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Retroviral Delivery of Platelet-Derived Growth Factor to Spinal Cord Progenitor Cells Drives the Formation of Intramedullary Gliomas

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Abstract

BACKGROUND—High-grade gliomas of the spinal cord are poorly understood tumors that are very commonly associated with bad outcomes. The transforming effects of platelet-derived growth factor (PDGF) on spinal cord glial progenitor cells may play an important role in the development of these tumors.

OBJECTIVE—To investigate the possible tumor-initiating effects of PDGF overexpression in the spinal cord, we delivered a PDGF retrovirus directly into the substance of the spinal cord.

METHODS—The spinal cords of wild-type adult rats were surgically exposed and injected with 10⁶ colony-forming units of a green fluorescent protein-tagged, PDGF-expressing retrovirus. A control virus was injected to assess the cell types that become infected during retroviral delivery to the spinal cord.

RESULTS—It was observed that PDGF overexpression in the spinal cord causes morbidity from high-grade intramedullary glioma formation between 27 and 49 days after PDGF retrovirus injection. Retroviral transduction was highly efficient with 100% of injected animals displaying the tumor phenotype. The tumors produced were highly proliferative, were locally invasive, and displayed the immunophenotype of virus-targeted glial progenitor cells (Olig2+PDGFR+NG2+GFAP−).

CONCLUSION—PDGF is capable of driving glial progenitor cells within the adult spinal cord to form high-grade gliomas. Further investigation of PDGF signaling in the spinal cord is needed to better understand and treat these devastating tumors.

Keywords

Astrocytoma; Ependymoma; Glioma; NG2; Platelet-derived growth factor; Spinal cord tumor; Stem cell

Malignant intramedullary spinal cord gliomas are uncommon neoplasms associated with high rates of morbidity and mortality.¹ The prognosis for patients with these tumors is largely unaffected by current therapeutic strategies.^{2,3} The likelihood of developing new,

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effective therapies is low without a more fundamental understanding of spinal cord tumor oncogenesis.

Multiple reports have offered evidence indicating a close relationship between growth factor-induced proliferation of glial progenitors and the development of intracranial gliomas.^{4,5} Specifically, platelet-derived growth factor (PDGF) has been shown to drive the proliferation of glial progenitors leading to the development of high-grade intracerebral gliomas in animal models.^{4,6–8} Recent evidence has also implicated PDGF in the development of intramedullary spinal cord gliomas. PDGF receptor expression has been identified in human spinal cord ependymoma, and transgenic animal models of PDGF-induced spinal cord gliomas have been reported.^{9,10}

Here we report a new model of intramedullary spinal cord glioma in which tumors are driven by PDGF. In this model, PDGF-B is expressed by white matter-resident glial progenitor cells after retroviral gene delivery. The advantages of this model include a straightforward delivery technique, rapid tumor development from intrinsic tissue, and 100% phenotype penetrance. Because this model utilizes adult, wild-type animals, it is the first to demonstrate the oncologic potential of the adult spinal cord.

MATERIALS AND METHODS

Retrovirus Production

Detailed methods describing the construction of PDGF-IRES-GFP and pNIT-GFP retroviruses have previously been reported.^{4,11} In brief, the PDGF retrovirus plasmid was created by ligating a 0.8 kb DNA fragment encoding PDGF-B to the MCS1 region of the pQ-MCS1-IRES-eGFP retrovirus vector. Virus particles were then produced by transient cotransfection of NIH-293T cells with structural protein (VSV-G, Gag, Pol)-encoding plasmids and either pQ-PDGF-IRES-eGFP or pNIT-GFP constructs. Virus was collected from the culture media 24 to 48 hours posttransfection, concentrated by centrifugation, and stored at -80°C . Intramedullary injection of 10^6 colony-forming units/mL was performed in all experiments. Reporter green fluorescent protein (GFP) fluorescence was used as an indicator of retroviral DNA integration and expression. Because the virus genome lacks the essential structural proteins for virus replication and packaging, no live virus is produced after initial infection.

Rat Surgery and Intramedullary Injections

Adult Fischer rats (Charles River Laboratories, Wilmington, Massachusetts) weighing 250 to 300 g were used for spinal intramedullary injections. Rat housing, maintenance, surgery, and sacrifice were all done in accordance with the standards of the Columbia University Institutional Animal Care and Use Committee. Before surgery, rats were anesthetized by induction in a Plexiglas chamber with 1 % to 3% isoflurane at a rate of 4L/min for 5 minutes. After induction, inhalational anesthesia was maintained by using a nasal-oral mask delivering 1% to 3% isoflurane at a rate of 1 to 2.5 L/min. The anesthetized rat was placed prone and fitted to a rodent stereotactic head frame (Stoelting Co., Wood Dale, Illinois) for stabilization. Hair covering the region of the midthoracic spine (localized by palpation of ribs) was then shaved and prepped with isopropyl alcohol. A midline 3- to 5-cm skin incision was made. With skin retracted laterally, sharp dissection through the thoracodorsal fascia down to the spinous processes was accomplished. Hemostasis was maintained with bipolar cautery. With the use of the operating microscope to aid visualization, a blunt subperiosteal dissection of paraspinal musculature was made over the thoracic vertebrae exposing 2 to 3 spinal laminae. With the use of rongeurs, a 2-level laminectomy was

performed exposing the dura covering the spinal cord. The dura was sharply incised and reflected laterally, liberating a small volume of CSF and exposing the spinal cord.

Once the spinal cord was exposed, a stereotactic head frame with a 33-gauge needle (Hamilton Co., Reno, Nevada) was positioned over the midline. The needle was slowly inserted into the cord to a depth of 2 mm in the midline. A 2- μ L volume of retrovirus was then delivered to the cord at a rate of 0.1 μ L/min. At the conclusion of virus injection, the thoracodorsal fascia and skin were closed with 4-0 Vicryl and 3-0 nylon, respectively.

Histology, Immunostaining, and Microscopy

For studies in which the PDGF-IRES-GFP retrovirus was delivered to the spinal cord, rats were sacrificed when they first displayed hind leg paresis ($n = 10$) by cardiac perfusions with 4% paraformaldehyde. For studies where control pNIT-GFP retrovirus was delivered to the cord, rats were either sacrificed 72 hours after injection ($n = 2$) or observed daily for signs of hind leg paresis until the study conclusion 65 days postinjection ($n = 3$). All spinal cords were removed en bloc and post-fixed in 4% paraformaldehyde for 24 hours. Histological preparation and immunostaining was performed as previously described.^{4,12} Hematoxylin and eosin, Mason trichrome, and immunoperoxidase staining was done on 5- μ m sections cut from paraffin-embedded tissue, and immunofluorescence was performed on 10- μ m cryosections. The following primary antibodies were utilized for tissue staining: rabbit anti-Ki67 (1:1000; Vector), rabbit anti-GFAP (1:500; DAKO), rabbit anti-olig2 (1:500; Chemicon), sheep anti-GFP (1:200; Abd Serotec), rabbit anti-PDGFR- α (1:500; Cell Signaling), mouse anti-smooth muscle actin (1:200; DAKO), and mouse anti-NG2 (1:500; Chemicon). Immunofluorescence analysis was performed using an Eclipse TE-2000 microscope (Nikon) running Metamorph imaging software (Molecular Devices).

Statistical Analysis

All statistical analyses were performed using Prism 4 (GraphPad Software). Kaplan-Meier survival curves were compared using the Mantel-Haenszel log rank test. Significance for all associations was set for $P < .05$.

RESULTS

PDGF Retrovirus Initiates Glioma Formation in the Adult Spinal Cord

To test the effect of PDGF overexpression within the spinal cord, we injected a PDGF-expressing retrovirus tagged with GFP (PDGF-GFP) into the spinal cords of adult wild-type rats. The exposure involved a 2-level laminectomy of the upper thoracic spine, a small durotomy, and then a slow infusion through a stereotactically guided needle. All rats tolerated virus delivery without appreciable neurological deficit and were seen to begin ambulating within minutes after discontinuation of inhalational anesthesia.

The PDGF-GFP virus was delivered to the spinal cords of a total of 10 rats over the course of 2 experiments (5 rats per experiment) with an end point of hind leg paresis. All rats ($n = 10$) were seen to develop hind leg paresis between 27 and 49 days postinjection (Figure 1). No significant difference ($P = .1$) in the paresis latency was detected between the 2 experiments performed. In contrast, no rat ($n = 3$) injected with the control (pNIT-GFP) retrovirus became symptomatic by the time of study conclusion 65 days postinjection ($P = .018$). At necropsy, each spinal cord injected with the PDGF-GFP virus evidenced an expansile intramedullary mass lesion on gross inspection (Figure 2A and 2C). Translucency, cyst formation, and destruction of the normal gray-white matter interface within the spinal cord were also apparent. Areas of direct GFP fluorescence were detected during *in situ*

illumination of the cord under a fluorescent dissecting microscope, suggesting a proliferation of infected cells (Figure 2B and 2D).

Histological examination of the intramedullary spinal cord lesions showed a highly cellular glial neoplasm with tumor cells aggregated in nests (Figure 3A and 3B). Septations with infiltrating tumor cells insinuated between tumor nests were seen as well. Mason trichrome and immunohistochemical staining for smooth muscle actin indicated that these septations were composed of collagen and proliferative endothelial cells (not shown). Architectural features such as the central canal and the gray matter were obliterated by tumor. The spinal cord white matter was destroyed or compressed peripherally and neoplastic cells were seen to be locally invasive (Figure 3B). The tumors were highly proliferative with frequent mitotic figures and Ki-67 indices averaging $30 \pm 6\%$ (Figure 3C). Necrosis, vascular proliferation within tumor nests, and hemorrhage were not seen. Of note, histological examination of spinal cords from all rats injected with the control (pNIT-GFP) retrovirus did not reveal evidence of tumor formation.

The percentage of neoplastic cells expressing GFP and thus infected with the PDGF retrovirus was variable. In high-power fields between 15% and 100% (mean, $40 \pm 34\%$) of tumor cells expressed GFP. Tumor nests showed fairly uniform expression of glial progenitor cell markers including: NG2, olig-2, and PDGFR α (Figure 4B–4D). Glial fibrillary acidic protein (GFAP) was not expressed by tumor cells (Figure 4A), but was seen in the intervening collagen-laden septations between tumor nests as well as in reactive astrocytes (not shown).

Retrovirus Delivered to the Adult Rat Spinal Cord Infects White Matter Progenitors

In order to assess the phenotypic characteristics of cells that become infected during stereotactic virus injections, we performed delivery experiments utilizing a control retrovirus. A pNIT-GFP control retrovirus that does not express PDGF was delivered to the spinal cord of adult rats using the same exposure, targeting, and injection techniques previously described. At 72 hours after injection, labeled cells were identified within the spinal cord white matter (Figure 5A–5D). These labeled cells demonstrate the immunohistochemical profile of glial progenitor cells with immunopositivity for NG2, PDGFR α , and Olig2 (Figure 5B'–D'). Consistent with the immunophenotype of spinal cord gliomas initiated by the PDGF-GFP retrovirus, no cells infected with control retrovirus expressed GFAP.

DISCUSSION

This report is the first to demonstrate the innate tumorigenic potential of the adult spinal cord. Tumors in this model are initiated by retrovirus-mediated overexpression of PDGF in the spinal cord. Whereas previously published intramedullary spinal cord tumor models have either utilized nonsyngeneic tumor cell lines^{13–16} or transgenic animals,¹⁰ this is the first report of a syngeneic spinal cord tumor model generated in wild-type adult animals. Histologically, the tumors observed are high-grade intramedullary gliomas. Through labeling experiments, we have identified glial progenitors in the spinal cord white matter with the immunophenotype Olig2+PDGFR α +NG2+GFAP– as the likely cells of origin. The immunophenotypic similarity between tumor cells and retrovirus-labeled cells suggest that tumors represent an unbridled expansion of glial progenitors. This model is promising for use in preclinical studies because it is facile, produces tumors over a very short time period, and demonstrates 100% phenotypic penetrance.

Intramedullary Spinal Cord Tumor Models

Salcman et al¹⁵ reported the first animal model of intramedullary spinal cord tumor in 1984. This was a canine model utilizing the intramedullary injection of cells derived from respiratory syncytial virus-induced brain tumors in beagles.^{17,18} Subsequent models have utilized injection of VX2 squamous-cell carcinoma cells into rabbit¹⁴ and 9L gliosarcoma or F98 glioma cells into rat^{13,16} spinal cords. These models demonstrate the feasibility of direct injections into the spinal cord, but they are of limited experimental value because they are based on cell lines from extraspinal tumors that have been altered by many years of passage in cell culture.

Recently, Hitoshi et al¹⁰ reported the development of spinal cord gliomas in a murine transgenic model where PDGF expression was driven by the GFAP promoter. This was the first report of a model in which intramedullary gliomas developed intrinsically from the substance of the spinal cord. This transgenic model is also predicated on aberrant PDGF expression in the spinal cord, and tumor cells from this model bear immunohistochemical markers of glial progenitors.

Nonetheless, there are important differences between the transgenic model and the retroviral model presented in this report. Tumors generated in the transgenic model are histologically different and express GFAP. Because the transgenic model involves a germline genetic alteration, it does not address the oncologic potential of the adult spinal cord. Tumor location, latency, phenotype, and penetrance were highly variable. In our retroviral model, stereotactic delivery to white matter spinal cord progenitors allows for precise spatial control of tumor development. Additionally, the high tumor formation efficiency, narrow temporal window for tumor formation, and consistent high-grade histopathology seen in our system makes it appealing for preclinical testing.

Our study does not attempt to address the role played by retroviral insertional mutagenesis in the formation of gliomas in the rat spinal cord. Whereas limited evidence suggests that retroviral insertional mutagenesis may facilitate PDGF-induced tumorigenesis in neural tissue, this mechanism is not thought to be sufficient for tumor initiation.¹⁹ After more than a decade of use in the context of fate-mapping studies in the rodent central nervous system (CNS), the pNIT retrovirus has never been shown capable of initiating gliomas.^{4,11,20–23}

Targeting Adult Glial Progenitors in the Spinal Cord

The white matter tracts of the adult mammalian spinal cord contain a population of slowly cycling progenitor cells postulated to play a role in repair after spinal cord injury.^{24–26} Bromodeoxyuridine-labeling experiments have identified NG2+ cells within the white matter as the most abundant cycling cells within the adult spinal cord.²⁷ The retroviral vector in this report preferentially infects NG2+ progenitors in the white matter, suggesting a possible role for PDGF-induced proliferation of spinal cord NG2+ white matter progenitors in human spinal cord tumorigenesis. The immunophenotype of targeted glial progenitors is recapitulated in the tumors formed, suggesting that spinal cord gliomas in this model represent an expansion of glial progenitors. Although progenitor marker staining is fairly uniform, tumors consist of both virus infected and uninfected cells, suggesting that both autocrine and paracrine PDGF signaling drives tumor formation.

Histological Divergence of PDGF-Driven CNS Tumors

Previous studies showing that induction of PDGF expression in the rodent forebrain and brainstem initiates the formation of gliomas suggest that PDGF signaling may be important in tumor formation throughout the CNS.^{4,6–8,20} The histology of these experimental intracranial tumors varied from oligodendroglioma to oligoastrocytoma to glioblastoma. By

the use of a transgenic system, it has been reported that a similar range of tumor grades can be generated in the spinal cord.¹⁰ In the model we present, the histology of tumors generated is consistent with a diagnosis of high-grade glioma. However, the features of this rodent spinal cord glioma are sufficiently unique that it cannot be categorized based on the current World Health Organization classification scheme.²⁸ The potential reasons for histological divergence across PDGF-driven tumors throughout the CNS are numerous and could include differences in the cell type expressing PDGF, the levels and/or timing of PDGF expression, or the effect of differences in the local environment.

CONCLUSION

The adult rat spinal cord contains a population of progenitor cells that initiate high-grade glioma formation in response to retrovirus-mediated PDGF overexpression. Our results provide further evidence that aberrant PDGF expression may play a role in the development of intramedullary gliomas in humans. Additional studies investigating the role of PDGF signal transduction and the genetic and environmental factors modulating its tumor-initiating effects are warranted.

ABBREVIATIONS

CNS	central nervous system
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
PDGF	platelet-derived growth factor

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COMMENT

The investigators present an interesting study showing that the direct injection of a PDGF-expressing retrovirus into the spinal cord substance of rats results in the formation of intramedullary tumors with 100% penetrance. Tracking studies suggest that the cells of origin of these intramedullary tumors are white matter progenitor cells. These investigators have previously shown that injection of this same PDGF construct into the brain also results in intracranial gliomas. Therefore, this spinal cord model recapitulates these intracranial studies, suggesting a common mechanism underlying the formation of gliomas in the brain and spinal cord. Differences in the microscopic pathology of the spinal cord tumors compared with the brain tumors suggest that the microenvironment of the spinal cord and brain may influence tumor growth patterns.

This model is of value because it represents the first syngeneic spinal cord glioma model in adult rats and, therefore, opens up avenues for studying the biology and therapies of intramedullary spinal cord tumors. However, a major shortcoming of this model, and one that may ultimately limit its applicability, is that the generated tumors do not mimic a spinal cord tumor pathology recognized by the World Health Organization. The tumors lack infiltration and are GFAP-negative, suggesting that they are not astrocytic. Although well demarcated, the tumors do not have the features of ependymomas. Therefore, this model does not reproduce the 2 most common spinal cord tumors known to arise in adults. Thus, when used in studies designed to test new therapies, questions will be raised regarding the applicability of the results to the human condition. Nevertheless, the authors are to be congratulated for their efforts to model this complex disease.

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FIGURE 1.

Kaplan-Meier plots of tumor-induced morbidity. The PDGF-GFP retrovirus was delivered to the thoracic spinal cords of a total of 10 rats during 2 experiments. All rats ($n = 10$) displayed hind leg paresis between 27 and 49 days after intramedullary delivery of the PDGF retrovirus. No rat ($n = 3$) injected with control retrovirus (pNIT-GFP) displayed hind leg paresis by conclusion of the study 65 days postinjection.

FIGURE 2.

Gross description of spinal cord tumors. Segmental thoracic spinal cord expansion and discoloration (arrow) are seen on gross inspection (A). Axial transection of the expanded spinal cord segment shows obliteration of normal architectural features, including the central canal and gray-white matter borders (C). Cystic regions were also evident. Corresponding GFP fluorescence of the in situ (B) and transected (D) cord indicate the presence of a massive expansion of virus-infected cells.

FIGURE 3.

Histological features of PDGF induced intramedullary glioma. Low power hematoxylin and eosin -stained axial section through the cord lesion shows nests of tumor cells interdigitated by less cellular septations (**A**). White matter tracts are distorted and compressed peripherally by the tumor. At higher power the tumor nests are seen to contain aggregates of pleomorphic, neoplastic glial cells (**B**). Infiltration of the surrounding white matter and the insinuated septations by neoplastic cells is also evident. The tumor is highly proliferative with immunoreactivity for Ki-67 averaging $30 \pm 6\%$ (**C**).

FIGURE 4.

Immunofluorescence analysis of tumors. Tumor cells within PDGF-induced spinal cord gliomas did not express the intermediate filament protein GFAP (**A**). However, GFAP immunoreactivity was prominent within the surrounding white matter and between tumor nests. The tumor cells markedly expressed the glial progenitor markers NG2, olig2, and PDGFR α (**B–D**). Within tumor nests, only a subset of neoplastic cells express GFP (**A'–D'**), indicating the presence tumor cells that are not infected with the PDGF retrovirus.

FIGURE 5.

Spinal cord glial progenitor cells become infected by retrovirus. Stereotactic injection of the control retrovirus pNIT-GFP into the spinal cord causes infection of cells (arrows) predominantly within the ventral white matter (A). The anterior spinal artery is indicated (arrowhead) for anatomic reference (4× objective). At higher power, individual virus infected cells immunoreactive for GFP (B–D) are shown to express markers of glial progenitors including NG2, PDGFR α , and olig2 (B'–D'). Scale bar size = 7 μ m.