Brainstem gliomas constitute up to 20% of childhood brain tumors; 80% of these lesions are diffuse intrinsic brainstem gliomas, which have the worst prognosis of any brain tumor in children.\textsuperscript{15,19,22,23,31} In comparison, brainstem gliomas in adults are much less common, accounting for < 2% of gliomas.\textsuperscript{20,29} Increasing evidence indicates that adult brainstem gliomas are different from the childhood subtypes. Overall, brainstem gliomas in adults are less aggressive than those in children, which in part results in a much better prognosis.\textsuperscript{20} The median survival of adults with intrinsic low-grade brainstem gliomas is 7.3 years, which is similar to that of patients with low-grade supratentorial gliomas. However, the median survival of children with intrinsic brainstem gliomas is < 1 year after diagnosis.\textsuperscript{22}

Different classifications of brainstem gliomas have been made based on location and imaging and pathological characteristics. According to location, brainstem gliomas have been subclassified into midbrain, tectal, pontine, medullary, and cervicomedullary gliomas. Based on neuroimaging results, brainstem gliomas can be classified as diffuse, focal, exophytic brainstem, and cystic gliomas. According to histopathological characteristics, brainstem gliomas are divided into low-grade (WHO Grades I and II) and high-grade (WHO Grades III and IV) gliomas. More recently, brainstem gliomas have also been separated into diffusely infiltrative brainstem gliomas, known for their relentless growth and bleak outcome, and focal discrete brainstem gliomas, which are normally associated with a favorable prognosis.\textsuperscript{17,19,23,28,32} For

**Object.** Brainstem gliomas are common in children and have the worst prognosis of any brain tumor in this age group. On the other hand, brainstem gliomas are rare in adults, and the authors of some clinical studies have suggested that this lesion behaves differently in adults than in children. In the present study, the authors test an orthotopic C6 brainstem glioma model in juvenile and adult rats, and investigate the biological behavior of this lesion in the 2 age groups.

**Methods.** The C6 glioma cells were stereotactically implanted into the pons of juvenile or adult male rats. Neurological presentation and survival time were recorded. Tumor proliferation and the number of apoptotic cells in brainstem gliomas of young and adult rats were determined by immunohistochemical staining with Ki 67 and terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate-mediated nick-end labeling assay.

**Results.** Striking differences in the onset of neurological signs, duration of symptoms, survival time, tumor growth pattern, tumor proliferation, and number of apoptotic cells were found between the gliomas in the 2 groups of rats. The lesions were relatively focal in adult rats but more diffuse in young rats. Furthermore, brainstem gliomas in adult rats were less proliferative and had more apoptotic cells than those in young rats.

**Conclusions.** The authors found that the C6 brainstem glioma model in young and adult rats closely imitates the course of brainstem glioma in humans both in neurological findings and histopathological characteristics. Their findings also suggest that different growth pattern and invasiveness of these lesions in children compared with that in adults could be due to different cellular environments in the 2 age groups, and warrants further investigation into the difference in the host response to brainstem gliomas in children and adults. (DOI: 10.3171/JNS/2008/109/11/0849)

**Key Words** • brainstem • glioma • progression • rat

**Abbreviations used in this paper:** DAPI = 4',6'-diamidino-2-phenylindole; ECM = extracellular matrix; PBS = phosphate-buffered saline; TUNEL = terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate-mediated nick-end labeling; WHO = World Health Organization.
diffuse lesions, surgical options are limited because even partial resection may lead to severe functional deficits. So far chemotherapy has not shown any benefit, and therefore, radiation remains the standard treatment for brainstem tumors. Patients with diffuse brainstem tumors have 2-year survival rates of 10–15%. In contrast, patients harboring focal brainstem tumors may benefit from surgery. Such lesions can be treated with radical surgery with a good outcome and prognosis.22,38

Generally, low-grade brainstem gliomas are considered to have a characteristic pattern of focal growth, while high-grade brainstem gliomas grow diffusely and aggressively.22,37 This is not always the case, however. Sparse histological data are available for diffuse brainstem gliomas because biopsy results do not alter the treatment strategy.1,10 Furthermore, low-grade gliomas such as fibrillary astrocytomas have also been found in association with diffuse brainstem gliomas.5 Diffuse brainstem gliomas constitute > 80% of brainstem gliomas in children, while the majority of brainstem gliomas in adults are focal.19,22 It is now appreciated that the genetic abnormalities associated with high-grade gliomas in children are different from those in adults, which may contribute to the differences in brainstem glioma growth patterns. In addition, the anatomical, histological, and immunological diversity between children and adults could also contribute to the different biological behavior of diffuse and focal brainstem gliomas in these 2 groups of patients. The present study was designed to investigate the differences in biological behavior of brainstem gliomas in children and in adults using an orthotopic C6 cell brainstem glioma model in rats.

Methods

Experimental Animals

Sprague–Dawley young male rats (3 weeks old, body weight 40–50 g, mean 45.6 g) and adult male rats (10 weeks old, body weight 325–350 g, mean 337 g) were purchased from Charles River Laboratories and maintained in a vivarium in 48 × 26.7 × 20–cm polycarbonate cages (2 rats/cage). The colony room was maintained at 23 ± 1°C under a 12-hour light/dark cycle beginning at 1800 hours. All rats had access to food and water ad libitum. All animal procedures were approved by the University of North Texas Health Science Center Animal Care and Use Committee.

Cell Culture and Surgical Procedure

The C6 cell line, originally cloned from an N-nitrosomethylurea transformed rat astrocyte cell line, was obtained from the American Type Culture Collection.3 The C6 cells were grown in Dulbecco Modified Eagle Medium with 10% fetal bovine serum and 100 μg/ml penicillin and streptomycin at 37°C with 5% CO2 in a humidified incubator. The cells were prepared for injection using the standard cell preparation protocol.

The brainstem glioma model was modified from the one previously described.27,40 The animals were anesthetized with ketamine (40 mg/kg) in combination with xylazine (10 mg/kg) administered intraperitoneally. With the head secured in a stereotactic frame, a midline incision was made and the lambda suture was identified. A bur hole (1-mm diameter) was drilled 0.5 mm to the right of and 1 mm posterior to the lambda. The C6 cells were trypsinized, washed twice, resuspended, and diluted in Dulbecco Modified Eagle Medium (without fetal bovine serum) to a concentration of 2.0 × 106 cells/15 μl. Using a Hamilton syringe with a 27-gauge needle (Hamilton Co.), C6 cells were then slowly implanted into the pons (10-mm depth from the dura mater in adult rats and 7 mm in young rats). To avoid back flow of the cells, the injection was made over a 5-minute period, after which the syringe was left in place for 5 minutes and then slowly withdrawn. The scalp was closed with 4–0–Vicryl sutures in the standard fashion. The rats were returned to their cages after recovery from anesthesia. All rats were weighed every other day for the first 2 weeks and then every day thereafter, and were evaluated for neurological deficits starting at 1 week after the implantation.

Assessment of Neurological Function

Abnormal behaviors and neurological signs associated with brainstem tumors were assessed every day starting 1 week after cell implantation, and the onset of paresis was recorded. A modified rotarod test was used to assess coordinated motor function.21 The accelerating rotarod is a motor-driven treadmill (Omnirotor treadmill; Omnitech Electronics) used to measure running coordination. The treadmill was set to accelerate gradually from 0 to 75 rpm for each trial. The total time of each trial was 150 seconds. The rotor consisted of a 7-cm-diameter nylon cylinder mounted horizontally. The cylinder rotated (by means of a microprocessor-controlled motor) with an acceleration of 0.5 rpm/second. In a given trial, 4 rats were placed on the cylinder, 1 rat in each compartment. The cylinder was made to rotate and a timer switch was simultaneously activated. Acceleration continued until either a speed of 75 rpm was reached or the last animal was unable to perform the running response and had fallen to the padded surface. When a rat landed on the surface, a photosensitive switch was tripped, and the timer for that compartment stopped. The elapsed time was recorded in tenths of a second as the measure of performance on each trial. The rotarod test was started 1 week after implantation of C6 cells. Rats received 2 sessions daily. Each session consisted of 4 trials with 10-minute intervals between successive trials and a minimum of 2 hours between daily sessions.

Tissue Preparation and Histological Studies

The rats were killed by intraperitoneal injection of a double dose of ketamine and xylazine. Immediately after death, the animals were perfused transcardially with 0.9% saline, followed by 4% formalin. The brains were harvested and postfixed in 4% formalin. The hindbrain was embedded in paraffin, and 7-μm coronal sections were cut with a microtome (Leica). The slides were then stained with H & E. The presence of tumor and the pattern of infiltration were assessed.

Ki 67 Assay. For immunohistochemical analysis, the slides were deparaffinized, rehydrated, and boiled in 10 mM citrate buffer (pH 6.0) for 10 minutes to expose the
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antigen epitope. Nonspecific binding sites were blocked by incubation of the slices with 10% normal goat serum in PBS-Tween (0.2%) for 1 hour at room temperature. The sections were incubated with primary antibody (rabbit anti–Ki 67 at 1:50 dilution; ABcam, Inc.) overnight at 4°C. Then the sections were washed with PBS and incubated with a secondary antibody at room temperature for 1 hour. After washing 3 times with PBS, a drop of antifade solution with DAPI was added, glass coverslips were mounted, and then the slides were viewed under a fluorescence microscope. For proliferation index, Ki 67–positive cells in brainstem gliomas of the young and adult rats were counted, the percentages of Ki 67–positive cells in 10³ tumor cells were calculated and statistically compared.

TUNEL Assay. The TUNEL assay was used to assess apoptosis of tumor cells by examining DNA fragmentation. Briefly, the slides were deparaffinized, rehydrated, fixed with 4% methanol-free formaldehyde solution in PBS, and incubated with 100 μl of 20 μg/ml proteinase K for 10 minutes at room temperature. A second fixation was performed after washing by immersing the slides in 4% methanol-free formaldehyde solution.Slides were washed again with PBS, and fragmented DNA in the apoptotic tumor cells was detected by adding fluorescein 12-dUTP to nicked ends of DNA (DeadEnd Fluorometric TUNEL System; Promega). Slides were incubated for 1 hour at 37°C, and the reaction was terminated with 2 × saline–sodium citrate buffer solution (Promega). Then a drop of antifade solution with DAPI was added to the slides, glass coverslips were mounted, and the slides were visualized under a fluorescence microscope, with green fluorescence correlating with DNA fragmentation. The percentage of TUNEL-positive cells was determined and statistically compared.

Statistical Analysis

All data are expressed as means ± standard error of the means. Kaplan–Meier survival curves for the 2 rat age groups were generated, and the survival of the 2 groups was compared using the log-rank test. The proliferation index and TUNEL-positive cells were analyzed with the Student t-test using commercially available software (Prism; GraphPad). The differences for each comparison were considered significant at a probability value < 0.05.

Results

The weight of the juvenile rats continued to increase in the first 2 weeks after cell implantation, although the increase was not as fast as predicted by the age-matched control data on Sprague–Dawley rats supplied by Charles River Laboratories. The weights of adult rats stopped increasing as soon as tumor cells were implanted. The weights of juvenile and adult rats began to decrease at 14 and 17 days after glioma implantation, respectively, consistent with their rotarod performance. Furthermore, the weights of the juvenile rats decreased more sharply than weights in the adult rats in the last week of testing (Fig. 1 upper and lower).

All adult rats presented the focal symptom of left forelimb paresis with a mean onset time of 14 days after cell implantation. The mild ipsilateral forelimb weakness usually progressed to severe paralysis within 5–7 days. In contrast, all young rats presented with symptoms of ataxia, multiple cranial nerve deficits, urinary and fecal incontinence, without evidence of focal neurological symptoms or signs in the early stage of disease. All symptoms were observed at the late stage (18 days postimplantation), and the rats’ conditions deteriorated rapidly within 2–3 days afterwards, indicating rapid tumor proliferation.

The Kaplan–Meier survival analysis suggests a different survival curve between young and adult rats with brainstem gliomas. The median survival time in young and adult rats was 20 and 23 days after glioma implantation, respectively; this difference reached statistical significance (Fig. 3).

Histopathological Characteristics of Brainstem Gliomas in Young and Adult Rats

Under gross examination, brainstem gliomas were observed in all young and adult rats implanted with C6. As shown in Fig. 4A, relatively focal lesions were evident in all adult rats with brainstem tumors. In the young rats, however, enlargement of the entire brainstem was noted, but no focal lesion were observed (Fig. 4D). Intratumoral hemor-
rhage was found in 2 of 7 adult rats and 6 of 10 young rats. All adult rats and 6 of 10 young rats developed obstructive hydrocephalus. Tumor location, boundary, incidence of intratumoral hemorrhage, and presentation of hydrocephalus in young and adult rats are summarized in Table 1.

The histological examination of adult rats revealed relatively demarcated lesions invading white matter. Abundant necrotic cells surrounded by palisading nuclei were consistently observed in the center of tumors. Endothelial proliferation was scarce with limited angiogenesis (Fig. 4B and C). Brainstem gliomas in young rats followed a more invasive, infiltrative, and diffusive course. Endothelial proliferation was evident, and alternating areas of angiogenesis were frequently observed (Fig. 4E and F).

Expression of Ki 67 was used to evaluate the degree of tumor proliferation in young and adult rats. As shown in Fig. 5A–C, the proliferation index of brainstem gliomas in young rats, calculated from the percentage of Ki 67-positive cells of $10^3$ tumor cells, was significantly higher than that of adult rats ($p < 0.001$). The TUNEL assay was performed to further assess apoptosis of tumor cells in young and adult rats with brainstem gliomas. There were many apoptotic tumor cells in the adult rats (Fig. 5D), but very few in the young rats (Fig. 5E and F; $p < 0.001$).

Discussion

All brainstem gliomas used to be regarded as malignant because their location rendered them inoperable. As modern technologies in neuroimaging and microneurosurgery have developed, favorable surgical outcomes for certain types of brainstem gliomas have been reported. However, surgical intervention has only been beneficial in focal brainstem gliomas. Little progress has been made in increasing the survival rates in patients with diffuse lesions, which constitute > 80% of brainstem gliomas in children. There has been significant progress in understanding the molecular pathogenesis of glioma, which has led to the discovery of novel therapies for malignant gliomas. Tezolozolomide has been effective in the treatment of cerebral glioblastoma multiforme in adults, but does not alter the poor prognosis associated with newly diagnosed diffuse brainstem gliomas in children, even when combined with radiotherapy. Currently, there is no effective therapeutic intervention available for diffuse brainstem gliomas because our understanding of the biological behavior of these lesions and the effect of host on the progression and invasion of brainstem glioma has lagged behind. Currently, diffuse brainstem gliomas have the worst prognosis of any brain tumor in children. The development of a satisfactory experimental model for brainstem glioma is critical to our understanding of the biological behavior of this tumor and the future discovery of novel therapies.

The C6 orthotopic glioma model is simple, reliable, and easily reproducible, and has been extensively used to study growth kinetics and mechanisms of angiogenesis, as well as the expression of growth factors and receptors involved in the progression of astrocytic tumor. Many studies indicate that the C6 glioma model best mimics the stages of early glioma progression in humans. Jallo et al. have recently reported on an experimental brainstem tumor model in neonatal rat pups and adult rats using suspensions of 9L and F98 tumor cells. In the present study, we modified this model by changing the coordinates from 1.4 mm to the right of and 1.0 mm anterior to the lambda to 0.5 mm to the right of and 1 mm posterior to the lambda to ensure that the lesion would be mainly in the pons. To investigate the difference between juvenile and adult brainstem glioma, we used 3-week-old and 10-week-old rats. The C6 glioma cells grew in all rats with a consistent location.

It has been suggested that the duration of preoperative history, as well as the prognosis, is influenced by patient age, tumor localization, growth pattern, and histopatho-
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Adult rats consistently had more focal neurological symptoms with a longer duration than young rats, suggesting that C6 cells in the young brainstem progress more aggressively than in the adult brainstem. In children with brainstem gliomas, the median duration was 6 months, whereas adult patients had a median history of 17 months. In the present study, we found that adult rats with brainstem tumors survived longer than young rats, even though the flexibility of brainstem and skull structure in young rats made distortion of the medulla and spinal cord junction possible, which could potentially extend survival time.

The difference in biological behavior of these lesions in juvenile and adult rats was also indicated by histopathological characteristics. In young rats there was a high degree of brainstem parenchyma invasion. In adult rats, the lesions grew relatively focally and displaced the long tract of the brainstem. Many intrinsic focal brainstem gliomas have an area of gliotic tissue because these lesions tend to displace rather than infiltrate neural elements; this provides a safety margin enabling aggressive resection. Our findings confirmed this characteristic of focal brainstem gliomas, and suggested that the anatomical, histological, or immunological diversity of brainstems between young and adult animals may affect the growth pattern and invasiveness of brainstem gliomas.

Although intratumoral hemorrhage is uncommon, symptomatic intratumoral hemorrhage may occur in nearly 20% of children after the diagnosis of diffuse brainstem glioma. The striking association between intratumoral hemorrhage and necrotic tumor areas suggests that bleeding is more likely in the areas in which the blood–brain barrier is disrupted. In the present study, intratumoral hemorrhage was more common in juvenile rats, indicating that this phenomenon is associated with the inherent biologic characteristics of this type of tumor.

Tumor cell invasion is defined as translocation of neoplastic cells through host cellular and ECM barriers. Invading glioma cells seem to follow distinct anatomic structures within the central nervous system. Tumor cell dissemination may occur along structures such as basement membranes of blood vessels or glial external limi- tants, which contain ECM proteins. Occasionally, invasive glioma cells are also found to migrate along myelinated fiber tracts of white matter. This behavior is most likely a consequence of constitutive extracellular ligand expression along the pathways of preferred dissemination; glioma cells may be able to use a multiplicity of matrix ligands to activate separate mechanisms for invasion. Our findings indicate that glioma cells in the young rat brainstem may have or create a more permissive cellular environment for the tumor to spread into the adjacent brain tissue.

Tumor progression is determined both by the rate of cell proliferation and the rate of cell death. The Ki 67 antigen is a prototypic cell cycle–related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle. This antigen is routinely used as a proliferation marker in brain tumors. In astrocytomas, Ki 67 expression is upregulated and correlates with tumor grade and clinical prognosis. We found that tumors in young rats had a higher proliferation rate than in adult rats. Furthermore, few tumor cells underwent apoptosis in the young rats, whereas in the adult rats, a fair number of apoptotic tumor cells were found. Therefore, the high proliferative rate and low level of apoptosis could contribute to faster tumor progression and more aggressive behavior in juvenile brainstem gliomas.

The present study was designed to reproduce the major characteristics of human brainstem gliomas in a rat ortho-topic C6 cell brainstem glioma model. Our findings show that this model simulates the major differences between brainstem gliomas in young and adult patients in terms of symptom duration, tumor growth pattern, and prognosis. However, there are limitations to the current study. The C6 glioma cell line was derived from nitrosomethylurea-in-

Fig. 4. Representative images and photomicrographs of brainstem gliomas in adult (A–C) and juvenile (D–F) rats. A: Photographic of the brainstem in an adult rat. The lesion is relatively focal with displacement of the long tract. B and C: Photomicrographs of brainstem cross-sections in an adult rat. Note highly cellular lesions invading the white matter with a relatively clear boundary. Abundant necrosis was consistently observed. D: In juvenile rats enlargement of the entire brainstem without focal lesion was observed. E and F: Juvenile brainstem cross-sections demonstrating infiltrative and diffuse lesion with a high degree of brainstem parenchyma invasion. Endothelial proliferation was evident and angiogenesis frequently observed. All photomicrographs are stained with H & E; original magnification × 1 (B and E), × 10 (C and F).
duced brain tumor in outbred rats, and intracranial growth of C6 cells results in the formation of glioblastoma after injection of these cells into Wistar and Sprague–Dawley rats.\textsuperscript{18} We could not rule out the role of immune response in this model as C6 cells are not syngeneic in outbred rats.\textsuperscript{5,30} In fact, immunogenicity has also been found in syngeneic glioma models, such as 9L cells in Fisher 344 rats and Gl261 cells in C57BL/6 mice.\textsuperscript{34,35} Nonetheless, our findings warrant further investigation of the different host responses between juvenile and adult brainstem glioma in humans, including angiogenesis, ECM, and reactive gliosis.

**Conclusions**

In conclusion, our modified brainstem tumor model in young and adult rats imitates the neurological findings and histopathological characteristics of brainstem gliomas in humans. Brainstem glioma in young rats is different from brainstem glioma in adult rats in terms of onset of neurological signs, symptom duration, survival time, tumor growth pattern, extent of tumor infiltration, proliferation, and apoptosis. Our results indicate that the growth pattern and invasiveness of brainstem glioma could depend not only on the malignancy of the tumor cells, but also on the host cellular environment. The different biological behaviors of brainstem glioma in young and adult could be attributed to the interaction of tumor cells and the host cellular environment. Because no model currently available exactly simulates human gliomas, our findings in this rat brainstem glioma model must be verified in future clinical studies. In addition, the use of this model of brainstem glioma could facilitate future studies and the discovery of novel therapies to treat diffuse disease.

**Disclaimer**

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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