Optic Nerve Glioma in Mice Requires Astrocyte Nf1 Gene Inactivation and Nf1 Brain Heterozygosity

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Abstract

Whereas biallelic neurofibromatosis 1 (NF1) inactivation is observed in NF1-associated gliomas, astrocyte-restricted Nf1 conditional knockout mice do not develop gliomas. These observations suggest that NF1 glioma formation requires additional cellular or genetic conditions. To determine the effect of an Nf1 heterozygous brain environment on NF1 glioma formation, we generated Nf1+/− mice lacking Nf1 expression in astrocytes. In contrast to astrocyte-restricted Nf1 conditional knockout mice, Nf1+/− mice lacking Nf1 in astrocytes develop optic nerve gliomas. This mouse model demonstrates that Nf1+/− cells contribute to the pathogenesis of gliomas in NF1 and provides a tool for the preclinical evaluation of potential therapeutic interventions for these tumors.

Introduction

Low-grade astrocytomas (gliomas) are common central nervous system tumors affecting children (1). Children with the inherited tumor predisposition syndrome neurofibromatosis 1 (NF1) are prone to the development of WHO grade I pilocytic astrocytomas (PAs), which predominantly involve the optic nerve and chiasm (2). Although these tumors are low-grade neoplasms, they can demonstrate aggressive biological features, diffusely infiltrate the optic pathway and neighboring hypothalamus, and culminate in visual loss and precocious puberty (2). Previous studies have shown that NF1-associated optic pathway gliomas involve biallelic inactivation of the Nf1 gene, whereas histologically similar sporadic PAs do not exhibit NF1 loss (3–6). In addition, PAs do not harbor genetic changes typical of diffuse fibrillary astrocytomas, such as inactivation of CDKN2A, TP53, or PTEN/MMAC1 or amplification of CDK4 or the epidermal growth factor receptor gene (7).

Current animal models of astrocytoma have focused on oncogenic events (e.g., constitutive RAS activation) or on combinatorial inactivating events involving p53 (reviewed in Ref. 8). These mice develop high-grade gliomas, which are histologically and biologically distinct from those observed in NF1 patients. Using an alternate approach, heterozygous Nf1 mutant mice are cancer prone but do not develop gliomas (9). Because mice lacking Nf1 expression do not survive embryonic development (9, 10), we developed a conditional knockout mouse in which the Nf1 gene was inactivated in astrocytes by embryonic day 14 using Cre/loxP technology (11). Glial fibrillary acidic protein (GFAP) Cre; Nf1+/− mice are viable and fertile and exhibit increased numbers of brain and optic nerve astrocytes, but they do not develop gliomas. The absence of glioma formation in these mice, even after 20 months of age, suggests that additional cellular or genetic events are necessary for glioma tumorigenesis in the setting of NF1. Because patients with NF1 are heterozygous for a germ-line inactivating Nf1 mutation, and Nf1 mutant mice develop neurofibroma, another NF1-associated nervous system tumor, only in the setting of constitutional Nf1 heterozygosity (12), we generated Nf1+/− mice lacking Nf1 expression in astrocytes. In this report, we describe this unique model of NF1-associated optic nerve glioma, in which Nf1+/− mice with conditional Nf1 inactivation in astrocytes develop low-grade optic nerve and chiasm astrocytomas.

Materials and Methods

Transgenic Mice. Nf1+/− mice (10) were bred with Nf1+/− mice (13) to produce Nf1+/− mice, which were subsequently crossed with GFAPCre; Nf1+/− mice (11) to generate GFAPCre; Nf1+/− mice. Optic nerves and brains from 8–12-month-old GFAPCre; Nf1+/− mice (n = 12) and 8–18-month-old GFAPCre; Nf1+/− mice (n = 20) were analyzed. We used control mice of genotypes Nf1+/−, Nf1+/−, and Nf1+/− at 8–12 months of age (n = 10).

Histopathology and Immunohistochemistry. Control and mutant mice were perfused transcardially with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Eyes, optic nerves, optic chiasms, and brains were dissected and postfixed in 4% paraformaldehyde overnight at 4°C before examination for gross anatomical changes and photography with a digital camera (Optronics) attached to a dissection microscope (Nikon). All specimens were then processed for paraffin embedding and sectioning in the Pharmacology Histology Core or Ophthalmology Histology Core at Washington University School of Medicine.

Serial 4-μm paraffin sections of the eyes and optic nerve heads, optic nerves and chiasms, and brains were stained with H&E and examined under the microscope for the presence of tumors or abnormal collections of cells by an experienced neuropathologist (A. P.). Immunohistochemistry was performed on adjacent paraffin sections with rat anti-GFAP (1:100; Zymed) and rabbit anti-Ki67 (1:1000; Novocastra) antibodies. We used microwave antigen retrieval and detected the primary antibodies with biotinylated secondary antibodies, followed by amplification with peroxidase-conjugated avidin (Vectastain Elite ABC Kit; Vector Laboratories) and treatment with 3,3′-diaminobenzidine or Vector VIP substrate kits (Vector Laboratories). For microglia, we used rabbit anti-L-CAM (1:200; American Type Culture Collection) and goat anti-MOG (1:100; Santa Cruz Biotechnology) antibodies. We used fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse immunoglobulin (Jackson Immunoresearch Laboratories). Control sections were stained with fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse immunoglobulin (Jackson Immunoresearch Laboratories).

Optic nerve and chiasm sections were deparaffinized and rinsed in PBS, followed by overnight incubation at 4°C with FITC-conjugated BSI-B4 (10 μg/ml) in PBS containing 0.5% Triton X-100. After subsequent washes in PBS, fluorescence images were examined using a confocal microscope (Nikon).
were recorded by digital photomicrography (Spot Advanced; Diagnostic Instruments, Sterling Heights, MI).

Proliferative indices were determined by counting the number of Ki67-labeled nuclei per 100 cell nuclei within the prochiasmatic optic nerves (areas 1 and 2) and the optic chiasm (area 3), using a 10-mm ocular grid at ×400 magnification.

**Magnetic Resonance Imaging.** Images were collected in an Oxford Instruments 4.7-Tesla magnet (33 cm, clear bore) equipped with 15-cm inner diameter, actively shielded gradient coils (maximum gradient, 18 G/cm; rise time, 100 μs). The magnet/gradient are interfaced with a Varian (Palo Alto, CA) INOVA console, and data were collected using a 1.5-cm outer diameter surface coil (receive) and a 9-cm inner diameter Helmholtz coil (transmit). Before the imaging experiments, the mice were anesthetized with isoflu-rane/O2 [4% (v/v)], and they were maintained on isoflurane/O2 [1.5% (v/v)] throughout the experiments. Diffusion tensor imaging data were acquired using a conventional spin-echo imaging sequence, modified by the addition of a Stejskal-Tanner diffusion-sensitizing gradient pair. Six images with different gradient directions were acquired with a b value of 785 s/mm², together with a reference spin-echo (b = 0) image. Other experimental parameters were 16 signal averages, time between scans (TR) = 0.75 s, echo time (TE) = 0.05 s, slice thickness = 0.5 mm, field of view (FOV) = 1.5 cm.

The diffusion tensor matrix was generated from a series of diffusion-weighted images, in which the primary diffusion parameters (three eigenvalues or diffusivities, λ₁, λ₂, and λ₃) were calculated by matrix diagonalization. These primary parameters were combined into two secondary parameters useful for describing water diffusion (15): (a) apparent diffusion constant (the mean of the three directional diffusivities); and (b) relative anisotropy (the normalized standard deviation of the three diffusivities). These parameter maps, together with conventional T2-weighted spin-echo images, were used to visualize the mouse optic nerves.

**Results and Discussion**

*Nf1* Inactivation in Astrocytes Alone Is Insufficient for Astrocytoma Formation. Whereas both *Nf1*+/− and GFAPCre; *Nf1*flx/flx mice exhibit increased numbers of brain astrocytes (11, 16, 17), neither strain develops astrocytoma *in vivo*. Similarly, *Nf1*−/− astrocytes have an incremental increase in cell-autonomous growth *in vitro* compared with *Nf1*+/− astrocytes, but they lack tumorigenic properties (11). Because our GFAPCre mice (both lines 1 and 8) exhibit robust Cre expression in astrocytes throughout the optic nerve and chiasm (data not shown), in addition to the brain and spinal cord (11), we sought to determine whether *Nf1* inactivation in optic nerve astrocytes was sufficient for optic nerve/chiasm tumor formation. Compared with *Nf1*+/− mice, GFAPCre; *Nf1*flx/flx mice demonstrate complete loss of neurofibromin expression in astrocytes (11), increased astrocyte numbers in the optic nerve, and minor increases in astrocyte proliferation. However, examination of the optic nerves from both *Nf1*+/− and GFAPCre; *Nf1*flx/flx mice failed to demonstrate any gross or microscopic evidence for optic nerve glioma. These results demonstrate that *Nf1* inactivation in astrocytes, beginning on embryonic day 14, confers a growth advantage *in vitro* and *in vivo* that is not sufficient for astrocytoma formation in the optic nerve and raise the possibility that constitutional *Nf1* heterozygosity in the supporting nonneoplastic cells is required for tumorigenesis.

*Nf1* Inactivation in Astrocytes Results in Optic Nerve/Chiasm Glioma Formation in the Context of *Nf1* Heterozygosity. In an effort to model the human condition, where patients are heterozygous for an inactivating germ-line *Nf1* mutation, we first injected *Nf1*-null postnatal day 2 neocortical astrocytes, generated by Cre adenovirus-mediated *Nf1*flx inactivation, into the brains of syngeneic *Nf1*+/− mice. None of the 22 mice that received the injection exhibited any evidence of glioma formation after 1 year (data not shown).

Second, we generated *Nf1*+/− mice lacking *Nf1* expression in astrocytes (GFAPCre; *Nf1*flx/flx mice). These mice are viable and healthy at 12 months of age, without obvious neurological abnormalities. Pathological examination of their brains failed to demonstrate astrocytoma (data not shown). These results suggest that *Nf1* inactivation, either alone or in combination with *Nf1* heterozygosity, does not result in parenchymal brain tumor formation.

Because gliomas in NF1 most typically involve the optic nerve, chiasm, and hypothalamus, we examined the optic nerves and chiasm in GFAPCre; *Nf1*flx/flx mice. All mice examined (n = 12) demonstrated areas of gross optic nerve and/or chiasm enlargement, which were not seen in either *Nf1*+/− or GFAPCre; *Nf1*flx/flx mice. These gross abnormalities were seen in mice as early as 8 months of age, although younger mice have not been systematically examined to date. We observed multiple patterns of gross pathology, including unilateral as well as bilateral optic nerve enlargement, with and without chiasmal involvement (Fig. 1). These patterns are highly reminiscent of those found in children with NF1.

Microscopic examination of these optic nerve masses demonstrated increased cellularity (Fig. 2, *top panel, A–G*) with collections of GFAP-immunoreactive astrocytes (Fig. 2, *top panel, I–K*). In addition to exhibiting elevated Ki67 proliferative indices (Fig. 3A, *a–c*), these proliferating individual or clustered cells exhibited enlarged, mildly to moderately atypical, hyperchromatic nuclei with small nucleoli, similar to their human counterparts (Ref. 1; Fig. 2, *top panel, D, H, and L*). As is typical for low-grade gliomas, there was no evidence of endothelial hyperplasia or necrosis. However, unlike human optic pathway PAs, these mouse gliomas lacked microcystic spaces, Rosenthal fibers, or eosinophilic granular bodies and shared some histological features with WHO grade II fibrillary astrocytomas. It should be noted that even within human NF1-associated PAs, portions of the tumor may be infiltrative, lack Rosenthal fibers, eosinophilic granular bodies, and protein droplets; and can be confused with low-grade WHO II fibrillary astrocytoma (18). In this respect, human pilocytic optic gliomas most often appear as glial tumors with alternating dense-spindled and loose microcystic...
areas (Fig. 2, bottom panel, A) with Rosenthal fibers and granular bodies (Fig. 2, bottom panel, B), but they may exhibit nuclear pleiomorphism (Fig. 2, bottom panel, C) with clustered atypical tumor nuclei (Fig. 2, bottom panel, D), reminiscent of the Nf1 mouse model. Therefore, it is difficult to determine with certainty whether the optic gliomas in our mouse model represent low-grade fibrillary astrocytomas, an otherwise rare subtype in NF1 patients, or an earlier stage of PA, lacking some of the more characteristic findings seen in human OPTIC NERVE GLIOMA IN Nf1 MUTANT MICE

Fig. 2. Histological analysis of optic pathway glioma in the GFAPCre; Nf1flox/mut mice (top panels). H&E staining (H&E) demonstrates enlarged optic nerves with areas of increased cellularity (C and G) in GFAPCre; Nf1flox/mut mice, compared with Nf1flox/flox (A and E) or GFAPCre; Nf1flox/flox mice (B and F). The areas of increased cellularity in the optic nerves of GFAPCre; Nf1flox/mut mice are highly enriched in GFAP-immunoreactive astrocytes (K), compared with control mice (J). GFAPCre; Nf1flox/mut mice exhibit moderate increases in astrocyte numbers in the chiasm and optic nerve (J). Many atypical nuclei were found clustered within the hypercellular optic nerve masses of GFAPCre; Nf1flox/mut mice. These atypical nuclei (arrows) are significantly larger than nonneoplastic cell nuclei (arrowheads), have an irregular contour, and are hyperchromatic (D and H). Mildly atypical nuclei (arrows) appear rarely in association with normal cell nuclei (arrowheads) in optic nerves from GFAPCre; Nf1flox/mut mice (L), and may represent reactive or hyperplastic astrocytes. Scale bar, 100 μm. Bottom panels: human pilocytic optic nerve gliomas typically appear as astrocytic tumors with alternating dense spindled and loose microcystic areas (A) with scattered brightly eosinophilic rod-shaped Rosenthal fibers and mulberry-shaped eosinophilic granular bodies (B), but may exhibit mild hypercellularity and nuclear pleiomorphism (C) with scattered individual and clustered atypical tumor nuclei (D) at the infiltrative edge of the same tumor, reminiscent of the Nf1 mouse model. Magnification, ×200 (A) and ×400 (B–D).

Fig. 3. Optic gliomas in GFAPCre; Nf1flox/mut mice contain proliferating astrocytes. A, Ki67 immunoreactivity was detected in clusters of cells corresponding to areas of increased cellularity, in prechiasmatic regions as well as along the optic nerves and in the chiasm of GFAPCre; Nf1flox/mut mice. The arrow points to a representative mitotic figure (b). The Ki67-labeled cells are GFAP-immunoreactive astrocytes as demonstrated by Ki67/GFAP double immunohistochemistry (c). No Ki67-labeled nuclei were identified in the Nf1flox/flox control mice (a). Scale bar, 400 μm. B, the Ki67 proliferative indices were tabulated for each optic nerve and chiasm from GFAPCre; Nf1flox/mut, GFAPCre; Nf1flox/flox, Nf1flox/mut, and Nf1flox/flox mice. C, Ki67 proliferative indices were determined in the prechiasmatic optic nerves (areas 1 and 2) and in the chiasm (area 3), denoted by the blue squares. D, microglia cells infiltrate the low-grade gliomas in GFAPCre; Nf1flox/mut mice. B. simplicifolia isoelectin (BSI-B4) histochemistry identifies microglial cells (arrowheads) in the optic nerve areas (primarily optic nerve and chiasm), corresponding to the gliomas in GFAPCre; Nf1flox/mut mice. In contrast, there were few, if any, microglia in the optic nerves of control mice. Nf1flox/mut and GFAPCre; Nf1flox/flox. V, vessel. Scale bar, 100 μm.
examples that had grown for several years before resection. Nevertheless, we have classified these collections of GFAP-immunoreactive astrocytes as neoplastic, based on their formation of grossly and radiologically (see below) recognizable tumor masses, the presence of nuclear clustering/atypia, and demonstrable cellular proliferation.

Because these tumors grow asymmetrically within the optic nerves in these mice, we determined the proliferative index for the prechiasmatic optic nerves (areas 1 and 2) and chiasm (area 3; Fig. 3C) for each of eight tumors. As shown in Fig. 3B, there was a spectrum of increased proliferative indices in GFAPCre; Nf1flox/mut mouse optic pathway tumors, ranging from 1% to 10%, using Ki67 as a proliferation marker, as has been reported for human low-grade astrocytomas (18). The highest proliferation rates were consistently observed in older GFAPCre; Nf1flox/mut mice (>10 months old). Lower proliferative indices were observed in GFAPCre; Nf1floxflox and Nf1+/− mice (1−2%), even after 18 months of age. No Ki67 labeling was observed in the optic nerve or chiasm of control Nf1floxflox mice.

Previous studies on another nervous system tumor in NF1, the peripheral nerve-associated neurofibroma, demonstrated that Nf1+/− mast cells infiltrate tumors composed of neoplastic Nf1−/− Schwann cells and likely contribute to the genesis of these benign tumors (12). In human optic nerve glioma, microglia are often observed at the periphery and within the tumor (19). To determine whether infiltrating Nf1+/− microglia were found in GFAPCre; Nf1flox/mut mouse optic nerve tumors, we performed lectin histochemistry using B. (Griffonia) simplicifolia agglutinin isolein-B4 (BSI-B4) to identify microglia. BSI-B4 labels α-galactose residues that are expressed on both resting and activated microglia cells, cells of the monocyte-macrophage lineage, and vascular endothelial cells. We observed increased numbers of microglia in the GFAPCre; Nf1flox/mut mouse optic nerve tumors compared with nonneoplastic optic nerve areas in these same mice or GFAPCre; Nf1floxflox; Nf1+/−, or Nf1floxflox mouse optic nerves. As reported for human low-grade gliomas (20), the microglia infiltration was variable from one optic nerve glioma to another in GFAPCre; Nf1flox/mut mice (Fig. 3D). Whereas microglia are associated with these mouse optic nerve gliomas, it remains to be determined whether Nf1+/− microglia, oligodendrocytes, or neurons directly contribute to the pathogenesis of Nf1-null astrocyte transformation.

Relevance of Nf1 Heterozygosity to NF1 Tumorigenesis. Recent studies by Zhu et al. (12) have demonstrated a role for Nf1+/− cells in the molecular pathogenesis of a related Nf1-associated tumor, the plexiform neurofibroma. In this model, Nf1+/− mast cells are hypothesized to elaborate factors that promote Nf1−/− Schwann cell transformation and neurofibroma formation. Studies on Nf1+/− mast cells (21), oligodendrocytes (22), and astrocytes (16, 17) have demonstrated that Nf1 heterozygosity results in abnormalities in cell migration, proliferation, and survival. These observations strongly support the emerging concept that Nf1+/− cells have unique properties, which might be important in the cellular pathogenesis of Nf1-associated tumors.

Development of a Preclinical Model for NF1-Associated Optic Nerve Glioma. All mice examined to date have gross and histopathological evidence of optic nerve glioma by 8 months of age, suggesting that this mouse might represent a preclinical model for NF1-associated optic pathway tumors. Because these tumors are routinely detected and followed by magnetic resonance imaging in children with NF1, we sought to determine whether small animal magnetic resonance imaging could detect abnormal optic nerve pathology in the living mouse. Diffusion tensor imaging was used to delineate tissue morphology and pathology (23), as has been used for human NF1-associated optic nerve gliomas (24). Using diffusion tensor imaging, we determined the location and size of the optic nerves in C57Bl/6 control, Nf1floxflox (data not shown), GFAPCre; Nf1floxflox, and GFAPCre; Nf1flox/mut mice, and we demonstrated the presence of abnormal tissue between the optic nerves only in GFAPCre; Nf1flox/mut mice (four of four mice imaged; age, 10–12 months; Fig. 4). Studies are in progress to determine the earliest time point when these tumors can be detected by magnetic resonance imaging.

In this report, we describe a mouse model of optic nerve glioma, the most common brain tumor seen in individuals affected with NF1. Whereas astrocyte Nf1 inactivation is insufficient by itself for glioma formation, in the context of an Nf1 heterozygous environment, optic nerve gliomas develop. These observations extend previous results on Nf1 mouse modeling of nervous system tumors and strongly implicate Nf1+/− cells in the cellular pathogenesis of NF1 brain tumor formation. Future studies aimed at defining the contributing Nf1+/− or Nf1−/− cells to glioma formation as well as characterizing the factors that promote Nf1−/− astrocyte transformation will lead to an improved understanding of the cellular and molecular events involved in the development of this benign tumor in patients with NF1.

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References


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