Targeting Cancer Stem Cells through L1CAM Suppresses Glioma Growth

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Abstract

Malignant gliomas are lethal cancers that display striking cellular heterogeneity. A highly tumorigenic glioma tumor subpopulation, termed cancer stem cells or tumor-initiating cells, promotes therapeutic resistance and tumor angiogenesis. Therefore, targeting cancer stem cells may improve patient survival. We interrogated the role of a neuronal cell adhesion molecule, L1CAM, in glioma stem cells as L1CAM regulates brain development and is expressed in gliomas. L1CAM+ and CD133+ cells cosegregated in gliomas, and levels of L1CAM were higher in CD133+ glioma cells than normal neural progenitors. Targeting L1CAM using lentiviral-mediated short hairpin RNA (shRNA) interference in CD133+ glioma cells potently disrupted neurosphere formation, induced apoptosis, and inhibited growth specifically in glioma stem cells. We identified a novel mechanism for L1CAM regulation in vivo, we targeted L1CAM in glioma cells before injection into immunocompromised mice or directly in established tumors. In each glioma xenograft model, shRNA targeting of L1CAM expression in vivo suppressed tumor growth and increased the survival of tumor-bearing animals. Together, these data show that L1CAM is required for maintaining the growth and survival of CD133+ glioma cells both in vitro and in vivo, and L1CAM may represent a cancer stem cell–specific therapeutic target for improving the treatment of malignant gliomas and other brain tumors. [Cancer Res 2008;68(15):6043–8]

Introduction

The cancer stem cell hypothesis posits that brain tumors contain a subset of neoplastic cells that propagate and maintain tumors through sustained self-renewal and potent tumorigenicity (1–6). Cancer stem cells can be enriched from many human brain tumor biopsy specimens using the cell surface marker CD133 (prominin1) although there are tumors that may contain CD133+ tumor-initiating cells (6, 7), albeit often with lower tumor potency. We previously showed that CD133+ glioma cells derived from human tumors are radioresistant (5), promote angiogenesis (6), and display greater tumorigenic potential in immunocompromised mice relative to CD133− glioma cells (5). Thus, targeting CD133+ glioma cell survival and protumorigenic behaviors by identifying novel molecular targets specific in cancer stem cells may improve patient outcome.

Molecular targets that are secreted or located on the cell surface are particularly enticing as antibody and small-molecule inhibitor therapies that do not have to cross the cell membrane and can be developed for clinical use. To select a potential target, we consulted the literature to select a cell surface protein that has been reported to be expressed in malignant gliomas and contribute to tumor malignancy. Based on these criteria, the neural cell adhesion molecule, L1CAM (L1, CD171), has been identified as a potential therapeutic target in neuro-oncology. L1CAM regulates neural cell growth, survival, migration, and axonal outgrowth and neurite extension during central nervous system development (8). Although the role of L1CAM in the normal adult nervous system is not well defined, L1CAM is overexpressed in gliomas and other solid cancers (9–15), including colorectal cancer where L1CAM is a prognostic indicator (15). Although L1CAM has many potential biological effects that can contribute to tumor formation and maintenance, recent studies suggest that overexpression of L1CAM in cancer can protect cells from apoptosis induced by nutrient deprivation (16) or chemotherapeutics (17). As glioma stem cells display a chemoresistant phenotype, we sought to determine if L1CAM mediates survival and tumorigenic potential of CD133+ glioma cell subpopulation.

Materials and Methods

Cell isolation and culture. Matched glioma cell populations enriched or depleted in cancer stem cells were isolated and cultured as previously described (5, 6) from human glioma surgical specimens (designated Txxx, Supplementary Table S1) or from the D456MG pediatric glioblastoma xenograft. Briefly, tumors were immediately dissected with removal of gross necrosis; washed in Earle's balanced salt solution; subjected to a papain digestion followed by tituration, filtering, and lysis of RBC in PBS/water (1:3) solution; and then cultured overnight (–12 h) in neurobasal medium (with B27 and epidermal growth factor and basic fibroblast growth factor at 20 ng/mL) for recovery of cellular surface antigens before cell sorting. Primary glioma tumor samples were obtained from patients undergoing resection in accordance with a protocol approved by the Duke University Medical Center Institutional Review Board. Normal human fetal neural progenitor cells were purchased from Lonza. To control for differences in medium conditions, all cells were cultured in stem cell medium for experiments.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Fluorescence-activated cell sorting analysis. Because papain digestion may cause loss of some cellular surface antigens such as CD133, it is critical to allow the isolated total tumor cells to reexpress their surface antigens in the neurobasal medium overnight (12 h) before sorting for CD133+ glioma cells. Tumor cell cultures were subjected to fluorescence-activated cell sorting (FACS) analysis and cell sorting after the antigen recovery period. Human-specific anti-CD133 (293C3) conjugated to allophycocyanin (APC) or anti-CD133 (293C3) conjugated to phycoerythrin (PE; Miltenyi) was used for cell sorting and FACS analysis as previously described (5, 6). Anti-LICAM-PE used for the FACS was generated using the Lightning-Link PE kit (Innova Biosciences) in combination with a GeneTex monoclonal antibody L1CAM-PE used for FACS analysis was generated using the Lightning-Link PE kit (Innova Biosciences). Anti-Olig2 goat IgG was obtained from R&D Systems. Anti-OCT4 monoclonal antibody (MAB-H01) was purchased from Millipore. Anti-p21 and anti-p27 antibodies were obtained from Cell Signaling. Immunoblotting was done as previously described (5).

In vivo tumor formation assays. Intracranial or s.c. transplantation of glioma stem cells into nude mice was done as described (5, 6). Glioma stem cells were infected with lentivirus expressing nontargeting shRNA or LICAM-targeting shRNA for 24 h, and then 10^3 cells per mouse of viable brain tumor stem cells transduced with control lentivirus or the LICAM-targeting lentivirus were transplanted into athymic Balb/c nu/nu mice through intracranial or s.c. injection. To establish glioma xenografts for lentiviral-mediated LICAM shRNA treatment, glioma stem cells were injected into the athymic nude mice (10^3 cells per mouse, 10 mice per group) first, and the same number of nontargeting lentivirus or LICAM-targeting lentiviruses were delivered to tumor sites through intratumoral injection once every 2 d.

Neurosphere formation efficiency. To determine the effect of knockdown of LICAM on the ability of glioma stem cells to form neurospheres, disaggregated cells were infected with nontargeting or LICAM-targeting lentivirus for 48 h and then plated into 96-well plates at a density of 1 cell per well via a flow cytometry cell sorter (FACS Aria, BD Bioscience) or through serial dilution. The percentage of wells with neurosphere formation was determined on day 10 as shown.

Immunofluorescent staining. CD133+ and CD133− glioma cells were fixed with 4% paraformaldehyde, washed with TBS, incubated with anti-LICAM monoclonal antibody (UJ127, GeneTex), and FITC-conjugated donkey anti-mouse IgG secondary antibody. Cells were costained with L1CAM monoclonal antibody (UJ127, GeneTex), and FITC-conjugated donkey anti-mouse IgG secondary antibody. Cells were contained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted with the anti-fade medium. Stained cells were examined under a fluorescent microscope (Zeiss Axiovert 200).

**Figure 1.** L1CAM is highly expressed on the surface of CD133+ glioma cells. A, flow cytometry showed that most CD133+ glioma cells isolated from surgical biopsy specimens were also L1CAM+. Total population fractions of CD133+ cells and L1CAM+ cells were very similar. Unsorted tumor cells from primary brain tumor samples were labeled with anti–CD133-APC and anti–L1CAM-PE, and then subjected to FACS analysis to determine CD133+ and L1CAM+ subpopulations. The total fractions of CD133+ cells and L1CAM+ cells in unsorted tumor cells were very similar in each case and there was significant overlap between the populations. B, immunofluorescent staining with L1CAM antibody showed that L1CAM was overexpressed on the cell surface of CD133+ glioma stem cells compared with matched CD133− cells. C, CD133+ cells from glioma surgical biopsy specimens including the anaplastic astrocytoma T3656 and the glioblastomas T3691, T3750, and T3359 expressed L1CAM protein at higher levels than matched CD133− cells by Western blot analysis. D, CD133+ glioma cells from the glioblastoma patient tumor specimens T3359, T3691, and T3359 expressed L1CAM protein at higher levels than matched CD133− normal neural progenitor cells (designated by lot number).
CD133+ brain tumor cells derived from the primary anaplastic astrocytoma T3565 and glioblastoma T3691 patient specimens was done using L1CAM lentiviral shRNA (L1) without effects by nontargeting control shRNA (NT) as determined by Western blotting. B, targeting L1CAM expression disrupted neurosphere formation of CD133+ brain tumor cells derived from a T3691 glioblastoma patient specimen or a D456MG pediatric glioblastoma xenograft. CD133+ cells infected with lentivirus expressing nontargeting shRNA formed neurospheres, whereas infection with lentivirus expressing L1CAM shRNA attenuated neurosphere formation. C, representative images of CD133+ and CD133− brain tumor cells isolated from the primary glioblastoma T3691 and normal neural progenitors stained with Annexin V–FITC (green) and DAPI (blue) after treatment with lentivirus expressing nontargeting shRNA or L1CAM shRNA. D, FACS analysis of apoptosis with Annexin V–FITC staining in CD133+ and CD133− brain tumor cells isolated from the primary glioblastoma T3691 and normal neurosphere stem cells confirmed that CD133+ brain tumor cells have a greater dependence on L1CAM for cell survival. *, P < 0.01; **, P < 0.001 with comparison to nontargeting shRNA.

**Annexin V–FITC staining.** Annexin V–FITC staining for detecting apoptosis in CD133+ and CD133− glioma cells and normal neural progenitor cells before and after L1CAM targeting for 48 h was done with the Annexin V–FITC Apoptosis Detection Kit (BD PharMingen) according to the manufacturer's instructions. The cells stained with Annexin V–FITC were analyzed by FACs, or fixed and stained with DAPI, and then examined under a fluorescent microscope (Zeiss Axiovert 200).

**Real-time PCR.** To examine the transcriptional regulation of L1CAM on Olig2 and p21WAF1/CIP1 in CD133+ cells, CD133+ glioma cells were treated with nontargeting lentivirus or L1CAM-targeting lentivirus for 48 h, and RNA samples were isolated from these cells using RNAeasy Kit (Qiagen), and then used for real-time PCR analysis with pairs of specific primers for Olig2, p21WAF1/CIP1, L1CAM, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; internal PCR control).

**Statistical analyses.** Descriptive statistics and significance were determined as described (5, 6).

**Results and Discussion.**

**L1CAM is differentially overexpressed in CD133+ glioma cells.** We examined L1CAM expression in CD133+ glioma cells derived from 12 human primary tumors (Supplementary Table S1) and one xenograft through complementary techniques. Using flow cytometry, we determined that the majority of CD133+ cells were doubly positive for L1CAM when isolated directly from human patient specimens or passaged in short-term cell culture (Fig. 1A; Supplementary Fig. S1A). In striking contrast, the vast majority of CD133− cells were L1CAM negative (>99% in T3565, T4121, and T4577; Fig. 1A; Supplementary Fig. S1B). In addition, the total percentage of CD133+ and L1CAM+ cells in patient glioma specimens were very similar (Fig. 1A; Supplementary Fig. S1B). L1CAM expression on the surface of CD133− cells, but not CD133+ cells, was further verified using immunofluorescence (Fig. 1B), and Western analysis confirmed that L1CAM protein was expressed at levels 18- to 42-fold higher in CD133+ cells from glioma patient specimens relative to matched CD133− tumor cells (Fig. 1C). In addition, CD133+ glioblastoma cells expressed L1CAM at significantly higher levels than normal neural progenitor cells derived from human fetal tissue that were enriched for CD133+ cells (Fig. 1D). Together, these data suggest that L1CAM is a surface glycoprotein specifically expressed by glioma stem cells.

**Targeting L1CAM decreases growth and survival of CD133+ glioma cells.** To assess the functional significance of the relative overexpression of L1CAM in CD133+ glioma cells compared with CD133− glioma cells and normal neural progenitors, we targeted L1CAM expression using lentivirus expressing shRNA directed against L1CAM, causing a 90% reduction of L1CAM expression relative to nontargeting control shRNA (Fig. 2A). As neurosphere formation is a key behavior of neural stem cells and brain tumor stem cells (1–6, 18) and is used as a measure of stem cell capacity for self-renewal, we determined neurosphere formation competence in cells with knockdown of L1CAM. Targeting L1CAM expression markedly decreased the ability of CD133+ glioblastoma cells to form neurospheres as indicated by the reduction in neurosphere formation efficiency (Fig. 2B; Supplementary Fig. S2A and B) and the size of the neurospheres formed (Fig. 2B). In addition, L1CAM knockdown resulted in significant growth inhibition in CD133+ glioblastoma cells, only modest attenuation in normal neural progenitors, and no effect on CD133− glioma cells (Supplementary Fig. S3). To determine if the reduction in neurosphere formation and growth of CD133+ glioma cells with L1CAM knockdown was due to decreased cell survival, we determined the percentage of apoptotic cells using Annexin V staining. Similar to the effects of L1CAM-directed shRNA on cell growth, L1CAM shRNA significantly increased the percentage of Annexin V−positive cells in CD133+ glioblastoma cells when compared with the effects of a nontargeting shRNA control (Fig. 2C; Supplementary Fig. S2C). In contrast, L1CAM knockdown had essentially no
significant effect on apoptosis in CD133+ glioblastoma cells (Fig. 2C; Supplementary Fig. S2C) and a moderate induction of apoptosis in normal neural progenitor cells (Fig. 2C and D), which was significantly less than that induced in CD133+ glioma cells. These data support a significant role for LICAM in maintaining the growth of CD133+ glioblastoma cells and suggest that targeting LICAM decreases CD133+ glioma self-renewal due to decreased survival.

Reduction of LICAM protein induces down-regulation of Olig2 and up-regulation of p21<sub>WAF1/CIP1</sub>. To delineate the molecular mechanisms through which LICAM regulates CD133+ glioma cell survival, we interrogated intracellular signaling pathways in LICAM knockdown cells with a focus on regulators of neural stem cell maintenance. As the neural stem cell transcription factor Olig2 was recently found to regulate neurosphere formation in vitro and glioma formation in vivo (19), we determined Olig2 expression in CD133+ glioma cells infected with lentivirus expressing no shRNA as a vector control, nontargeting control shRNA, or LICAM shRNA. LICAM knockdown reduced Olig2 protein expression in CD133+ glioma cells derived from primary glioma surgical specimens and a glioblastoma xenograft (Fig. 3A-i, B). The suppression of Olig2 induced by LICAM knockdown is expected to have specific effects on CD133+ glioblastoma cells, as minimal Olig2 was detected in matched CD133+ glioma cells (Fig. 3A-ii). In contrast, LICAM reduction did not alter the protein levels of other stem cell transcription factors such as Oct4 in CD133+ glioma cells (data not shown). As Olig2 may mediate its growth effects by suppressing expression of the cyclin-dependent kinase inhibitor p21<sub>WAF1/CIP1</sub> (19), we further determined the effect of LICAM shRNA on p21<sub>WAF1/CIP1</sub> expression. Knockdown of LICAM in CD133+ glioblastoma cells led to a specific up-regulation of p21<sub>WAF1/CIP1</sub>, but not p27<sub>KIP1</sub>, protein (Fig. 3B). Real-time PCR further confirmed that LICAM knockdown was associated with Olig2 down-regulation and p21<sub>WAF1/CIP1</sub> up-regulation at the RNA level (Fig. 3C). These data suggest that the poor growth and survival of CD133+ glioma cells with LICAM knockdown results, at least in part, from the loss of Olig2 expression and resulting increase in p21<sub>WAF1/CIP1</sub>. This model was further supported by data demonstrating that Olig2 overexpression rescued CD133+ glioblastoma cells from LICAM shRNA-induced reductions in cell growth (Fig. 3D). Thus, our data provide the first evidence that LICAM can mediate cancer stem cell self-renewal and survival by regulating Olig2 expression with associated changes in the downstream effector, p21<sub>WAF1/CIP1</sub>.

Targeting LICAM in CD133+ glioma cells suppresses glioma growth in vivo and increases survival of mice bearing glioma xenografts. As LICAM shRNA-mediated decreases in CD133+ glioma cell growth were associated with reduced Olig2 expression, we infected CD133+ glioma cells isolated from the glioblastoma patient specimen T3982 with lentiviral-mediated shRNA for 48 h. L1CAM knockdown in CD133+ cells isolated from the glioblastoma patient specimen T3982 dramatically and specifically down-regulated Olig2 expression with associated changes in the downstream effector, p21<sub>WAF1/CIP1</sub>. These data suggest that the poor growth and survival of CD133+ glioma cells with LICAM knockdown results, at least in part, from the loss of Olig2 expression and resulting increase in p21<sub>WAF1/CIP1</sub>. This model was further supported by data demonstrating that Olig2 overexpression rescued CD133+ glioblastoma cells from LICAM shRNA-induced reductions in cell growth (Fig. 3D). Thus, our data provide the first evidence that LICAM can mediate cancer stem cell self-renewal and survival by regulating Olig2 expression with associated changes in the downstream effector, p21<sub>WAF1/CIP1</sub>.
and Olig2 is critical for the growth of gliomas in vivo (19), we next sought to determine if targeting L1CAM expression could attenuate the tumorigenic potential of CD133+ glioma cells. To accomplish this goal, L1CAM expression was modulated in CD133+ glioma cells before implantation into immunocompromised mice (Fig. 4A and B; Supplementary Fig. S4). Knockdown of L1CAM in CD133+ cells derived from glioma surgical biopsy specimens before intracranial injection increased survival. *, P < 0.002 with comparison to nontargeting control. CD133+ cells isolated from brain tumor patient specimens and infected with nontargeting shRNA or L1CAM shRNA before injection were implanted into the brains of immunocompromised mice as in A. C, representative images of coronal sections of brains of mice implanted with CD133+ brain tumor cells isolated from the T3837 glioblastoma patient specimen and then infected with lentivirus expressing nontargeting shRNA or L1CAM shRNA. Identical numbers of CD133+ brain tumor cells (10^6 per mouse) were implanted into mouse brains to establish intracranial tumors. After 5 d to allow tumor engraftment, lentivirus expressing nontargeting shRNA or shRNA directed against L1CAM were delivered to the tumor site through direct injection (once every other day for 12 d). D, targeting L1CAM in vivo through lentiviral-mediated shRNA suppressed intracranial tumor growth of the established brain tumor xenograft and significantly increased the survival of mice bearing intracranial glioma xenografts (*, P < 0.004).

Figure 4. Lentiviral shRNA targeting of L1CAM suppresses tumor growth and increased survival of mice bearing intracranial brain tumor xenografts. A, representative images of brains of immunocompromised mice implanted with CD133+ cells isolated from the glioblastoma specimen T3691 and infected with nontargeting shRNA or L1CAM shRNA before intracranial implantation. Forty-eight hours after infection, identical numbers of viable cells (10^5 per mouse) were implanted and brains examined on day 17 after injection. Gross images and coronal sections from representative brains bearing glioma xenografts are displayed. B, knockdown of L1CAM in CD133+ cells isolated from the glioblastoma patient specimen T3691 before intracranial implantation increased survival. *, P < 0.002 with comparison to nontargeting control. CD133+ cells isolated from brain tumor patient specimens and infected with nontargeting shRNA or L1CAM shRNA before injection were implanted into the brains of immunocompromised mice as in A. C, representative images of coronal sections of brains of mice implanted with CD133+ brain tumor cells isolated from the T3837 glioblastoma patient specimen and then infected with lentivirus expressing nontargeting shRNA or L1CAM shRNA. Identical numbers of CD133+ brain tumor cells (10^6 per mouse) were implanted into mouse brains to establish intracranial tumors. After 5 d to allow tumor engraftment, lentivirus expressing nontargeting shRNA or shRNA directed against L1CAM were delivered to the tumor site through direct injection (once every other day for 12 d). D, targeting L1CAM in vivo through lentiviral-mediated shRNA suppressed intracranial tumor growth of the established brain tumor xenograft and significantly increased the survival of mice bearing intracranial glioma xenografts (*, P < 0.004).
tumorigenic subpopulations for novel therapeutic targets. We have discovered a novel molecular target that had not been linked to CD133⁺ glioma cells, the cell adhesion molecule L1CAM. The dramatic overexpression of L1CAM in CD133⁺ brain tumor cells, the increased survival of mice bearing gliomas in which L1CAM has been targeted, and the decreased percentage of CD133⁺ cells in tumors treated with lentivirus expressing L1CAM shRNA all suggest that targeting L1CAM may be useful as a cancer stem cell-directed therapy. As there is a very small percentage of (<1%) L1CAM⁺ cells that target L1CAM may be useful as a cancer stem cell–directed therapy. Studies will be needed to determine if L1CAM itself may be useful as a cancer stem cell marker in studies will be needed to determine if L1CAM itself may be useful as a cancer stem cell marker in vitro and immunohistochemical studies. Regardless of whether L1CAM can be used to prospectively identify cancer stem cells, our studies provide evidence that L1CAM should be considered for further exploitation in therapeutic development and biological investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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