A review of astrocytoma models

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Despite tremendous technical improvements in neuroimaging and neurosurgery, the prognosis for patients with malignant astrocytoma remains devastating because of the underlying biology and growth characteristics of the tumor. However, our understanding of the molecular bases of these tumors has greatly increased due to study findings involving operative specimens, astrocytoma predisposing human syndromes, teratogen-induced animal and established human astrocytoma cell lines, and more recently transgenic mouse models. Appropriate small-animal models of spontaneously occurring astrocytomas, which replicate the growth and molecular characteristics found in human tumors, are essential to test the relevance and interactions of these molecular aberrations. In addition, it is hoped that relevant molecular targets will eventually be therapeutically exploited to improve patient outcomes. Appropriate animal models are also essential for testing these novel biological therapies, before they are brought to the clinic, requiring a large investment of time and money. In this paper, various astrocytoma models are discussed, with emphasis on transgenic mouse models that are of great interest to laboratory investigators.

KEY WORDS • astrocytoma • glioblastoma multiforme • mouse • gene therapy

Malignant astrocytoma represents one of the most devastating tumors affecting children and adults. Surgery and adjuvant conventional radio- and chemotherapy have had minimal effect on changing the poor prognosis, which remains at a median range of only 9 to 12 months. Malignant astrocytomas are composed of pleomorphic, hyperproliferative infiltrative astrocytes, with areas of necrosis, increased tumor angiogenesis and regions of blood–brain barrier breakdown. These heterogeneous pathological characteristics pose major obstacles to the effective management of gliomas. In addition to pathological heterogeneity, there is associated molecular heterogeneity, the expression, interactions, and functional importance of which we have just begun to decipher. It is hoped that this increased understanding of the molecular pathogenesis of astrocytomas will lead to novel therapeutic targets, which can be exploited in combination with a variety of genetic, pharmacological, and other approaches to improve the overall clinical prognosis. In tandem with our increasing understanding of the molecular pathogenesis and development of novel biological therapies of malignant astrocytomas, there is a need to develop appropriate models in which to test these novel therapies. Indeed, many adjuvant therapies, in which promise in various existing in vitro and in vivo models of astrocytomas has been demonstrated, have been applied with little success in patients; however, there has been a significant cost in terms of time, patient psychological and occasional physical morbidity, and economic issues. These failures are partly based on the fact that we sometimes short-circuit the rigors of the scientific investigational process because of the terminal nature of the disease and because of our own enthusiasm to obtain a cure. This has contributed to cynicism and nihilism expressed by the public and clinicians regarding the management of malignant astrocytomas. Hence, it is important that we expend the time and effort to develop and test our novel therapies in appropriate preclinical models that most accurately reproduce the clinical, histological, and molecular characteristics found in human astrocytomas; it is hoped that this will translate into improved success in subsequent clinical trials.

Currently available models of astrocytomas include in vitro cell lines derived from human malignant astrocytomas as well as chemically or virally induced rat or mouse astrocytomas. These cell lines have certainly served to in-
Two molecular pathways have been proposed for GBMs. Models discussed later in this review (Fig. 1). At least genes as well as activation of critical oncogenes provide slowly emerging: the loss of specific tumor suppressor 2 Neurosurg. Focus / Volume 8 / April, 2000 First, patients can present de novo, with the tumor char-
some). The corresponding knockout mouse models. These include NF1 and NF2, TSC, and the Li–Fraumeni cancer syndrome. Unfortunately, the corresponding knockout mice do not develop astrocytomas, perhaps reflecting a combination of the low incidence of astrocytomas in these familial syndromes, the requirement of additional genetic aberrations for developing astrocytomas, and the limitations and differences in mouse models as compared with humans. Despite the limitations, ongoing research is still required to develop spontaneously occurring small-animal models of astrocytomas that mimic the pathological and molecular profile of human astrocytomas; additionally, research in these animal models has the potential to advance our ability to treat and investigate this disease. Ideal animal models of human astrocytomas should fulfill the following criteria: 1) tumors should arise from astrocytes; 2) tumors should be transformed as characterized by immortalization in vitro and the ability to be transplanted into syngeneic or immunocompromised hosts in vivo; 3) tumors should develop in small inexpensive animals in which there are relatively short and predictable induction times; 4) tumors should share similar molecular profiles with human astrocytomas; and 5) tumors should be responsive to known therapies (radio- and chemotherapy) that have demonstrated efficacy in human tumors.

MOLECULAR PATHOGENESIS OF ASTROCYTOMA

A molecular pathogenic profile of astrocytoma is slowly emerging: the loss of specific tumor suppressor genes as well as activation of critical oncogenes provide the rationale for development of the transgenic mouse models discussed later in this review (Fig. 1). At least two molecular pathways have been proposed for GBMs. First, patients can present de novo, with the tumor characterized by deletions on chromosome 10 harboring at least two tumor suppressor genes (PTEN/MMAC1 [a dual specific and lipid phosphatase]) and Deleted in Malig-
result of cardiovascular developmental abnormalities, the heterozygous Nf1 mouse model (+/-) develops a variety of tumors in late adult life but not astrocytomas.32 However, closer examination of the Nf1 heterozygote brains reveals the increased proliferation of astrocytes as compared with wild-type littermates,26 with proliferation inhibited by Ras effector pathway blockade. Hence, although examination of the Nf1 mouse model may not yield much information with regards to astrocytomas, the derivative astrocytes with reduced neurofibromin expression have increased Ras pathway activation. The Nf1 heterozygote mice that have been mated with other relevant genetically engineered mice demonstrate genetic cooperativity between Nf1 and cell cycle regulatory genes with respect to astrocyte growth regulation. For example, crossing of the Nf1 mice with the p53-deficient mice has been recently been reported to lead to development of neurofibromas.3

Neurofibromatosis Type 2

Neurofibromatosis Type 2 is another autosomal-dominant cancer predisposition syndrome that is distinct from NF1. The prevalence and incidence of NF2 is 10-fold less common than NF1; however the incidence of astrocytomas, ependymomas, and meningiomas affecting the brain and spinal cord is comparatively higher. Two groups of investigators cloned the NF2 gene on chromosome 22q, encoding a protein termed either “merlin” or “schwannomin.” Merlin/schwannomin shares similarities with members of the protein 4.1 family, including ezrin, radixin, and moesin, which link cell glycoproteins to the actin cytoskeleton.24 The normal function of merlin/schwannomin and how it relates to cellular transformation is not as well known as that of neurofibromin but is being actively investigated. That Nf2 homozygous knockout mice (-/-) die early during embryogenesis (secondary to a failure to induce mesoderm formation), suggests that merlin is essential for the development of extraembryonic structures.43 The Nf2 heterozygotes (+/-) are cancer prone and develop highly metastasizing osteosarcomas, lymphomas, lung adenocarcinomas, hepatocellular carcinomas, and fibrosarcomas, although, not the classic schwannomas or the associated gliomas found in patients with NF2.44

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cently, mice, in which there was overexpression of a dominant inhibitory isoform of merlin specifically found in Schwann cells by using the myelin P0 promoter, developed schwannomas involving the trigeminal nerve, spinal ganglia, uterus, stomach, intestine, and pancreas. Breeding the Nf2 heterozygote mice with other relevant genetically altered mice may yet yield models of gliomas, and this remains an area of ongoing research.

**Tuberous Sclerosis Complex**

Tuberous sclerosis complex is another rare autosomal-dominant neurocutaneous syndrome in which patients develop hamartomatous lesions in the nervous system and other organs. Individuals with TSC develop a special type of astrocytoma, termed subependymal giant-cell astrocytoma. Two genes are responsible for TSC, TSC1 and TSC2, which code for hamartin and tuberin, respectively. Tuberin has been shown to function as an inactivating protein for Rap1 (another Ras-like protein that is transforming in certain cell types), which is similar to neurofibromin’s role in regulating Ras. The role of this Rap1 signaling pathway in glioma pathogenesis is underscored by our observations that approximately two thirds of all transformed astrocytes also express TSC2. In this model, EGFRvIII has been expressed in both -/- astrocytes and -/--deficient astrocytes grown in culture from these mice do become transformed when injected either subcutaneously into immunocompromised mice or intracranially into syngeneic recipients.

**Li–Fraumeni Cancer Syndrome**

Li–Fraumeni cancer syndrome is a prevalent familial syndrome resulting from germline mutations in the p53 tumor suppressor locus, a common tumor suppressor gene lost in a variety of sporadic human cancers including gliomas. Astrocytomas, the third most common tumor type in patients with Li–Fraumeni cancer syndrome, occur early in age and quickly become malignant in nature. Although p53 knockout mice (hetero- or homozygous) have not been informative model for astrocytomas, these rats do not develop astrocytoma or subependymal giant-cell astrocytomas.

**NONTRANSGENIC MODELS OF ASTROCYTOMAS**

Spontaneous brain tumors found in nonhuman primates and rodents are extremely rare. The most common astrocytic tumor is the VM murine astrocytoma arising in an inbred mouse strain with an incidence of 1.5% at 600 days. In an effort to develop nontransgenic models for human brain tumors, three approaches have been conducted using: 1) primary astrocytoma cells derived from patient or from rat astrocytomas; 2) retrovirus injection; or 3) chemical mutagenesis. Xenografts obtained from established human astrocytoma cell lines or tumor explants into immunocompromised rodents have likewise been used. These established cell lines suffer from being clonal in nature, whereas tumor explants have been shown to have low and variable implantation rates, unpredictable latencies, and loss of invasive growth over time. Cell lines obtained from chemically induced astrocytomas have also been used, including C6, 9L, RG2, and T9 rat glioma cells. Unfortunately, these chemically induced models are histologically and genetically different from human astrocytomas. For example, an allogeneic major histocompatibility complex has recently been identified in the C6 rat astrocytoma (a tumor cell line), which could explain the phenomenon in which some C6 tumors in the immunocompetent rats regress spontaneously even without therapeutic intervention. Therefore, we believe that the C6 astrocytoma model in the immunocompetent rat should no longer be used for therapeutic studies, and the available data obtained in this model need to be critically reinterpreted.

Retroviruses that induce brain tumors include members of the Rous sarcoma virus family and simian sarcoma viruses in marmosets, whose oncogenic properties are caused by the overexpression of PDGF-B (v-sis). In a recently published model, injected recombinant retrovirus was used to achieve cell type–specific brain expression. In this model, EGFRvIII has been expressed in both wild-type mice and those aberrant for relevant cell cycle regulatory genes such as p53, p16, retinoblastoma, and CDK4. A variety of proliferating, GFAP immunoreactive astrocyte populations were induced that expressed the inoculated transgene. In these experiments, astrocyte-like cells expressing EGFRvIII did not form tumors unless they also overexpressed CDK4. No cooperativity was demonstrated between EGFRvIII and p53. In addition, either loss of INK4a (p16) or overexpression of CDK4 permitted the infected astrocyte-like cells to grow without senescence. These results indicate that genetic cooperativity between growth factor and cell cycle regulatory pathways is required for astrocyte transformation in this model. It is not clear, however, whether this astrocytic cell cultures have not been tested for their ability to form tumors in another host. Although this retroviral infection strategy does not lead to germline mouse astrocytoma models, such as those potentially derived from transgenic models for use in preclinical therapeutic testing, it
A review of astrocytoma models has the strength to test quickly the interactions among the known genetic aberrations found in malignant human astrocytomas, as well as to shed light onto their ontogeny. These retroviral studies are extremely important and complement the transgenic strategies in the proceeding section.

**TRANSGENIC ASTROCYTOMA MODELS**

Although the knockout mouse models associated with cancer predisposition syndromes discussed in the preceding sections have not yielded useful models of astrocytomas, several other knockout mouse models with disruption of the genes that are lost in sporadic astrocytomas are of interest. Mice lacking p16, a CDK inhibitor that is found on chromosome 9p that is involved in the retinoblastoma-regulated cell cycle pathway, and that is commonly mutated in human astrocytoma specimens and derived cell lines (Fig. 1), develop a variety of malignancies but not astrocytomas. These mice provide a useful background on which additional genetic alterations associated with astrocytomas and GBMs, such as overexpression of EGFRVIII transduced by retroviral injection, leads to development of astrocytoma-like lesions.39 Cross-breeding experiments with these p16 (-/-) mice with other genetically altered mice are ongoing and may yield additional astrocytoma models. A second gene of interest on chromosome 9p is p14ARF, which is also commonly mutated in astrocytomas, and is involved indirectly in the p53 pathway by its regulation of MDM2.30 A small proportion of p14ARF knockout mice develop gliomalike lesions presumably due to increasing MDM2 levels that lead to sequestration and secondary inhibition of p53 function.34 The PTEN/MMAC1 on chromosome 10q, a dual specific and lipid phosphatase that is lost in the majority of GBMs, has also been targeted in mice. The PTEN null (-/-) mice are embryonically lethal, whereas heterozygous (+/-) mice develop a plethora of tumors but not astrocytomas.58 Current studies in which we use a variety of strategies, are underway to knockout PTEN/MMAC1 specifically in astrocytes, to avoid the embryonic lethality. These knockout models of astrocytoma-specific genetic alteration have not resulted in useful models of astrocytomas; however, they may augment and complement strategies in which the mouse genome has been altered for a gain of function in genetic aberration associated with astrocytomas, such as those used in our laboratory with ES cells.

**Embryonic Stem Cell–Mediated Transgenesis**

In the last two decades, the advent of novel and very efficient techniques to manipulate the mouse genome, such as in mouse ES cells, has given us the capability of tailoring the mouse genes and genomes at will. We have reached the stage at which geneticists studying mice believe there is no limitation to creating phenocopies (or genocopies) of any mutations or chromosomal aberrations identified in human diseases. Embryonic stem cells are derived from an early (blastocyst-stage) embryo and can be maintained in culture as undifferentiated, pluripotent cells under the proper growth conditions. A broad spectrum of strategies has been designed to create genomic alterations in these cells. When the genetically altered ES cells are injected into a host blastocyst, or aggregated within a morula-stage embryo, they have the capacity to contribute to all tissues of the resultant chimeric mouse (Fig. 2). Most important, they can contribute to germ cells and transmit the genetic mutations in vivo, allowing development of established mouse lines in which the altered gene(s) are carried.47,49,52

Embryonic stem cell–mediated transgenesis has several advantages over the standard pronuclear DNA injection routinely used to create transgenic models. Conventional pronuclear DNA injection frequently results in multiple-copy integration of a transgene, which can result in variation of transgene expression, whereas ES cell–mediated transgenesis provides a higher frequency of low-copy numbers or even single copy of transgene integration.38,49 In addition, transgenic models can be tested in vitro for cell type–specific expression by using in vitro ES cells–differentiation assays, as exemplified by astrocytic lineage differentiation in which retinoic acid is used in the mouse astrocytoma models derived in our laboratory (unpublished data). Finally, the ES cell–mediated transgenic approach also allows us to avoid the problem, found in a number of cases, in which expression of the transgene is lethal, because it involves chimera production with transgenic ES cells contributing to different levels in the animals.

To examine specifically the effects of a genetic alteration in a certain cell type, such as an astrocyte, cell-specific promoters can be used in conventional or ES cell transgenesis. In the nervous system, tumors formed with tissue-specific promoters in transgenic mice include: 1) pineoblastomas (tryptophan hydroxylase promoter); 2) primitive neuroectodermal tumors (tyrosine hydroxylase promoter); 3) oligodendrogliomas (myelin basic promoter and S-100 promoter); 4) neuroblastoma and ganglioneuromas (dopamine-β-hydroxylase promoter); and 5) gonadotrophic hormone–releasing tumors leukinizing hormone/follicle–stimulating hormone promoter.53

**Transgenic Models of Astrocytomas**

The GFAP promoter has been used to express oncoproteins specifically in astrocytes, such as the SV40 large T antigen and v-src.46 The GFAP–SV40 large T transgenic mice were shown to develop aggressive nonastrocytic brain tumors with hyperplasia of the choroid plexus.10 In GFAP–v-src transgenic mice abnormal nests of proliferating astrocytes are formed by 2 weeks of age, and these astrocytes later evolve into overt malignant astrocytomas in the brain and spinal cord in 14% of mice by 65 weeks of age.66 Hemizygosity for p53 or retinoblastoma, achieved by crossing these mice with the respective p53 and retinoblastoma knockout mice, was not shown to increase the incidence or shorten the latency of astrocytic tumors in these GFAP–v-src mice.40 These transgenic astrocytomas have histological features similar to human GBMs, including expression of VEGF and angiogenic endothelial-specific receptors such as flt-1, flk-1, tie-1 and tie-2.62,66

We have used the GFAP promoter to express relevant transgenes in astrocytes, by using the ES cell strategy outlined previously (Fig. 2). Based on our prior work in
which we found that one of the main signaling pathways
activated by PDGFRs and EGFRs overexpressed in
human GBMs involves activation of Ras,
we have created a mouse GBM model by overexpressing onco-
genmic Ras (V12 Ras) in astrocytes using the GFAP promoter
in ES cells (by retinoic acid) and using selection markers positive clones expressing the transgene are
selected. These transfected ES cells undergo aggregations and are transferred into pseudopregnant mice to create chimeric embryos
composed of cells arising from the transfected and wild-type ES
cells, with the transgene only being expressed in a tissue-specific
manner, such as occurs in astrocytes in the presence of the GFAP
promoter. These chimeras are then crossed with normal mice to
propagate stably the transgene in a germline fashion. This and
other transgenic strategies have led to derivation of mouse astrocy-
toma models, which replicate the pathological and molecular pro-
file found in human astrocytomas.

astrocytomas. Derivative astrocytoma cells obtained in
these mice are tumorigenic when inoculated into naïve
syngenic or immunocompromised mice. Cytogenetic
analysis revealed consistent clonal aneuploidies of several
chromosomal regions syntenic with comparable loci altered in human astrocytomas. For example, the transgenic mouse astrocytoma cells harbored trisomy of mouse chromosome 10, an area that contains human chromosome 12q, which is the second most commonly amplified region in human GBMs.

Direct assay conducted to determine protein expression revealed decreased expression of p16, p19/p14ARF, p53, and PTEN and overexpression of proteins such as EGFR, MDM2, and CDK4, which is similar to findings in human malignant astrocytomas.

In addition, these GFAP-V12Ras transgenic mice have been shown to possess genetic cooperativity, as they develop malignant astrocytomas within 2 to 3 weeks when crossed with transgenic mice in which expression of EGFRvIII in astrocytes occurs. These mice were developed using a similar strategy with the GFAP promoter in ES cells but, by themselves, did not result in astrocytoma formation (unpublished results). Additional crosses with mice harboring knockout of relevant astrocytoma genes such as p53 and retinoblastoma are ongoing and may modulate the occurrence of the GBMs in these mice. Furthermore, the mice and derived astrocytoma lines are responsive to biological therapies targeting the Ras pathway, such as with farnesyl transferase inhibitors, which have shown promise in a variety of human tumors including, as found in our own studies, astrocytomas. The molecular–pathological similarities with human GBMs, in addition to some of these early, promising studies, leads us to believe that this transgenic mouse GBM model may serve to increase further our knowledge of the molecular pathogenesis, as well as serve as an appropriate preclinical model of human malignant astrocytomas.

CONCLUSIONS

The generation of spontaneously occurring mouse models of astrocytomas, based on the molecular pathogenesis of human astrocytomas, would greatly advance our ability to treat human astrocytomas by serving as informative preclinical models in which to test novel therapeutic agents. The transgenic models with multiple alterations in growth factor and cell cycle regulatory pathways can be used to advance our understanding of the molecular pathogenesis of astrocytomas. Biological therapies, such as inhibitors of receptor tyrosine kinases, inhibitors of signaling pathways such as Ras, or antiangiogenesis strategies, are already being conducted in early clinical trials. The transgenic models mimicking the histology and molecular pathogenesis should markedly enhance our ability to test these and other novel agents, with an increased likelihood of therapeutic success in subsequent clinical trials.

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