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NG2-expressing glial precursor cells are a new potential oligodendroglioma cell initiating population in *N*-ethyl-*N*-nitrosourea-induced gliomagenesis

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Gliomas are the most common primary brain tumor affecting human adults and remain a therapeutic challenge because cells of origin are still unknown. Here, we investigated the cellular origin of low-grade gliomas in a rat model based on transplacental exposure to *N*-ethyl-*N*-nitrosourea (ENU). Longitudinal magnetic resonance imaging coupled to immunohistological and immunocytochemical analyses were used to further characterize low-grade rat gliomas at different stages of evolution. We showed that early low-grade gliomas have characteristics of oligodendroglioma-like tumors and exclusively contain NG2-expressing slow dividing precursor cells, which express early markers of oligodendroglial lineage. These tumor-derived precursors failed to fully differentiate into oligodendrocytes and exhibited multipotential abilities *in vitro*. Moreover, a few glioma NG2+ cells are resistant to radiotherapy and may be responsible for tumor recurrence, frequently observed in humans. Overall, these findings suggest that transformed multipotent NG2 glial precursor cell may be a potential cell of origin in the genesis of rat ENU-induced oligodendroglioma-like tumors. This work may open up new perspectives for understanding biology of human gliomas.

Introduction

Glioma represents the most common and heterogeneous group of brain tumors emerging in adult human central nervous system. These tumors are traditionally classified according to their histopathology and the cell type markers they express and lead to a complex classification theme. Nevertheless, glioma can roughly be subdivided into three major subgroups of different grades: (i) astrocytomas: pilocytic (grade I), diffuse (grade II), anaplastic (grade III) and glioblastoma multiform (grade IV), (ii) oligodendrogliomas (grade II and grade III) and (iii) mixed oligoastrocytomas. This diversity of glioma correlates with a wide spectrum of mutated or affected cell signaling pathways observed in these neoplasms and also probably due to their different 'cell of origin' (1). Moreover, in many cases, tumors that share a similar morphology and phenotype may have very different prognoses and responses to therapies. This underlines the need to redefine the criterions of the tumor classification, which now will have to deal not only with a molecular and cellular analysis of the tumor bulk but also on a better understanding of how tumors emerge, evolve over time and what cell types they arise from. Several recent studies have identified a tumorigenic CD133+ subpopulation of cancer cells regarded as tumor stem cells within glioblastoma multiform responsible for the initiation and development of those heterogeneous aggressive brain tumors (2–5). The presence of cancer stem cells in glioblastoma multiform may also explain the resistance of these tumors to radiotherapy and chemotherapy (6). In contrast to glioblastomas, oligodendrogliomas are more sensitive to radiotherapy and alkylating agent chemotherapy (7,8). This variable therapeutic responsiveness has raised the hypothesis that oligodendroglioma and glioblastoma may have distinct origins. Transgenic mouse and rat models using controlled ectopic expression of oncogenes in either precursor or maturing astrocytes have been used to model human glioblastoma, astrocytomas, oligodendrogliomas or oligoastrocytomas, depending on the oncogenic stimuli (9–16). These studies highlight the potential genetic mutations, which could be responsible for tumor initiation and development. On the other hand, recent studies have shown that signals from environment can also initiate and mediate tumor growth, as nestin-positive neonatal neural progenitors retrovirally overexpressing platelet-derived growth factor (PDGF)-B generate pure oligodendroglioma, whereas targeting GFAP-expressing glial progenitors gives rise to several types of tumors (17,18). In order to complement these genetically induced tumor studies, we here investigated the cell of origin of low-grade glioma in a far known rat model which spontaneously develops glioma in response to transplacental exposure to the DNA alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) (19–23). ENU-induced tumors have morphological and biological features similar to human glioma (22,24). ENU-induced tumors mimic the evolution and development of a brain tumor upon a sporadic exposure to carcinogens and neoplasms appear after several months of latency, thus helping with the understanding of early events occurring during gliomagenesis. In this study, we performed longitudinal magnetic resonance imaging (MRI) analysis of ENU-induced glioma over a period of 11 months combined with full characterization of tumor cells at different developmental stages. We identified a new slow dividing NG2 cell population, expressing early markers of oligodendroglial lineage and compromised in their differentiation, in low-grade glioma. *In vitro*, these cells exhibited multipotential abilities. In postnatal/adult brain, NG2-expressing cells are strongly associated with fast dividing oligodendrocyte progenitor cells (OPC) (25). However, heterogeneity of NG2+ cell population has been demonstrated in several occasions. Bouslama-Oueghlani *et al.*(26) provided evidence that two types of NG2+ cells are present in developing brain, one highly proliferating and able to myelinate axons and one almost quiescent with a reduced ability to produce myelin. Recent studies have also demonstrated that early postnatal NG2$^+$ cells are able to form passageable neurospheres and to differentiate into neurons *in vitro* (27) as well as to contribute to postnatal neurogenesis in developing hippocampus and olfactory bulb (28,29). We also previously found that NG2 marker is expressed in multipotent neurospheres derived from adult subventricular zone (SVZ) (30). We propose a potential contribution of these multipotent NG2+ glial precursors in low-grade gliomagenesis and recurrence. These findings may help to understand several features observed in human oligodendroglioma and to design new therapies to cure glioma.

Abbreviations: bFGF, basic Fibroblast Growth Factor; CNTF, ciliary neurotrophic factor; ENU, *N*-ethyl-*N*-nitrosourea; GFAP, glial fibrillary acidic protein; MRI, magnetic resonance imaging; OPC, oligodendrocyte progenitor cells; PDGF, platelet-derived growth factor; RMS, rostral migratory stream; SVZ, subventricular zone.

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Materials and methods

Antibodies

The following primary antibodies were used: IQGAP1 (H109, rabbit, 1/500^e, Santa Cruz Biotechnology, Inc., Tebu-bio, France), Nestin (clone rat-401, mouse, 1/500^e, Developmental Studies Hybridoma Bank, Iowa City, IA), NG2 (mouse or rabbit, 1/500^e, Upstate, Euromedex, France), Olig2 (rabbit, 1/500^e, a generous gift from Dr. H. Chneiweiss, INSERM U114, Paris), GFAP (rabbit, 1/500^e, Dako Cytomation, S.A, France), Sox10 (rabbit, 1/500^e, a generous gift from Dr Wegner, Erlangen University, Germany), Nkx2.2 (mouse, 1/100^e, Developmental Studies Hybridoma Bank, Iowa City, IA), A2B5 (mouse, 1/3, home made hybridoma production), O4 (mouse, 1/3, home made hybridoma production), βIII-tubulin (clone Tuj-1, rabbit, 1/1000^e, Eurogentec, France), Ki67 (mouse, 1/100^e, Novocastra Laboratories Ltd., AbCys, S.A, France), Collagen IV (goat, 1/1000^e, Southern Biotech, Birmingham, AL). Secondary antibodies conjugated to cyanin 3 or cyanin 5 were from Jackson Laboratories (Interchim, France), secondary antibodies conjugated to Alexa Fluo 488 were from Molecular Probes (Invitrogen, France) and biotinylated anti-goat secondary antibody was from Southern Biotech (Birmingham, AL).

Chemo-induced tumorigenesis

All procedures on animals were approved by the Rhône-Alpes Committee for Animal Experimentation Ethics (CREEA). OFA Sprague–Dawley pregnant rats were obtained from Charles River Laboratories (France). Pregnant rats were injected intravenously via the tail vein with 60 mg/kg Ethylnitrosourea (Sigma–Aldrich) at gestational day E19. Five independent ENU injections were done over a period of 3 years, including two to four pregnant animals per experiment.

MRI of rat brains

MRI images were obtained with a 2.35T magnet using a volume coil for transmission and a surface coil for reception. Anesthetized rats were placed in a cradle and their head was maintained with ear bars and a bite bar. For each animal, 20 contiguous 1 mm thick slices were acquired using a T2-weighted spin echo sequence (TR/TE = 2000/80 ms, matrix = 256 × 192 or 128 × 128, two accumulations, Field of View = 30 × 30 mm^2).

Tumor growth profile

Tumor volume was calculated using Image J software. Tumor surface (millimeter square) was measured on MRI images according to MRI resolution. Tumor volume was calculated by analyzing the surface of the tumor on successive MRI slices multiplied by slice thickness (1 mm). Two-way analysis of variance statistical analysis was done using Sigma Stat software. Holm–Sidak tests were used for *post hoc* multiple comparisons.

Immunohistochemistry/immunofluorescence

Animals (3–12 months old) were deeply anesthetized with 5% isoflurane and killed by decapitation. Brains were immediately frozen in isopentane at −80°C. Cryosections (10 μm) were cut and postfixed in 4% paraformaldehyde. Sections were colored with hematoxylin and eosin for histological analysis. Alternatively, cryosections were permeabilized in Tris-buffered saline-0.2% Triton and blocked in Tris-buffered saline-5% goat serum. After overnight incubation with primary antibodies, sections were stained with secondary antibodies and counterstained with nuclear marker Hoechst 33258 (1 μg/ml) when desired. Images were obtained with a Carl Zeiss Axiovert 200M microscope and with a Leica (TCS SP2) confocal microscope.

Cells or glioma explants were fixed using 4% paraformaldehyde and procedures were the same as brain slices except that incubation times for primary and secondary antibodies were reduced to 1 h each. Antibodies for cell surface markers, such as A2B5 and O4, were added to culture medium for 1 h before cell fixation.

Oligodendroglioma-derived cell culture

After MRI localization, tumors were excised from freshly dissected rat brains. They were cut in explants and plated onto poly-L-lysine-treated glass coverslips, either in proliferation medium with 10 ng/ml PDGF and 1 μg/ml bFGF or in differentiation medium where PDGF and bFGF were replaced by 30 ng/ml T3, 5 ng/ml CNTF and 5 μm Forskolin [as described in Deloulme *et al.* (41) and Kondo and Raff (29)]. After 4–8 days in culture, cells were fixed in 4% paraformaldehyde. Additionally, a piece of the tumor was frozen in isopentane at −80°C immediately after dissection for immunohistological characterization.

Rat brain tumor irradiation

Irradiation was performed at the Biomedical beamline of the European Synchrotron Radiation Facility. Irradiation was performed with monochromatic synchrotron radiation, emitted from a wiggler multipole magnet. The energy used was 80 keV with a bandwidth of $\Delta E/E = 10^{-3}$. The dose delivered to tumors was 15 Gy. The irradiated volume covered a whole hemisphere and it was performed in tomotherapy mode, i.e. rotating the animal in the beam. The animals were anesthetized for irradiation and held vertically in a plastic frame, which kept their heads fixed. Beam dimensions were 1 mm vertical and 10 mm horizontal. Therefore, multistage irradiations were performed to cover the total volume of the brain hemisphere, typically 15 mm in length and 10 mm in diameter.

Results

Spatial and temporal emergence and development of rat chemically induced brain tumors

In this study, we used a well-established experimental model of brain tumors in which rats exposed *in utero* to a single dose of a mutagen, the ENU preferentially develop brain tumors (19,22,24). This model has also the advantage of retracing basic events of carcinogenesis following a random exposure to mutagens or chemicals. The latency before appearance of brain tumor enables to address early events occurring in brain tumor genesis. Moreover, the drug is rapidly degraded *in vivo* thus avoiding any side effects caused by inflammatory response (31). Six to eleven months after exposure, some exposed animals exhibited severe neurological symptoms and the postmortem examination revealed the presence of peripheral tumors and/or highly invasive brain tumors. These animals were no further investigated. We focused on exposed rats, which showed no pathological evidence of tumor formation, hypothesizing that these might represent earliest stages in tumor occurrence. Longitudinal T2-weighted MRI along with measurements of water diffusion (i.e apparent diffusion coefficient) was used to visualize the spatial and temporal emergence of asymptomatic brain tumors. Several brain tumors within a single animal were frequently observed. At 6 months of age, asymptomatic brain tumors showed sizes ranging between 250 and 1200 μm^3 and exhibited a homogenous hypersignal on T2-weighted scans (Figure 1a–d). Subsequently, 4 to 5 months later (i.e at 10-11 months of age) ENU-induced tumors evolved in different ways and allowed us to distinguish 3 tumor subtypes (Types 1-3) according to MRI pictures (Figure 1 a–d), tumor growth profiles (Figure 1d) and mitotic index (Figure 1e). Type 1 glioma (67%) and Type 2 glioma (17.5%) maintained a homogenous hypersignal on T2-weighted scans (Figure 1a–b). Type 1 gliomas were characterized by a linear growth over time with a doubling volume every 2 to 3 months (Figure 1d). Type 1 gliomas were mainly located in the corpus callosum (CC) (45%) and in germinative areas (30%) including the anterior subventricular zone and the rostral migratory stream (Supplemental Figure 1a). Type 2-induced glioma followed the same diagram (Figure 1b) but grew almost 3 times faster than Types 1 to increase 10-fold in size over a period of 5 months (Figure 1d). Type 2 gliomas were predominantly located in the corpus callosum (29%) and the cortex (33%) (Supplemental Figure 1b). Type 3 gliomas (15%) adopted a 2 phase-pattern, with a slow growth for the first 9 months followed by an exponential growth profile (Figure 1d). MRI analysis of Type 3 tumors revealed heterogenous T2-weighted images which ended up with a large hypersignal in tumor cores, surrounded by a hyposignal ring. Type 3 gliomas were predominantly observed in the cortex (67%) (Supplemental Figure 1c). In agreement with their growth profiles, 11 months after ENU exposure, Type 3 gliomas have the highest mitotic index and Type 2 gliomas present 3 times more Ki67+ cells compared to Type 1 neoplasms (Figure 1e).

Histological characterization showed that Type 1 tumors have homogenous cellular density within tumor bulks with a normal vascular network (Figure 1f). Type 2 neoplasms exhibited a mixed population of normal rounded shape and hyperchromatic nuclei with an increased density of blood vessels (Figure 1g). Type 3 gliomas exhibit malignant features with a large necrosis along with a very high developed vasculature (Figure 1h).

Slowly proliferating rat glioma display features of oligodendroglioma

We have shown previously that the most aggressive rat glioma (Type 3) has histological and immunophenotypic characteristics that resemble human glioblastoma (5). Both rat and human glioblastoma are characterized by niches of tumorigenic cells, which express nestin