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Brain Tumor Stem Cells From an Adenoid Glioblastoma Multiforme

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Abstract

Brain tumors may arise from and contain cancer stem cells (CSCs) capable of self-renewal, proliferation, and differentiation that recapitulate the parent tumor. These CSCs are thought to be important in gliomagenesis. Detection of CSCs invading the adjacent brain regions is important for the diagnosis and effective treatment of glioblastoma multiforme (GBM). A 57-year-old man presented with an adenoid GBM, and underwent resection of the tumor. Multipotent, self-renewing cells derived from the human adenoid GBM were isolated and identified with the tumor-derived stem cell surface antigen CD133 from whole autopsied patient's brain. Tumorsphere culture and flow cytometric analysis revealed that 1.02–2.32% of the cells were positive for CD133. Transplantation of cultured tumorspheres into the mice brain resulted in the formation of well-defined tumor masses after 12 weeks. The histological and immunohistochemical characteristics of the xenograft were identical to those of the parent tumor. Examination of the patient's brain at autopsy showed CD133-positive cells were identified in the brain regions adjacent to the tumor, suggesting that CD133-positive CSCs might be localized to the vascular niche. Methods to localize CSCs may open new approaches for the treatment of brain tumors.

Key words: adenoid glioblastoma, cancer stem cell, CD133, niche

Introduction

Glioblastoma multiforme (GBM) is the most lethal type of brain tumor. GBM is highly infiltrative and the margins may not be readily identifiable, so many GBMs cannot be completely resected.⁷⁾ The classification of GBMs has remained largely unchanged for nearly 80 years, and the prognosis continues to be based on histological findings such as vascular proliferation, presence of poorly undifferentiated cells, nuclear atypia, brisk mitotic activity, and necrosis.⁵⁾ Similarities between stem cells and cancer cells in capacity for self-renewal and multipotentiality have suggested the concept of cancer stem cells (CSCs). The definition criteria for CSCs include extensive self-renewal in vitro and cancer initiation in vivo.^{9,10,12)} The concept of CSCs has led to a re-examination of the classic theories regarding gliomagenesis.^{10,12)} The properties of CSCs may have diagnostic implications, so a new classification system is needed to reflect the morphology and pheno-

type of brain tumors, as well as the progenitors, prognosis, and treatment response.

The present study investigated the distribution of CSCs in the resected adenoid GBM of a 57-year-old man, the histological character of the CSCs, and the distribution of CSCs in the brain examined at subsequent autopsy.

Materials and Methods

A 57-year-old man presented with a 3-month history of aphasic speech. Initial computed tomography and magnetic resonance imaging revealed a 5-cm heterogeneous left temporal lesion with enhancement by contrast medium (Fig. 1A). He underwent left temporal craniotomy for resection. Histological examination of the resected tumor showed nests of atypical epithelial-like cells separate and distinct from the malignant glial cells (Fig. 1B). The adenoid portion demonstrated focal tubular patterns like adenocarcinomas and numerous mitoses within the adenoid

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areas. The malignant glial area consisted of atypical glial cells with highly pleomorphic astrocytic nuclei, occasional multinucleated cells, and gemistocytes with necrosis. Distinct areas of the tumor consisted of atypical spindle cells within dense collagenous stroma with occasional atypical mitotic cells. Areas showing epithelial differentiation showed positive for wide cytokeratin (CK20; AE1/AE3) (Fig. 1C) and negative for epithelial membrane antigen (EMA) (Fig. 1D). They also expressed nestin, glial fibrillary acidic protein (GFAP), and β III-tubulin (Fig. 1E-G). MIB-1 (Ki-67) staining showed a high degree of proliferation (Fig. 1H). The histological diagnosis was adenoid GBM. After obtaining his written prior informed consent, a portion of the surgical specimen was submitted for tumorsphere culture under sterile conditions. The patient died 15 months after the tumor removal. Autopsy of the whole brain was performed with the written prior informed consent of his family.

Tumor was removed including left-temporal tip, and used for tumorsphere culture including tumor core, tumor margin, and adjacent brain. An hour after tumor removal, tissues were minced with scissors, incubated in 0.05% trypsin (GIBCO-Invitrogen, La Jolla, Calif., U.S.A.) in 0.1 mM ethylenediaminetetra-acetic acid (20 min, 37°C), washed, cells were dissociated in phosphate-buffered saline ($\times 3$) to eliminate cell debris, and triturated in the same solution in a fire-polished Pasteur pipette. Cells passed through a 100- μ m strainer (Falcon, Oxnard, Calif., U.S.A.) were then seeded (1×10^5 /ml) into Falcon culture flasks and grown in medium containing Dulbecco's modified Eagle's medium (DMEM)-F12 (GIBCO-Invitrogen), penicillin G, streptomycin sulfate, B-27 (GIBCO-Invitrogen), recombinant human fibroblast growth factor-2 (20 ng/ml; R&D Systems, Minneapolis, Minn., U.S.A.), and recombinant human epidermal growth factor (20 ng/ml; R&D Systems).^{4,6,8,15} The medium was replaced every 3 days with identical fresh medium. Spheres were counted under a phase-contrast microscope (Olympus IMT-2, Tokyo). Passage was by trituration in a fire-polished Pasteur pipette; the cells were reseeded into fresh proliferation medium and maintained in a 37°C incubator with 95% air and 5% CO₂. To investigate tumorsphere self-renewal capacity, modified limiting dilution assays were performed.^{4,8,15} Briefly, dissociated cells were plated in 96-well plates (Falcon) in 200 μ l volumes of DMEM-F12 medium with growth factors. Cultures were fed 20 μ l of medium with growth factors every 2 days and the percentage of wells without tumorspheres was calculated and plotted against the number of cells per well.

Tumor samples were fixed in 4% paraformaldehyde,

paraffin embedded, cut into 3 μ m sections. For hematoxylin-eosin staining, sections were first stained with Mayer's hematoxylin (1 min) and then counterstained with alcoholic eosin. For immunohistochemical studies, endogenous peroxidase was neutralized with 3% H₂O₂ in methanol (15 min) after antigen retrieval in citrate buffer in a microwave at 500 W (15 min). Sections were blocked with 1% bovine serum albumin in tris-buffered saline, then treated with primary antibodies for overnight at 4°C as follows; anti-human nestin (mouse monoclonal antibody [mAb], 5 μ g/ml; R&D Systems) for neural stem cells, anti-human MIB-1 (Ki-67) (mouse mAb, 1:50; DAKO, Glostrup, Denmark) for proliferative indices, anti-GFAP (mouse mAb, 1:500; DAKO) for astrocytes, anti-human β III-tubulin (mouse mAb, 1:500; Chemicon, Temecula, Calif., U.S.A.) for neurons, anti-human EMA (mouse mAb, 1:100; DAKO), and anti-human cytokeratin AE1/AE3 (mouse mAb, 1:50; DAKO). After treatment with biotinylated secondary antibody and horseradish peroxidase-linked streptavidin (LSAB2 kit; DAKO), color reactions were performed with peroxidase-substrate 3,3'-diaminobenzidine hydrochloride (DAKO), and counterstained with Mayer's hematoxylin.

Immunocytochemistry of tumorspheres was carried out as described previously^{4,6,8,15} using anti-nestin (rabbit polyclonal antibody [pAb], 1:200; Chemicon) and anti-CD133 (mouse mAb, 1:10; Miltenyi Biotec, Auburn, Calif., U.S.A.) for brain tumor stem and progenitor cells, with anti- β III-tubulin (mouse mAb, 1:200; Chemicon) and with anti-GFAP (rabbit pAb, 1:500; DAKO); and then with Alexa fluorophore-conjugated secondary antibodies (1:1000; Molecular Probes, Eugene, Ore., U.S.A.). Aliquots of CD133-positive cells were evaluated by flow cytometry with a fluorescent activated cell sorter Calibur machine (BD Biosciences, San Jose, Calif., U.S.A.), using CD133/2 (293C3)-allophycocyanin antibody (mouse mAb; Miltenyi Biotec) according to the manufacturer's recommendation.

Our experimental procedures involving animals followed the guidelines of the Animal Experimental Committee of Gifu University. Tumorigenicity was determined by orthotopically injecting CSCs derived from the brain tumor as described previously.^{4,8,15} After 20 weeks of primary culture, cells were injected into the right flank of non-obese, diabetic, severe combined immunodeficiency mice and 2 μ l of 1×10^8 cells/ml cell suspension in proliferation medium (total 10^4 cells) was delivered into the right striatum (0.2 μ l/min) by stereotactic injection through a Hamilton syringe, all under general anesthesia. The mice were sacrificed at 12–14 weeks post-injection depending on the injected cell line.

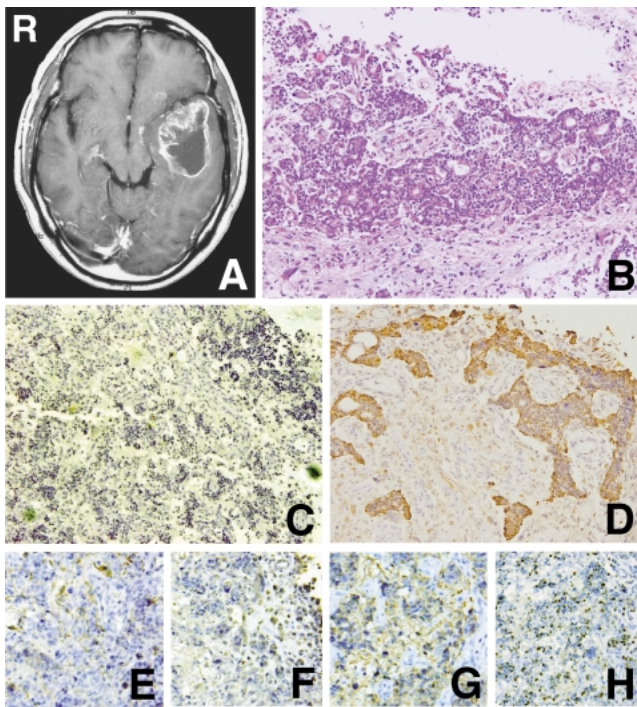


Fig. 1 A: Axial T₁-weighted magnetic resonance image with gadolinium demonstrating a left temporal mass. B: Photomicrograph showing a glandular structure and diffuse infiltration of glial-like cells at the periphery of the tumor. Hematoxylin and eosin stain, $\times 100$. C-H: Areas showing epithelial differentiation are positive for wide cytokeratin (CK20; AE1/AE3) (C) and negative for epithelial membrane antigen (EMA) (D). The expression of glial fibrillary acidic protein (GFAP) is reduced and the astrocytic nature of the glandular structures is unclear (E). The tumor expressed the cytoplasmic primitive intermediate filament nestin (F) and the neuronal marker β III-tubulin (G), and manifested a high proliferative index (MIB-1) (H). C: Cytokeratin stain, $\times 100$; D: EMA stain, $\times 100$; E: GFAP stain, $\times 100$; F: nestin stain, $\times 100$; G: β III-tubulin stain, $\times 100$; H: MIB-1 stain, $\times 100$.

Results

Tumorsphere culture formed neuronal sphere-like aggregates within 7 days. Sphere-like aggregates continuously increased and could be passaged for more than 3 years by refeeding fresh medium twice a week (Fig. 2A). The proliferative activity of these cells increased during the late subculture stages compared with earlier passages (Fig. 2B); this phenomenon was previously reported by Galli et al.¹⁾ The spheres resembled tumor-derived spheres and

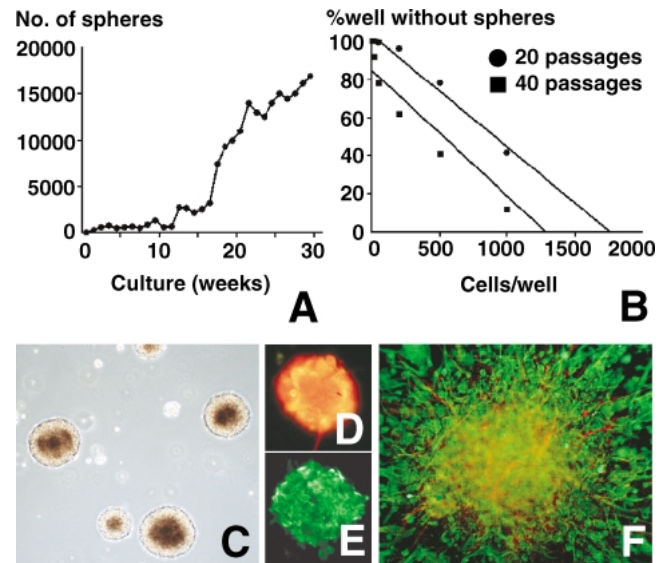


Fig. 2 A: Time-dependent sphere formations. B: Limiting dilution analysis. Sphere cells were dissociated and plated in 96-well. The final cell dilutions ranged from 1 to 1000 cells/well. The percentage of wells not containing spheres for each cell plating density was calculated and plotted against the number of cells per well. C-F: Photomicrographs showing tumorspheres derived from an adenoid glioblastoma, and cells immunostained for progenitor and stem cell markers CD133 (D) and nestin (E). Immunocytochemical analysis of differentiated tumor-derived spheres showing positive staining for glial marker glial fibrillary acidic protein (green) and/or neuronal marker β III-tubulin (red) (F). C: Phase contrast, $\times 50$; D-F: fluorescence immunocytochemistry, $\times 50$.

were immunoreactive for CD133 and nestin (Fig. 2C-E), indicating that they contained undifferentiated stem cells. Flow cytometry revealed that 1.02–2.32% of the cells in spheres were positive for CD133. Differentiation with fetal bovine serum showed the cells expressed glial and neuronal markers (Fig. 2F). A portion of these cells returned to serum-free proliferation medium containing growth factors grew morphologically undifferentiated spheres with high self-renewal capacity.⁴⁾

Orthotopic transplantation of cultured tumorspheres after 20 passages resulted in well-defined tumor masses appearing after 12 weeks (Fig. 3A). The transplanted spheres recapitulated the properties and the general profile of the parent tumor, and glandular structures mimicking adenocarcinoma were also present (Fig. 3B). Detailed immuno-

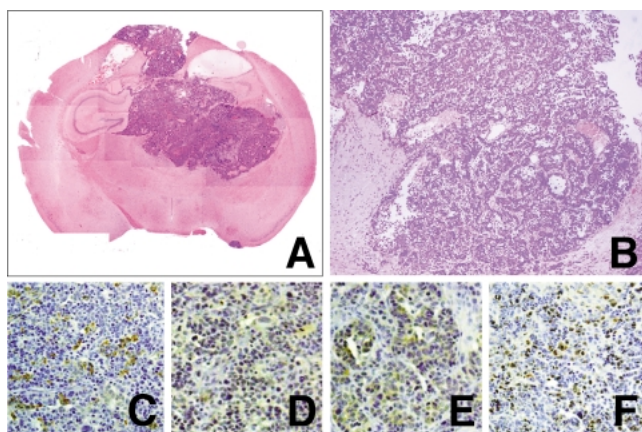


Fig. 3 A: Photograph showing the gross appearance of the orthotopic xenograft from an adenoid glioblastoma. Hematoxylin and eosin stain, $\times 50$. B: Photomicrograph showing that the xenograft had similar histological features to the original tumor phenotype including the glandular structures. Hematoxylin and eosin stain, $\times 100$. C-F: The xenograft evidenced glial fibrillary acidic protein (GFAP) (C), nestin (D), β III-tubulin (E), and a high proliferation index (MIB-1) (F). The phenotype of the xenograft matched well with that of the original human tumor (Fig. 1). C: GFAP stain, $\times 100$; D: nestin stain, $\times 100$; E: β III-tubulin stain, $\times 100$; F: MIB-1 stain, $\times 100$.

histochemical analysis of the transplanted tumors and original human tumor showed that the xenografts also expressed nestin, β III-tubulin, and GFAP; and had similar MIB-1 (Ki-67) index to the original tumor (Fig. 3C-F).

After his death (Fig. 4A), the whole brain was examined for detection of CD133-positive cells using immunohistochemistry method. Sections were made from tumor core, tumor margin, edematous area, basal ganglia, subventricular zone, and several parts of gray and white matters (Fig. 4B). In our experience, CD133-positive cells could be detected only in the brain regions adjacent to the tumor, and not be in the tumor core, subventricular zone, or normal brain, including the contralateral hemisphere (Fig. 4C, D).

Discussion

Adenoid GBM is a rare, atypical variant of GBM.^{5,11} These tumors contain foci with glandular and ribbon-like epithelial structures, and the differential diagnosis between primary adenoid GBM and metastatic tumors, derived, for example, from

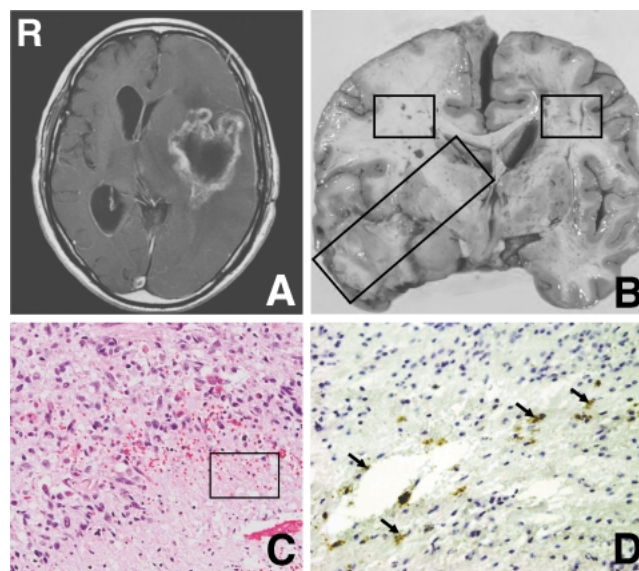


Fig. 4 A: Axial T_1 -weighted magnetic resonance image with gadolinium demonstrating a left temporal mass, 1 month before his death. B: Photograph of the patient's brain at autopsy. Boxes showing the pathological sections for detection of CD133-positive cells. C: Photomicrograph showing tumor cells spread into the adjacent brain. Hematoxylin and eosin stain, $\times 100$. D: CD133-positive tumor cells (arrows) in the adjacent brain. CD133 stain, $\times 200$.

adenocarcinoma, can be difficult.¹¹ In the present case, the tumor consisted mainly of glandular structures and exhibited features of atypical GBM at the periphery, so the diagnosis was adenoid GBM.

CD133 is a marker of neural stem cells in the adult central nervous system as well as brain CSCs.^{13,14,16} The biological function of CD133 is not well understood, but CD133-positive cells may be considered as a possible target for cancer therapy.¹⁴

Tumorsphere culture of samples from the present case formed cell aggregates containing 1–2% of cells immunopositive for CD133. We considered that these aggregates were tumorspheres.^{1–3,13,14} Transplantation showed that these tumorspheres recapitulated the phenotypically complex properties of the parent tumor. These findings indicated that this atypical variant of GBM was organized from a progenitor population that contained multipotent, long-term self-renewing, population-expanding cells that remained viable even after several months in culture, as found for classical GBM.^{1,13,14}

In our experience, CD133-positive CSCs could be identified in the brain adjacent to the tumor in autopsied whole brain. Detection of CSCs invading ad-

acent brain regions is important for the diagnosis and effective treatment of GBM, so new imaging techniques for determining the extent of CSC spread are necessary to allow total tumor resection.

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Commentary

In the present study, the authors isolated cancer stem-like cells from a patient with adenoid glioblastoma. Since these cells formed spheres and were immunoreactive to CD-133 and nestin differentiated into both glias and neurons, they were considered to be cancer stem cells (CSCs). These cells, when transplanted into the mouse brain, formed tumors that were histologically and immunohistochemically identical to the original tumor. Recently, CSCs have been identified in various types of cancers and can be considered as the new target for cancer therapy. Because of the low curability of glioblastoma even after ad-

vanced radio-chemotherapy, CSCs are an attractive target for glioma treatment. Investigating the characteristics of CSCs obtained from patients, as reported in the present study, is especially important to develop novel treatment strategies to make a breakthrough in glioblastoma treatment whose prognosis has not remarkably improved during the last three decades.

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The use of surgical specimens in establishing tumor-sphere culture and orthotopic xenograft models offers great promise not only as a complementary diagnostic tool, but also as a means to characterize tumor stem cells. This is especially important in cases when the initial tumor is a rare pathological entity, like the adenoid glioblastoma presented in this study.

The authors successfully cultured tumor-derived spheres which retained differentiation potential, had a high self-renewal capacity and upon orthotopic

transplantation into mice, mirrored the initial tumors including the glandular structures. These spheres can thus be considered to contain adenoid-glioblastoma initiating cells and comparing them to tumorspheres established from classic glioblastoma could shed new light on the biology of this subtype.

This experimental line could also be used to determine the location of tumor-initiating cells within the surgical specimen. The authors mention that tumor core, tumor margin and adjacent brain were used for tumorsphere culture, and that these regions had a different CD133 status, as revealed by post-mortem analysis. It would be interesting to know whether tumorspheres/secondary tumors can be established from all of these locations and if so, whether they are similar or not. Furthermore, it is important to consider what they can do next by using this good model system.

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