturing GSCs (7-9). Cells were passaged and the media replaced every 4 days. Temozolomide (#T2744; Tokyo Chemical Industry) and the IDO1 inhibitor 1-methyl-L-tryptophan (1-MT) (#447439; Sigma-Aldrich Japan) were used. IDO1-inhibited cell lines were generated with 10 μM 1-MT added into the medium during each passage.

Western blotting. Protein expression analysis was performed using western blotting according to the previously described methods (18). Briefly, $1x10^4$ cells from each cell line were seeded onto a 10-cm diameter dish, and the cells were harvested 72 h later to extract the proteins. Anti-IDO1 antibody, (#66528-1-Ig; Proteintech Japan) anti-Nestin antibody (#29285-1-AP; Proteintech Japan), anti-Nanog antibody (#67255-I-Ig; Proteintech Japan), anti-Sox2 antibody (#11064-1-AP; Proteintech Japan), and anti-GFAP antibody (#60190-1-Ig; Proteintech Japan) were used as primary antibodies. Anti-β-actin antibody (#sc-47778; Santa Cruz Biotechnology) used as a loading control.

Cell counting assay. Cell proliferation inhibition assays were also conducted as described previously (18). Briefly, $1x10^4$ cells were seeded onto a 10-cm diameter dish, and 24 h later, the medium was replaced with fresh medium containing varying concentrations of temozolomide. After 72 h, cells were harvested and counted using a Z1 Coulter Counter® (Beckman Coulter). Temozolomide concentrations used were 0, 0.1, 1, 10, 100, and 1,000 μM. Additionally, complementary cell viability analysis was performed using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies) according to the manufacturer's instructions. A total of $1x10^4$ cells were seeded into each well of a 96-well plate. Cells were treated with the same concentrations of temozolomide and assessed at the same time points.

Statistical analysis. Statistical analysis was performed using unpaired Student's t-test and one-way analysis of variance followed by Tukey's post-hoc test with IBM SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of IDO1 inhibition on cell morphology and adhesion. The morphology of each cell line was observed under a fluorescence microscope (Fig. 1A). Glioblastoma cell line U-87MG, cultured in serum-containing medium, had adhered to the culture dish and proliferated (Fig. 1A, top-left). Rev-U-87MG, cultured in serum-free medium for more than 2 weeks, had formed floating spheres (Fig. 1A, top-center), a characteristic of stem cells. Rev-U-87MG-IDO(-), cultured with 10 μM 1-MT for over 2 weeks to inhibit IDO1, had formed smaller spheres (Fig. 1A, top-right). The concentration of 1-MT to be administered continuously was determined based on preliminary experiments. The preliminary experiments were designed to determine the maximum concentration of 1-MT that could be continuously administered without inducing reduction in the cell number (data not shown), and 10 μM was selected as the concentration. After removing the medium, adherent cells were collected using trypsin, and cell numbers were counted. Rev-U-87MG-IDO(-) had formed a significantly greater number of adherent cells than Rev-U-87MG ($P<0.01$) (Fig. 1B, top). GSC lines 0125-GSC and 0222-GSC, cultured in serum-free medium, had also formed floating spheres (Fig. 1A, middle/bottom-center). However, 0125-GSC and 0222-GSC spheres, cultured in serum-containing medium for more than 2 weeks, had dissolved and the cells became adherent, so forming the DGC lines 0125-DGC and 0222-DGC (Fig. 1A, middle/bottom-left). Similarly, 0125-GSC-IDO(-) and 0222-GSC-IDO(-), cultured with 10 μM 1-MT for more than 2 weeks, formed smaller spheres (Fig. 1A, middle/bottom-right). 0125-GSC-IDO(-) and 0222-GSC-IDO(-) formed significantly higher numbers of adherent cells than in their respective controls ($P<0.01$) (Fig. 1B, middle and bottom).

Effects of IDO1 inhibition on proteins expression. Western blot analysis was used to examine the protein expression of IDO1, stem cell markers (Nestin, Nanog, and SOX2) and astrocyte marker (GFAP) in each cell line. The IDO1-inhibited cell lines Rev-U-87MG-IDO(-), 0125-GSC-IDO(-), and 0222-GSC-IDO(-) showed significantly reduced IDO1 expression compared to Rev-U-87MG, 0125-GSC, and 0222-GSC (Fig. 2A). These IDO1-inhibited cell lines also showed significantly reduced expression of stem cell markers (Fig. 2B), along with increased expression of astrocyte marker (Fig 2C). Additional experiments using 1-MT at concentrations of 1 and 5 μM further demonstrated that IDO1 suppression and the subsequent reduction in stem cell marker expression occurred in a concentration-dependent manner (Fig. S1). These findings suggest that continuous treatment with 10 μM 1-MT for more than 2 weeks effectively inhibited IDO1 expression which in turn reduced the expression of stem cell markers along with increased the expression of astrocyte marker, indicating that IDO1 inhibition promotes the differentiation of GSCs.

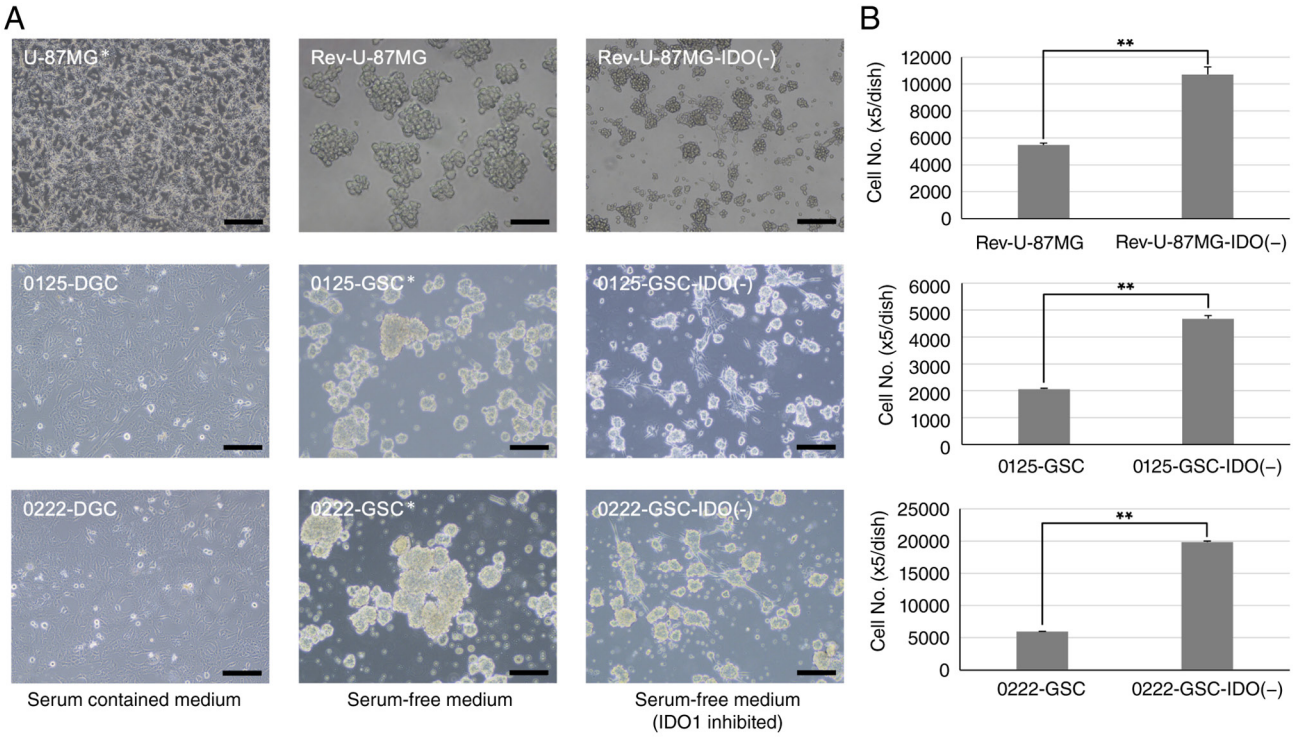

Figure 1. Morphology and cell adherence in each cell line. (A) Morphology of the cell lines used in this study. Top row shows the commercially available human glioblastoma cell line U-87MG (left), and the derived lines Rev-U-87MG (center) and Rev-U-87MG-IDO(-) (right). Rev-U-87MG was generated by culturing U-87MG in serum-free medium for 2 weeks and formed spheres. Rev-U-87MG-IDO(-) was established by culturing Rev-U-87MG with 10 μM 1-methyl-L-tryptophan added at each passage for 2 weeks and formed smaller spheres. Middle and bottom rows show newly established glioma stem-like cell lines 0125-GSC/0222-GSC (center), and their derived lines 0125-DGC/0222-DGC (left) and 0125-GSC-IDO(-)/0222-GSC-IDO(-) (right). 0125-DGC and 0222-DGC were generated by culturing 0125-GSC and 0222-GSC in serum-containing medium for 2 weeks, and adhered to the culture dish with no sphere formation. 0125-GSC-IDO(-) and 0222-GSC-IDO(-) were established by culturing 0125-GSC and 0222-GSC with 10 μM 1-methyl-L-tryptophan at each passage for 2 weeks, resulting in reduced sphere formation. Scale bars=0.1 μm. (B) Number of adherent cells in each cell line. Compared to Rev-U-87MG (top), 0125-GSC (middle), and 0222-GSC (bottom), the number of adherent cells was significantly increased in Rev-U-87MG-IDO(-) (top), 0125-GSC-IDO(-) (middle), and 0222-GSC-IDO(-) (bottom). Cells were cultured in 10-cm dishes. After removal of floating cells, adherent cells were detached using trypsin and counted with a Z1 Coulter Counter® (Beckman Coulter, Brea, CA, USA). **P<0.01. DGC, differentiated glioblastoma cell; GSC, glioma stem-like cell; IDO1, indoleamine 2,3-dioxygenase 1.

Effects of IDO1 inhibition on temozolomide sensitivity. Based on these results, the drug sensitivity of each cell line to temozolomide was examined. Rev-U-87MG exhibited reduced sensitivity (indicating increased resistance) to temozolomide compared to the parent U-87MG, with significant differences observed at 1 and 10 μM (both P<0.05) (Fig. 3A, solid and dotted lines). However, Rev-U-87MG-IDO(-) showed increased sensitivity (indicating reduced resistance) compared to Rev-U-87MG, with a significant difference observed at 100 μM (P<0.01) (Fig. 3A, dotted and dashed lines). The half-maximal inhibitory concentration (IC_{50}) values of temozolomide were 0.5 μM for U-87MG, 16.0 μM for Rev-U-87MG, and 0.9 μM for Rev-U-87MG-IDO(-). Similarly, 0125-DGC showed significantly higher sensitivity to temozolomide compared to the parent 0125-GSC line at 0.1, 1, 10, and 100 μM (all P<0.01) (Fig. 3B, solid and dotted lines). 0125-GSC-IDO(-) also showed enhanced drug sensitivity compared to the parent 0125-GSC, with significant differences observed at 10 and 100 μM (both P<0.01) (Fig. 3B, dotted and dashed lines). The IC_{50} values of temozolomide were 250.6 μM for 0125-GSC, 0.2 μM for 0125-DGC, and 106.7 μM for 0125-GSC-IDO(-). The differentiated line 0222-DGC was significantly higher sensitive compared to 0222-GSC at 0.1 and 1 μM (both P<0.01) (Fig. 3C, solid and dotted lines). 0222-GSC-IDO(-) showed increased sensitivity compared to 0222-GSC, with significant differences at 0.1, 1, and 100 μM (P<0.05, P<0.01, and P<0.05, respectively) (Fig. 3C, dotted and dashed lines). The IC_{50} values of temozolomide were 155.4 μM for 0222-GSC, 117.6 μM for 0222-DGC, and 12.2 μM for 0222-GSC-IDO(-). Consistent results were also obtained through comprehensive quantification using a CCK-8-based metabolic activity assay to assess the sensitivity of 0125-GSC and 0125-GSC-IDO(-) cells to temozolomide (Fig. 3D). 0125-GSC-IDO(-) showed enhanced drug sensitivity compared to the parent 0125-GSC, with significant differences observed at 1, 100, and 1,000 μM (P<0.05, P<0.01, and P<0.05, respectively) (Fig. 3D, dotted and dashed lines). These findings indicate that GSCs exhibit greater resistance to temozolomide compared to DGCs. However, IDO1 inhibition in GSCs reduces this resistance, supporting the hypothesis that IDO1 inhibition promotes GSC differentiation and decreases chemoresistance.

Discussion

This study demonstrated that inhibition of IDO1 expression in GSCs leads to a loss of stem cell characteristics and promotes differentiation. Induction of differentiation through IDO1 inhibition also reduced temozolomide resistance in GSCs.

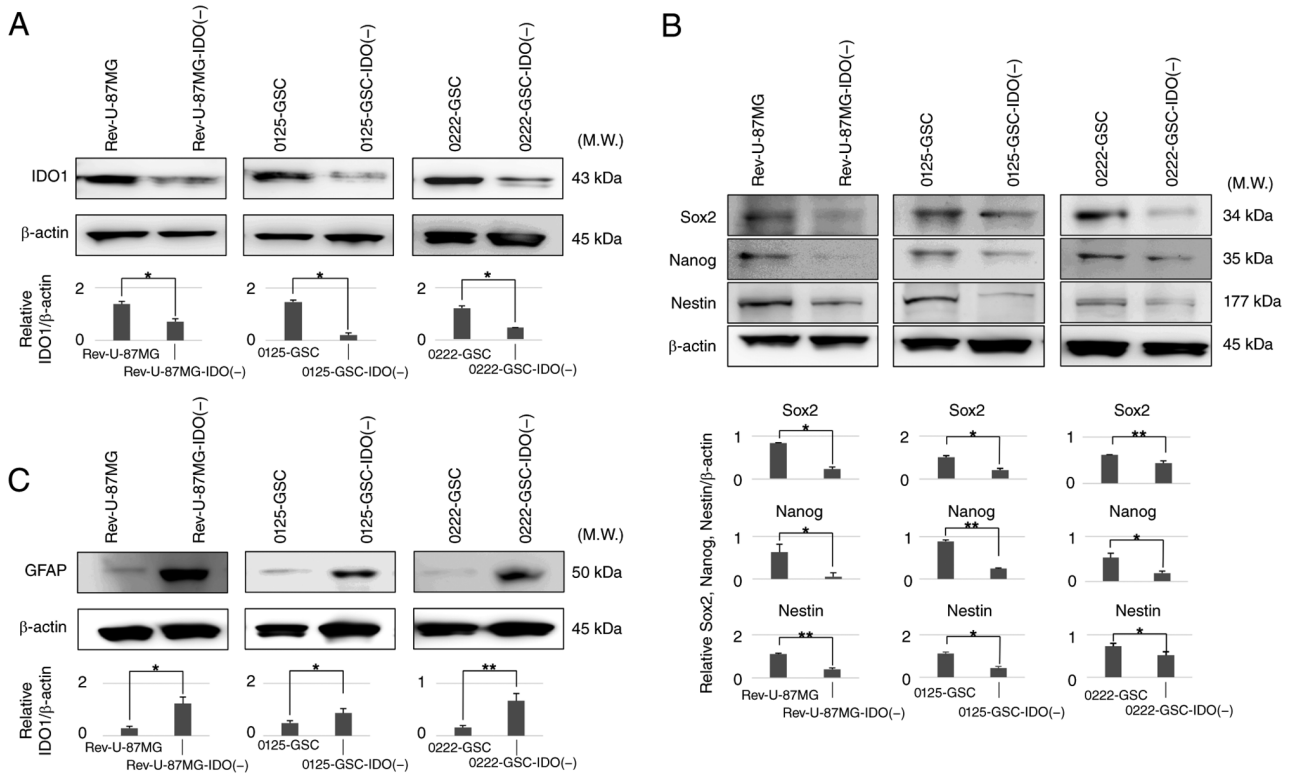

Figure 2. Western blot analysis of protein expression of (A) IDO1, (B) stem cell markers Sox2 (top), Nanog (upper middle) and Nestin (lower middle), and (C) astrocyte marker GFAP in each cell line. (A) Compared to glioma stem-like cell lines Rev-U-87MG, 0125-GSC, and 0222-GSC, IDO1 expression was significantly reduced in the IDO1-inhibited cell lines Rev-U-87MG-IDO(-), 0125-GSC-IDO(-), and 0222-GSC-IDO(-). *P<0.05. (B) Similarly, expression of the stem cell markers Sox2, Nanog, and Nestin was significantly decreased in Rev-U-87MG-IDO(-), 0125-GSC-IDO(-), and 0222-GSC-IDO(-) compared to their respective stem cell-like glioma cell lines. *P<0.05; **P<0.01. (C) Conversely, expression of the astrocyte marker GFAP was significantly increased in Rev-U-87MG-IDO(-), 0125-GSC-IDO(-), and 0222-GSC-IDO(-) compared to their respective stem cell-like glioma cell lines. *P<0.05; **P<0.01. GSC, glioma stem-like cell; IDO1, indoleamine 2,3-dioxygenase 1.

Immunotherapy has recently gained attention as a novel strategy for the treatment of malignant gliomas. Among the targets under investigation, IDO1, a tryptophan-metabolizing enzyme, has drawn particular interest (10,12,15,16,19). Glioblastoma cells show high levels of expression of IDO1, which suppresses antitumor immunity by catabolizing tryptophan, an amino acid critical for immune cell function (12). IDO1 inhibitors enhance the effectiveness of immunotherapy in a murine glioblastoma model (19), and combined treatment with IDO1 inhibitors and temozolomide also achieves synergistic effects (10). Furthermore, IDO1 expression is particularly high in glioblastoma patients with poor prognosis (16), and is significantly higher in World Health Organization grade III and IV gliomas compared to lower grade I or II gliomas (15). IDO1 expression is clearly associated with glioblastoma malignancy, but few studies have specifically addressed the relationship between IDO1 and GSCs, which are critical drivers of glioblastoma aggressiveness. Our previous study analyzed IDO1 expression in various cell types, including DGCs and GSCs, and found that IDO1 expression was significantly higher in GSC lines than in DGC lines (8).

Recent reports have shown that interferon-beta (IFN-β) expression is upregulated in IDO1-knockout mice (20). IFN-β is known to promote cell differentiation and exert antitumor effects (6,7,17). Therefore, we hypothesized that IDO1 inhibition leads to increased IFN-β expression, which in turn promotes the differentiation of GSCs. Differentiation of GSCs or undifferentiated glioblastoma cells could enhance their susceptibility to chemotherapy and/or radiotherapy, so overcoming the inherent treatment resistance of glioblastoma. This concept formed the basis for our present study. However, the relationship between IDO1 and IFN-β was not verified in the present study. Beyond IFN-β signaling, the mechanisms by which IDO1 inhibition promotes differentiation remain to be elucidated and should be addressed in future studies.

The present study observed that the culture conditions affected the differentiation status of each cell line. U-87MG cultured in serum-free medium (Rev-U-87MG) formed spheres, which is characteristic of neuron and/or glioma stem cells. We previously reported that this change promotes higher expression of stem cell markers (8). In contrast, the present study found continuous treatment with 1-MT suppressed sphere formation and reduced expression of stem cell markers [Rev-U-87MG-IDO(-)]. Similarly, 0125-GSC and 0222-GSC cultured in serum-containing medium (0125-DGC and 0222-DGC) formed no spheres. We previously reported that this change leads to reduced expression of stem cell markers (8). Inhibition of IDO1 in 0125-GSC and 0222-GSC led to similar reductions in both sphere formation and stem cell marker expression [0125-GSC-IDO(-) and 0222-GSC-IDO(-)]. These results clearly indicate that IDO1 inhibition promotes differentiation of GSCs.

Moreover, the present study demonstrated that IDO1 inhibition-induced differentiation reduces resistance to

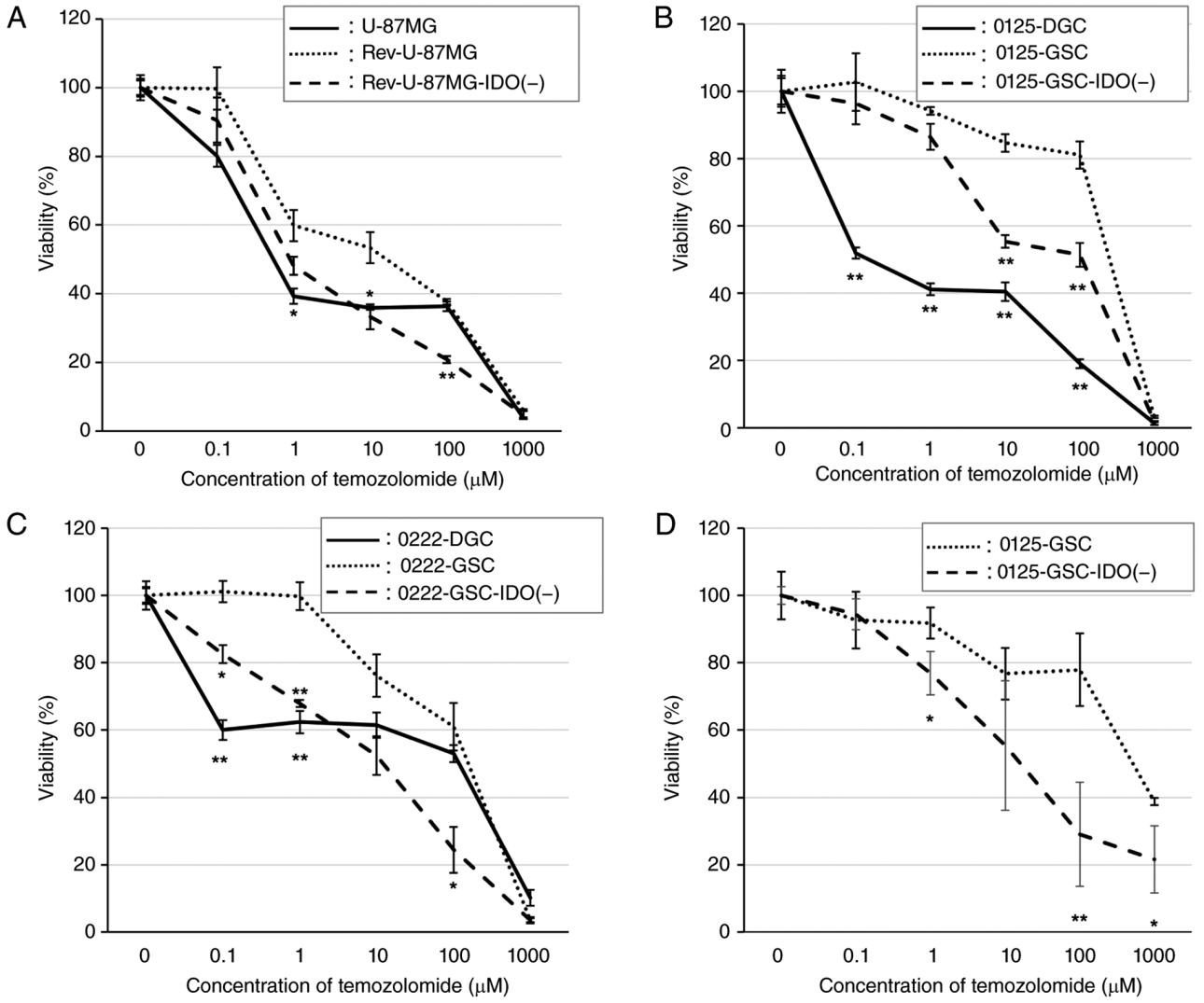

Figure 3. Drug sensitivity to temozolomide was evaluated in each cell line. A total of $1x10^4$ cells were seeded into 10-cm dishes, and the medium was replaced with fresh medium containing varying concentrations of temozolomide 24 h later. After 72 h, cells were harvested and counted using a (A-C) Z1 Coulter Counter or analyzed using (D) Cell Counting Kit-8. (A) Rev-U-87MG exhibited significant reduction of sensitivity for temozolomide compared to U-87MG at 1 and 10 μM. Rev-U-87MG-IDO(-) showed significant increase of sensitivity for temozolomide compared to Rev-U-87MG at 100 μM. *P<0.05; **P<0.01. (B) 0125-DGC exhibited significant sensitivity for temozolomide compared to 0125-GSC at 0.1, 1, 10, and 100 μM. 0125-GSC-IDO(-) showed significant sensitivity for temozolomide compared to 0125-GSC at 10 and 100 μM. **P<0.01. (C) 0222-DGC exhibited significant sensitivity for temozolomide compared to 0222-GSC at 0.1 and 1 μM. 0222-GSC-IDO(-) showed significant sensitivity for temozolomide compared to 0222-GSC at 0.1, 1, and 100 μM.*P<0.05; **P<0.01. (D) 0125-GSC-IDO(-) showed significant sensitivity for temozolomide compared to 0125-GSC at 1, 100, and 1,000 μM. *P<0.05; **P<0.01. GSC, glioma stem-like cell; IDO1, indoleamine 2,3-dioxygenase 1.

temozolomide in GSCs. Rev-U-87MG exhibited increased resistance compared to U-87MG, but this resistance was attenuated in Rev-U-87MG-IDO(-). Likewise, 0125-GSC and 0222-GSC showed decreased resistance after IDO1 expression was inhibited. These findings suggest that differentiation-inducing therapy via IDO1 inhibition enhances drug sensitivity in GSCs.

Importantly, the differentiation-inducing approach examined in this study is not intended to replace temozolomide therapy but rather to complement and enhance its therapeutic efficacy. Differentiation therapy has long been explored in hematological malignancies, but no effective method has yet been established for glioblastomas. Some molecules, such as bone morphogenetic proteins or FOXO3, have been studied for their potential to induce glioblastoma differentiation, clinical translation remains limited (21-23). However, IDO1 has not previously been considered a target for differentiation-inducing therapy. IDO1 has been extensively studied from the perspective of immunotherapy, but few studies have investigated its relationship with GSCs or as a target for differentiation-inducing therapy. The present findings provide the first evidence supporting the use of IDO1 inhibition as a novel strategy to induce differentiation and overcome chemotherapy resistance in glioblastomas.

Various limitations and future prospects for this research need to be discussed further. In the present study, IDO1 was only inhibited by pharmacological action via continuous administration of 1-MT. To establish the specificity of IDO1 more clearly, future studies employing genetic approaches, such as IDO1 knockdown or knockout cell models, are required. Also, the mechanistic basis by which IDO1 inhibition leads to the loss of stem cell character was not experimentally validated

in the present study, so further studies will be required to elucidate the intracellular signaling pathways linking IDO1 inhibition to the loss of stem cell character. Furthermore, other effects of inhibiting IDO1 in glioblastoma remain to be investigated. In particular, whether IDO1 inhibition affects additional malignant phenotypes, such as migration and invasion, was not evaluated in the present study and is therefore beyond the scope of the current dataset; accordingly, our conclusions are limited to differentiation-related changes and altered drug sensitivity. To further clarify the mechanism of synergistic effects with temozolomide, the apoptotic pathway and the mechanisms of cell death must be elucidated. In addition, proliferation and apoptosis were not directly evaluated in the present GSCs models. In this study, IDO1-inhibited cell lines were established by continuous pre-treatment with 1-MT to minimize the effects of acute cytotoxicity. Although previous studies have reported that treatment with 1-MT alone does not significantly affect in vitro cell viability under standard culture conditions (10), the possibility that cytostatic or cytotoxic effects may contribute to the observed phenotypic changes cannot be completely excluded.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

KN performed the experiments, generated all of the data presented in the results and figures, prepared the figures and drafted the original manuscript. SY conceived the study, acquired funding, developed the study methodology, managed the project, and reviewed and edited the manuscript. YA performed the experiments together with KN, and contributed to the collection of all the data presented in the results and figures. YO conceived the study together with SY, and contributed to the generation of the data presented in the results and figures. ES performed the experiments together with KN, and contributed to the collection of all the data presented in the results and figures. AY managed and supervised the project, reviewed and edited the manuscript, and contributed to the analysis and interpretation of the data. KN and SY confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Nihon University School of Medicine (approval no. 2026-09; Tokyo, Japan).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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