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LABORATORY INVESTIGATION

Unique microenvironmental responses to PDGF stimulation in brain and spinal cord gliomas determine tumor phenotype

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Abstract Injection of a PDGF-B expressing retrovirus into the subcortical white matter of adult rats induces the rapid formation of brain tumors that have the histological features of glioblastoma. In contrast, when the same retrovirus is injected into the spinal cord of adult rats the resulting tumors are more indolent and display a unique histology characterized by nests of tumor cells separated by a dense vascular network without areas of necrosis. To study whether these differences are determined by the tumor cell of origin or due to microenvironmental influences, we conducted a series of transplantation experiments. Cells were independently isolated from PDGF-induced brain and cord tumors then subsequently transplanted into naive rat forebrains and spinal cords. The resulting tumors were characterized by histological analysis, marker expression profiling, PDGFR subtyping, and latency to tumor-induced morbidity. Tumor phenotypes were found to be consistently predicted by the tissue into which they were transplanted rather than by the tissue of origin. These results suggest that tumor microenvironment rather than the tumor cell of origin may be the primary determinant of glioma phenotype in the model presented.

Keywords Brain tumor · Glioma · Glioblastoma · Neural progenitors · PDGF · Spinal cord tumor

Introduction

The histology and aggressiveness of gliomas can often be predicted based on their location within the central nervous system (CNS). Glioblastoma multiforme (GBM) is the most common intrinsic tumor occurring in the cerebrum but is rare in the spinal cord where low grade astrocytomas and ependymomas predominate. The mechanisms underlying these anatomic predilections are not known, however, their study offers a new paradigm with which to investigate glioma pathobiology. Previous studies from our lab and others suggest that abnormal PDGF signaling may initiate gliomagenesis in a subset of both brain and spinal cord tumors, however, the determinants of phenotypic differences remain unclear [1–3]. One interesting possibility, that has bearing on therapeutic approaches, is that the tumor microenvironment plays an important role [4, 5].

Retroviral induction of platelet-derived growth factor (PDGF) expression has been shown to initiate distinct gliomas in the spinal cord and forebrain that bear the common marker signature Olig-2+NG-2+PDGFRα+GFAP− [1, 2]. Although generated by infection of closely related cells with the same retroviral vector, tumors develop with distinct histological features predicted by the anatomic location in which they arise. PDGF induces subcortical white matter progenitors to rapidly produce tumors bearing all the histological hallmarks of glioblastoma, whereas it induces spinal cord progenitors to produce

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a unique glioma phenotype that is distinct from glioblastoma.

In this study, we show through a series of transplantation experiments in adult rats that the glioma microenvironment plays a significant role in determining tumor phenotype in both the forebrain and the spinal cord. We further provide evidence that this finding is regulated by a tissue-specific expansion of recruited progenitor and vascular cells that express different PDGF receptors.

Materials and methods

Retrovirus production

The retroviruses used were described previously and consist of a 0.8 kb fragment encoding PDGF-B-hemagglutinin (HA) ligated into the MCS1 region of the retroviral vector pQ-MCS1-IRES-eGFP [1, 2, 6]. Replication-deficient viruses with vsv-G coats were generated from these constructs. The control pNIT-GFP retrovirus that does not express PDGF was similarly constructed. Viral titers were determined in colony-forming units (CFU) by incubating 293T glioma cells with serial dilutions of retrovirus. At 48 h after infection, GFP+ cell clusters were counted. The CFU were calculated by multiplying the number of GFP+ cell clusters by the dilution factor.

Forebrain injections

Retrovirus was injected into the forceps minor of the corpus callosum (coordinates 2 mm lateral, 2.5 mm rostral, 3.5 mm deep to bregma) in adult Sprague–Dawley rats using a Hamilton syringe attached to a 33 gauge needle (5 µl at 0.2 µl/min). The injection of tumor cells was performed using the same protocol including identical volume, flow rate, and stereotactic coordinates.

Spinal cord injections

Details of injection into the rat spinal cord have previously been reported [2]. Briefly, a two-level laminectomy and durotomy was performed, exposing the dorsal aspect of the spinal cord. Intramedullary injections were performed using a 33-gauge needle positioned over the midline. The needle was slowly inserted into the cord to a depth of 2 mm. A 3 µl volume of retrovirus or tumor cells was then delivered to the cord at a rate of 0.2 µl/min.

Histology, immunofluorescence, and microscopy

Animals were cared for in accordance with the standards of the Columbia University Institutional Animal Care and Use Committee. Rats were sacrificed with lethal doses of ketamine upon appearance of tumor-induced morbidity which may include paresis, seizures, posturing, periorbital hemorrhage or other signs. Tissues were fixed by means of cardiac perfusion with 4 % paraformaldehyde. Hematoxylin and eosin (H&E) stains were performed on 5 µm sections cut from paraffin embedded tissue. Immunofluorescence analysis was performed on 10 µm cryosections with the following antibodies: rabbit anti-Olig2 (1:500, Millipore), chicken anti-GFP (1:1000, Invitrogen), mouse anti-Ng2 (1:500, Millipore), mouse anti-PDGFR beta (1:500, Chembio), rabbit anti-PDGFR alpha (1:500, Millipore), and mouse anti-SMA (1:200, DakoCytomation). Stained sections were examined and photographed using a Zeiss Axiophot 200 fluorescent microscope equipped with an Axiocam (Oberkochen, Germany) and OpenLab imaging software (Improvision, Lexington, MA). Cell counts were manually performed on at least three high-powered fields per section. All tumors were analyzed microscopically and representative micrographs were taken.

Tumor dissociation

Fresh tumors were dissected at the time of animal sacrifice and tumor cells were isolated as previously described [1]. A total of 1×10^5 cells were re-suspended in Opti-Mem (Gibco) and re-injected into the rat brain or spinal cord within 2 h.

Statistical analysis

All statistics were performed using GraphPad Prism 4 software (La Jolla, CA). Survival curves were analyzed using the method of Kaplan and Meier. The Fisher's test was used to compare categorical data and the unpaired *t* test was used for continuous variables. All injection groups included five animals per experiment. A p value of less than 0.05 was considered significant.

Results

PDGF induces the formation of histologically and clinically distinct gliomas in the brain and spinal cord

To investigate the differences in glioma development within the forebrain and the spinal cord, we compared PDGF-B-GFP retrovirus induced gliomagenesis in these two regions of the adult rat CNS. We have previously shown that both in the brain and in the spinal cord, PDGF induces the formation of malignant gliomas [1, 2].

However, injection of PDGF retrovirus into the brain results in tumors with the histological features of GBM (glomeruloid vascular proliferation and palisading necrosis) whereas injection of PDGF retrovirus into the spinal cord results in tumors with a unique histology characterized by small clusters of tumor cells separated by a dense, vascular network (Fig. 1a, b). Furthermore, PDGF-induced brain and spinal cord tumors are observed to have different clinical characteristics; animals with brain tumors become symptomatic significantly earlier than animals with spinal cord tumors (22–25 vs. 36–57 days-post injection, $p < 0.05$) (Fig. 1c).

Retrovirus selectively infects resident progenitor cells in the brain and spinal cord

Although the histological features of PDGF induced brain and spinal cord tumors are distinct, these tumors are remarkably similar in terms of progenitor marker expression [1, 2]. Having previously determined that subcortical injection of a GFP-tagged retrovirus (pNIT-GFP) preferentially infects white matter progenitors that differentiate into oligodendroglia [1], we proceeded with injection of the same control retrovirus into the spinal cord. Immunofluorescence analysis showed that, as in the brain, retrovirus targets resident progenitor cells of the spinal cord identified by the marker signature NG2+Olig2+PDGFRα+GFAP− (Supplementary Fig). Due to the relative rarity of these cells in the spinal cord, attempts to recover them for in vitro analysis were not successful. Nonetheless, their expression profile suggests that, like their supratentorial counterparts, they are oligodendroglial precursors.

PDGF receptor subtyping reveals the unique expression profiles of brain and spinal cord gliomas

While PDGF-induced brain and spinal cord gliomas seemingly arise from the same progenitor cell of origin and display a similar marker signature, we sought to uncover possible differences in these tumors by performing PDGF receptor subtyping. Interestingly, double immunofluorescence staining

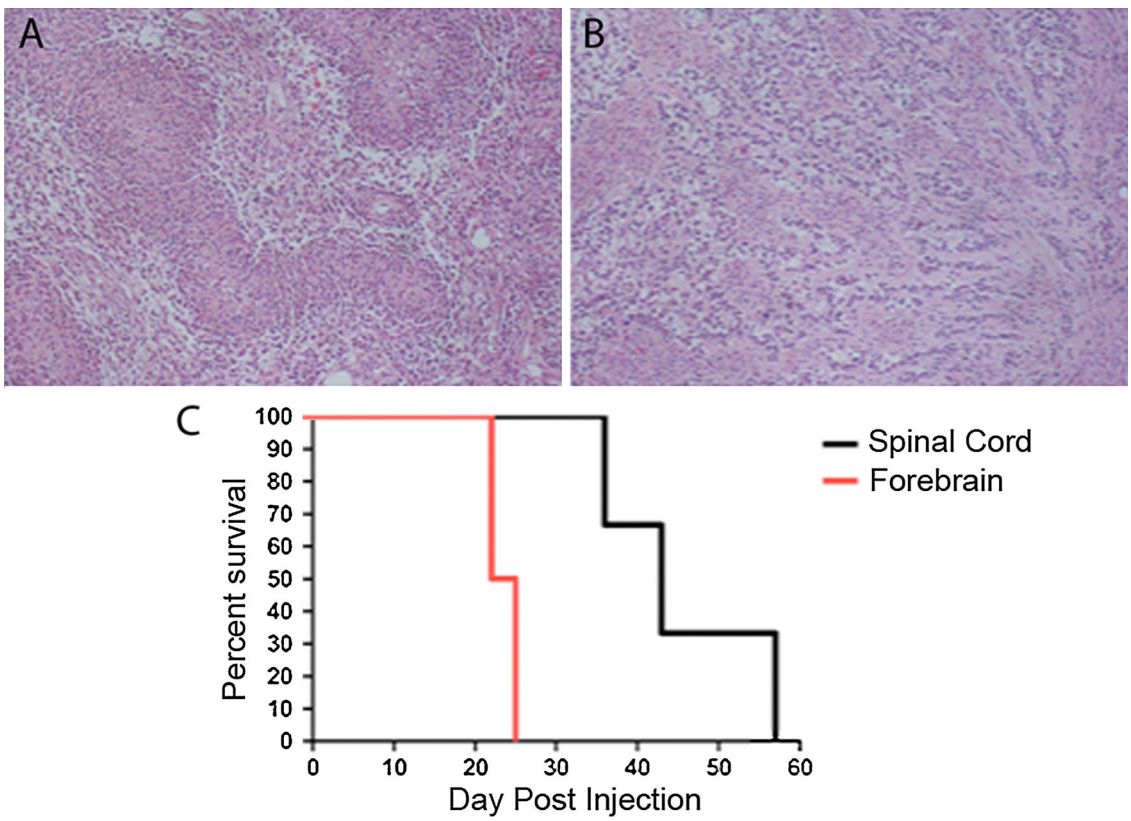

Fig. 1 PDGF induces the formation of distinct gliomas in the brain and spinal cord. H&E staining of PDGF-induced gliomas from the brain demonstrate that they histologically resemble glioblastoma with prominent areas of palisading necrosis and glomeruloid vascular proliferation (**a**). PDGF-induced gliomas that form in the spinal cord are histologically different from GBM and exhibit small clusters of tumor cells separated by a dense vascular network (**b**). Kaplan–Meier curves show that gliomas formed by PDGF retrovirus injection into the forebrain becomes symptomatic earlier ($p < 0.05$) than gliomas initiated by PDGF retrovirus injection into the spinal cord (**c**). Scale ×10 magnification

for PDGFRα and PDGFRβ showed unique patterns of labeling within each tumor type (Fig. 2). In the forebrain tumors, a small population of PDGFRβ+ cells coalesced into isolated clusters surrounded by a large population of PDGFRα+ cells. In contrast, the spinal cord tumors had significantly more PDGFRβ+ cells creating nests around PDGFRα+ cells. The PDGFRα+ cell population has been previously noted to be composed of glial progenitors [1, 2]. We additionally show that the trabecular networks seen on histological analysis of spinal cord gliomas correspond to tumor-associated vasculature as identified by smooth muscle actin (SMA) staining. These location specific patterns of PDGF receptor expression and tumor architecture held true in all cases analyzed.

Glioma phenotype is predicted by the local tissue environment and not the tissue of origin

Whereas in the model presented we have shown that retrovirally infected cells in the brain and in the spinal cord are very similar, we next tested the hypothesis that differences in PDGF-induced brain and spinal cord gliomas are due to microenvironmental rather than cell of origin differences. During a series of tumor transplantation experiments it was observed that when either brain or spinal cord glioma cells were injected into the rat forebrain, infiltrating tumors developed with glomeruloid vascular proliferation and palisading necrosis (Fig. 3a, b). In contrast, transplantation of brain or spinal cord glioma cells into the rat spinal cord resulted in tumors composed of small clusters of cells separated by a dense, vascular network (Fig. 3c, d). In other words, transplantation of tumor cells to the brain results in GBM, while transplantation of tumor cells to the spinal cord results in non-GBM gliomas. These findings appear to be independent of the tissue (brain or spinal cord) of origin.

We next examined whether the clinical behavior of transplanted tumors—as judged by the rapidity of tumor-induced morbidity—was more predictable based on the tissue of origin or the environment into which they were transplanted. Kaplan–Meier analysis shows significantly shorter latency to animal morbidity after transplantation to the forebrain ($N = 5$, median 19 days) as compared to transplantation to the spinal cord ($N = 5$, median 35 days) regardless of the tissue of origin ($p = 0.0027$). Similar analysis showed no correlation between the tissue of origin and the latency to animal morbidity ($p = 0.5$). Thus, the environment in which a tumor grows and develops appears to be more important than the cell or tissue of origin for determining tumor behavior.

PDGF-B initiates a microenvironment-dependent response from recruited progenitors and tumor vascular cells

Previous studies from our lab have shown that PDGF-induced brain gliomas are composed of a significant

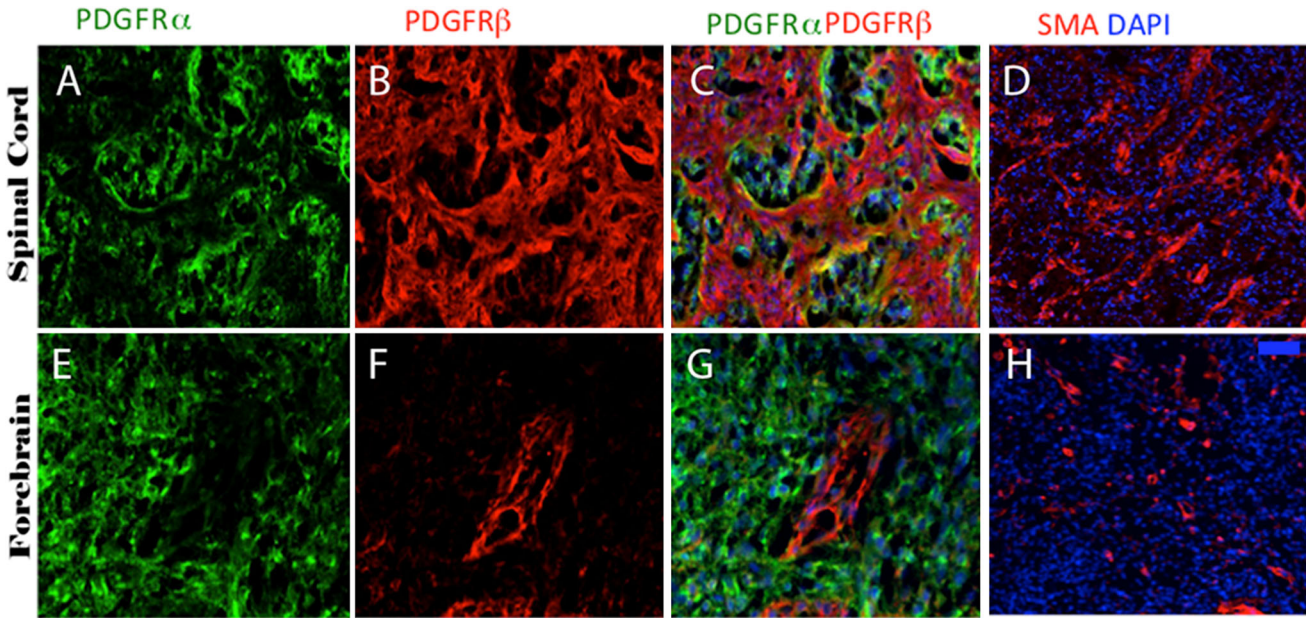

Fig. 2 PDGF receptors are uniquely expressed in brain and spinal cord gliomas. PDGF-induced spinal cord gliomas exhibit an abundance of PDGFRβ+ cells which create nests around a smaller population of PDGFRα+ cells (**a**–**c**). In contrast, PDGF-induced forebrain gliomas are predominantly composed of PDGFRα+ cells with smaller populations of PDGFRβ+ cells intermingled (**e**–**f**). SMA immunofluorescence indicates that the dense cellular network of septations seen most prominently in PDGF-induced spinal cord gliomas is vascular in nature (**d**, **h**). Scale bar 70 microns

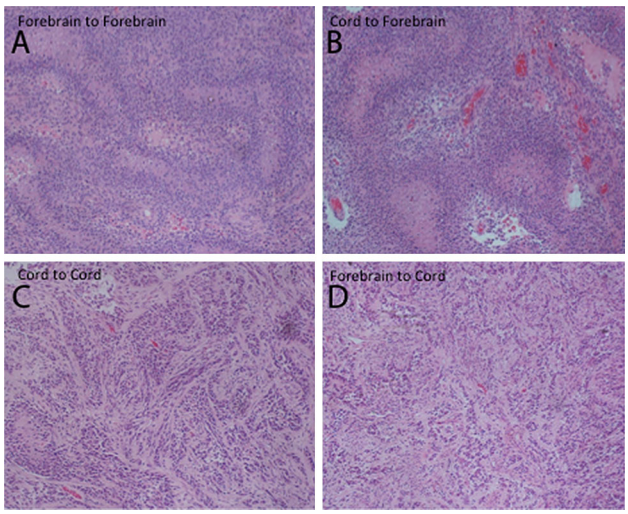

Fig. 3 Glioma histology is determined by the local environment rather than the tissue of origin. The transplantation of glioma cells into a tumor-naive rat forebrain results in the formation of tumors that resemble glioblastoma, regardless of whether the tumor cells were recovered from a PDGF-induced spinal cord or a brain glioma (**a**–**b**). Analogous experiments in which glioma cells are transplanted to the spinal cord show that the resulting tumors retain the characteristics of PDGF-induced spinal cord gliomas, regardless of whether the tumor cells were recovered from a PDGF-induced spinal cord or a brain glioma. The spinal cord tumors are distinct from forebrain tumors and are composed of small clusters of neoplastic glial cells surrounded by a prominent network of vasculature (**c**–**d**). Scale ×10 magnification

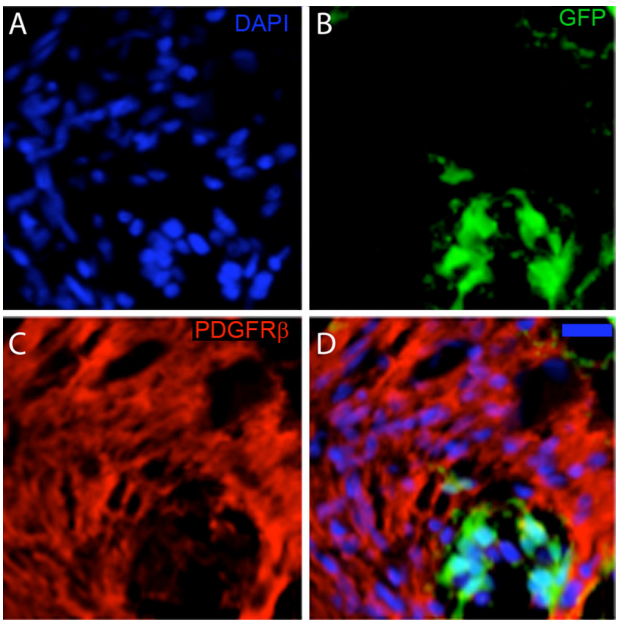

Fig. 4 PDGF retrovirus recruits but does not infect PDGFRβ+ cells. High power micrograph shows DAPI stained cell nuclei (*blue*) in a PDGF-induced spinal cord glioma (**a**). PDGF-GFP retrovirus infected cells (*green*) consist of a minority of cells in this tumor (**b**). PDGFRβ+ vascular cells (*red*) interdigitate between nests of non-vascular neoplastic glioma cells (**c**). These PDGFRβ+ tumor vascular cells do not become infected by retrovirus (**d**). Scale bar 35 microns

population of recruited progenitor cells [1, 4, 5]. Unlike progenitor cells that have been directly transformed by PDGF retrovirus infection, these recruited progenitors respond predominantly to environmental cues, namely, paracrine PDGF stimulation. Taking advantage of the PDGF retrovirus GFP tag, one can reliably distinguish virally infected [progenitor] cells from uninfected cells that are recruited to the tumor. Our experiments verify that GFP expression (i.e. retroviral targeting) is limited to glial progenitors and is in no case expressed in the PDGFRβ+ vasculature (Fig. 4).

Analysis of the recruited cell population revealed no significant differences when tumors were compared based on the tissue of origin ($p = 0.93$). Conversely, significant differences in the constitution of the recruited cell population were observed when tumors were grouped by the transplanted location ($p = 0.0095$). In tumors transplanted to the spinal cord, 40–60 % of recruited cells were shown to be PDGFRα+ glial progenitors while an equal number were PDGFRβ+ vascular cells. In comparison, tumors transplanted to the forebrain had 75–80 % of recruited cells being PDGFRα+ glial progenitors and 20–25 % of recruited cells being PDGFRβ+ vascular cells. This data suggests that the spinal cord microenvironment fosters a more balanced cellular response than the brain to paracrine PDGF stimulation with approximately equal numbers of PDGF responsive progenitor and vascular cells being recruited to form the tumor. Similar analysis showed that the proportion of recruited (GFP-) versus retrovirally infected (GFP+) progenitor cells was significantly ($p = 0.0095$) higher in tumors derived from transplantation to the brain as compared to those derived from transplantation to the spinal cord (Fig. 5). Thus the brain microenvironment seems to have a greater capacity to promote cellular recruitment than that of the spinal cord.

Discussion

Despite the fact that white matter progenitors in the spinal cord and in the cerebrum are extremely similar in terms of marker expression, morphology, and biological behavior [7–9], their induction by retrovirally encoded PDGF-B results in very different tumors. In the cerebrum, a rapidly expanding malignant glioma bearing all the histological features of glioblastoma results, whereas in the spinal cord, a less aggressive glioma with a prominent vascular network results. Since the transforming agent—PDGF retrovirus—is the same and the infected cells are essentially the same, we hypothesized that differences in the microenvironment may account for the observed phenotypic divergence.

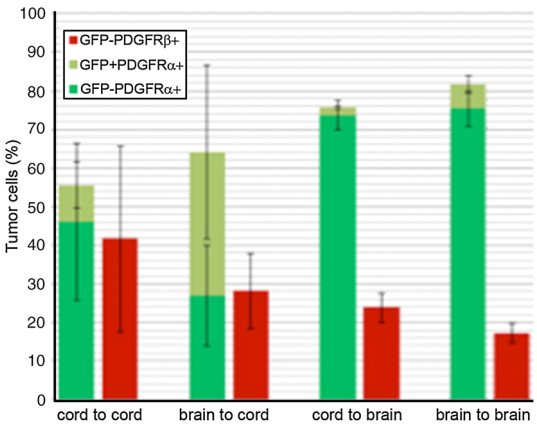

Fig. 5 Forebrain microenvironment promotes greater progenitor cell recruitment in gliomas than the spinal cord microenvironment. PDGF-induced gliomas are composed of three populations of PDGF-responsive cells. The progenitor cell constituents include those that are locally infected by the PDGF-GFP retrovirus (GFP+ PDGFRα+) and those that are recruited from the surrounding microenvironment (GFP-PDGFRα+). The non-progenitor, vascular cell constituents are similarly recruited from the microenvironment and express PDGFRβ (GFP-PDGFRβ+). Progenitor cell recruitment is significantly elevated in gliomas that grow in the brain as compared to those that grow in the spinal cord. Conversely, the PDGF responsive vasculature is more prominent within tumors that grow in the spinal cord as compared to those that grow in the brain. These observations are independent of the tumor tissue of origin

Indeed, the findings of this report suggest that glioma microenvironment can be a very powerful determinant of tumor histology and clinical behavior. In the model presented, microenvironment appears to supersede cell of origin as the primary determinant for tumor phenotype in some instances.

Our experiments show that when glioma cells are transplanted, tumor phenotypes are consistently and reliably predicted by the tissue into which they are transplanted. More surprisingly, tumor phenotype was not predicted by the tissue of origin. Spinal cord tumor cells and brain tumor cells transplanted into the brain produce the glioblastoma phenotype; brain tumor cells and spinal cord tumor cells transplanted into the spinal cord produce the spinal cord tumor phenotype. This indicates that microenvironmental factors play a decisive role in determining the kind of glioma that develops after a transforming event.

The experimental tumors generated by retroviral PDGF induction can be considered to be composed of two populations of PDGF responsive cells, namely progenitor cells and vascular cells. These cell populations respond to paracrine PDGF signaling through the PDGFRα and the PDGFRβ receptors, respectively. An advantage of the retroviral system we utilize is that recruited cells can be easily distinguished allowing additional classification of tumor cell constituents including: (1) infected PDGFRα+ progenitors, (2) uninfected and recruited PDGFRα+ progenitors, and (3) uninfected and recruited PDGFRβ+ vascular cells. The relative contributions of these constituent populations to the tumor mass differed according the anatomical microenvironment in which they grew. In the transplantation experiments, brain tumors were dominated by an expansion of uninfected, recruited PDGFRα+ progenitors, whereas spinal cord tumors contained approximately equal numbers of progenitor and vascular cells. The spinal cord tumors had a smaller proportion of recruited progenitors than did the brain tumors. The proportions of these cell types, like tumor histology and clinical behavior, were consistently predicted by the host tissue into which tumor cells were transplanted and not predicted by the tissue of origin.

Although not tested, it is possible that differences in microenvironmental paracrine PDGF signaling in the brain and spinal cord may play a role in determining tumor phenotype. Regarding tumor composition specifically, it is also possible that the increased progenitor recruitment seen in brain gliomas results from increased availability of recruitable progenitors in the brain compared to the spinal cord. Although, NG2+PDGFRα+ progenitors appear evenly distributed throughout the adult rat CNS, these cells are 2–3 times more prevalent in white matter than in gray matter [7]. Thus the number of potentially recruitable progenitors is much greater in the area of the brain targeted by retrovirus injections compared to the area of the spinal cord that is targeted.

Cell recruitment has become increasingly viewed as a key component of glioma growth, development, and clinical progression [10–15]. PDGF tumor models provide evidence that a subset of recruited cells may in fact become transformed, becoming independently capable of initiating gliomagenesis. Although it is not clear if such a mechanism is at play in this study, the capability of recruited progenitors from the local environment to profoundly impact tumor phenotype is evident.

Conclusion

PDGF retrovirus induces histologically and clinically distinct gliomas in the brain and spinal cord. Glioma phenotype is predicted by the local tissue environment in which the tumor grows and not by the tissue of tumor origin. The differential response to retroviral PDGF induction in the brain and spinal cord may result from microenvironment-specific differences in the number and type of PDGF-responsive cells available for recruitment.

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