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Cell surface Nestin is a biomarker for glioma stem cells

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ABSTRACT

Cancer stem cells (CSCs) are the most aggressive cell type in many malignancies. Cell surface proteins are generally used to isolate and characterize CSCs. Therefore, the identification of CSC-specific cell surface markers is very important for the diagnosis and treatment of malignancies. We found that Nestin (a type VI intermediate filament protein), like the glioma stem cell (GSC) markers CD133 and CD15, exhibited different levels of expression in primary human glioblastoma specimens. Similar to our previous finding that cytoplasmic Nestin is expressed as a cell surface form in mouse GSCs, the cell surface form of Nestin was also expressed at different levels in human GSCs. We isolated cell surface Nestin-positive cell populations from human GSCs by fluorescence-activated cell sorting FACS analysis, and observed that these populations exhibited robust CSC properties, such as increased tumorsphere-forming ability and tumorsphere size. Mechanistically, we found that DAPT, a γ-secretase (a multi-subunit protease complex) inhibitor, reduced the proportion of cell surface Nestin-positive cells in human GSCs in a time- and dose-dependent manner, without significant changes in total Nestin expression, implying that a post-translational modification was involved in the generation of cell surface Nestin. Taken together, our data provides the first evidence that cell surface Nestin may serve as a promising GSC marker for the isolation and characterization of heterogeneous GSCs in glioblastomas.

1. Introduction

Glioblastoma multiforme (GBM, WHO grade IV) is the most aggressive malignancy of the central nervous system with a median survival time of only 12–15 months despite vigorous treatments such as surgical resection, radiotherapy, and chemotherapy [1]. Recently, glioma stem cells (GSCs), a subpopulation of glioma cells [2], were identified as the main cause of tumor propagation or tumor recurrence after anti-cancer therapy [3–5].

A number of cell surface markers are generally used to isolate and characterize cancer stem cells (CSCs) [6]. In GBM, 2 cell surface markers, CD133 [7] and CD15 [8], are generally used to isolate and characterize GSCs. CD133 is a glycoprotein that specifically localizes to the outer cellular membrane [9,10], and is expressed in hematopoietic stem cells [11], endothelial progenitor cells [12], neural stem cells, and brain tumors [10,13]. However, several recent reports have indicated that CD133 may not be a robust marker for GSCs [14–18]. CD15 is a carbohydrate adhesion molecule that is also known as stage-specific embryonic antigen 1 (SSEA1) [19], and is expressed in embryonic or adult central nervous system stem cells [20,21], leukemias [22], and GBM [23].

Many cellular factors, including transcriptional factors (e.g., Sox2, Nanog, and Oct3/4) [24], cytoskeletal proteins (e.g., Nestin) [25], post-transcriptional factors (e.g., Musashi 1) [25], and Polycomb transcriptional suppressors (e.g., Bmi1 and Ezh2) [26,27], are also considered GSC markers. However, in contrast to CD133 and CD15, these cellular factors are not useful for the isolation of live GSCs from tumor tissues given their intracellular localization, such as in the nucleus or cytoplasm.

During the early developmental stage of the central nervous system, Nestin is primarily expressed in neural progenitor/stem cells [28]. The Nestin protein is mainly localized in the cytoplasm and functions as a type VI intermediate filament with a high molecular weight (240 kDa) [29]. We have previously reported that an ~60-kDa N-terminal isotype of Nestin (hereafter, referred to as cell surface Nestin) is expressed on the outer cellular membrane of Id4-driven murine GSCs [30]. Here, we report the expression of cell surface Nestin in human GBM specimens and human GSCs, the isolation of live cell surface Nestin-positive GSCs, characterization of their self-renewal property, and a plausible mechanism underlying the generation of cell surface Nestin.

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2. Materials and methods

2.1. Conditions and reagents for GSC suspension culture

All human GSCs (X01 and X02 [31], GSC3, GSC4, GSC5, GSC8, AC17, AC20, 84NS, 528NS, MD13, MD30, 1123NS [32,33]) were established from patients with GBM, except X03 GSCs derived from patient with WHO grade III oligoastrocytoma [31]. All GSCs were grown in DMEM/F12 medium (Lonza) supplemented with modified N2, B27, penicillin/streptomycin (1%; Lonza), epidermal growth factor (EGF, 20 ng/mL; R&D Systems), and basic fibroblast growth factor (bFGF, 20 ng/mL; R&D Systems). EGF and bFGF were replaced every 3 days, as described previously [34]. GSCs were treated with γ-secretase inhibitor, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (LY-374973; Sigma–Aldrich).

2.2. Immunofluorescence

Paraffin-embedded human GBM sections were blocked with 10% donkey serum, and then simultaneously stained with primary antibodies against CD133 (1:20, #AC133; MACS), CD15 (1:200, #559045; BD Pharmingen), or Nestin (1:200, #N5413, which recognizes an N-terminal epitope of Nestin; Sigma–Aldrich) for 12 h at 4 °C. After binding of fluorescent protein-conjugated secondary antibodies, green-fluorescent Alexa Fluor 488 goat anti-rabbit IgG (1:400, #A11008; Invitrogen) and red-fluorescent Alexa Fluor 594 goat anti-mouse IgG (1:400, #A11005; Invitrogen) for 2 h at 20 °C, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 μg/mL) for 5 min at 20 °C. Human glioma specimens were collected after protocol approval by the Institute for Biomedical Research Ethics Committee, Samsung Medical Center. Fluorescent images were obtained using a confocal laser scanning microscope. The levels of Nestin, CD15, and CD133 expression were quantified using the Zeiss LSM Image Browser software.

2.3. Flow cytometry and fluorescence-activated cell sorting

To detect the proportions of cell surface Nestin-positive cells, live GSCs were directly incubated with a primary antibody against Nestin (1:200, #N5413; Sigma–Aldrich) for 30 min at 4 °C. GSCs were fixed with 4% paraformaldehyde and permeabilized in 0.15% saponin for 30 min at 4 °C. The permeabilized GSCs were incubated with primary antibody against Nestin for 30 min at 4 °C to identify cells expressing total Nestin (cytoplasmic and cell surface form). After binding of biotinylated goat anti-rabbit IgG (1:400, #BA-1000; Vector) for 20 min at 4 °C, streptavidin–phycoerythrin (1:1000, #554061; BD Pharmingen) was added for 10 min at 4 °C before fluorescence-activated cell sorting (FACS) analysis (BD FACSCalibur and BD FACSAria).

2.4. Single-cell sphere formation assay

After sorting with an anti-Nestin antibody (#N5413), live GSCs were seeded at a density of 1 cell per well in 96-well plates and grown under suspension culture conditions, as described in the "*Conditions and reagents for GSC suspension culture*" section. At day 14, the tumorsphere numbers and sizes were determined with a light microscope.

2.5. Statistics

Data were analyzed using two-tailed Student's *t*-tests. *P* values <0.05 were considered statistically significant.

3. Results

3.1. Cell surface Nestin expression in human primary GBM specimens and GSCs

To assess whether Nestin was expressed at the surface of cells expressing the GSC marker CD133 or CD15 in primary human GBM specimens, we performed an immunofluorescence (IF) assay on paraffin-embedded tissue sections from 5 human GBM specimens using antibodies against Nestin, and CD133 or CD15. Four out of 5 GBM specimens exhibited varying proportions of cells co-expressing Nestin and CD133 or Nestin and CD15 (Fig. 1A). Interestingly, 1 GBM specimen only expressed cell surface Nestin, and no CD133 or CD15 (Fig. 1B). Quantification of IF images revealed that an average of 13% (range, 6–28%) cells were cell surface Nestin-positive, 22% (range, 0–58%) cells were CD15-positive, and 4% (range, 0–7%) cells were CD133-positive in the 5 GBM specimens (Fig. 1C). These data suggest that cell surface Nestin may be one of the reliable GSC markers and may serve as an unique GSC marker in GBMs that are devoid of CD133-positive and CD15-positive GSCs.

To determine whether cell surface Nestin was expressed in human GSCs, we performed flow cytometric analysis on 14 primary GSC lines—AC17, X02, 528NS, GSC4, GSC3, MD30, X03, 83NS, MD13, GSC5, GSC8, X01, 1123NS, and AC20—using an anti-Nestin antibody, and found that the proportion of cell surface Nestin-positive GSCs ranged from 1.4% to 70% (Fig. 2), which is similar to the proportions of CD15-positive and CD133-positive cells in human GSCs [8].

3.2. Cell surface Nestin-positive cells have robust tumorsphere-forming ability

To determine the biological significance of cell surface Nestin-positive GSCs, we first isolated cell surface Nestin-positive and -negative MD30, 528NS and GSC8 by FACS analysis using the anti-Nestin antibody. The green (P3) and blue (P4) dots in the FACS plot represent cell surface Nestin-positive and -negative cells, respectively. Representative images show single cell-derived tumorspheres of cell surface Nestin-positive and -negative GSC8 cells grown in serum-free medium containing EGF and bFGF for 14 days (Fig. 3A). Because GSCs can form floating clonal colonies (referred to as tumorspheres) and sustain their self-renewal property when grown in suspension in serum-free medium containing defined growth factors, such as EGF and bFGF [35], we incubated cell surface Nestin-positive and -negative GSCs under suspension culture conditions. As shown in Fig. 3B, cell surface Nestin-positive MD30, 528NS, and GSC8 GSCs formed significantly larger tumorspheres than cell surface Nestin-negative GSCs, implying higher proliferation ability of cell surface Nestin-positive GSCs. In addition, the single-cell sphere formation assay revealed that 43% (range, 31–57%) cell surface Nestin-positive GSCs were able to generate tumorspheres, whereas only 14% (range, 7–23%) cell surface Nestin-negative GSCs formed tumorspheres (Fig. 3C), indicative of a higher tumorsphere-forming ability in cell surface Nestin-positive GSCs. These findings suggest that cell surface Nestin is an useful GSC marker for GSC isolation and characterization.

3.3. γ-Secretase regulates the generation of cell surface Nestin in human GSCs

Nestin is a typical cytoplasmic intermediate filament protein. However, our previous study demonstrated that cell surface

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