Animal Model

Astrocytes Give Rise to Oligodendrogliomas and Astrocytomas after Gene Transfer of Polyoma Virus Middle T Antigen *in Vivo*

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The cells of origin for oligodendrogliomas and astrocytomas are not known but are presumed to be oligodendrocyte and astrocyte precursors, respectively. In this paper we report the generation of mixed gliomas from *in vivo* transformation of glial fibrillary acidic protein (GFAP)-positive cells (differentiated astrocytes) with polyoma virus middle T antigen (MTA). MTA is a powerful oncogene that activates a number of signal transduction pathways, including those proposed to be involved in gliomagenesis, and has been shown to induce tumors in many cell types. We have achieved transfer of MTA expression specifically to GFAP⁺ cells *in vivo* using somatic cell gene transfer, and find resultant formation of anaplastic gliomas with mixed astrocytoma and oligodendroglioma morphological features. We conclude that GFAP-expressing astrocytes, with appropriate signaling abnormalities, can serve as the cell of origin for oligodendrogliomas, astrocytomas, or mixed gliomas. (Am J Pathol 2000, 157:1031–1037)

The most common forms of human infiltrating gliomas fall into two morphological variants, astrocytomas and oligodendrogliomas.¹ Gliomas that exhibit a combination of these morphologies, oligoastrogliomas or mixed gliomas, also occur and are being diagnosed with increasing frequency. Both oligodendrogliomas and astrocytomas vary in grade, with tumors of lower grade tending to progress to higher grades over time. The most aggressive of these, grade D oligodendroglioma and glioblastoma multiforme, respectively, have the worst prognoses and may be dif-

ficult to distinguish histologically. Analysis of specific mutations has been more complete for astrocytomas than for oligodendrogliomas, but chromosomal studies have shown differences between these two lesions, with astrocytomas tending to have deletions on chromosomes 10 and 17 and oligodendrogliomas frequently having deletions on chromosomes 1 and 19.² Preliminary limited genetic analyses of mixed oligoastrocytomas suggest that some of these tumors may be purely oligodendroglial or astrocytic despite the mixed phenotypic appearance, whereas others may be true mixed-lineage tumors. From these data it is not clear whether these two morphologies represent different diseases that arise from different cell types or two ends of a spectrum of differentiation. The cells of origin for any of the gliomas are not known.

To investigate the ability of these two tumor morphologies to arise from a single cell type, we used the RCAS/tv-a system, which allows cell type-specific gene transfer in mice. We have previously reported this system for glia-specific gene transfer *in vivo*, which allows us to investigate the effects of both individual mutations and combinations of mutations on gliomagenesis in mice.³ This system utilizes replication-competent avian leukosis virus (ALV) splice acceptor (RCAS) viral vectors, which are derived from the avian retrovirus, ALV subgroup A, and a transgenic mouse line (Gtv-a) that produces TVA (the receptor for ALV-A) from the astrocyte-specific promoter for the gene encoding glial fibrillary acidic protein (GFAP; Figure 1). GFAP-expressing astrocytes from Gtv-a mice are susceptible to infection and gene transfer by RCAS vectors both *in vivo* and *in vitro*. Gene transfer is most efficient in this system when avian viral producer cells are injected intraparenchymally, which not only places producer cells in close proximity to astrocytes, but also induces astrogliosis with increased expression of GFAP, and therefore tv-a. These cells survive for a few

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Figure 1. *Gtv-a* transgenes and RCAS-MTA. **A:** Transgene expressing the ALV subgroup A receptor, TVA from the GFAP promoter. MP-1 is a portion of the mouse protamine gene that provides an intron and polyA site. **B:** RCAS vector carrying polyoma virus middle T antigen. The spliced message expressing the MTA sequence is illustrated below.

days and infect adjacent TVA-expressing astrocytes. We have used this system to show that infection of GFAP⁺ cells with RCAS carrying the coding sequence for basic fibroblast growth factor causes glia to proliferate, migrate over long distances, and assimilate into the normal brain structure without tumor formation.³ We have also previously demonstrated that gene transfer of a constitutively active form of the EGFR can cooperate with mutations that disrupt cell cycle arrest pathways to induce glioma-like lesions in mice.⁴ In this study we describe the use of this system to transfer expression of the potent viral oncogene, middle T antigen (MTA), specifically to GFAP-expressing cells in postnatal mice.

MTA has been shown to activate a number of signal transduction pathways. These pathways are also activated by the tyrosine kinase receptors—endothelial growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and IGFR—that have been shown to be activated in gliomas. MTA stimulates Shc (leading to Ras activation), PI3 kinase (leading to Akt activation), and Src^{5–7} and may activate other pathways as well (Figure 2). MTA expression has been shown to result in the formation of a number of tumor types in

Figure 2. Signaling pathways downstream of polyoma virus middle T antigen. Some of the known signal transduction pathways activated by EGFR and middle T antigen are diagramed above. Additional pathways may be activated by this oncogene product, as illustrated.

transgenic mice, including hemangiosarcomas,⁸ primary bone tumors,⁹ neuroblastomas,¹⁰ and salivary,¹¹ mammary,¹² and thyroid carcinomas.¹³ Infection of rat embryo brain cultures with viral vectors carrying MTA resulted in cells causing rapidly fatal hemangiosarcomas when injected into nude mice, possibly due to gene transfer to endothelial cells or secondary to effects of MTA on progenitor cell types found in the embryonic brain.¹⁴

In this study we have used MTA expression to achieve neoplastic transformation of astrocytes *in vivo*, and find that MTA gene transfer to GFAP⁺ cells gives rise to anaplastic mixed gliomas but not glioblastomas. The morphological features of these lesions have many similarities in common with human gliomas, including the ability to display both oligodendroglial and astrocytic phenotypic characteristics. We conclude that sufficient stimulation of signaling pathways in GFAP⁺ cells can give rise to tumors with both astrocytic and oligodendroglial character, and, therefore, astrocytes have the potential to act as cells of origin for both of these classical glioma histologies.

Methods

Constructs

The *Gtv-a* transgene is a 2.2-kb fragment of the GFAP promoter driving expression of the quail *tv-a* cDNA and a fragment from the mouse protamine gene (MP-1) supplying an intron and signal for polyadenylation.

RCAS-MTA, the gene encoding polyoma middle T antigen, was excised as a 1.4-kb *Bam*HI fragment from pUHD-MT (a gift from Michele Fluck, Michigan State University), blunted with the Klenow fragment of T4 DNA polymerase, and ligated into RCAS-Y (RCAS with a linker DNA containing *Not*I-PmeI-PacI inserted in place of the *Cl*aI cloning site) that had been linearized with PmeI.

Mice

Production of the *Gtv-a* mouse line has been described.³ The *Gtv-a* mouse line was originally generated from an FVB/N crossed with a C57B6 X BALB/c F1. The *Gtv-a* founder was then bred to an FVB/N to generate F1 progeny that have subsequently been interbred to maintain the transgenic line. The genetic backgrounds of the *tv-a* transgenic mice used for infection were therefore mixes of FVB/N, 129, and C57BL6.

Cell Culture

DF-1 cells, an immortalized line of chicken cells, were a generous gift from Doug Foster of the University of Minnesota¹⁵ and were grown in Dulbecco's modified Eagle's medium with 5% fetal calf serum, 5% calf serum, 1% chicken serum, and 10% tryptose phosphate broth (Gibco BRL). DF1 cells transfected and infected with RCAS-MTA show clear transformation with a rounded, refractile appearance.

Infection of Transgenic Mice

DF-1 cells infected with and producing RCAS vectors were harvested by trypsin digestion and pelleted by centrifugation. The cell pellets were resuspended in approximately 50 μ l of medium and placed on ice. Using a 10- μ l gas-tight Hamilton syringe, a single intracranial injection of 1 μ l containing 10^4 cells was made in the right frontal region of newborn mice, just anterior to the striatum, with the tip of the needle just touching the skull base.

Brain Sectioning and Immunohistochemical and Immunofluorescent Staining

Animals were sacrificed at 4 to 9 weeks of age and the brains fixed in 4% formaldehyde, 0.4% glutaraldehyde, 1 $\times$ PBS for 36 hours. The sections were then treated with 10% hydrogen peroxide/70% methanol for 15 minutes to inactivate endogenous peroxidases. The sections were blocked with 1% goat serum in Tris-buffered saline, pH. 8.0, with 0.1% Tween (TBST) solution for 20 minutes followed by a one hour incubation at room temperature after the addition of mouse monoclonal antibodies to human GFAP (Boehringer). The sections were washed extensively with TBST, antibody staining was visualized with peroxidase-conjugated anti-mouse antibody (ABC, Vector), and the sections were mounted on glass slides.

For immunofluorescence, after being deparaffinized in xylene and treated with 0.05% saponin, the tissue sections (5 μ m in thickness) were incubated with mouse monoclonal anti-polyoma virus Tag (Ab-4, Oncogene) at 1 μ g/ml. Immunoreactions were visualized with fluorescein isothiocyanate-conjugated anti-mouse F(ab')₂ (Boehringer Mannheim).

Results

Development and Characterization of a Vector, RCAS-MTA, for Expression of Polyoma Virus Middle T Antigen

The polyoma middle T antigen cDNA was inserted into the RCAS vector to generate RCAS-MTA. This vector is replication-competent in avian cells but will only infect mammalian cells expressing the RCAS receptor tv-a.¹⁶ Generation of a producer cell line for RCAS-MTA was achieved by transfection of the chicken fibroblast cell line, DF-1.¹⁵ Transfection of DF-1 cells with RCAS-MTA resulted in transformation, with refractile, rapidly growing cells having little or no contact inhibition of growth. Infection of cultured tv-a expressing astrocytes with a viral stock of RCAS-MTA generated by DF-1 cells also resulted in a rapidly proliferating population of cells with poor contact inhibition (data not shown).

MTA Gene Transfer to GFAP⁺ Cells in Vivo

Gtv-a transgenic mice were injected at birth with 1 ml containing 10^4 DF-1 cells producing RCAS-MTA. All infected mice were sacrificed at 9 weeks, or earlier if they showed signs of macrocephaly and lethargy, and the brains were fixed in formalin. The brains were cut coronally in 5-mm sections and embedded in a single paraffin block. A single H&E-stained tissue section from each block, representing one mouse, was used as a screen for glioma formation. Of the 33 mice injected, 9 had lesions determined to be gliomas. The majority of these were asymptomatic, although 3 developed macrocephaly and lethargy requiring sacrifice before 9 weeks. The reason for absence of glioma formation in the majority of mice is unknown but may be due in part to the mixed genetic background of this mouse line and to the possibility that

Figure 3. Gliomas arising from intracerebral infection of *Gtv-a* mice with RCAS-MTA. Transfer of MTA gene to *Gtv-a* astrocytes *in vivo*. Newborn *Ntv-a* mice were injected in the right frontal lobe with DF-1 cells producing RCAS-MTA and sacrificed between 4 and 8 weeks of age. **A:** Glioma infiltrating the corpus callosum (arrow). **B:** Higher power image of diffuse infiltrating anaplastic astrocytoma with mitotic figures (arrows). Original magnifications, $\times 40$ (**A**) and $\times 200$ (**B**).

Figure 4. Invasion of normal brain structures by glioma cells. H&E-stained paraffin sections illustrating secondary structures of Scherer in this MTA-induced glioma. **A:** Perineuronal satellitosis of tumor cells around two large neurons (**arrows**). **B:** Mitosis of glioma cell adjacent to smaller neuron (**arrow**). **C:** Intrafascicular spread of glioma cells along white matter tracts. **D:** Perivascular satellitosis of glioma cells around blood vessel (**arrow**). Original magnifications, $\times 400$ (**A** and **B**) and $\times 200$ (**C** and **D**).

some of the mice may not have been productively infected with the RCAS-MTA virus. Some variability in resistance to glioma formation may be inherent to the genetic backgrounds present in the mouse population or the length of time the mice were observed before histological analysis of their central nervous systems (CNS).

*Characteristics of Gliomas Induced by MTA Gene Transfer in *Gtv-a* Mice*

The lesions had regions of increased cell density, vascular proliferation, and nuclear pleomorphism. A number of the lesions had sufficient size to exert mass effect and significantly distort the adjacent brain. Mitotic figures were frequently found, indicating a rapidly proliferating tumor. The lesions invaded the adjacent brain and infiltrated through white matter tracks in a similar fashion to that seen with human gliomas. In Figure 3, a glioma is seen spreading bilaterally across the corpus callosum,

similar to a human "butterfly" glioma. Further evidence of similarity between this mouse model and human gliomas is the presence of secondary structures of Scherer in Figure 4, illustrating perineuronal and perivascular satellitosis as well as intrafascicular spread of tumor cells through the normal brain parenchyma.

In some areas, the morphological features of the tumors were consistent with astrocytic differentiation: oval nuclei and abundant eosinophilic cytoplasm with fibrillary cytoplasmic processes that showed strong immunopositivity for GFAP. In the same lesions, other regions of the glioma exhibited features classically associated with oligodendroglial differentiation, with regular round nuclei, cleared cytoplasm (perinuclear halos), and a lack of immunopositivity for GFAP. Immunostaining for GFAP also revealed scattered immunopositive cell types with the phenotypic characteristics of mini-gemistocytes (micro-gemistocytes) and gliofibrillary oligodendrocytes, which are two GFAP⁺ cell types that are commonly accepted as

Figure 5. Microscopic and immunohistochemical character of gliomas arising from MTA expression in GFAP⁺ cells. **A:** Glioma with histology consistent with oligodendroglioma adjacent to **B** regions consistent with anaplastic astrocytoma. **C:** GFAP staining, demonstrating lack of staining in the oligodendroglioma regions with the exception of interspersed reactive astrocytes. **D:** Diffuse expression in the astrocytoma regions. **E** and **F:** Two examples of GFAP⁺ mini-gemistocytes within the oligodendroglial component (**arrows**). Original magnifications, $\times 200$ (**A–D**) and $\times 400$ (**E** and **F**).

part of the morphological spectrum of oligodendrogliomas (Figure 5). No areas of necrosis or tumor pseudopalisading were found even in the largest of these lesions. The features of these gliomas, therefore, correspond to those exhibited by human gliomas that have been traditionally diagnosed as anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic mixed oligoastrocytoma. Features of other primary CNS neoplasms, such as primitive neuroectodermal tumors or ependymomas, were not seen in any of these lesions.

We used an antibody directed against MTA to demonstrate expression of the virally transduced MTA gene product in the tumor cells. As expected, the lesions stained with this antibody and were detected by immunofluorescence (Figure 6).

Discussion

MTA Activates Signal Transduction Pathways

Although there is no evidence for MTA's role in gliomagenesis, some of the pathways activated by MTA (Shc-Ras, PI3K-Akt, and Src) are the same as those activated by the tyrosine kinase receptors implicated in gliomagenesis, eg, EGFR, PDGFR, IGFR, and fibroblast growth factor receptor (FGFR). Moreover, although the genes encoding Ras and Akt are not mutated in gliomas, their activities have been shown to be markedly elevated in these tumors.^{17,18} In these ways, MTA activity mimics the function of mutations found in gliomas and, in that way is a functional model of the genesis of gliomas in humans. Additionally, MTA may activate other unknown

Figure 6. Immunofluorescent staining of glioma for expression of middle T antigen. MTA-induced glioma stained for H&E (**A**), immunofluorescent stain for MTA expression (**B**), and control secondary antibody only (**C**). Adjacent normal brain from same animal stained with H&E (**D**), immunofluorescent stain for MTA (**E**), and control secondary antibody only (**F**). Original magnifications, $\times 200$.

pathways that could play a part in tumor formation from astrocytes or other cell types. MTA is a potent oncogene and has been shown to cause many types of tumors depending on the cell type in which it is expressed. Therefore, MTA tumorigenesis is not specific to a particular tumor histology; rather, MTA can convert specific cell types to their neoplastic counterparts by the activation of a number of signaling pathways. We have used this oncogene to achieve tumorigenesis from GFAP⁺ cells and to determine the tumor type or types that arise from this defined group of cells.

The Cellular Origin of Astrocytomas and Oligodendrogliomas

Much debate has occurred concerning the cellular origin of the gliomas. It has been proposed that gliomas of oligodendroglial character arise from oligodendrocyte precursors or from precursors to both oligodendrocytes and astrocytes similar to O2A progenitors,¹⁹ although these results have not been duplicated. There is some evidence that less differentiated cells are more prone to neoplastic transformation and therefore more likely to give rise to gliomas.⁴ There is, however, mounting evidence that under appropriate conditions, GFAP⁺ cells can give rise to cells with morphology resembling oligodendrocytes³ and even neurons²⁰ *in vivo*, implying that these cells can modulate their differentiation characteristics. In this paper, we demonstrate that astrocytes can give rise to gliomas with mixed morphological character. We are unable to determine whether these tumors are clonally derived, and therefore do not know whether a single cell can give rise to both astrocytomas and oligodendrogliomas. Our data show only that GFAP-expressing astrocytes, as a population, are capable of giving rise to either astrocytomas or oligodendrogliomas. This transformation occurs with activation of the signal transduction pathways stimulated by mutations found in human gliomas. These data imply that substantial similarity may exist between these two histological diseases. The cell of origin for human astrocytomas and oligodendrogliomas is unknown; however, our data provide evidence that under the appropriate conditions differentiated astrocytes may be capable of giving rise to both of these tumor types as well as mixed gliomas.

Lesions with the above described features arise from GFAP⁺ cells in our mice; however, these tumors show no characteristics of other glial or neuroectodermal tumor types, indicating that the neoplastic counterpart for astrocytes may be limited to astrocytomas and oligodendrogliomas. In contrast, glioblastomas appear to arise from nestin-expressing progenitors in mice after combined transfer of genes encoding activated Ras and Akt.¹⁷ Although we have not performed this experiment, if our MTA-induced gliomas were to be observed for longer periods of time they potentially could progress to glioblastoma multiforme, similar to what is seen in the human disease.

MTA-Induced Gliomagenesis Does Not Require Additional Experimental Disruption of the Cell Cycle Arrest Pathways

Previous studies have shown that induction of glioma-like lesions in mice by gene transfer of an activated form of EGFR requires disruption of cell cycle arrest pathways,⁴ and human gliomas have both signal transduction abnormalities and disruption of the cell cycle control pathways.²¹ These data imply that cell cycle arrest disruption may be required for gliomagenesis. However, MTA appears to induce gliomas in mice in the absence of experimentally induced cell cycle arrest disruption, even though EGFR and MTA activate a number of the same signaling pathways.

The reason for this apparent discrepancy is not known, but a number of possibilities exist. For example, excessive activation of the relevant signal transduction pathways may result in disruption of the cell cycle as a direct downstream consequence of the signal transduction abnormalities. MTA is much more efficient at activating these pathways than EGFR and therefore, unlike MTA, EGFR alone is insufficient to accomplish the required effect on the cell cycle. Alternatively, MTA may have direct effects on the cell cycle by unknown pathways by which it is capable of inducing gliomas on its own. Finally, tyrosine kinase receptors may activate not only the transformation-promoting pathways but inhibitory pathways as well. These inhibitory pathways may require cell cycle arrest pathways to achieve their effect. For whatever reason, additional experimental disruption of the cell cycle control pathways appears to be required to achieve a transformation by EGFR but not by MTA.

Mutations found in human gliomas frequently result in activation of signal transduction pathways or disruption of cell cycle arrest. Oligodendrogliomas have recently been shown to have frequent deletions of chromosomes 1p and 19q, the genes involved in these deletions have not been identified. Although the functional outcome of these chromosomal abnormalities in human oligodendrogliomas is not yet known, given our current understanding of glioma biology, one reasonable hypothesis is that signal transduction pathways may be affected by these deletions. Our model generates tumors with all of the histological characteristics of oligodendrogliomas by activation of appropriate signal transduction pathways via a different mechanism than is seen in the human disease. Our model, therefore, would not meet a strict definition of oligodendroglioma if it were to include identical genomic alterations.

The data presented in this report imply that although the DNA mutations that are found in these tumors may differ, oligodendrogliomas and astrocytomas may be closely related lesions at opposite ends of a spectrum of differentiation, rather than discrete and separate entities. The gene expression patterns that induce the morphological character of a given human glioma are currently unknown. However, gliomas located on both ends of this morphological spectrum in mice appear capable of arising from astrocytes as their cell of origin.

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