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Elevated Invasive Potential of Glioblastoma Stem Cells

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Abstract

Glioblastomas (GBMs) are the most lethal and common types of primary brain tumors. The hallmark of GBMs is their highly infiltrative nature. The cellular and molecular mechanisms underlying the aggressive cancer invasion in GBMs are poorly understood. GBM displays remarkable cellular heterogeneity and hierarchy containing self-renewing glioblastoma stem cells (GSCs). Whether GSCs are more invasive than non-stem tumor cells and contribute to the invasive phenotype in GBMs has not been determined. Here we provide experimental evidence supporting that GSCs derived from GBM surgical specimens or xenografts display greater invasive potential *in vitro* and *in vivo* than matched non-stem tumor cells. Furthermore, we identified several invasion-associated proteins that were differentially expressed in GSCs relative to non-stem tumor cells. One of such proteins is L1CAM, a cell surface molecule shown to be critical to maintain GSC tumorigenic potential in our previous study. Immunohistochemical staining showed that L1CAM is highly expressed in a population of cancer cells in the invasive fronts of primary GBMs. Collectively, these data demonstrate the invasive nature of GSCs, suggesting that disrupting GSCs through a specific target such as L1CAM may reduce GBM cancer invasion and tumor recurrence.

Keywords

Glioblastoma; cancer stem cell; invasion; L1CAM

1. Introduction

Glioblastoma multiformes (GBMs) are among the most aggressive and least successfully treated solid tumors [1,2]. More than 14,000 new cases of GBM occur in the United States every year and most patients die within 16 months of diagnosis [1,3]. Despite multimodal therapies, the treatment of GBM remains essentially palliative due to rapid tumor recurrence. The poor prognosis for GBM patients has remained almost unchanged for decades, underscoring the challenges in effectively treating these lethal cancers. The fundamental problem of these malignancies is their highly infiltrative nature and extreme

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resistance to conventional treatments. Current therapy for GBM includes surgical resection, radiotherapy, chemotherapy such as temozolomide treatment and targeted molecular therapies [4]. However, aggressive invasion of GBM cancer cells into brain tissue often prevents complete surgical resection and contributes to therapeutic resistance, which leads to fatal tumor recurrence. The cellular and molecular mechanisms associated with GBM invasion are not well defined. The development of more effective treatments clearly requires new insight into the mechanisms underlying the GBM invasion and recurrence.

It has been well recognized that most GBMs contain heterogeneous tumor cells in varied differentiation status. Increasing evidence has supported that a subpopulation of cancer cells sharing stem cell properties within a GBM has potent capacity of tumor propagation [5–8]. These cells were called glioblastoma stem cells (GSCs) or tumor-propagating cells that have been characterized by several groups including ours [5–7,9–13]. GSCs are functionally defined by self-renewal, cell differentiation potential, and tumor propagation *in vivo*. GSCs display greater tumorigenic potential than matched non-stem tumor cells when xenotransplanted into the brains of immuno-compromised rodents [6,9,12,13]. GSCs have been implicated in several malignant behaviors associated with GBM tumor progression. We demonstrated that GSCs express elevated levels of vascular endothelial growth factor (VEGF) to promote tumor angiogenesis [10]. Other group showed that GSCs express an additional pro-angiogenic ligand SDF-1 (stromal-derived factor-1, also known as CXCL12) [14]. In addition, we found that GSCs contribute to GBM radioresistance by preferential activation of the DNA damage checkpoint and the enhanced DNA repair capacity [9], while other groups showed relative resistance of GSCs to chemotherapies such as temozolomide [15,16]. Although the origin of GSCs is not clearly defined, GSCs share similar properties with normal neural stem/progenitor cells (NPCs) that endow GSCs with key traits in carcinogenesis. These properties include enhanced potentials for proliferation, angiogenesis and modulating immune responses [17]. It has been hypothesized that GSCs are more invasive than matched non-stem tumor cells, but direct experimental evidence addressing this issue has been lack so far. Here we show experimental data demonstrating that GSCs display greater invasive potential *in vitro* and *in vivo* than matched non-stem tumor cells derived from primary GBMs and xenografts.

2. Materials and Methods

2.1. Isolation and culture of glioblastoma-derived tumor cells

Glioblastoma stem cells (GSCs) and non-stem tumor cells (Non-stem TCs) were derived from GBM xenografts or surgical specimens and cultured as previously described [9,13]. De-identified GBM specimens were collected from Cleveland Clinic Brain Tumor Center. GBM tumors were disaggregated using the Papain Dissociation System (Worthington Biochemical Corp.). Total tumor cells were recovered in stem cell culture medium for at least 6 hours to allow re-expression of surface markers and then sorted by FACS (Fluorescence-Activated Cell Sorting) based on the presence of CD133 (Milenyi Biotech) or CD15 (SSEA-1, BD Bioscience). The GSC phenotype was confirmed by functional assays of self-renewal (serial neurosphere formation), stem cell marker expression, differentiation potential and tumor propagation as described in our previous studies [9,13].

2.2. In vitro matrigel invasion assay

The *in vitro* cell invasion assay was performed using MatrigelTM-coated invasion chamber (BD Bioscience) as previously described [10]. Cells were added to the upper chamber of the MatrigelTM coated prehydrate polycarbonate membrane filter. After incubation for 24 hour, the non-invaded cells from the upper side of the filter were scraped using moist cotton swab.

The invaded cells in the reverse side of the filter were fixed and stained with hematoxylin and eosin (H&E), and then counted under an inverted microscope.

2.3. Intracranial transplantation to establish GBM xenografts

Intracranial transplantation of GSCs or non-stem tumor cells into athymic/nude immunocompromised mice was performed as described [9,13]. Briefly, isolated GSCs or non-stem tumor cells (1×10^4 to 2×10^5) were transplanted into the right frontal lobes in brains of athymic BALB/c nu/nu mice to establish GBM xenografts. Mouse brains implanted with GSCs or non-stem tumor cells were harvested for analysis on day 5 or 35 after tumor cell transplantation. Prior to the collection of mouse brains bearing GBM tumors, cardiac perfusion with PBS followed by perfusion with 4% PFA (Paraformaldehyde, Sigma-Aldrich) was performed. GBM xenografts were fixed with 4% PFA overnight at 4°C, post-fixed in 70% ethanol, embedded with paraffin and cut for histological analysis.

2.4. Immunoblot analysis

Immunoblot analysis was performed as previously described [9,13,22]. The anti-L1CAM antibody (Clone UJ127, mAb, Lab Version or Genetex) and the antibodies against MMP16 (Abcam, ab73877), ADAMTS1 (Santa Cruz), SEMA3C (R&D System), SOX2 (Millipore), and α -tubulin (Sigma-Aldrich) were used for the immunoblotting.

2.5. Immunofluorescent and Immunohistochemical staining

Immunofluorescent (IF) staining of GSC tumorsphere or the differentiated cells was performed as previously described [9,22]. Briefly, frozen tumorsphere sections were fixed in 4% paraformaldehyde (PFA), incubated with primary antibodies [α -L1CAM (Lab Version), α -SOX2 (Millipore)] overnight at 4°C, and then incubated with the fluorescence-labeled secondary antibody for 1 hour at room temperature. Nuclei were counterstained with DAPI. To validate the differential potential of GSCs, cells were induced for differentiation in α -MEM with 10% FBS and then immuno-stained with antibodies against the astrocyte marker GFAP (Covance), oligodendrocyte marker Galc (Millipore) and the neuronal marker TUJ1 (Covance) by IF staining as described [9]. Stained cells or sections were viewed and examined under a fluorescent microscope (Leica DMI3000B) or confocal microscope (Leica TCS SP5). Immunohistochemical (IHC) staining to examine L1CAM expression in GBM tumor tissues was performed with an ABC kit using DAB detection as described previously [10].

2.6. Statistical analysis

All quantified data were statistically analyzed and presented as mean $\pm$ standard deviation. The difference between experimental groups was assessed by one way ANOVA or one way ANOVA on ranks tests.

3. Results

3.1. Glioblastoma stem cells are invasive than matched non-stem tumor cells in vitro

To compare the invasive potential between glioblastoma stem cells (GSCs) and matched non-stem tumor cells, we performed the matrigel invasion assay *in vitro*. GSCs isolated from primary GBM surgical specimens or xenografts were validated for the enrichment of stem cell-like cancer cells by functional assays of self-renewal, multi-lineage differentiation potential, and tumor propagation *in vivo*. These GSCs formed tumorspheres (Fig. 1A) that expressed the GSC marker SOX2 (Fig. 1B), and displayed differentiation potential into different cell lineages expressing makers for neurons (TUJ1+), astrocytes (GFAP+) and oligodendrocytes (Galc+) (Fig. 1C). Isolated GSCs and non-stem tumor cells were then

assessed for their invasive potential by examining cell migration through an artificial extracellular matrix (BD Matrigel). We found that both GSC populations derived from a GBM surgical specimen (CCF2170) and a xenograft (D456MG) showed more cells migrated through the matrigel gel than matched non-stem tumor cell populations (Fig. 1D). Quantified data confirmed that GSCs had more (4–5 folds) cells migrated through the matrigel than matched non-stem tumor cells (Fig. 1E). These data suggest that GSCs are significantly more invasive *in vitro* than matched non-stem tumor cells from the same GBM tumor.

3.2. Glioblastoma stem cells display greater invasive capacity than non-stem tumor cells *in vivo*

Aggressive invasion of cancer cells into brain tissue is one of most malignant characteristics of GBMs. To further compare the invasive potential of GSCs and non-stem tumor cells *in vivo*, we transplanted GSCs or matched non-stem tumor cells (Non-stem TCs) into brains of athymic BalbC nu/nu mice through intracranial injection, and monitor tumor cell dispersal into brain tissue at early and late stages after transplantation. In this experiment, we used GSCs and non-stem tumor cells from a pediatric GBM xenograft (D456MG), as non-stem tumor cells derived from this line of GBM xenograft can form tumor when a large number of cells are used for intracranial transplantation as demonstrated in our previous study [10]. At early stage (5 days) after tumor cell transplantation, we observed massive invasion of cancer cells into surrounding normal tissue in the brains implanted with GSCs, (Fig. 2A). Strikingly, these cancer cells had spread far away from the original injection site on day 5 after tumor cell transplantation (Fig. 2A). In contrast, in the brains implanted with non-stem tumor cells, most tumor cells were limited in the injection site and no cancer cell dispersal was detected 5 days after cell transplantation (Fig. 2B). Consistently, at late stage (35 days) after intracranial transplantation, we detected aggressive invasion of cancer cells that diffusely infiltrated into normal tissue in mouse brains implanted with GSCs (Fig. 2C). But we rarely detected cancer cell invading into the mouse brains implanted with non-stem tumor cells (Fig. 2D), even these non-stem tumor cells also formed small tumors in the brains. These data demonstrate that GSCs display greater invasive potential than matched non-stem tumor cells. In addition, GSCs grew much larger tumors than non-stem tumor cells in mouse brains (Fig. 2E and 2F), confirming that GSCs with greater invasive capacity are also much more tumorigenic than matched non-stem tumor cells.

3.3. Increased expression of invasion-associated proteins in glioblastoma stem cells

We have shown that GSCs are much more invasive than matched non-stem tumor cells *in vitro* and *in vivo*. To define the molecular mechanisms underlying the increased invasive potential of GSCs, we analyzed the differential expression of invasion-associated proteins in GSCs and matched non-stem tumor cells derived from five GBM surgical specimens and six xenografts through micro-array. We identified several invasion-associated proteins that were differentially expressed in GSCs relative to non-stem tumors in the majority of GBM samples (Supplementary Table S1). Notably, several metalloproteases including MMP16, MMP14, ADAM19, ADAMTS2, ADAMTS9 and ADAMTS1 were commonly more expressed in GSCs than matched non-stem tumor cells. Immunoblot analysis confirmed the differential expression of several invasion-associated proteins in GSCs relative to non-stem tumor cells (Fig. 3). These proteins include MMP16 (Matrix metalloproteinase 16), ADAMTS1 (a disintegrin and a metalloprotease with thrombospondin motifs 1), SEMA3C (Semaphorin3C) and L1CAM (cell adhesion molecule). MMP16 has been demonstrated to be involved in cancer invasion [18]. A recent study showed that cleavage of Semaphorin3C by ADAMTS1 promotes cell migration [19]. Interestingly, L1CAM surface molecule has been shown to be highly expressed in the invasive fronts of colon cancers and promote cancer progression in several tumors [20,21]. These proteins may be also involved in GSC

invasion. We are currently evaluating the functional significance of these proteins in GSC invasion to determine the critical regulators or pathways that mediate GSC invasion. It is likely that multiple proteins may be associated with the increased invasion potential of GSCs.

3.4. L1CAM, a cell surface protein preferentially expressed in GSCs, is highly expressed in the invasive fronts of primary GBMs

Our previous studies demonstrated that L1CAM is differentially expressed in GSCs relative to non-stem tumor cells and L1CAM play critical roles to maintain tumorigenic potential and promote radioresistance of GSCs [11,22]. As GSCs display greater invasive potential than non-stem tumor cells, and L1CAM overexpressed in a number of solid tumors has been linked to cancer invasion, we examined L1CAM expression pattern in primary GBMs using immunohistochemical (IHC) staining. We found that L1CAM expression was enriched in a population of cancer cells in the invasive fronts of human primary GBM tumors (Fig. 4). Although a subpopulation of cancer cells inside tumors also expressed L1CAM, the invasive fronts showed many more cells with high levels of L1CAM (Fig. 4, right panels). In addition, immunofluorescent (IF) staining further confirmed that many more L1CAM-positive cells were localized in the invasive fronts than the center region of primary GBMs (Supplementary Fig. S1). These data demonstrate that L1CAM is highly expressed in a subpopulation of cancer cells the invasive fronts of primary GBMs, suggesting that preferential expression of L1CAM in GSCs may be closely associated with the elevated invasive potential of GSCs in GBM tumors.

4. Discussion

GBMs are among the deadliest of all cancers. Despite therapeutic advances in treating other cancers, the median survival of GBM patients remains less than 16 months. Aggressive invasion of cancer cells into brain tissue is one of lethal characteristics of GBMs, which greatly contributes to the inability to achieve cures through surgical intervention. Although GBM cancer cells rarely metastasize beyond the central nervous system (CNS), GBM tumor cells often infiltrate into normal brain tissue or spine cord and recur rapidly in a local fashion despite maximal surgical resection and other treatments [1,2]. Current conventional treatments can not fully eliminate the infiltrated resistant cancer cells that lead to rapid tumor relapse. These resistant cancer cells invading into brain tissue are likely to be enriched with GSCs. In this study, we provide direct evidence demonstrating that GSCs have greater invasive potential than matched non-stem tumor cells. Normal neural stem/progenitor cells (NPCs) arise in the periventricular zones and often migrate along white matter pathways to repopulate the olfactory bulb and the injured areas, supporting a critical role for migratory behaviors of normal neural stem cells. The invasive phenotype of GSCs appears to be a natural extension of a neural stem or progenitor cell program. Our studies demonstrating invasive property of GSCs may provide us a new strategy to develop an effective therapeutics targeting one of the most malignant characteristics of GBMs. Our previous studies showed that therapeutic resistance of GBM is conferred by the selective survival of those highly tumorigenic GSCs [9]. Thus, targeting GSCs through a specific target may inhibit cancer invasion, overcome therapeutic resistance and reduce tumor recurrence, which may lead to a significant improvement of GBM treatment.

We have identified several invasion-associated proteins that are differentially expressed in GSCs relative to non-stem tumor cells. Although further evaluation of these proteins in mediating GSC invasion will be important, we have shown that a cell surface molecule L1CAM was highly expressed in a subpopulation cancer cells in the invasive fronts of primary GBM tumors. L1CAM was originally identified as a cell adhesion molecule in the nervous system during development [23,24]. This protein can interact with other EGFR,

Neuropilin-1, $\alpha 5 \beta 1$ and $\alpha v \beta 3$ integrins, and several extracellular matrix proteins [20,23]. L1CAM mediated cellular signaling plays critical roles in regulating cell adhesion, migration, survival, and cancer cell invasion. Overexpression of L1CAM has been correlated with tumor progression and metastasis of several solid cancers including malignant gliomas [20]. Ectopic expression of L1CAM in cancers increased cell motility and invasiveness *in vitro* as well as tumor growth in nude mice [20,21]. Interestingly, the extracellular domain of L1CAM can be released through proteolytic cleavage by ADAM10, ADAM17 (a disintegrin and metalloprotease) and plasmin. Soluble L1CAM can also stimulate cell migration, invasion, survival and growth through autocrine or paracrine binding to integrins. Both membrane-bound and soluble forms of L1CAM enhance protection of ovarian carcinoma cells [25]. Importantly, L1CAM has been shown to be highly expressed at the invasive fronts of colon cancers, and it is a target of β -catenin signaling [21]. Furthermore, L1CAM was found to play a critical role in mediating chemoresistance in pancreatic cancers through interaction with $\alpha 5$ integrin [26]. Our previous study demonstrated that L1CAM is preferentially expressed in GSCs and is critical to maintain the tumorigenic potential of GSCs [11]. Most recently, we showed that L1CAM regulates DNA damage checkpoint response and enhances radioresistance of GSCs through nuclear translocation of the L1CAM intracellular domain (L1-ICD) that mediates NBS1 upregulation via c-Myc [22]. In this study, we found that L1CAM expression is enriched in a population of cancer cells in the invasive fronts of GBMs. Based on this new data and our previous studies, we propose that GSCs expressing L1CAM in the invasive fronts may be relatively resistant to radiation and other therapies. Thus, these infiltrated GSCs in the invasive fronts may be responsible for rapid tumor recurrence after conventional treatments. Collectively, our studies suggest that targeting invasive and resistant GSCs through a specific molecular target such as L1CAM may effectively reduce GBM cancer invasion and recurrence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Glioblastoma stem cells (GSCs) are invasive than matched non-stem tumor cells *in vitro*.

(A) Representative images of GSC tumorspheres. GSCs derived from a primary GBM specimen (CCF2170) were cultured in neurobasal stem cell medium for seven days to form tumorspheres. (B) Immunofluorescent staining of SOX2 (a stem cell transcription factor) and L1CAM (a surface molecule of GSC) on frozen sections of GSC neurospheres. SOX2 was labeled in red, L1CAM in green, and nuclei were counterstained with DAPI in blue. (C) Immunofluorescent staining of GFAP (astrocyte marker), Galc (oligodendrocyte marker) or TUJ1 (neuronal marker) in the differentiated cells derived from GSCs. Isolated GSCs from a primary GBM (CCF2170) were induced for differentiation for 7 days and then immunostained with antibodies against GFAP, Galc or TUJ1 (green). Nuclei were counterstained with DAPI (blue). (D) *In vitro* matrigel invasion assay of GSCs and matched non-stem tumor cells from two GBMs. The relative invasive capacity of GSCs and matched non-stem tumor cells (Non-stem TCs) derived from D456MG GBM xenograft and CCF2170 primary GBM were examined in the BD Matrigel gel. Cells migrated through the matrigel were stained and photographed. (E) Quantified data from (D) shows that GSCs had significantly more cells migrated through the matrigel than matched non-stem tumor cells *in vitro*. Data are means $\pm$ SD (n=3). *, $p < 0.001$.

Fig. 2.

GSCs displayed greater invasive capacity than matched non-stem tumor cells *in vivo*. (**A** and **B**) Aggressive cancer invasion into brain tissues at early stage (day 5) after GSC transplantation into mouse brains. GSCs and non-stem tumor cells (Non-stem TCs) derived from a GBM xenograft (D456MG) were transplanted into mouse brains through intracranial injection (10,000 cells/mouse). Mouse brains harvested 5 days after tumor cell transplantation were examined for cancer cell dispersal through histological analysis.

Aggressive cancer cell invasion (indicated by red arrows) into brain tissues were detected in mouse brains implanted with GSCs. But none cancer cell dispersal was found in the brains implanted with non-stem TCs, and all cells stayed in the injection site (indicated by a black arrow). (**C** and **D**) Infiltration of cancer cells into normal tissues in mouse brains implanted with GSCs at late stage (day 35) after cell transplantation. GSCs and non-stem tumor cells (Non-stem TCs) isolated from a D456MG GBM xenograft were transplanted into mouse brains through intracranial injection (200,000 cells/mouse). Mouse brains were harvested 35 days after cell transplantation and then examined for cancer cell invasion through histological analysis. Diffuse infiltration of cancer cells (indicated by red arrows) into normal tissues were detected in brains implanted with GSCs but not in those with non-stem tumor cells, although D456MG non-stem tumors also grew small tumors (indicated by a black arrow) in mouse brains. (**E** and **F**) Representative images of brain cross sections (H & E stained) from mouse brains harvested on day 39 after cell transplantation described in (**C** and **D**). GSCs formed much larger tumors than matched non-stem tumor cells in mouse brains. Red arrows indicate large infiltrative tumors in the brain implanted with GSCs, and a black arrow indicates a small tumor in the brain implanted with non-stem tumor cells.

Fig. 3.

Immunoblot analysis shows increased expression of several invasion-related proteins in GSCs relative to matched non-stem tumor cells. GSCs and matched non-stem tumor cells (Non-stem TCs) isolated from a primary GBM (CCF1992) and a GBM xenograft (T3359) were analyzed for the expression of invasion-associated proteins including L1CAM, MMP16, ADAMTS1 and SEMA3C, and the GSC marker SOX2. The sizes of molecular markers are indicated on right side.

Fig. 4.

Immunohistochemical (IHC) staining shows that L1CAM is highly expressed in a subpopulation of cancer cells in the invasive fronts of primary GBMs. Representative images of L1CAM staining (in brown) in two GBMs (CCF1863 and T4121) are shown. The marked invasive fronts with small squares were enlarged in right panels to show L1CAM expression in the invasive fronts (indicated by arrows). The tumor sections were counterstained with hematoxylin. T, tumor tissue, N, normal brain tissue.