Monitoring of Tumor Growth and Post-Irradiation Recurrence in a Diffuse Intrinsic Pontine Glioma Mouse Model

Viola Caretti1–3; Ilse Zondervan2,3; Dimphna H. Meijer3,8; Sander Idema2,3; Wim Vos4; Bob Hamans6; Marianna Bugiani4; Esther Hullemen1–3; Pieter Wesseling7; W. Peter Vandertop2; David P. Noske2,3; Gertjan Kaspers1; Carla F.M. Molthoff5; Thomas Wurdinger2,3,9

1 Department of Pediatric Oncology, 2 Department of Neurosurgery, 3 Neuro-oncology Research Group, 4 Department of Pathology, 5 Nuclear Medicine & PET Research, VU University Medical Center, Amsterdam. 6 Department of Radiology, 7 Department of Pathology, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands. 8 Department of Cancer Biology, Harvard Medical School and Dana-Farber Cancer Institute, 9 Molecular Neurogenetics Unit, Department of Neurology, Massachusetts General Hospital and Harvard Medical School, Boston, Ma.

Abstract

Diffuse intrinsic pontine glioma (DIPG) is a fatal malignancy because of its diffuse infiltrative growth pattern. Translational research suffers from the lack of a representative DIPG animal model. Hence, human E98 glioma cells were stereotactically injected into the pons of nude mice. The E98 DIPG tumors presented a strikingly similar histopathology to autopsy material of a DIPG patient, including diffuse and perivascular growth, brainstem- and supratentorial invasiveness and leptomeningeal growth. Magnetic resonance imaging (MRI) was effectively employed to image the E98 DIPG tumor. [18F] 3′-deoxy-3′-[18F]fluorothymidine (FLT) positron emission tomography (PET) imaging was applied to assess the subcutaneous (s.c.) E98 tumor proliferation status but no orthotopic DIPG activity could be visualized. Next, E98 cells were cultured in vitro and engineered to express firefly luciferase and mCherry (E98-Fluc-mCherry). These cultured E98-Fluc-mCherry cells developed focal pontine glioma when injected into the pons directly. However, the diffuse E98 DIPG infiltrative phenotype was restored when cells were injected into the pons immediately after an intermediate s.c. passage. The diffuse E98-Fluc-mCherry model was subsequently used to test escalating doses of irradiation, applying the bioluminescent Fluc signal to monitor tumor recurrence over time. Altogether, we here describe an accurate DIPG mouse model that can be of clinical relevance for testing experimental therapeutics in vivo.

INTRODUCTION

Diffuse intrinsic pontine glioma (DIPG) is the most common and malignant form of primary brainstem tumors. DIPG is a major cause of pediatric brain tumor-related death (9, 14), whereas focal brainstem glioma presents a more circumscribed growth and a better prognosis (8). DIPG generally develops in the pons region of the brainstem, where tumor cells diffusely invade the parenchyma, spreading throughout the brainstem and often into the cerebellum, spinal cord and to supratentorial brain regions (32). Of note, a recent study reported a high incidence of leptomeningeal dissemination and the importance to monitor this tumor component in DIPG patients (26).

Palliative radiotherapy is the standard treatment but it only provides temporary relief of symptoms and is mostly followed by fast recurrence of tumor growth (9). Overall survival is about 10 months from the time of diagnosis (14) and less than 10% of patients survive longer than 2 years (29). Different factors account for such a dismal prognosis in DIPG. First, resective surgery is not possible without serious morbidity because of its diffuse nature and the anatomical complexity of the brainstem (1). Second, in the past decades no chemotherapeutical scheme has proved successful in conferring a survival advantage (14). Third, radiotherapy is not curative and effective radiosensitizers have not been identified yet. Fourth, a better understanding of the biology, and subsequently the possibility to develop and test targeted treatment strategies, has been hampered by: (i) the significant reduction in biopsy sample acquisition since the advent of high-quality magnetic resonance imaging (MRI); (ii) the low number of autopsies performed on DIPG patients (4); and (iii) the low number of pre-clinical studies carried out on this type of cancer.

Until now only a few studies have been published on brainstem glioma models and none of them demonstrated specific targeting of the pontine region of the brainstem. Additionally, the brainstem...
glioma rodent models developed by using 9L, F98, or C6 rat glioma cells (16–19, 30), human glioma cell lines, i.e., U87 and U251 and primary human glioma cells maintained as neurosphere cultures (15, 28), failed to show the typical diffuse and infiltrative DIPG growth. Two recently published rodent brainstem glioma models, developed by genetically overexpressing platelet-derived growth factor (PDGF) (2, 20), presented a more invasive phenotype, but still did not resemble the robust diffuse infiltrative growth observed in DIPG patients.

In the present study, we developed an orthotopic DIPG mouse model presenting diffuse and infiltrative growth by injecting human adult E98 glioma cells in the pontine region of the brainstem of nude mice. Direct comparison of the E98 DIPG model with autopsy material of a DIPG patient revealed a similar histopathology. The E98 DIPG model was also used to investigate MR- and positron emission tomography (PET) imaging modalities. In addition, we stably modified the E98 DIPG cells with expression cassettes for firefly luciferase (Fluc) and the fluorescence reporter mCherry (E98-Fluc-mCherry), allowing bioluminescence imaging (BLI), while preserving the infiltrative DIPG phenotype. Finally, we monitored the response of the E98-Fluc-mCherry DIPG tumors to different doses of irradiation (IR) via BLI.

METHODS

E98 in vivo models

Female athymic nude mice (age 6–8 weeks; Harlan, Zeist, the Netherlands) were kept under specific pathogen-free conditions in air-filtered cages and received food and water ad libitum. All animal experiments were approved and performed according to the guidelines established by the VU University Ethical and Scientific Committees on animal experiments. E98 cells were originally obtained from a surgical glioma specimen of an adult patient. The E98 human glioma mouse model has been previously described (6). E98 glioma cells were cultured as adherent monolayers in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen). After two passages, the cells were transduced with a lentiviral vector containing firefly luciferase (Fluc) and mCherry, as described elsewhere (21, 31). The E98-Fluc-mCherry xenograft model was established by transplantation of 3 ¥ 10⁶ cells, after <10 passages in vitro. Tumor growth was monitored via charge-coupled device (CCD) camera imaging and by caliper measurements. For stereotactic surgery mice were sedated using gas anesthesia (1.5 L O₂/minute and 2.5% isoflurane). A total of 0.5 ¥ 10⁶ E98 glioma cells were injected stereotactically in a volume of 5 μL (5). The skull of the mouse was exposed and a small burr hole (0.5 mm) was made using a high speed drill at the appropriate stereotactic coordinates. E98 or E98-Fluc-mCherry glioma cells were injected at a speed of 2 μL per minute into the pons with a 26-gauge (2 mm, point style AS) Hamilton syringe (Bonaduz, GR, Switzerland), after which the needle was removed at a speed of 0.5 mm/minute. After closing the scalp, mice were placed on a warming pad and returned to their cages after full recovery. Upon discomfort and >15% weight loss, mice were sacrificed and brains were removed and formalin-fixed or snap frozen.

Immunohistochemistry (IHC)

Sections of 5 μm were cut from mouse brains. IHC and hematoxylin and eosin (H&E) staining were performed according to standard procedures. For IHC antibodies against human vimentin (clone V9, VUMC, Amsterdam, the Netherlands) and mouse glucose transporter-1 (Glut-1; DakoCytomation, Glostrup, Denmark) were used after antigen retrieval with citrate. Omission of primary antibody was used as negative control. Envision²HRP and Liquid DAB (DakoCytomation) were used for visualization.

MR- and PET imaging

MR- and PET imaging

Mice were anesthetized as described earlier and BLI was performed 10 minutes after i.p. injection of 150 μL p-Luciferin (150 mg/kg body weight). Photon counts were recorded over 5 minutes using a cooled CCD camera with no illumination, using the Xenogen IVIS Lumina System (Xenogen Corp., Alameda, CA, USA). A light image of the animal was taken in the chamber using dim polychromatic illumination. Following data acquisition, post-processing and visualization was performed using image display and analysis suite, as defined by the LivingImage software (Xenogen Corp.). For visualization purposes, bioluminescence images were fused with the corresponding white light surface images in a transparent pseudocolor overlay.

For the IR study, 13 days after stereotactic surgery mice were sacrificed and brains were removed on the basis of BLI signal intensities into four groups with comparable mean Fluc activity. Mice were anesthetized using ketamine and xylazine and received single cranial IR using Clinac D/E (Varian Medical Systems, Palo Alto, CA, USA), 6 MV, dose rate 300 MU/min. The body of the mouse was shielded using lead. Three mice per group received a dose of 2, 6 or 10 Gy, whereas three control mice were not treated.

In vivo BLI and IR

Mice were anesthetized as described earlier and BLI was performed 10 minutes after i.p. injection of 150 μL p-Luciferin (150 mg/kg body weight). Photon counts were recorded over 5 minutes using a cooled CCD camera with no illumination, using the Xenogen IVIS Lumina System (Xenogen Corp., Alameda, CA, USA). A light image of the animal was taken in the chamber using dim polychromatic illumination. Following data acquisition, post-processing and visualization was performed using image display and analysis suite, as defined by the LivingImage software (Xenogen Corp.). For visualization purposes, bioluminescence images were fused with the corresponding white light surface images in a transparent pseudocolor overlay.
RESULTS

Development of a human E98 DIPG mouse model presenting a diffuse infiltrative phenotype

We first determined whether the pons of nude mice could be targeted via stereotactic injection of human E98 glioma cells. The following coordinates were selected: −0.8 mm on the y plane, −1 mm on the x plane and −5 mm on the z plane (Figure 1A–C). First, E98 cells were injected and mice were directly sacrificed once the stereotactic injection was ended; the pons was successfully targeted (Figure 1D).

To determine whether E98 glioma cells injected into the pons formed tumors and to characterize the development of such tumors, three groups of nude mice underwent stereotactic surgery and were sacrificed 8 (n = 3), 16 (n = 3) and 28 (n = 3) days after injection. Brains were harvested and tissue sections were stained with H&E and anti-human vimentin in order to identify human E98 glioma cells and to distinguish single infiltrating E98 cells from the mouse glial cells, in particular reactive astroglial cells. E98 DIPG tumors developed in all mice and consistently showed diffuse infiltrative growth in the pons as determined by H&E (Figure 2A–C), and human vimentin immunoreactivity (Figure 2E–G), which is visible in more detail in Figure 2I–K and M–O. In the E98 DIPG model, areas of compact growth were present in the leptomeninges in all E98 DIPG mice analyzed (Figure 2F) and occasionally within the ventricles (~20% of cases) (Figure 2M), in particular inside the aqueduct of Sylvius and the fourth ventricle. The E98 DIPG mouse model showed key histological features of human glioma such as nuclear atypia, cellular pleomorphism and increased mitotic activity.

At day 8 after injection the tumors were already evident (Figure 2A and E). No clear cut border separated the tumor from the surrounding parenchyma; instead, single cells invading the adjoining tissue were clearly visible (Figure 2I, arrows). Compact E98 foci were identifiable in the fourth ventricle (Figure 2M). Interestingly, tumor cells from these foci started invading the tegmentum of the pons (Figure 2M, arrows). At day 16 the tumors had increased in dimension (Figure 2B and F) and two different components were identifiable, a more solid core area and an invasive front. The core area corresponded to the original tumor focus, visible at day 8 (Figure 2I), where small islands of proliferating tumor cells continued to form (Figure 2I, asterisk). An invasive front composed of groups of cells as well as single cells invading the pons tissue were also observed (Figure 2J). Moreover, compact components were visible in the subarachnoid space (Figure 2F). The neoplastic pontine tissue was compressed both by the intraparenchymal and the leptomeningeal tumor mass, which seemed to invade through permissive routes, such as the white matter tracts in the middle cerebellar peduncle (Figure 2N). Between day 16 and 28 the tumors underwent major expansion, with more than 60% of the pons being diffusely invaded by the E98 cells (Figure 2C and G). Even 28 days after injection the invading fronts with infiltrating single cells were still present, as shown in a view of the second cerebellar lobe (Figure 2K, arrows), and in the area between the initial tumor core and the subsequently grown tumor spreading in the pons parenchyma (Figure 2O).

In conclusion, we were able to develop an E98 DIPG mouse model presenting 100% take rate. Additionally, the stereotactic coordinates selected and the use of gas anesthesia allowed us to reproducibly target the pons of nude mice, to achieve full recovery of the mice within half an hour from tumor cell injection and to report no surgery related mortality.

For comparison, E98 glioma cells were also injected in the supratentorial region (Figure 2D and H) of the brain of nude mice (n = 3). Here the infiltrative component was preferentially present in major white matter fiber tracts (Figure 2L), as previously...
The supratentorial tumor component in the hypothalamic area revealed less pronounced margins (Figure 2P), whereas in the E98 DIPG model the diffuse component infiltrated throughout the pontine parenchyma (Figure 2K and O).

The orthotopic E98 DIPG mouse model resembles the histopathology of clinical DIPG

Next the histopathological characteristics of the E98 DIPG tumors were directly compared with autopsy material from a DIPG patient. The tumor of the DIPG patient presented the typical diffuse growth in the basis (Figure 3A and B, arrowheads), as well as in the tegmentum pontis (Figure 3E and F, arrowheads). The tumor of the DIPG patient, originally located in the pons, invaded supratentorial (data not shown) and other infratentorial structures, by means of direct intraparenchymal infiltration and leptomeningeal dissemination. Figures 3J and 3N (arrowheads) show human DIPG cells invading the medulla oblongata via intraparenchymal infiltration.

Leptomeningeal and intraventricular dissemination occurred throughout the brain, including the fourth ventricle, the cerebellum and ventral part of the medulla oblongata (Figure 3E, I and M, respectively, indicated by asterisks). A similar direct infiltration of the surrounding parenchyma was also observed in E98 DIPG tumors (Figure 2K and O). The same applies to the leptomeningeal dissemination (Figure 2F) with secondary infiltration of the underlying parenchyma from the compact subarachnoid component, which was observed in both the E98 model (Figure 2N) and the patient (Figure 3M, arrows).

Furthermore, perivascular tumor dissemination was detected throughout the brain of the clinical DIPG case (Figure 3G), as well as in the preclinical E98 DIPG model (Figure 3H), providing permissive routes to invade distal brain areas (10). Interestingly, perivascular migration appeared to be a route for the tumor cells located in the subarachnoidal space to invade the brain parenchyma both in the clinical (Figure 3K) and E98 (Figure 3L) DIPG tumors. Additionally, vascular proliferation was detected in the intraparen-

---

Figure 2. E98 diffuse intrinsic pontine glioma (DIPG) tumor development. Gross appearance of a representative E98 DIPG tumor at 8 (A,E), 16 (B,F) and 28 (C,G) days after E98 glioma cells implantation. E–G. Areas enclosed by the two dotted squares are magnified in I,M,J,N,K,O,D,H. Gross appearance of a supratentorial E98 tumor; the areas enclosed by the two dotted squares are magnified in L,P. Size bar = 1 mm (A–H), 125 μm (I), 200 μm (J,K,L,P), 500 μm (M,O) and 62.5 μm (N).
Chymal and leptomeningeal (Figure 3O and P) tumor parts, both in the human and E98 DIPG tumors where also the preexisting leptomeningeal vessels may have been incorporated into the subarachnoid tumor component.

Endothelial expression of Glut-1 is considered to be a marker for the integrity of the blood–brain barrier (BBB) (13). In the human autopsy material and in the E98 DIPG tumors the majority of blood vessels in the intraparenchymal diffuse tumor component stained positive for Glut-1 (Figure 3C and D, respectively).

MR- and PET imaging of the E98 DIPG mouse model

The applicability of MRI and PET was investigated as imaging modalities for the E98 DIPG tumors in the pons of mice. Two mice underwent MR-scans at 28 days after stereotactic implantation of E98 glioma cells in the pons, together with a healthy control mouse (Figure 4). The pons of the control mouse was clearly visible (Figure 4A, arrows). MR-images of the E98 DIPG-bearing mice revealed the presence of tumors in the pons region (Figure 4B). MRI scans also showed E98 DIPG tumor invasion of the midbrain, cerebellum, medulla oblongata and thalamus (Figure 4B). The sagittal and transversal MRI views revealed enlargement of the mouse pons (Figure 4B, upper and central panels), resembling the human situation (Figure 4C). The coronal MRI scan (Figure 4B, mouse 1, lower panel) clearly reproduces the H&E- and vimentin-stained tissue sections from the same mouse (Figure 2C and G).

To test whether PET could be used to monitor E98 DIPG metabolic activity, we first determined the uptake of the $[^{18}{}F]FLT$ tracer...
in E98 cells in vitro. [18F]FLT tracer uptake and retention, an indicator of cell proliferation (25), increased proportionally to E98 cells number (Supporting Information Figure S1). Next [18F]FLT PET imaging was performed in E98 tumor bearing mice to investigate whether the proliferative activity of E98 tumors could be monitored in vivo. First, the tracer was administered to mice bearing s.c. E98 tumors (n = 4) in both flanks. Figure 4D (left panel) shows a representative PET image of a mouse bearing bilateral s.c. E98 tumors, presenting [18F]FLT tumor uptake (arrows). [18F]FLT PET was also performed in mice 21 days after injection of E98 cells in the pons (n = 3) or in the striatum (n = 3). Although post-mortem analysis of the brains did reveal the presence of E98 tumors in these locations (data not shown), the resolution (2.3–2.7 mm) of this HRRT PET scanner was not high enough to significantly measure [18F]FLT tracer activity in E98 tumors in the mouse brain (Figure 4D, right panel).

BLI of E98-Fluc-mCherry DIPG tumors
In order to establish DIPG tumors in mice that could be monitored longitudinally, E98-Fluc-mCherry cells were engineered. More than 99% of the cells expressed the red fluorescent protein mCherry (Figure 5A, insert), and transduction efficiency remained constant after multiple passages. Additionally, the BLI signal correlated directly to E98-Fluc-mCherry cell number (Figure 5A). E98-Fluc-mCherry cells were injected stereotactically into the pons of nude mice. Mice injected with E98-Fluc-mCherry cells showed an increasing Fluc signal over time, indicating DIPG tumor engraftment and growth (Figure 5B and C). After sacrificing the mice, brain slices were analyzed by fluorescence microscopy to identify mCherry positive E98-Fluc-mCherry cells and results were confirmed by IHC. All mice analyzed showed mCherry expression throughout the pons (Figure 5D). However, IHC evaluation revealed a more compact phenotype (Figure 5E), resembling that of focal pontine glioma. At best a limited invasive growth instead of diffuse infiltration was detected, even though the duration before onset of clinical signs and weight loss were similar to the parental E98 cells. In Figures 5F and G we show the tumor that developed with the most invasive margins.

To determine whether the focal phenotype of the E98-Fluc-mCherry tumors was caused by in vitro culture prior to intracranial injection, E98-Fluc-mCherry cells were implanted s.c. in the flank of nude mice. The Fluc activity of the E98-Fluc-mCherry s.c. tumors correlated with the caliper measurements (Figure 6A and B). After 3 weeks, E98-Fluc-mCherry cells were isolated from the s.c. tumors and transplanted directly into the pons of nude mice. Again, these mice showed an increasing Fluc signal over time (Figure 6C and E). Interestingly, after the s.c. transplantation step, the brains analyzed by IHC revealed a major diffuse and infiltrative phenotype (Figure 6F and G), and in addition the perivascular and leptomeningeal growth (Figure 6H), which was already observed in the parental E98 model. Figure 6I depicts single cells invading the second cerebellar lobe, similar to the parental E98 DIPG xenografts (Figure 2K). The E98-Fluc-mCherry focal and diffuse...
pontine tumors presented tumor growth in all injected mice and a reproducible tumor phenotype. Finally, BLI allowed the identification of supratentorial brain invasion as well as the identification of spinal cord metastases (Figure 6D).

**IR treatment of nude mice bearing E98-Fluc-mCherry DIPG**

Radiotherapy is the standard treatment for DIPG. Hence to prove the feasibility of the E98-Fluc-mCherry DIPG model for therapeutic testing, an IR dose–response experiment was performed. The doses used are 2, 6 and 10 Gy, as described previously (2). The BLI signal, detected by CCD camera imaging, decreased in all irradiated mice, whereas the non-treated tumors continued to grow (Figure 7A and B). The 6 and 10 Gy doses showed a similar effect on DIPG tumor recurrence, ie, resumption of DIPG tumor growth approximately 13 days after IR. The 2 Gy dose had a less strong effect resulting in tumor recurrence 7 days after IR. Similarly, survival analysis showed that mice irradiated with 2 Gy survived longer than non-treated mice, but less compared with mice that received a dose of 6 or 10 Gy (Figure 7C).

**DISCUSSION**

The most important hallmark of DIPG is its extreme infiltrative diffuse phenotype, which is also the major obstacle to effective treatment. While resective surgery may constitute a treatment option for focal brainstem tumors because of their compact phenotype, such treatment is not possible for DIPG. Another hallmark is the specific anatomical location where DIPG presents its epicenter, the pontine region of the brainstem, as revealed by MRI of DIPG patients at diagnosis. Hence, such a diffuse infiltrative growth pattern and the specific anatomical location where DIPG develops should be represented in a robust DIPG animal model. Brainstem
animal models previously developed show a more compact phenotype or lack the specific DIPG anatomical location in the pons. Such models represent brainstem gliomas with a more focal growth rather than the diffuse pattern characteristic of DIPG. Here, to the best of our knowledge, we report for the first time the development of a DIPG specific mouse model and not of a brainstem glioma model. In particular, this study was constantly centered over a direct comparison with the histopathology and clinical data of human DIPG with the ultimate goal to develop a mouse model presenting clinical relevance.

We characterized the histopathology of the E98 DIPG xenografts at different time points during their growth, demonstrating a robust infiltrative growth pattern present since day eight after tumor cells injection until mice were sacrificed. Such a detailed description provides a useful histopathological reference for future studies employing this model. This especially holds true as we directly compare the E98 DIPG mouse model with autopsy material of a DIPG patient, highlighting salient common characteristics such as tumor brainstem- and supratentorial invasiveness and perivascular and leptomeningeal growth. A recent study on MRI neuroaxis surveillance revealed that 56% of DIPG patients presented leptomeningeal dissemination and a shorter overall survival than patients with localized disease (26). The E98 DIPG model would also allow investigation of the leptomeningeal component.

The E98 DIPG xenografts presented here are a very accurate phenotypical model for this type of glioma, although it has to be noted that they do not necessarily represent the genotype of this disease. Recently two studies reported for the first time genomic profiling of DIPG tumor samples (23, 33). Although DIPG appeared to be heterogeneous on chromosomal level, we identified discrepancies in chromosomal aberrations between E98 (6) and the DIPG samples analyzed (23, 33). E98 tumors revealed chromosomal loss of 2q, 4q, 9p and 10q, while DIPG tumors showed loss of 13q, which is in common with pediatric high-grade astrocytoma (HGA), and loss of 1p, 17p, 14q, 18p and 22q. E98 tumors presented gain of 7 and 19p, while DIPG and HGA showed gains in 1q and 9q, whereas only DIPG showed gain of 10p and 17q (23, 33). Hence, a future genotypic improvement over the E98 DIPG model presented here may be the use of primary glioma cells derived from DIPG patients. Although the development of suited human DIPG cell lines has not been reported yet, such cells could be injected following the stereotactic approach described here.

Another caveat of this study is the impaired immune system of nude mice. While genetic mouse models better recapitulate the

---

**Figure 6.** Transplanted E98-Fluc-mCherry glioma cells develop diffuse intrinsic pontine glioma (DIPG) tumor. **A.** Charge-coupled device (CCD) camera images of a mouse bearing s.c. E98-Fluc-mCherry tumor. **B.** Corresponding quantification of the s.c. Fluc activity and tumor volume. **C.** CCD camera images of mice bearing E98-Fluc-mCherry DIPG. Days refer to days after stereotactic surgery. **D.** CCD camera images of drop metastasis (left) and supratentorial invasion (right). **E.** Quantification of the Fluc activity in (C). **F.** Vimentin IHC of a representative E98-Fluc-mCherry DIPG tumor (Mouse 1). **G.** Higher magnification of (F), tumor cells diffusely invade the pons parenchyma (arrowheads). **H.** Midbrain (Mouse 1) where diffuse DIPG invasion is visible (arrowheads). **I.** Cerebellum. The asterisk indicates leptomeningeal growth, the arrow perivascular growth and the arrowheads diffuse intraparenchymal invasion. Size bar = 1 mm (F), 556 μm (G), 250 μm (H) and 100 μm (I).
impact that inflammatory or stromal cells have in the pathogenesis of tumors, xenograft mouse models allow the use of human cell lines. Although thus far other human glioma cell lines (15, 28), once injected into the brainstem of rodents, did not show an infiltrative diffuse phenotype, as shown for the E98-Fluc-mCherry glioma cells, we cannot exclude that other cancer cell lines could give rise to such phenotype.

While characterizing the E98 DIPG xenografts and human DIPG we found Glut-1, a BBB marker (13), to be positive both in the human DIPG autopsy material and in the E98 DIPG tumor, indicating a functional BBB, and suggesting that tumor cells propagate around pre-existing vessels that retain normal expression of BBB markers (11). These results suggest that the BBB in the E98 and patient DIPG tumors remains intact in areas of diffuse tumor growth. Interestingly, DIPG tumors often show only limited contrast enhancement on MRI, as expected by the extreme diffuse and infiltrative nature of the DIPG cells growing in areas with an intact BBB. Furthermore, a reason for the inefficiency of systemic chemotherapy in DIPG patients may be the lack of effective drug delivery caused by an intact BBB in the diffuse DIPG areas. These results warrant further investigation of the role of the BBB in DIPG.

The applicability of MRI and PET imaging for the E98 DIPG mouse model was also investigated. Here we showed that MR-imaging of E98 DIPG tumors is feasible, allowing DIPG tumor localization within the different anatomical structures of the mouse brainstem along with DIPG invasiveness of other brain structures. In addition, future studies could employ MRI contrast agent to further investigate the function of the BBB in E98 DIPG xenografts, also during preclinical investigation of novel treatments for this disease. [$^{18}$F]FLT PET imaging using the HRRT can be used to determine proliferative activity in s.c. E98 tumors, but not in the E98 orthotopic brain tumors. For this purpose the use of the more sensitive micro-PET or micro-PET-computed tomography (CT) will need to be investigated (25).

Next, E98 glioma cells were genetically engineered to express Fluc and mCherry to allow BLI monitoring of tumor engraftment, growth and response to therapies. Interestingly, by injecting cultured E98-Fluc-mCherry cells into the pons of nude mice, focal pontine gliomas developed, whereas injection after an intermediate s.c. passage gave rise to typical DIPG tumors, presenting the same histopathology as the parental E98 DIPG tumors. These results suggest that specific environmental stimuli play a role in the ability of glioma cells to invade the surrounding tissue. Previous studies

Figure 7. E98-Fluc-mCherry diffuse intrinsic pontine glioma (DIPG) response to irradiation (IR) monitored by bioluminescence imaging (BLI). A. Quantitation of Fluc activity in treated and control groups, results are presented as mean ± standard deviation (SD). B. Charge-coupled device (CCD) camera images of a representative mouse from each group. C. Survival analysis of A. Day 0 refers to the day mice were irradiated.
on primary human DIPG cells demonstrated that serial s.c. passaging in mouse flanks allowed retention of tumor invasiveness upon subsequent implantation intracranially (12). This versatility of the E98-Fluc-mCherry glioma cells could be used to investigate differences in biology and treatment strategies between focal brainstem tumors and DIPG tumors.

As a proof of principle for the applicability of BLI monitoring in future studies, an IR dose–response study was performed. In most DIPG patients, IR causes temporary DIPG regression and partial relief of symptoms. However, recurrence of the tumor always occurs within a period of months. The challenge for translational research in DIPG is the discovery of effective sensitizers able to potentiate or prolong the effects of radiotherapy, preventing DIPG recurrence. The E98-Fluc-mCherry DIPG model may be suited for this purpose as it responds to IR while presenting subsequent tumor recurrence.

CONCLUSION

We have characterized a unique orthotopic DIPG mouse model using human adult E98 glioma cells. Although the E98 DIPG model may not fully represent the genotypic hallmarks of human DIPG, this model does present the characteristic diffuse infiltrative phenotype of human DIPG, and DIPG recurrence after IR treatment, one of the major obstacles in the treatment of this devastating disease. Additionally, we have demonstrated that this model can be useful for studies on DIPG growth and for preclinical testing of potential therapeutic approaches.

ACKNOWLEDGMENTS

We are grateful to Petra van der Stoop, Tonny Lagerweij, Marco Barazas (Neuro-oncology Research Group), Paul van der Valk and Inge Greeuw (Department of Nuclear Medicine & PET Research) for skilled technical support. We thank Dannis G. van Vuurden and Marc H.A. Jansen (Department of Pediatric Oncology) for support and the MR-imaging and Phil Koken (Department of Radiation Oncology Research Group), Paul van der Valk and Barazas (Neuro-oncology Research Group), Paul van der Valk and Balzarini J, Spreeuw enberg MD et al (2008) [18F]FDG and [18F]FLT uptake in human breast cancer cells in relation to the effects of chemotherapy: an in vitro study. Br J Cancer 99:481–487.


REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. FLT uptake analysis in vitro. E98 cells were plated in triplicates in 24-well plates 2 days prior to radiotracer addition. Medium was replaced with DMEM without L-glutamine, and after 4 h of incubation, 1 MBq well−1 of FLT was added to each well and incubated for 60 minutes. Cells were harvested and cell-bound radioactivity was measured in a Compugamma gamma counter.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.