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The growth of glioblastoma orthotopic xenografts in nude mice is directly correlated with impaired object recognition memory

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HIGHLIGHTS

- Cognitive impairment is present in about 80% of patients with brain tumors.
- Animal behavior models are necessary to study these alterations.
- Object recognition task is efficient to test behavior in glioblastoma mouse model.
- Cognitive alterations were related with tumor size and appeared before clinical signs.
- This test is useful to evaluate the efficacy of new therapies against glioblastomas.

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ABSTRACT

Cognitive dysfunction is found in patients with brain tumors and there is a need to determine whether it can be replicated in an experimental model. In the present study, the object recognition (OR) paradigm was used to investigate cognitive performance in nude mice, which represent one of the most important animal models available to study human tumors *in vivo*. Mice with orthotopic xenografts of the human U87MG glioblastoma cell line were trained at 9, 14, and 18 days (D9, D14, and D18, respectively) after implantation of 5×10^5 cells. At D9, the mice showed normal behavior when tested 90 min or 24 h after training and compared to control nude mice. Animals at D14 were still able to discriminate between familiar and novel objects, but exhibited a lower performance than animals at D9. Total impairment in the OR memory was observed when animals were evaluated on D18. These alterations were detected earlier than any other clinical symptoms, which were observed only 22–24 days after tumor implantation. There was a significant correlation between the discrimination index (*d2*) and time after tumor implantation as well as between *d2* and tumor volume. These data indicate that the OR task is a robust test to identify early behavior alterations caused by glioblastoma in nude mice. In addition, these results suggest that OR task can be a reliable tool to test the efficacy of new therapies against these tumors.

1. Introduction

Primary malignant brain tumors are very aggressive and devastating, and survival rates are still very poor, particularly for patients with glioblastoma multiforme (reviewed by Kuijlen et al. and Vauleon et al. [1,2]). Severe brain dysfunction, which is manifested by neurological and cognitive impairments, is present in more than 80% of patients with brain tumors [3,4] and is more prominent in these patients than in those with extracranial malignancies [5]. Cognitive deficits vary from patient to patient as well as with the site of the lesion. Remarkably, these differences are eliminated or reduced after surgical ablation of the tumor [3].

Patients with high-grade glioma evaluated eight or eighteen months after surgery show deterioration in attention and psychomotor speed. In addition, cognitive decline was shown to be more prominent in patients with tumor recurrence, and their performance was already worse at baseline [6]. Verbal memory evaluated before treatment was also positively associated with survival duration in patients with malignant gliomas [7], indicating that the tumor *per se* affects cognition, and that neurocognitive deficit is a negative prognostic factor.

The treatment of brain tumors with chemotherapy or radiotherapy can alleviate cognitive deficits but, unfortunately, can also cause

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behavioral side effects in up to 70% of patients (reviewed by Crossen et al., Ricard et al. and Dietrich et al. [8–10]). Over the last few years, a growing number of studies have assessed strategies to prevent or treat cognitive deficits in patients with brain tumors. The approaches range from pharmacological prevention and treatment of cognitive deficits to multifaceted cognitive rehabilitation [11].

Currently, there is a need to determine whether the cognitive dysfunction observed in patients with brain tumors can be replicated in an experimental animal model. Such a model would be an extremely useful tool for studies on the treatment of brain tumors and their associated dysfunctions. The novel object recognition (OR) paradigm is a simple learning paradigm that is suitable to investigate declarative memory in rodents. This task [12] relies primarily on the animal's innate exploratory behaviors in the absence of externally applied rules or reinforcement. In addition, both short (STM) and long-term memories (LTM) can be evaluated with this task [13,14]. The preference for novelty is revealed by the tendency of the animal to spend more time exploring the novel *versus* the familiar stimulus [15]. This behavior can be easily quantified and utilized to study simple recognition memory as well as more complex temporal, and episodic-like memory in rodents [16,17].

In this study, we explored recognition memory in Foxn1^{nu} (*nu*/*nu*) mice using the OR task. These mice are the most used animal model to study the molecular aspects of human tumors as well as for the preclinical development of anti-cancer therapies. Indeed, OR was used to search for memory deficits in nude mice bearing orthotopic xenografts of the human U87MG glioblastoma cell line. Correlations between behavioral performance and tumor growth allowed for the validation of OR as an experimental model for the assessment of cognitive decline during tumor growth.

2. Materials and methods

2.1. Cell and tumor culture

The human U87MG glioblastoma cell line was obtained from ATCC and cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum in a humidified atmosphere with 5% CO_2 at 37 °C.

2.2. Orthotopic glioma model

Male 8–10 week-old Balb/C Foxn1^{nu} (*nu*/*nu*) mice, herein designated *nude* mice, were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice were anesthetized by intraperitoneal (i.p.) administration of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). U87MG cells (5×10^5) were resuspended in saline solution and implanted in the right striatum using a 26-gauge needle attached to a 10 μL Hamilton syringe, which was then attached to a Harvard 22 syringe pump (Harvard Apparatus, MA). The site of implantation followed coordinates from bregma in mm: Antero-Posterior: +1.0; meso-lateral: +2.0; and dorso-Ventral: −3.5. Neurological symptoms were assessed by modified neurological scores [18] as follows: grade 0, no symptoms; grade 1, tail weakness or tail paralysis; grade 2, hind leg paraparesis or hemiparesis; grade 3, hind leg paralysis or hemiparalysis; and grade 4, complete paralysis (tetraplegia), moribund stage or death. Animals were euthanized using CO_2 saturation when they presented grades 3 to 4.

The National Institute of Health (USA) and institutional guidelines for animal welfare and experimental conduct were followed. This study was approved by the Animal Care and Use Committee of Fundação Antonio Prudente, Hospital A. C. Camargo (025/08). A total of 45 animals were used in this study.

2.3. Object recognition task

OR experiments were conducted in an open-field arena ($30\times25\times20$ cm) built from polyvinyl chloride plastic. Stimulus objects were made of plastic. There were several copies of each object, which were used interchangeably. The open-field arena and the stimulus objects were cleaned thoroughly between trials to ensure the absence of olfactory cues. Animals (n = 39) were habituated to the arena by allowing them to explore it freely for 20 min per day for 4 days in the absence of any other behaviorally relevant stimulus. After habituation, 31 mice were xenografted with U87 cells and together with the control group (n = 8) (not exposure to any procedure) were subjected to OR 9 days latter (D9). Mice were placed in the arena containing two identical objects (denoted by a_1 and a_2) and left to explore them freely for 5 min. Test sessions of 5 min duration were performed 90 min (STM) and 24 h later (LTM). For this purpose, one of the objects used during the training phase was randomly replaced by novel ones, which were denoted by b (90 min) or c (24 h), and exploratory behavior of the mice toward familiar and novel objects was quantified. From the 31 xenografted mice, 7 animals were euthanized at D9 to have their tumors measured. After 14 days post-tumor implantation (D14) the 24 xenografted animals remained and the control group were submitted to a second OR training session using two identical new objects (denoted by d_1 and d_2); 90 min and 24 h later, a test phase was performed by replacing one of the objects with novel ones (denoted e and f). From the xenografted group of mice, 12 were euthanized to have their tumors measured. The last OR training session was conducted with the 12 remained xenografted animals 18 days post-tumor implantation (D18) and with control animals using two identical new objects (denoted g_1 and g_2) and a test phase was performed by replacing one of the objects by novel ones (denoted h and i) 90 min and 24 h later. All animals were euthanized and tumors measured in the xenografted group. Exploration was defined as sniffing or touching the stimulus object with the nose and/or forepaws. Sitting on or going around the objects was not considered exploratory behavior. A discrimination index (*d2*) was calculated for each animal and expressed by the ratio Tx − Ty/(Tx + Ty), where Tx = time spent exploring the novel object x, and Ty = time spent exploring the familiar object y.

2.4. Determination of tumor volume and histology

Brains from mice xenografted with U87 cells were removed from the cranial cavity. The tumors formed were encapsulated and presented a consistence very different from the brain tissue allowing their isolation using a stereomicroscope at magnification of 10×. Tumor volume (mm^3) was determined using width (a) and length (b) measurements ($V = a^2 \times b/2$, where $a \leq b$). An additional group of mice (n = 6) were xenografted with U87 cells and after 9 (n = 2), 14 (n = 2) and 18 (n = 2) days post-tumor implantation, animals were euthanized and their brains fixed in 4% paraformaldehyde. Serial criosections (10 μm) were stained with eosin–hematoxylin in order to evaluate tumor localization. Sections were imaged using DAKO ChromaVision Systems ACIS III.

2.5. Statistical analyses

Data were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey's test and t test. The Pearson's correlation coefficient was used for correlation analysis, and values were calculated using GraphPad Prism 6.0 Software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. A temporal cognitive deficit is detected in nude mice bearing orthotopic glioblastoma xenografts

Nude mice were implanted with 5×10^5 U87MG cells in the striatum and tested for recognition memory retention at 9, 14, and 18 days after tumor implantation (D9, D14, and D18, respectively). At the sample