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Long-term maintenance of brain tumor stem cell properties under at non-adherent and adherent culture conditions

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Abstract

There is increasing evidence for the presence of cancer stem-like progenitors in malignant brain tumors. This subpopulation of progenitor cells, the so-called "cancer stem cells (CSCs)", may play a pivotal role in brain tumor initiation, growth, and recurrence. Here we describe the establishment of one permanent brain tumor stem cell line that able to form new spheres after culture under adherent monolayer conditions and to recapitulate the properties of the original tumor upon transplantation into immunodeficient mice. Re-formed spheres retained their stem cell properties and isolated single CSCs from these spheres formed spheres/tumors even after long-term cultures (over 2 years). These data suggested that a small population of CSCs preserved its stem cell properties even after serial passages under non-adherent/adherent culture conditions. Evaluation of underlying metabolic events and assessment of the biological features of these viable cell lines will yield useful knowledge on the *in situ* behavior of brain tumors.

Keywords: Brain tumor stem cell; Cell line; Glioma; CD133; Long-term culture; cDNA array

There is increasing evidence that malignant brain tumors arise from and contain cancer stem-like progenitors with the potential for self-renewal and multi-lineage differentiation that recapitulates the phenotype of the original tumor [1–4]. As this subpopulation of stem-like progenitors, designated cancer stem cells (CSCs), may play an important role in tumorigenesis and tumor recurrence, effective cancer treatments must target and eliminate this population [5–8]. Therefore, functional analysis and a detailed understanding of CSCs and their role in tumor pathogenesis are critical.

We isolated and characterized multipotent, self-renewing cells derived from fresh human GBM [9]. We recognized the need for a model that can be used to study CSCs from human gliomas and postulated that CSCs derived from individual patients would serve this purpose better than established cell lines. Patient-specific CSC lines that can re-form new non-adherent spheres from adherent monolayer cells represent are a powerful tool for the study of CSC biology and may be exploitable for the development of therapies targeted at specific patients [3]. Based on these considerations we focused our efforts on establishing permanent cell lines derived from the CSCs of individual patients' tumors. Here we report the properties of our X01GB CSC cell line and demonstrate that these cells retained the biological characteristics of the original tumor.

Materials and methods

Primary sphere formation. Our study was approved by the Medical Review Board of Gifu University. Tumor-sphere cultures were performed as described previously with some modification in medium containing

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DMEM-F12 (GIBCO-Invitrogen, La Jolla, CA), penicillin G, streptomycin sulfate, B-27 (1:50; GIBCO-Invitrogen), recombinant human FGF-2 (20 ng/ml; R&D Systems, Minneapolis, MN), recombinant human EGF (20 ng/ml; R&D Systems), and leukemia inhibitory factor (1000 U/ml) [9].

Characterization of tumor spheres serially passaged in monolayer culture. Tumor spheres were seeded into poly-L-lysine (Sigma, St. Louis, MO)-coated 24-well dishes (Falcon) with DMEM-F12 and B27 (1:50) containing 10% fetal bovine serum (FBS) but no growth factors. After they attached and grew in monolayers, cells were washed with PBS to remove floating cells and only adherent tumor-sphere colonies were treated with 0.05% trypsin and 0.1 mM EDTA. Dissociated cells (1×10^5/ml) were transferred to 60-mm Falcon dishes containing the same medium. Upon reaching subconfluency, they were transferred to 100-mm dishes. Performing the same procedure once or twice a week, the cultures were serially transferred.

Immunocytochemistry and flow cytometry. Immunocytochemistry of tumor-spheres was as described [9]. Antibodies used were as follows: human anti-nestin (rabbit polyclonal antibody (pAb), 1:200; Chemicon, Temecula, CA) and CD133 (mouse monoclonal antibody (mAb), 1:10; Miltenyi Biotec, Auburn, CA) for neural stem- and progenitor cells, with anti-β III-tubulin (mouse mAb, 1:200; Chemicon) for neurons, with anti-glial fibrillary acidic protein (GFAP; rabbit pAb, 1:500; DAKO, Glostrup, Denmark) for astrocytes, with anti-galactocerebroside (GalC; mouse mAb, 1:200; Chemicon) for oligodendrocyte; and then with Alexa fluorophore-conjugated secondary antibodies (1:1,000; Molecular Probes, Eugene, OR).

Aliquots of CD133-positive and negative cells were evaluated by flow cytometry with a FACS Calibur machine (BD Biosciences, San Jose, CA), using CD133/2 (293C3)-APC antibody (mouse mAb, Miltenyi Biotec) according to the manufacturer's recommendation.

Transplantation into immunodeficient mice. Our experimental procedures involving animals followed the guidelines of the Animal Experimental Committee of Gifu University. Tumorigenicity was determined by injecting brain tumor-derived CSCs orthotopically into non-obesity diabetic-severe combined immunodeficient (NOD-SCID) mice [9].

Histochemical analysis of brain tissues. HE staining and immunohistochemistry of paraffin-embedded tissues was performed as previously described [9]. The following antibodies were used as primary antibodies: anti-human nestin (mouse mAb, 5 μg/ml; R&D Systems) for NSC, with anti-human Ki-67 (mouse mAb, 1:50; DAKO) for proliferative indices, with anti-GFAP (mouse mAb, 1:500; DAKO) for astrocytes, and with anti-human βIII-tubulin (mouse mAb, 1:500; Chemicon) for neurons.

RNA preparation. The preparation of cRNA, hybridization, and scanning of the microarrays were according to the manufacturer's protocol (Affymetrix, Santa Clara, CA); analysis was on both a spectrophotometer and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was extracted from each sample by using TRIZOL reagent (Invitrogen); this was followed by passage through an RNeasy spin column (Qiagen, Valencia, CA) and amplification with RiboAmp RNA Kits (Arcturus Engineering, Mountain View, CA) according to the manufacturer's protocol. Amplified RNA (7.5 μg) was labeled with Cy5-dUTP (experimental RNA) or Cy3-dUTP (Stratagene, La Jolla, CA) using Superscript II reverse transcriptase (Invitrogen).

Microarray procedure and data analysis. Labeled cRNA was hybridized to the Affymetrix Human Genome U133 Plus 2.0 Genechip with 60 rpm rotation for 16 h at 45 °C. After hybridization, the microarrays were washed in buffer containing biotinylated anti-streptavidin Ab (Vector Laboratories, Burlingame, CA) and stained (10 min at 25 °C) with streptavidin-PE (final concentration 10 μg/ml; Molecular Probes). Subsequently, the microarrays were washed, restained with streptavidin-PE, and washed again before measuring fluorescence at 570 nm in the Affimetrix GeneChip Scanner 3000. All of the microarrays were examined for surface defects, grid placement, background intensity, housekeeping gene expression, and the 3′/5′ ratio of probe sets from genes of varying length (signal 3′/5′ ratio <3).

Initial data analysis was performed using Affymetrix Microarray Suite 5.0 to determine gene expression levels. Data analysis was conducted using Affymetrix GeneChip operating software, and fold-changes in the values for genes were calculated as the ratio of the signal values of the adherent X01GB monolayer cells to non-adherent X01GB sphere cells. Only gene expression changes with 2-fold significance were considered.

Results

Maintenance of tumorigenicity and sphere-forming capacity of original CSCs after long-term culture under in adherent conditions

In this study, we used X01GB-CSCs from a GBM patient [9]. In differentiation medium containing serum, the X01GB spheres quickly attached to the culture dishes; we observed the outgrowth of cells and the extension of cell processes beyond the core of the spheres. When we serially passaged these monolayer cells in differentiation medium, they acquired a flat, fibroblast-like cell shape (Fig. 1A, left) and contained βIII-tubulin- and GFAP double-positive cells (Fig. 1A, right). After 50 passages, X01GB monolayer cells injected i.c. into mice continue to survive, however, unlike cells from tumor-forming non-adherent spheres, they failed to form tumor masses (Fig. 1B). We hypothesized that the adherent monolayer cells harbored a limited number of tumorigenic cells and examined their ability to recapitulate the features of the parental tumor after heterotopic transplantation. Therefore, we injected 1×10^7 cells i.p. into immunodeficient mice. As shown in Fig. 1C and D, the heterotopic xenografts manifested the features of the original brain tumor. X01GB tumor stem cells retained their stem cell properties, irrespective of whether they derived from non-adherent spheres or adherent monolayers. Thus, our experimental system yields a practically sufficient supply of GBM monolayer cell lines that retain their tumor stem cell properties.

After 100 days in of monolayer culture, we returned some of the X01GB cells to serum-free proliferation medium containing growth factors. The cells formed non-adherent spheres again and their number increased over the subsequent 70-weeks period (Fig. 2A). The cells also migrated out on the culture dish surfaces (Fig. 2B) and transplanted spheres recapitulated the parental tumor (Fig. 2C). During serial passage, the population of CD133-positive cells among the monolayer cells derived from primary spheres decreased; flow-cytometry indicated that only 0.06% of the cells remained positive for CD133 in adherent monolayer cultures (Fig. 2D). On the other hand, CD133-positive cells reappeared in the newly formed non-adherent spheres and their number exceeded that found in the original tumor spheres; 12.7% of these cells were CD133-positive compared to 2.53% in the primary spheres. Although we used varying numbers of cells were used for *in vivo* tumor formation, the minimal number of CD133-positive cells required for GBM formations in mice tended to be constant (Table 1); it was 2530 for primary spheres, 6000 for adherent monolayer cells, and 1000 for secondary-sphere cells. On the other hand, in the case of secondary sphere cells, 100 CD133 positive cells were not