B7-H1 is correlated with malignancy-grade gliomas but is not expressed exclusively on tumor stem-like cells

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Human glioblastoma is well known for its capacity to interfere with effective antitumor immune responses. B7-H1 is the third member of the B7 family that plays important roles in tumor immune evasion. Recent studies have shown that brain tumor stem-like cells (TSCs) contribute to tumorigenesis and radioresistance. However, the relationship between B7-H1 and the clinical behavior of brain TSCs remains unclear. In the present study, we report that B7-H1 is correlated with the malignancy grade of astrocyte tumors. B7-H1 was significantly upregulated at the growing edge of the tumors. Immunostaining and flow cytometric analysis indicate that B7-H1 was expressed primarily by Ki67-negative tumor cells. In vitro, tumors cultured under medium favoring the growth of neural stem cells were able to form spheres, along with expression of neural stem/progenitor cell markers. These cells were able to differentiate into different neural lineages when cultured in differentiation medium, indicating that these cells have TSC characteristics. We also found that B7-H1 was expressed, but not exclusively on CD133-positive stem cells. Interestingly, we found that CD133-negative tumor cells also had the capacity to form brain tumors. Our data establish a correlation between the expression of the negative costimulatory molecule B7-H1 and the malignancy grade of human gliomas, suggesting that B7-H1 may be a novel tumor marker and target for therapy, although it is not expressed exclusively on brain TSCs. Neuro-Oncology 11, 757–766, 2009 (Posted to Neuro-Oncology [serial online], Doc. D08-00272, March 5, 2009. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2009-014)

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Cancer immunosuppression is one of the hallmark features of glioblastoma multiforme (GBM), which is the most notorious and highly malignant brain tumor that continues to defy current treatment strategies. Improvement in conventional treatment modalities has not significantly changed patient survival; experimental research concerning the nature of immunosuppression in gliomas is being carried out extensively. B7-H1 (PD-L1, CD274) is the third member of the B7 family that has been described to negatively regulate T-cell functions by engagement with PD-1, a CD28 homolog. B7-H1 can be detected in several types of cancer cells and contributes to immunoresistance, possibly by apoptosis induction of cytotoxic T-lymphocytes (CTLs), which express PD-1 (B7-H1 receptor) after ligation to B7-H1+ tumor cells. A molecular shield or barrier hypothesis was also proposed to describe the immunoresistance of B7-H1+ tumor cells to antigen-specific cytolytic T-cells. Two recent studies using immunohistochemistry and reverse transcriptase polymerase chain reaction (PCR) techniques showed B7-H1 expression in gliomas and its correlation with the tumor
malignancy grade.\(^\text{17,18}\) Although these findings are consistent with the opinion that brain tumors can escape from immune surveillance through the B7-H1 pathway, the relationship of B7-H1 to the proliferative behavior of brain tumors, tumor infiltration of CD8\(^+\) T-cells, and the distribution (tumor core or tumor edge) of B7-H1 is uncertain. In addition, our previous study showed that another negative costimulatory molecule, B7-H4, could be expressed on brain tumor stem-like cells (TSCs), a minority fraction of the entire brain tumor (e.g., gliomas or medulloblastomas) cell population, which expressed the neural stem cell surface marker CD133 and had more malignant properties, such as tumorigenesis, radioresistance, and chemoresistance, than did their CD133\(^-\) counterparts.\(^\text{19–24}\) Whether B7-H1 can also be expressed on TSCs remains largely unknown.

In the present study, we found that B7-H1 was correlated with the malignancy grade of human astrocytic tumors, but preferentially in the nondividing tumor cells. It was upregulated at the growing edge of the tumors and had a negative correlation with tumor-infiltrating CD8\(^+\) T-cells. In addition, we found that B7-H1 was expressed in a population of CD133\(^+\) cells sorted by flow cytometry (FCM). However, both CD133\(^+\) and CD133\(^-\) cells could initiate tumor formation after implantation into severe combined immunodeficiency (SCID) mice. We found that these secondary glioma cells also expressed B7-H1 protein. Our findings suggest B7-H1 may not only be a novel tumor marker and target for human glioma therapy but also have implications for understanding the clinical behavior of brain TSCs.

### Materials and Methods

#### Immunohistochemistry

Human astrocyte tumor specimens were obtained from patients that received surgical resection at the Department of Neurosurgery, Huashan Hospital, Fudan University (Shanghai, China). Tumors were classified according to WHO guidelines\(^\text{25}\) by two individual attending neuropathologists. Patients ranged from 3 to 72 years of age (median age, 45 years). The 48 astrocytic tumors included pilocytic astrocytoma (WHO grade I, \(n = 12\)), astrocytoma (WHO grade II, \(n = 12\)), anaplastic astrocytoma (WHO grade III, \(n = 12\)), and glioblastoma multiforme (WHO grade IV, \(n = 12\)). Immunohistochemistry was performed on these frozen sections. Peroxidase activity was blocked with 1% H\(_2\)O\(_2\), and sections were rinsed thoroughly in PBS after each step. Negative controls were obtained by omitting the primary Abs or using control immunoglobulins. Reactivity was detected with a DAB (diaminobenzidine) VectaStain Elite Kit (Vector Laboratories, Burlingame, CA, USA), and positive staining was detected as a brown coloration of the tissues. To study the relationship between B7-H1 and infiltration of CD8\(^+\) T-cells, CD8 staining was also evaluated with anti-CD8 (RPA-T8, ebioscience; 1:20) in the same frozen sections.

B7-H1\(^+\) cells were counted in six to eight areas of maximal labeling using an eyepiece grid covering an area of 0.0625 mm\(^2\) under ×400 magnification. Vessels and blood cells were excluded from analysis. Evaluation was performed independently by two researchers with greater than 90% concordance. To explore the relationship between B7-H1 expression and CD8\(^+\) T-cells, the number of CD8\(^+\) cells per 1,000 total nuclei was counted in the frozen sections from the same tumor stained for B7-H1.

#### Western Blot Analysis

All patients were divided into two groups based on tumor differentiation: high-grade gliomas (WHO grade III and IV, \(n = 24\)) and low-grade gliomas (WHO grade I and II, \(n = 24\)). Tumor tissue was removed and defined into whole tumor tissue, core, and edge sections with the aid of neuron-navigation and intraoperation MRI scans. The core and edge of tumors were identified according to the preoperative images: if contrast MRI showed tumors with obvious enhancement, lesions located at the margin of enhancement were regarded as tumor edge (1-cm diameter, including some peritumor edema). If there was no tumor enhancement, tumor edge was identified based on T2-weighted images. Tissues were homogenized in lysis buffer consisting of 0.25% sodium deoxycholate, 50 mM Tris (pH 8.5), 10 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% Nonidet P-40 with 1× protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO, USA). The suspension was sonicated for 10 s to facilitate protein solubilization and incubated for 1 h at 4°C. The samples were then centrifuged at 16,000 g for 30 min. The protein concentration of the supernatant was measured using the protein concentration assay kit (Bio-Rad, Hercules, CA, USA). Proteins were separated by sodium dodecyl sulfate gel electrophoresis and electrotransferred onto nitrocellulose membranes. Membranes were incubated successively with blocking solution, anti-B7-H1 primary Ab (R&D Systems, Minneapolis, MN, USA), and HRP-linked secondary Abs. After substrate reaction, the image was developed using Image-Pro Plus software (version 5.1; Media Cybernetics, Silver Spring, MD, USA). The expression level of B7-H1 protein was quantitated by densitometry. If B7-H1 was detected in the whole tumor tissue of gliomas, the protein levels of the tumor cores and the corresponding edges were then compared.

#### Culture of Tumor Cells and Spheres

Human astrocytic tumor specimens (\(n = 6\)) and medulloblastoma specimens (\(n = 2\)) were obtained from patients undergoing surgical resection. Patients' ages ranged from...
9 to 69 years, with a median age of 49 years. Demographics of this group of patients are described in Table 1. All examined tumors were primary brain tumors except one recurrent glioma (BT6). The protocol for this study was approved by Huashan Hospital Institutional Review Board. Brain tumor tissues were washed with cold PBS, cut into very small pieces, and incubated in Accutase solution (Chemicon, San Diego, CA, USA) at 37°C with continuous stirring for 30 min. The supernatant containing liberated cells was collected and washed with PBS, and 1 × 10^6 cells/ml were plated in 75-cm² culture flasks containing Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium (Gibco, Grand Island, NY, USA) supplemented with penicillin/streptomycin sulfate, B-27 (Gibco), recombinant epidermal growth factor (EGF; 20 ng/mL; Chemicon), and recombinant human fibroblast growth factor-2 (FGF-2; 20 ng/mL; Chemicon). The cultures were subsequently incubated at 37°C in a humidified 5% CO₂ incubator, and fresh FGF-2 and EGF were added twice a week. Tumor spheres were separated by trituration through a fire-polished pipette and reseeded into the fresh culture medium. All brain TSCs were also cultured in the DMEM/F12 medium supplemented with 10% serum but without FGF-2 and EGF (differentiation medium) for multipotency analysis after 7–14 days.

Immunocytochemistry and FCM Analysis of B7-H1 in Tumor Cell Lines

For immunostaining, tumor cells were plated onto cover slips precoated with poly-l-lysine (Sigma, St. Louis, CA, USA) and cultured in neurobasal medium for 24 h. Cells were fixed with 4% paraformaldehyde and were then incubated in blocking buffer (2% horse serum, 0.2% Triton X-100, 0.1% BSA in PBS) for 1 hr at room temperature. Cover slips were incubated with primary anti-B7-H1 Ab (MIH1, 1:200; ebioscience) overnight at 4°C and then visualized with fluorescein isothiocyanate (FITC)-conjugated goat antimouse (1:250), (3) mouse monoclonal antihuman-specific nestin Ab (Chemicon; 1:1,000), (4) rabbit polyclonal anti-TUC-4 Ab (Chemicon; 1:200), (5) rabbit polyclonal antihuman CD133-biotin Ab (Miltenyi Biotec, Bergisch Gladbach, Germany; 1:50), (6) affinity-purified goat polyclonal antidoublecortin (anti-TOAD/Ulip/CRMP family-4) Ab (Zymed Laboratories; 1:200), (7) rabbit polyclonal anti-glial fibrillary acidic protein (anti-GFAP) Ab (Zymed Laboratories, San Francisco, CA, USA; 1:200), (8) mouse monoclonal anti-β III-tubulin Ab (Chemicon; 1:400), and (9) rabbit polyclonal antihuman Ki67 Ab (clone MIB-1; Zymed Laboratories; 1:50). Staining was visualized using appropriate secondary Abs, including FITC-conjugated goat antimouse, FITC-conjugated goat antirabbit, FITC-conjugated rabbit antigoat, rhodamine-conjugated goat antimouse, rhodamine-conjugated goat antirabbit, antibiotin-FITC, and rhodamine-conjugated rabbit antimouse antibiotin-FITC, and rhodamine-conjugated rabbit anti-TOAD/Ulip/CRMP family-4 (anti-TUC-4) Ab (ebioscience; 1:5). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI).

In order to explore whether the expression of B7-H1 is affected by the different culture medium, we performed surface and intracellular B7-H1 FCM analysis with cells cultured under stem cell medium or serum medium (differentiation medium: DMEM/F12 + 10% serum for 1–2 weeks). Briefly, 100-μl aliquots of 10^6 cells were placed in FCM tubes and incubated with phycoerythrin (PE)-Cy7-conjugated anti-B7-H1 Ab (ebioscience; 1:5) or isotype control Abs (ebioscience; 1:5) on ice for 30 min in the dark. Cells were washed twice with FCM buffer (3 ml PBS, 0.1% NaN₃, 5% fetal bovine serum) and resuspended in 0.5 ml 1% formalin/PBS solution. For intracellular B7-H1 staining, Fix & Perm cell permeabilization buffer (Caltag Laboratories, Invitrogen Corp., Carlsbad, CA, USA) was used according to the manufacturer’s protocol. Analysis was performed on a FACSCalibur system (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). We collected 10,000–30,000 cells and analyzed them with WinMDI software 2.8 (Scripps Research Institute, Flow Cytometry Core Facility, Kellogg School of Science and Technology, La Jolla, CA, USA). Forward and side scatter plots were used to exclude debris/dead cells from further analysis.

Expression of Neural Stem Cell Proteins and Ki67 in Brain Tumor Cells In Vitro

Tumor cells were dissociated and stained as described previously.21–24 Primary Abs included (1) mouse monoclonal antihuman CD133-biotin Ab (Miltenyi Biotec, Bergisch Gladbach, Germany; 1:50), (2) rabbit polyclonal anti-CD133 Ab (Abcam, Cambridge, MA, USA; 1:250), (3) mouse monoclonal antihuman-specific nestin Ab (Chemicon; 1:200), (4) rabbit polyclonal anti-TUC-4 Ab (Zymed Laboratories; 1:200), (5) rabbit polyclonal anti-Sox related HMG box gene 2 (anti-Sox2) Ab (Abcam; 1:100), (6) affinity-purified goat polyclonal antidoublecortin (anti-DCX) Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200), (7) rabbit polyclonal anti-glial fibrillary acidic protein (anti-GFAP) Ab (Zymed Laboratories, San Francisco, CA, USA; 1:200), (8) mouse monoclonal anti-β III-tubulin Ab (Chemicon; 1:400), and (9) rabbit polyclonal antihuman Ki67 Ab (clone MIB-1; Zymed Laboratories; 1:50). Staining was visualized using appropriate secondary Abs, including FITC-conjugated goat antimouse, FITC-conjugated goat antirabbit, FITC-conjugated rabbit antigoat, rhodamine-conjugated goat antimouse, rhodamine-conjugated goat antirabbit, antibiotin-FITC, and rhodamine-conjugated rabbit anti-

Table 1. Tumor and patient characteristics

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Patient Age (Years) and Sex</th>
<th>Diagnosis</th>
<th>Site of Tumor</th>
<th>Percent CD133⁺ Cells</th>
<th>Percent B7-H1⁺ Cells</th>
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<tr>
<td>BT1</td>
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<td>Anaplastic astrocytoma</td>
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<td>Medulloblastoma</td>
<td>Cerebellum</td>
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<td>50.40</td>
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<td>9, male</td>
<td>Medulloblastoma</td>
<td>Cerebellum</td>
<td>1.10</td>
<td>5.36</td>
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mouse Abs. DAPI (Chemicon) was used for cell nuclei counterstaining. Controls included omitting or preabsorbing primary Ab or no secondary Ab. Fluorescence signals were detected using a Nikon fluorescence microscope at excitation/emission wavelengths of 535/565 nm (rhodamine, red) and 470/505 (FITC, green). Results were recorded with a digital camera. Corresponding images were subsequently fused using Adobe Photoshop computer software version 8.0.1 (Adobe Systems, San Jose, CA, USA) to confirm colocalization of markers.

Double-Labeling of B7-H1 with Neuronal Marker Proteins and Ki67

Double-labeling of brain tumor cells to detect coexpression of B7-H1 antigen with early stem cell protein and Ki67 was performed as previously described,20 using the primary and secondary Abs listed above. FCM analysis was also performed to detect surface B7-H1 expression on Ki67+ tumor cells. Ki67 antigen was stained with intracellular label technique using Fix & Perm cell permeabilization reagent.

Interferon-γ Treatment

Lyophilized recombinant human interferon-γ (IFN-γ; PeproTech, Rocky Hill, NJ, USA) was reconstituted in PBS containing 1% BSA at a final concentration of 10 μg/ml, aliquoted, and stored at −20°C. IFN-γ was added to the culture media 2 days after tumor dissociation to achieve a final concentration of 100 ng/ml. Expression of B7-H1 on CD133+ and CD133− cells was evaluated 48 h later with FCM.

FCM Cell Sorting and In Vivo Tumor Model

Cells from tumors BT1 and BT7 were dissociated and resuspended in PBS containing 0.5% BSA. CD133+ cells were sorted with FCM (Beckman Coulter Epics ALTRA, Fullerton, CA, USA) using EXPO32-MultiCOMP software after labeled with CD133/1-phenylethanol (Miltenyi Biotech). Cell purity was >98% as confirmed by FCM (FACScalibur; Becton Dickinson) using CD133/1-PE (phycoerythrin) or isotype control Ab (PE-Mouse IgG1, ebioscience). To evaluate the tumorigenicity of brain TSCs, CD133+ or CD133− cells were injected subcutaneously into the right armpit (1 × 107 cells) or into the right striatum (1 × 105 cells) of SCID mice (n = 3 each). The tumor size in the right armpit was monitored by palpation and caliper measurement, and tumor volume was calculated as (length × width2)/2. Hematoxylin and eosin (H&E) staining and immunohistochemical staining using Abs against nestin, GFAP, and DCX were performed on the primary tumor sections and secondary xenografts 27 days after subcutaneous implantation or 30 days after intracranial injection. Proliferation index was defined as the number of Ki67+ cells divided by the total number of cells in the evaluated area of primary and secondary gliomas and was expressed as percentage. Secondary tumor cells from CD133+ or CD133− cell xenografts were also cultured for B7-H1 analysis.

Statistical Analysis

The Mann-Whitney test was used for assessing the differences in B7-H1 expression between high-grade gliomas and low-grade gliomas, the relationship between the number of CD8+ cells and B7-H1 expression (<20% or >20%), and differences in percentages of B7-H1+ cells between CD133+ and CD133− tumor cells. The difference in B7-H1 expression between the glioma’s edge and core, the level of B7-H1 under different culture conditions (stem cell medium or standard medium), B7-H1 expression in Ki67+ and Ki67− cells, and B7-H1 expression before and after IFN-γ treatment were evaluated by the Wilcoxon signed rank test. The difference of proliferation index between CD133+ and CD133− xenografts was assessed by t-test. A p-value < 0.05 was considered statistically significant. All statistical analyses were done using SPSS for Windows software (version 13.0; SPSS Inc., Chicago, IL, USA).

Results

B7-H1 Is Correlated with the Malignancy Grade of Human Astrocytic Tumors and Has a Negative Correlation with Tumor Infiltration CD8+ T-Cells

To explore the expression of B7-H1 in astrocytomas, we first performed B7-H1 immunohistochemistry on frozen sections. As illustrated in Fig. 1A, B7-H1 expression was confined to the cytoplasm and/or cell surface of tumor tissues. Interestingly, we found that tumor-infiltrating lymphocytes in GBM also expressed B7-H1. To further examine whether B7-H1 might be a candidate tissue biomarker for gliomas, we compared the levels of B7-H1 protein expression in high-grade gliomas (n = 24) with low-grade gliomas (n = 24) and the expression level of this protein in the tumor’s core versus edge. Western blot showed that the level of B7-H1 was significantly higher in high-grade than in low-grade gliomas (Fig. 1B, middle; p < 0.001, Mann-Whitney U-test). B7-H1 protein was not detected in 12 samples of low-grade gliomas, and the expression level was similar to that of the normal autopsy tissues (data not shown). We also observed increased expression of B7-H1 in the tumor edge compared to the tumor core (Fig. 1B, left; p < 0.001, Wilcoxon signed rank test).

We assessed the relationship between low (<20%) versus high (>20%) B7-H1 expression and CD8+ T-cells in all clinical specimens. The mean number of CD8+ cells in the low B7-H1 expression group was 87 ± 28 (n = 27), versus 48 ± 26 (n = 21) in the high B7-H1 expression group (Fig. 1B, right; p = 0.005, Mann-Whitney U-test), indicating a negative correlation between B7-H1 expression in brain tumors and the number of tumor-infiltrating CD8+ CTLs (Fig. 1C).

B7-H1 Is Not Expressed Exclusively on TSCs

Eight human astrocytoma and medulloblastoma cell lines were cultured in media favoring the growth of stem cells in order to identify the expression of B7-H1
Brain TSCs. Immunocytochemistry and FCM analysis indicated that all eight brain tumor cell lines expressed B7-H1 only on the cell surface (Table 1, Fig. 2). Tumor cells expressed higher levels of surface B7-H1 under serum medium (DMEM + 10% serum) than under stem cell medium (Fig. 2B; p < 0.05).

The tumor spheres displayed nonadherent forms when cultured in the proliferation medium. Immunocytochemical analysis showed the tumor spheres contained TUC-4+, Sox2+, nestin+, and CD133+ cells (data not shown). However, tumor spheres became adherent and showed differentiated morphology when cultured in the differentiation medium. A sphere derived from a single CD133+ cell was dissociated, and cells were able to differentiate into three neural lineages. FCM analysis using anti-CD133 Ab showed that 0.92%–9.85% of...
tumor cells were CD133\(^+\) cells (Table 1). Sorted CD133\(^+\) cells were confirmed by immunocytochemistry (data not shown). CD133 protein was predominantly located in the membrane of these cells. Double immunostaining indicated that CD133\(^+\) cells consisted of both Ki67\(^+\) and Ki67\(^-\) cells. In addition, sorted CD133\(^+\) cells also contained nestin\(^+/\)Ki67\(^+\) cells and nestin\(^+/\)Ki67\(^-\) cells, suggesting that some TSCs were proliferative and others were quiescent. CD133\(^-\) cells also expressed nestin, TUC-4, and DCX (data not shown), suggesting that CD133\(^-\) cells may contain some tumor progenitor cells.

Next, we examined whether brain TSCs could express B7-H1. We observed B7-H1\(^+\) cells could express stem cell/progenitor cell markers such as nestin, CD133, TUC-4, and DCX (Fig. 3A). We then examined whether tumor cell expression of B7-H1 might be associated with cell proliferation. Double immunolabeling was performed using Abs against Ki67 antigen, which bound to the nuclear proteins in the G1, S, G2, and M phases of the cell cycle,\(^{26}\) together with Ab against B7-H1. As shown in Fig. 3B, most B7-H1 was expressed by Ki67\(^+\) tumor cells in vitro. This finding was further confirmed by FCM analysis, and Wilcoxon signed rank test showed that Ki67\(^+\) cells expressed a higher level of B7-H1 protein (Fig. 3B; \(p < 0.05\)). Taken together, these data indicate that brain TSCs derived from gliomas and medulloblastomas are either dividing or nondividing, and that the negative costimulatory molecule B7-H1 can be expressed in a subpopulation of brain TSCs (mostly nondividing cells).

To investigate whether TSCs preferentially express B7-H1, we double-labeled CD133 and B7-H1 antigens both in the resting state and after exposure to IFN-\(\gamma\) treatment. IFN-\(\gamma\) treatment significantly upregulated B7-H1 expression in brain tumor cells (either CD133\(^+\) or CD133\(^-\)) cells 48 h later (Fig. 4A,B); FCM analysis showed that an average of 42% of CD133\(^+\) cells and 30% of CD133\(^-\) cells expressed B7-H1 constitutively (Fig. 4C). Although CD133\(^+\) cells tended to express higher levels of B7-H1 than did CD133\(^-\) cells, this difference was not significant between CD133\(^+\) and CD133\(^-\) tumor cells with or without IFN-\(\gamma\) treatment (\(p > 0.05\)).

**Expression of B7-H1 in Secondary Glioma from CD133\(^+\) and CD133\(^-\) Xenografts**

To evaluate the tumorigenicity of CD133\(^+\) and CD133\(^-\) cells and to determine whether secondary tumor cells could also express B7-H1, CD133\(^+\) or CD133\(^-\) cells...
isolated from a patient with left temporal GBM (tumor BT1; Fig. 5A) were implanted into the right armpit or into the right striatum of SCID mice. After around 4 weeks, a large glioblastoma-like subcutaneous mass was detected in the SCID mice implanted in the armpit with either CD133$^+$ or CD133$^-$ cells (Fig. 5B), suggesting that both CD133$^+$ and CD133$^-$ cells were tumorigenic. However, CD133$^+$ cells produced larger tumors than did CD133$^-$ cells; the average volumes of tumor initiated by CD133$^+$ and CD133$^-$ cells were 10.48 cm$^3$ and 2.25 cm$^3$ in 27 days, respectively. CD133$^+$ or CD133$^-$ cells could also initiate gliomas after 30 days when implanted into the right striatum. However, the former had enhanced tumor growth and vascularity (Fig. 5C). CD133$^+$ cell-induced GBMs had a higher proliferation index compared with CD133$^-$ xenografts ($p < 0.05$). H&E staining indicated that the pathological features of CD133$^+$ and CD133$^-$ xenografts resembled the primary tumor. As shown in Fig. 5B, recultured CD133$^+$ and CD133$^-$ xenografts showed similar B7-H1 expression (24.62% vs. 25.65%).

Discussion

In the present study, we first investigated B7-H1 expression in vivo in brain tumor biopsies, including high- and low-grade gliomas. We observed B7-H1 expression was restricted to the cytoplasm and/or cell surface. Statistical
analysis showed that the level of B7-H1 correlated with the malignancy grade of human astrocytomas. Our findings from Western blotting is consistent with the results from Winterle et al. and Wilmotte et al., who used immunohistochemistry to confirm that B7-H1 was a good tissue biomarker for gliomas. In addition, we found some novel properties of B7-H1 in glioma that might relate to its invasive nature. First, tumor-infiltrating lymphocytes in malignant glioma also expressed B7-H1 (Fig. 1A). These B7-H1+ lymphocytes might further deliver inhibitory signals to attenuate host immune response through T:T interactions, either in the microenvironment of the tumor or after recycling back to the circulation. Second, the level of B7-H1 expression was significantly higher at the growing edge of the tumor than in the tumor core (Fig. 1B). Upregulation of B7-H1 at the edge of the tumor might contribute to the high malignancy and the escape from immune surveillance by (1) aiding in inhibiting CTL activity in malignant gliomas (“molecular shield”) during invasion into adjacent brain tissue, and (2) inducing surrounding CTL apoptosis (“kiss of death”) so that they either cannot enter into the core of the tumor or have impaired function. These findings, in addition to the fact that major histocompatibility complex molecule expression is downregulated in the invading edge of the gliomas, might partially explain the mechanisms of immunosuppression and the highly invasive nature of gliomas.

Fig. 5. CD133+ and CD133− glioma xenografts (BT1) both showed histological features of primary glioblastoma and expressed similar levels of B7-H1 protein, but CD133+ cells had more tumor growth capability and a higher proliferation index. (A) Sagittal contrast-enhanced MR image revealed temporal space-occupying tumor with heterogeneous enhancement. Hematoxylin and eosin (H&E) staining showed histological features of glioblastoma multiforme (GBM). Gial fibrillary acidic protein (GFAP), Ki67, doublecortin (DCX), and nestin were expressed by the primary tumor. (B) Tumors formed in severe combined immunodeficiency mice after subcutaneously inoculation of 1 × 10^6 CD133+ or CD133− cells in 27 days (left three panels). The adjacent pleura and lung tissue were infiltrated with CD133+ cells (arrow). CD133+ cells had more tumor growth capability based on the secondary tumor size. B7-H1 was expressed at the similar degree in secondary glioma cells (right). Red histogram, B7-H1 expression in CD133+ xenograft cells (25.65%); green histogram, B7-H1 expression in CD133+ xenograft cells (24.62%); black histogram, isotype control. (C) CD133− or CD133+ cells could also initiate gliomas in 30 days when implanted into the right striatum. However, the latter had enhanced tumor growth and vascularity (left). CD133+ cell–induced glioblastoma had a higher proliferation index compared to the CD133− xenografts (middle and right, p < 0.05).
All the examined tumor cells could constitutively express surface, but not intracellular, B7-H1 when they were cultured in vitro under medium conditions favoring the growth of neural stem cells (Table 1, Fig. 2). Given the cytoplasmic and/or membranous staining pattern of B7-H1 in vivo (Fig. 1A), we hypothesized that complex tumor microenvironmental signals that transferred among tumor cells, antigen-presenting cells, and T-cells in vivo induced B7-H1 membranous expression. Surprisingly, the same types of cells had different proportions of B7-H1 expression when grown in stem cell medium containing growth factors such as EGF compared with non-stem-cell culture medium (p < 0.05). At least two possible mechanisms could explain this discrepancy. First, mitogenic EGF and FGF might decrease B7-H1 expression, which reflects the possibility that B7-H1 might be differentiatated protein. Second, serum might increase expression of B7-H1 in vivo (Fig. 1A), we hypothesized that complex tumor microenvironmental signals that transferred among tumor cells, antigen-presenting cells, and T-cells in vivo induced B7-H1 membranous expression. Surprisingly, the same types of cells had different proportions of B7-H1 expression when grown in stem cell medium containing growth factors such as EGF compared with non-stem-cell culture medium (p < 0.05). At least two possible mechanisms could explain this discrepancy. First, mitogenic EGF and FGF might decrease B7-H1 expression, which reflects the possibility that B7-H1 might be differentiatated protein. Second, serum might increase expression of B7-H1.

We analyzed the expression of neuronal stem cell markers and multipotency of our candidate TSCs and found they were immunoreactive to TUC-4, Sox2, nestin, and CD133. Similar to neural stem cells, all of the TSCs differentiated into neural lineages when cultured under differentiation conditions (data not shown). These findings are consistent with previous studies2–4 and confirmed the existence of TSCs in our samples. Ki67 antigen, which binds to nuclear proteins in the G1, S, G2, and M phases of the cell cycle, represents cell proliferative status. Ki67 tumor cells, which represent the G0 phase cells, have been proven to be less vulnerable to injury by radiation and/or chemotherapy.5–8 In our study, we noted that TSCs were not all or not always dividing (data not shown); Ki67 TSCs formed a significant proportion of the population. This subset of TSCs might be unresponsive to therapy because they represent tumor stem cells within the resting G0 phase of the cell cycle. We observed that the majority of B7-H1 was expressed on these resting cells (Fig. 3B). This might facilitate B7-H1 tumor cells to survive after multimodality antitumor therapy, such that they were able to deliver an inhibitory signal to T-cells and revive. This finding might support the fact that downregulation of B7-H1 expression was found in the proliferative medium induced by EGF (Fig. 2).

More data from double immunolabeling support our hypothesis that TSCs could express B7-H1 (Fig. 3A). IFN-γ treatment was able to significantly upregulate B7-H1 expression on brain tumor cells (either CD133+ or CD133 cells; Fig. 4), which has already been reported in other murine and human tumors.9–11 However, not all TSCs expressed B7-H1. FCM analysis for B7-H1 and CD133 expression showed an average of 42% of CD133+ cells and 30% of CD133 cells expressed this molecule (Fig. 4). Although CD133+ cells tended to express higher levels of B7-H1 than CD133+ cells, this difference was not significant with or without IFN-γ treatment. This might indicate that TSCs are not “better” at utilizing the B7-H1 pathway to escape immune surveillance. However, we could not exclude the possibility that the B7-H1 expression levels on TSCs might be underestimated due to the existence of CD133– TSCs.55 Furthermore, in this study we analyzed the data from only four GBMs: two anaplastic astrocytomas and two medulloblastomas, which may result in bias.

Previous studies have revealed that as few as 100 CD133+ cells are sufficient for the formation of human brain tumors in SCID mice;21 therefore, in this study we did not test how few cells are needed to drive tumors, but rather evaluated the tumorigenicity of CD133+ and CD133 tumor cells by subcutaneous (106) and intracranial (105) injection into nude mice. We found that CD133+ glioma cells could also form large lesions (Fig. 5B). We then examined whether CD133+ tumor cells contained special subpopulations that behaved like TSCs. Because no surface marker is available to differentiate CD133+ TSCs from CD133+ non-stem cells, experiments similar to those done with the purified CD133– TSC subset were not feasible. A recent report by Beier et al.35 showed that CD133+ tumor cells had tumorigenicity when 106 or 105 tumor cells were implanted into the brains of SCID mouse. In addition, our findings showed that CD133+ tumor cells express neural stem/progenitor markers. Taken together, CD133+ tumor cells might contain a small subpopulation of brain tumor progenitor cells that retain the capacity of proliferation and, therefore, initiate tumor formation in mice. Beier et al.35 also found that about 0.5%–2% of CD133+ cells were able to proliferate and form new adherent spheres. The cells in these spheres had multilineage differentiation capacity, which they referred to as CD133+ TSCs. Although it is not clear how many CD133+ TSCs were required to induce tumor formation in vivo, in this study tissue sections of CD133+ and CD133+ xenograft resembled original tumor histology (Fig. 5C). Further FCM analysis of recultured CD133+ and CD133+ xenografts showed they both expressed similar levels of B7-H1 (24.62% vs. 25.65%; Fig. 5C).

These findings have some important clinical implications. First, B7-H1 is a novel and good tissue biomarker for brain malignancies. Aggressive resection of the “normal” edge of gliomas needs to be considered whenever possible. Second, TSCs are able to express B7-H1, but not exclusively so. This is of paramount importance for understanding how these tumors form, expand, and escape from immune surveillance and may indicate key cellular and molecular mechanisms to be investigated for diagnostic and therapeutic purposes. Third, future studies are required to determine the significance of these negative costimulatory molecules in other cancer cell types to broaden the significance of the data reported here.

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References