Cyclin D1 and Cdk4 Mediate Development of Neurologically Destructive Oligodendroglioma

Daniel Ciznadia1,2, Yuhui Liu1,3, Stephanie M. Pyonteck2, Eric C. Holland2,3, and Andrew Koff1,3

Abstract

Although the molecular changes that characterize gliomas have been studied, the pathogenesis of tumor development remains unclear. p21 contributes to gliomagenesis by stabilizing cyclin D1–cdk4 kinase complexes, suggesting that cyclin D1 and cdk4 may also be required for glial tumor development. In this study, we used a mouse model to attempt to confirm this hypothesis, finding that cyclin D1 and cdk4 played active roles in not only the tumor but also the tumor microenvironment. Loss of cdk4 blocked tumor development, but loss of cyclin D1 did not prevent gliomas from developing. Instead, loss of cyclin D1 impeded progression to higher stages of malignancy. Enforcing expression of cyclin D1 was insufficient to correct the progression defect observed in cyclin D1–deficient animals. In contrast, restoration of cdk4 in the cdk4-deficient animals restored cell proliferation and tumor formation, although at lower tumor grades. Notably, the failure of tumors in the cyclin D1- and cdk4-deficient animals to progress to higher grades was correlated with a failure to fully activate microglia in the tumor microenvironment. Moreover, when platelet-derived growth factor–transformed glial cells were engrafted orthotopically into the mice, the tumors that formed progressed to high grades in wild-type mice but not cyclin D1–deficient animals. Together, our findings establish that the cyclin D1–cdk4 axis is not only critical in glioma cells but also in stromal-derived cells in the surrounding tumor microenvironment that are vital to sustain tumor outgrowth. Cancer Res; 71(19); 6174-83. ©2011 AACR.

Introduction

Despite impressive advances in imaging, diagnosis, and treatment, neurologically destructive glial brain tumors remain among the most lethal and intractable forms of cancer, with a median prognosis of less than 18 months (1). Although a number of critical molecular alterations underpinning gliomas have been defined, the ways in which these changes orchestrate tumor formation are still unclear. One of the hallmarks of cancer is the capacity for autonomous cell growth through the elaboration of autocrine signaling loops. In malignant oligodendroglioma, overexpression of platelet-derived growth factor (PDGF) and its cognate receptor are genetically to this activity (12). An important prediction stemming from this work is that cyclin D1 and cdk4 should also be required for tumor development.

In most cell types, the cyclin D1–cdk4 axis is the primary gateway through which mitogenic information is channeled. Extracellular growth signals result in the de novo synthesis of cyclin D1 and assembly with its catalytic partner, cdk4, prior to accumulation of the complex in the nucleus (5–7). Although the CIP/KIP family of cell-cycle inhibitors had been reported to associate with active cyclin D1–cdk4/6 complexes in cycling cells (8), stabilizing (9) and anchoring them in the nucleus (10, 11), whether this activity contributes to cell proliferation is controversial. The strongest evidence that cdk inhibitor–dependent assembly and stabilization of cyclin D1–cdk4 can contribute to cell proliferation came from a revolutionary approach in tumor modeling, in which the critical role of the cdk inhibitor p21 in PDGF-induced glioma was mapped genetically to this activity (12). An important prediction
cyclin D1- and cdk4-deficient mice, as well as when tumor cells were transplanted into cyclin D1 knockout mice. We suggest that cyclin D1–cdk4 is a critical regulator of microglial activation, and that, in its absence, these cells are unable to support the development of glial tumor cells to more malignant states.

Materials and Methods

Mice
Cyclin D1, cdk2, and cdk4 knockout mice were generously provided by Piotr Sicinski (Dana-Faber Cancer Institute), Mariano Barbacid (CNIO, Spain), and Hiroaki Kiyokawa (Northwestern University), respectively (13–15).

Cell culture, transfection with RCAS viruses, re and in vivo transduction of glial cells
The culture of chicken DF-1 fibroblasts used to propagate RCAS viruses, transfection of these cells with RCAS viral plasmids, and intracranial injections used to infect nestin-positive glial stem and progenitor cells have all been described previously (12).

Histology and immunohistochemistry
Morbid animals were euthanized and the brains removed and fixed overnight in 10% neutral buffered formalin (Fisher). The brains were dehydrated in graded alcohol, embedded in paraffin, and 5-μm sections used for analysis. Sections were stained with hematoxylin and eosin (H&E) or used for immunohistochemical analysis done on an automated Ventana machine (Discovery; Ventana Medical Systems Inc.). The antibodies we used are indicated in the Supplementary Methods section.

Generation of whole-brain lysates
As soon as animals showed signs of morbidity, they were euthanized, and the brains rinsed in PBS, snap-frozen in liquid nitrogen, and ground into a fine powder using a mortar and pestle. Lysates were prepared by sonication in 50 mmol/L Tris-Cl, pH 7.4; 250 mmol/L NaCl; 5 mmol/L EDTA; 0.5% NP-40; 10 μg/mL leupeptin; 10 μg/mL aprotinin; 10 μg/mL soybean trypsin inhibitor; 2 mmol/L phenylmethylsulfonylfluoride. Clarified lysates (13,000 × g, 5 minutes, 4°C) were aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C.

Western blotting
Twenty to 80 μg of whole-brain lysate was resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore) for immunoblotting as described (8), except that bound antibodies were detected with western SuperSignal Pico ECL chemiluminescence (Pierce).

Statistical analysis
Log-rank analysis was used to assess differences in survival on the basis of Kaplan–Meier curves. As considerable regional heterogeneity exists within glial tumors, differences in tumor cell proliferation (as determined by Ki-67 staining) were examined using a logit model, with generalized estimating equations to correct for correlations within mice.

Results

Cyclin D1 contributes to PDGF-induced gliomagenesis
Given the exciting discovery that the cdk inhibitor p21 facilitated the nuclear accumulation of cyclin D1–cdk4 complexes to drive the development of PDGF-induced glioma (12), we wanted to probe this further by asking whether cyclin D1 and cdk4 were required for tumor formation. Consistent with this possibility, we found cyclin D1 was overexpressed in both human glioma as well as PDGF-driven tumors developing in nestin-tvA mice (Supplementary Fig. S1). Thus, we first set out to determine whether cyclin D1 was required for gliomagenesis. To accomplish this, we crossed the nestin-tvA transgene into a cyclin D1 knockout background (14). These mice were subsequently challenged with RCAS-PDGF and followed for 11 weeks for symptoms of glioma (macrocephaly, lethargy, and seizures). Previous studies had shown that more than 90% of wild-type nestin-tvA mice developed a glial tumor by this time when challenged with RCAS-PDGF (12). The absence of cyclin D1 reduced the onset of glioma-associated morbidity in a dose-dependent manner (Fig. 1A). Somewhat surprisingly, these animals developed histologically identifiable glial tumors at the same frequency as wild-type animals (Fig. 1B). It is known from studies of the human disease that tumor grade is a strong prognostic marker of survival in glioma patients (CBTRUS registry: www.cbtrus.org); thus, we looked at the grade of those tumors that developed in wild-type and cyclin D1 knockout mice.

Glial tumors from wild-type and cyclin D1 knockout mice were graded according to criteria from the World Health Organization (ref. 16, Supplementary Fig. S2). In wild-type mice, greater than 80% of the tumors were moderate or high grade. In contrast, all of the tumors in cyclin D1 knockout mice were low grade (Fig. 1C). This was confirmed by examining 2 other parameters correlated with histologic grade: tumor cellularity (Fig. 1D) and proliferation (Fig. 1E). The difference in proliferation remained apparent even in a comparison of the low-grade gliomas arising in cyclin D1 knockout animals with the few low-grade gliomas observed in wild-type animals (data not shown). Taken together, these results highlight a critical role for cyclin D1 in the progression of PDGF-induced glial tumors from low-grade to more malignant states.

Activity of cdk4 is essential for gliomagenesis
The importance of cdk4 activity for the growth of glioma has been suggested by pharmacologic inhibition of cdk4 in cell lines and in mice carrying human GBM xenografts (17–19). To assess whether cdk4 contributes to glioma formation, we challenged nestin-tvA wild-type and cdk4 knockout animals (13) with RCAS-PDGF and monitored them for symptoms of glial tumors. Strikingly, removal of cdk4 abolished glioma-associated morbidity (Fig. 2A) and tumor formation (Fig. 2B). Although we did not detect tumors by H&E staining, we observed scattered cyclin D1–positive cells and,
occasionally, an isolated focus of such cells (data not shown). These foci did not stain with Ki-67 (data not shown). Although such foci were generally not seen in sections from uninfected animals, we could not say whether they might progress into tumors if they coalesced in some fashion. To rule out the possibility that the number of target cells that could be infected by RCAS-PDGF was reduced in cdk4−/− mice, we confirmed that nestin-positive progenitors were expressed in the brains of these animals at levels equivalent to those seen in wild-type animals (Fig. 2C and D). Thus, cdk4 was critical for the rapid and timely development of glial tumors.

Glial-specific expression of cdk4 restores progression of PDGF-induced glioma in cdk4 knockout mice

We next asked whether we could reconstitute tumor development by restoring cdk4 expression in nestin-positive progenitors. To accomplish this, we challenged cdk4 knockout mice with RCAS vectors expressing PDGF and cdk4. Glial cell–specific expression of cdk4 did not change glioma-associated morbidity from the level observed in the cdk4 knockout animals (Fig. 3A); however, these mice did develop glial tumors at the same frequency as wild-type animals (Fig. 3B). The bulk of the tumor cells were positive for an oligodendrocyte marker, OLIG2, although tumors also contained trapped A

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Figure 1. Cyclin D1 contributes to PDGF-induced gliomagenesis. A, Kaplan-Meier curve: The indicated mice were followed for 11 weeks following infection with RCAS-PDGF at postnatal days 1 to 3: wild type (n = 27); cyclin D1+/–; n = 43, cyclin D1+/+ (n = 18); cyclin D1−/+ versus cyclin D1−/−; log rank, P = 0.02. B, gross tumor formation was determined by H&E stain. The percentage of wild-type and cyclin D1−/− mice with histologically identifiable glioma is shown. C, oligodendrogliomas from wild-type (n = 24) and cyclin D1−/− knockout (n = 14) mice were graded as low (L), moderate (M), or high (H) according to Kleihues and colleagues (16). D, tumor cellularity: due to the diffuse and invasive nature of glioma, cellularity is analyzed as a distribution of the number of nuclei within each ×400 field of tumor. The total number of nuclei within each of 120 fields from 4 wild-type and 4 cyclin D1−/− tumors was counted and plotted. E, Ki-67 staining: sections from 3 wild-type and cyclin D1−/− knockout mice were stained and examined microscopically (representative images are shown on the right). Cell counts were obtained in 90 fields. The number of Ki-67 positive and total nuclei per field was quantified using MetaMorph, with the ratio of these 2 numbers designated the Ki-67 labeling index. cyclin D1+/+ versus cyclin D1−/−, P = 0.01.
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neurons (NeuN positive) and astrocytes [glial fibrillary acidic protein (GFAP) positive; Fig. 3C]. These staining patterns and the characteristic nuclear haloing of the tumor cells indicated that these were oligodendrogliomas. Although the Ki-67 index in the reconstituted tumor cells was similar to that seen in wild-type tumor cells (Fig. 3D), these tumors were still low grade based on their histologic appearance and cellularity (Fig. 3E). Together, these results indicate that cdk4 promotes gliomagenesis, both by increasing the proliferation of nestin-positive progenitors and through another mechanism, unrelated to its expression in glial tumor cells.

Sequestration of p27 by cdk4 is not sufficient to explain its tumor-promoting activity

Our results strongly indicate that both cyclin D1 and cdk4 contribute to glial tumor formation. One of the mechanisms by which cyclin D1–cdk4 facilitates proliferation is through sequestration of the cell-cycle inhibitor, p27 (13, 20–22). Thus, we asked whether removal of p27 in cdk4 knockout mice would similarly reestablish PDGF-driven gliomagenesis. Whereas p27 deficiency enhanced glioma formation and associated morbidity in this model (23), ablation of one or both alleles of p27 in a cdk4 knockout background does not restore gliomagenesis, and no readily identifiable tumors were found in any mouse in this cohort (data not shown). Given that sequestration of p27 by the cyclin D1–cdk4 complex is thought to relieve inhibition of the cyclin E–cdk2 kinase (24), we also examined whether cdk2 was required for gliomagenesis. Studies by others have indicated a role for cdk2 in the proliferation and expansion of the glial cell lineage (25–27). However, deletion of cdk2 did not profoundly affect morbidity, glial tumor formation, or grade (Supplementary Fig. S3). In sum, these results suggested that neither sequestration of p27 by the cyclin D1–cdk4 complex nor the presence of cdk2 played an essential role in glial tumor formation.

Glial-specific reconstitution of cyclin D1 does not restore progression of PDGF-induced glioma to more malignant stages

Given that we could restore tumor development in cdk4 knockout animals by reconstituting the tumor cell-of-origin with cdk4, but were unable to restore the malignancy of such cells, we asked whether glial cell–specific reexpression of cyclin D1 would affect malignant progression of tumors in cyclin D1 knockout animals following challenge with RCAS-PDGF. For these experiments, we made use of a nuclear-stabilized and activated mutant of cyclin D1, D1T286A, in which mutation of threonine-286 to alanine prevents its phosphorylation and nuclear export by GSK3β (12, 28). The progression of disease in wild-type mice expressing this allele of cyclin D1 and PDGF is accelerated compared with mice expressing PDGF alone (data not shown). However, coinfection with RCAS-PDGF and RCAS-D1T286A did not accelerate morbidity of animals any more than infection with RCAS-PDGF alone (Fig. 4A). All of the tumors arising in the coinfected knockout animals expressed cyclin D1 protein (Fig. 4A, inset). Furthermore, tumor cell–specific reconstitution of cyclin D1 did not affect the grade of the tumors. These mice developed low-grade tumors based on histology, cellularity, and cell proliferation (Fig. 4B–E). Therefore, it seems that
although cyclin D1–cdk4 can promote the initial formation of glial tumors in a tumor cell–specific manner, progression to higher grades depended on another role of these proteins, one not carried out within the tumor cell.

Expression of PDGFR-α and downstream PDGF signaling is unaffected by the deletion of cyclin D1

Glioma formation and progression is dose dependent, and the amount of PDGF signaling dictates tumor development and grade (29). We therefore interrogated the levels of PDGFR-α expression in glial tumors arising in wild-type and cyclin D1 knockout mice to determine whether the difference in tumor grade might reflect altered expression of PDGFR. PDGFR-α was expressed in both the tumors arising in wild-type and cyclin D1 knockout mice (Supplementary Fig. S4A). Consistent with this, phosphorylation of ERK1/2 (Ras pathway effector) and p70S6K (Akt pathway effector) was not affected by cyclin D1 deficiency (Supplementary Fig. S4B). Thus, the ability of glial tumor cells to respond to PDGF at the level of the receptor seems to be unaffected by the absence of cyclin D1. Collectively, this further enforces the notion that tumor cell–specific expression of either cyclin D1 or cdk4 was insufficient to correct the defects in morbidity and malignancy in the respective knockout animals, leading us to consider the possibility that the absence of these genes might perturb some aspect of the microenvironment in which glioma arise.

The activation of tumor-associated microglia is perturbed in cyclin D1- and cdk4-deficient mice

The development of cancer relies on the dynamic interplay between the mutant tumor cell and other normal cells in the microenvironment (30). Because glial cell–specific
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expression of either cyclin D1 or cdk4 was insufficient to correct the defects in morbidity and malignancy in the respective knockout animals, it was possible that the absence of these genes affected recruitment or activation of another cell type in the tumor microenvironment. Given the complete absence of tumors arising in nestin-tvA;cdk4 knockout animals, it was possible that the absence of cyclin D1 did not prevent the establishment of a coherent tumor vasculature.

We also detected GFAP-positive astrocytes (Fig. 5A; i and ii), NeuN-positive neurons (Fig. 5A; iii and iv), and Iba1-positive microglia (Fig. 5B) in the tumors regardless of genotype. However, whereas the number and intensity of the GFAP- and NeuN-positive cells in tumors was comparable between wild-type and mutant mice, the morphology and intensity of the Iba1-stained cells were very different.

We noted that the TAMs in tumors arising in cyclin D1 knockout animals had a predominantly ramified appearance, with processes extending away from the cell body and into the adjacent stroma (Fig. 5B). This is typical of cells residing in a

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surveillance state (36). On the other hand, TAMs in tumors arising in wild-type mice were far more compact and ameboid, indicative of fully engaged and reactive cells (36). We then looked at the expression of the cathepsins X, H, and S, as well as the CSF1 receptor, additional markers associated with the activation of microglia (37–39). The intensity of each of these markers was reduced in the tumors arising in cyclin D1 knockout mice compared with those seen in wild-type mice (Fig. 5C). In addition, TAMs in gliomas arising in tumor-cell reconstituted cyclin D1 and cdk4 knockout mice in which the cyclin D1–cdk4 axis has been reconstituted in the tumor cell-of-origin were assessed for microglia activation by Iba1, cathepsin X, H and S and CSF1R immunohistochemistry. Activation status of TAMs: TAMs in cyclin D1 knockout gliomas express and secrete reduced levels of cathepsin X.

Figure 5. The absence of cyclin D1 does not affect the infiltration of stromal-derived cells into glial tumors but alters the activation state of TAM. A, tumors from wild-type and cyclin D1 knockout animals symptomatic for glioma were fixed in formalin and stained with antibodies to GFAP (reactive astrocytes; i and ii) and NeuN (neurons; iii and iv). B, TAMs, marked by Iba1 staining. C, gliomas arising in the cyclin D1 knockout mice and cyclin D1 and cdk4 knockout mice in which the cyclin D1–cdk4 axis has been reconstituted in the tumor cell-of-origin were assessed for microglia activation by Iba1, cathepsin X, H and S and CSF1R immunohistochemistry. Activation status of TAMs: TAMs in cyclin D1 knockout gliomas express and secrete reduced levels of cathepsin X.

Although there is a strong correlation between the activation of TAMs and tumor progression in this model as well as others (40–42), whether this is a consequence of tumor progression or contributes to tumor progression is not clear. To address this question, we attempted to carry out transplantation experiments. We isolated tumor cells from moderate-grade tumors arising in nestin-tvA;Ink4a/Arf knockout mice challenged by infection with RCAS-PDGF and transplanted these orthotopically into the brains of wild-type and cyclin D1 knockout recipients. Nine wild-type mice and 5 cyclin D1–deficient recipient animals were followed for 6 weeks and tumors isolated from morbid animals or at the end of the experiment. Although we found no difference in morbidity between wild-type and cyclin D1–deficient recipient animals, 7 of the 9 gliomas that arose in the wild-type animals were high-grade, and the resident TAMs were fully activated based on their morphology and staining with Iba1 and cathepsin X (Supplementary Fig. S5). On the other hand, 3 of the 4 tumors that arose in cyclin D1 knockout animals were moderate grade, and the activation of the TAMs appeared between the resting state observed in cyclin D1 knockout mice and the activated state seen in the wild-type mice (Supplementary Fig. S5). Because microglia are derived from resident cells of the central nervous system, and not hematopoietic cells infiltrating from the circulation (43, 44), we were unable to ask whether reconstituting cyclin D1–deficient animals with wild-type microglia was able to support tumor progression to higher states of malignancy in cyclin D1 knockout mice. However, on the basis of the outcomes above, we concluded
that cyclin D1 and cdk4 are required for maturation and activation of microglia, an event that contributes to the progression of this disease.

Discussion

Malignant gliomas remain an almost invariably destructive and rapidly lethal form of cancer, despite substantial progress in the development of new diagnostic and treatment modalities. It is clear that a more detailed understanding of the underlying molecular and cellular basis of this disease is required to identify critical new targets that might be amenable to therapeutic intervention.

The impact of the cyclin D1–cdk4 axis on tumor development and progression resides in its ability to phosphorylate the Rb tumor suppressor, which pushes cells through the G1–S phase boundary (13, 21, 24, 45). Deregulation of the cyclin D1–cdk4 cell-cycle axis is a common feature in glioma, with levels of proliferation increasing as tumors progress toward higher states of malignancy (46, 47). However, direct evidence that cyclin D1 and cdk4 can contribute to gliomagenesis has been lacking.

Using the RCAS-PDGF/nestin-tvA model of glioma, we found that both cyclin D1 and cdk4 are necessary for glioma development and progression. Whereas cdk4 knockout mice were completely refractory to glioma, tumors formed in cyclin D1 knockout animals. Reconstituting cyclin D1 or cdk4 expression in the nestin-positive progenitors that give rise to the tumors could increase the proliferation of the tumor cells in low-grade lesions; however, this was insufficient to support progression to higher grade. Likewise, the stroma of cyclin D1-deficient mice was unable to support progression of transplanted tumor cells. This tumor cell–independent stromal effect was correlated with a failure of the TAM to enter a strongly activated state, both by morphologic and immunohistochemical criteria. On the basis of these observations, we concluded that cyclin D1 and cdk4 have roles in both the tumor cell and in the development of the surrounding stroma.

The traditional view of cancer as an autonomously growing aggregation of mutant cells has been superseded by one in which the tumor acts more insidiously, actively subverting the surrounding tissue to support its growth and proliferation. This is especially true in glial tumors, in which development, progression, and, ultimately, invasion relies on extracellular cues derived from stromal cells, including astrocytes, neurons, and brain macrophages recruited into the growing tumor mass (30, 41). This dynamic interplay between tumor and stroma, coordinated by the tumor cells themselves, creates a permissive environment in which progression to malignancy is favored. For example, brain microglia stimulated by exposure to glioma cell conditioned media or infiltrating into tumor sites have been shown to actively secrete numerous factors, including immunosuppressive cytokines such as TGF-β, mitogens, cathepsins, and metalloproteinasases (42, 48, 49). Such microglia are polarized toward a phenotype that is progrowth and immunosuppressive, leading to a state in which tumor cell growth and diffusion throughout the brain parenchyma becomes more likely (50). All of these processes can contribute to the ability of glial tumor cells to grow and invade into surrounding tissue, while simultaneously avoiding clearance by immunologic sentinels in the brain (39, 51, 52). With TAMs comprising an active component of the tumor microenvironment, understanding the mechanisms that govern the responsiveness of these cells to external cues is important.

Our results suggest that cyclin D1–cdk4 can play 2 roles in the development of glioma: one, a tumor cell–autonomous role driving proliferation and the other, regulating the activity of nontumor cells in the microenvironment, which can also affect malignant progression. Microglia are known to show a burst of cyclin D1 and cdk4 expression that precedes activation and correlates with proliferation and migration into regions exhibiting damage to neural integrity (53, 54). Consistent with this, we found that cyclin D1 and cdk4 knockout TAMs exhibited a ramified morphology (characterized by multiple processes extending from the central cell body into the adjacent tissue) associated with a reduced state of activation. Furthermore, other markers of TAM activation, including cathepsin X, H, and S and CSF1R expression were reduced in knockout tumors relative to wild-type gliomas. Hence, in the absence of cyclin D1 or cdk4, TAMs may be unable to evolve into fully activated cells, which impedes progression to increased malignancy. How this occurs remains unclear. It is possible that cyclin D1 and cdk4 are required in TAMs or, alternatively, are required in other stromal cells which facilitate activation of TAMs. In vitro experiments on isolated cells and the use of specific genetically engineered mouse models may clarify this in the future.

Other investigators have independently shown that the cdk4 inhibitor drug, PD0332991, currently in phase II clinical trials, may be effective in halting the progression of glioma cell lines and xenografts. We are in the process of addressing whether PD0332991 can inhibit disease progression or reduce tumor burden in mice that have developed oligodendroglioma in situ and whether this is due to effects in the tumor cell, the microglia, or both. Current therapeutic strategies typically focus on direct inhibition of glial tumor proliferation and growth, even though compromising macrophage activity can also enhance chemotherapeutic efficiencies in other systems (reviewed in ref. 55). Thus, cdk4 inhibitor therapies may be useful in targeting both the tumor cell and modulating the activity of a stromal cell type that is critical to supporting malignant progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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