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 (d_2) and time after tumor implantation as well as between d_2 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.

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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 pre-clinical 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% CO₂ 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 μ L 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 CO₂ 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 × 25 × 20 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 later (D9). Mice were placed in the arena containing two identical objects (denoted by a₁ and a₂) 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 euthanatized 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 d₁ and d₂); 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 euthanatized 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 g₁ and g₂) 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 euthanatized 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 (d_2) was calculated for each animal and expressed by the ratio $T_x - T_y / (T_x + T_y)$, where T_x = time spent exploring the novel object x, and T_y = 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 euthanatized and their brains fixed in 4% paraformaldehyde. Serial criosections (10 μ m) 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

Fig. 1. A temporal memory deficit in nude mice with orthotopic glioblastoma xenografts. U87MG cells (5×10^5) were resuspended in saline solution and implanted in the right striatum of nude mice. Nine days after tumor implantation (D9), the animals ($n = 31$) were exposed to a training phase (A) for 5 min to two identical objects (a_1 and a_2). The test phase was performed after 90 min or 24 h and the mice were exposed to a familiar (a_1) and a novel object (b or c , respectively) to assess for object recognition (OR). From these animals 7 were euthanized for tumor volume evaluation. (C) A second training phase was performed with the remaining mice ($n = 24$) 14 days after tumor implantation (D14) using a new set of objects (d_1 and d_2), followed by a test phase performed after 90 min or 24 h with the familiar object (d_1) and a novel object (e or f , respectively). Twelve animals were euthanized for tumor volume evaluation. (E) A third training phase was performed on the remaining animals ($n = 12$) 18 days after tumor implantation (D18) with a new set of objects (g_1 and g_2), followed by a test phase performed after 90 min or 24 h with the familiar object (g_1) and a novel object (h and i , respectively). A control group of mice without tumors ($n = 8$) was tested in parallel using the same objects (B, D, and F). Data are presented as a mean ($\pm$ SEM) of the percentage of time exploring a particular object over the total time of object exploration. * $p < 0.0001$, in one-way analysis of variance (ANOVA) followed by a Tukey's test. (G) Total exploration time(s) measured in mice from the control group and from groups D9, D14, and D18. Gray bars represent control mice and the black ones represent nude mice bearing tumors. Data are presented as mean ($\pm$ SEM) of the total time exploration.

phase animals from both tumor-implanted and control groups evaluated on D9, D14 and D18 spent the same amount of time exploring the two identical objects (a_1 and a_2 ; d_1 and d_2 ; g_1 and g_2 respectively)

(Fig. 1A–F). Tumor implanted animals at D9 (1A) as well as control group (1B) spent more time exploring the novel object (b , c) than the familiar one (a) either 90 min ($p < 0.0001$) or 24 h ($p < 0.0001$) after

training (measured by the % of the total time used to explore familiar or novel objects). The D14 mice were still able to discriminate between familiar (d) and novel (e, f) objects (Fig. 1C), but their performance was significantly lower than that on D9 either at 90 min ($p < 0.0001$) or 24 h ($p < 0.0001$) post-training (Fig. 1A and C). At D18, the mice were no longer able to form or retain OR memory (Fig. 1E) and, consequently, spent the same amount of time exploring the novel (h, i) and the familiar (g) objects during the test sessions.

As shown in Fig. 1B, D, and F, the control nude mice that were tested for OR at the same time as the animals with tumors and using the same set of objects successfully learned the OR task when tested for short term memory (90 min after training) or long-term memory (24 h after training). These results demonstrated that nude mice present a normal performance for the OR task, and that this task can be repeated with the same group of animals at least 3 times with intervals of 4–5 days without interfering with their performance.

There were no differences in the total exploration time (defined as the sum of the time that animals explored both objects in test phase) among mice implanted with tumors (black bars) at D9, D14, D18 (Fig. 1G) or among control mice at D9 and D14 (gray bars). The control group presented a difference in the total exploration time at D18 when compared to the other groups; this difference is due to a higher variability between animals within this time point and has no impact on overall data. Together, these results indicate that tumor growth impairs OR memory without affecting exploratory activity.

3.2. Time-dependent tumor growth, absence of major ventricular and hippocampal compression and lack of clinical symptoms

In order to evaluate tumor growth, we established a novel approach to determine the volume of tumors. The U87 xenograft tumors presented an encapsulated growth, which is restricted to the area of implantation. Remarkably, the consistence of the tumors is very different from the brain tissue allowing tumor straightforward and complete removal and isolation from the rest of the tissue. This approach permits a precise measurement of tumor volume and is less time consuming than performing tumor measurements on histological serial sections. As expected, tumor size from mice tested on D9, D14 or D18 was statistically different (Fig. 2A, $p < 0.05$) and presented a linear growth (Fig. 2B).

A second group of animals ($n = 6$) was used to evaluate tumor localization and size within the brain. U87 cells were xenografted and after 9, 14 and 18 days the brains were removed and processed for histological evaluation using eosin–hematoxylin. As depicted in Fig. 2C (a panels), tumors presented an antero-basal growth from the injection site (AP +1.0; ML +2.0; DV −3.5) and neither ventricular nor hippocampal compression was observed (Fig. 2C, b and c panels). These mice were also evaluated for clinical parameters according to a five-point scale (Section 2.2) [18,19] and all of the mice were graded 0, which indicated a complete absence of clinical symptoms. The first clinical signs, tail weakness or tail paralysis (grade 1), was observed in 40% of animals at day 23 post-tumor implantation, which is later than the alterations in OR described here. Therefore, changes in OR performance (Fig. 1) were observed several days before the presence of any clinical symptoms.

3.3. Negative correlation between the discrimination index and time after tumor implantation

The correlation between the discrimination index ($d2$, which is the ratio between the time spent exploring the new object minus the time exploring the familiar object and the total time of exploration) and the time of tumor implantation was also evaluated. Control animals (gray symbols) showed positive $d2$ values throughout all test sessions performed either 90 min (Fig. 3A) or 24 h post-training (Fig. 3B), which indicated that they were discriminating between new and familiar objects. At D9, the mice implanted with tumors (black symbols)

showed similar $d2$ values when compared to control animals either at 90 min or 24 h post-training (Fig. 3A and B). Animals with tumors at D14 presented lower $d2$ than D9 group at 90 min post-training (Fig. 3A, $p < 0.0001$) but did not differ from D9 group at 24 h post-training or from the control group (Fig. 3B).

Mice at D18 were unable to discriminate between novel and familiar objects, and their $d2$ was significantly lower than the control group (Fig. 3A and B, $p < 0.005$). D18 animals also presented a lower $d2$ when compared to those at D9 (Fig. 3A and B, $p < 0.005$).

A significant negative correlation index (r) was found between $d2$ and the time after the implantation of tumor cells when the test phases were performed at either 90 min (Fig. 3C) or 24 h post-training (Fig. 3D). We also evaluated the correlation index (r) between $d2$ and the time after the implantation of tumor cells using only mice that were tested in all time points (D9, 14 and 18) and we still observed a significant negative correlation index in animals which performed the 90 min test (Fig. 3E). However, the correlation index for the 24 h test was not significant (Fig. 3F).

3.4. Negative correlation between the discrimination index and tumor volume

The discrimination index also correlated to the tumor volume (mm^3) at D9, D14, and D18. A moderate but significant correlation between $d2$ and tumor volume was observed when test phases were performed 90 min (Fig. 4A) post-training. However, the correlation did not reach statistical significance when test phases were performed 24 h post-training (Fig. 4B). These data indicate that the decrease of OR memory retention at 90 min post-training (STM), but not at 24 h post-training (LTM), is correlated to the increase of tumor volume.

4. Discussion

The present study established a memory impairment that correlates with the growth of orthotopic xenografts of the human glioblastoma in nude mice. These mice have a mutation in the transcription factor forkhead box N1 (Foxn1), which is expressed in a thymic epithelial cell lineage responsible for thymopoiesis [20]. Indeed, the mice represent one of the most recognized experimental models for studying human tumors *in vivo* due to their deficiency in mature T cells and inability to establish an immune response.

Very few behavioral studies have been performed to date with systemic immune deficient mice. Yang and colleagues have shown that nude mice bearing U87-MG tumors have sensorimotor dysfunction, which is observed in very late stages of brain tumor progression [21]. Conversely, our work presents evidences that specific cognitive deficits (determined by OR task) in tumor-bearing mice occur in early stages of tumor progression. Kipnis et al. [22] demonstrated that SCID mice (BALB/cByJSmn-Prkdc-scid) as well as nude mice (BALB/c/OLA) presented impaired cognitive activity for spatial learning when evaluated by the Morris water maze (MWM) test. This behavioral alteration was reversed by replenishment with T cells, indicating the importance of the integrity of the adaptive immune system in cognitive functions. The present data show that despite a possible alteration at spatial learning [22], nude mice are still able to perform OR task properly.

The MWM is a task for rodents that employs a variety of sophisticated mnemonic processes. These processes encompass the acquisition of relevant visual and spatial cues that are subsequently processed, consolidated, stored, and then retrieved in order to successfully navigate and thereby locate a hidden platform to escape from the water [23]. This spatial learning task involves mainly the hippocampus, but a number of studies have assessed that other brain structures are involved with MWM performance, such as nucleus accumbens (movement directions), and caudate nucleus (place-reward information), lateral mammillary and thalamic nuclei (head direction), posterior parietal

Fig. 2. Growth and localization of U87MG tumors in mouse brain. (A) Nude mice were euthanized at D9 ($n = 7$), D14 ($n = 12$), and D18 ($n = 12$), and tumors were removed from brain tissue using a stereomicroscope at magnification of $10\times$. Tumor volume was determined using the formula $(\text{length} \times \text{width}^2) \times 0.5$. * $p < 0.005$, ANOVA followed by Tukey's test. (B) The Person's correlation between tumor volume (mm^3) and days after tumor implantation shows $r = 0.6032$ and $p = 0.0003$. (C) Serial representative images from brains of an additional group of animals ($n = 6$) after 9, 14 or 18 days of tumor implantation. Each panel lane depicts coronal brain slices of the same animal and tumor area presents an increased HE staining: a – anterior end; b – ventricular region; c – hippocampus.

cortex (local panoramic view system), subiculum, entorhinal cortex and superior colliculus (orientation toward specific cues) [24]. In this study, the OR task was used to evaluate memory by measuring the ability of the animals to discriminate between novel and familiar objects. OR also involves hippocampus activation [13], but several studies reported that hippocampal lesions do not affect object recognition [25,26] indicating that memory processing requires the activation of different areas of the brain, such as peri-posthinal cortex [27]. A large advantage of OR over MWM is that it does not require spatial learning [28]. In addition, OR requires little training and is also far less stressful and arousing than tasks based on negative reinforcement, such as the hidden platform version of the water maze. As cognitive impairment, OR task measures a specific trait of declarative memory, the ability to judge a prior occurrence [15,25]. Declarative memory requires few exposures to the task to be learned, while non-declarative memory acquisition often has to be more extensive [25]. At least two cognitive components could be viewed in declarative memory, familiarity and recollection, which have been extensively studied in learning processes [15,25,29]. Therefore, differences between previous data showing impaired memory in nude mice [22] and our current results could be due to the type of task (MWM and OR, respectively) used to evaluate these mice.

Data presented here showed that nude mice were able to learn the OR paradigm of 3 consecutive training and testing phases with a 4–5 day interval. The performance of a previous OR task did not interfere with the next one, but a different set of objects had to be used in each round of OR. Remarkably, the total exploration time of control groups and tumor implanted animals on D9, D14, and D18 did not change, demonstrating that animals bearing tumors maintain exploratory and locomotor activities during these experimental times. In addition, this result also showed that mice do not tire out or habituate with repetitions of the task.

A negative correlation was found between $d2$ at 90 min (STM) and at 24 h post-training (LTM) and the time after tumor implantation as well as between $d2$ at 90 min post-training and tumor volume, indicating that tumor growth affects the recognition memory of mice as measured by their capacity to discriminate between new and familiar objects. However, there was no correlation between $d2$ at 24 h post-training (LTM) and tumor volume. These data indicate that STM can be directly affected by tumor volume while LTM impairment depends on other factors besides that. STM and LTM are biochemically distinct entities and they could be differently affected by tumor volume. LTM but not STM requires gene expression and protein synthesis [30] and different

Fig. 3. Negative correlation between the discrimination index and time after tumor implantation. Index of discrimination $d2$ in the object recognition task in animals implanted with 5×10^5 U87MG cells and evaluated after 9 (D9, $n = 31$), 14 (D14, $n = 24$), or 18 (D18, $n = 12$) days (black symbols). Naïve animals without tumors (gray symbols, $n = 8$) were exposed to the OR task in parallel with D9, D14, and D18 mice. The $d2$ was determined at (A) 90 min and (B) 24 h post-training. Each symbol represents one animal, and the bars represent the mean for each group $**p < 0.0005$; $*p < 0.005$, t test. Linear regression between $d2$ evaluated (C) 90 min and (D) 24 h post-training and days after U87MG cell implantation ($r = -0.5996$, $p < 0.0001$ and $r = -0.3261$, $p < 0.01$, respectively, Pearson's correlation test). Linear regression between $d2$ evaluated (E) 90 min and (F) 24 h post-training and days after U87MG cell implantation in animals that were evaluated at all times, D8, D14 and D18 ($n = 12$; $r = -0.5514$, $p < 0.001$; $r = -0.2935$, $p = 0.082$, respectively). Each symbol represents one animal.

signaling pathways are necessary for the acquisition of each one of these memories [31]. The mechanisms associated with the impairment of STM but not LTM by tumor volume are presently unknown and deserve further investigation.

The impact of brain tumors on cerebral function has been largely discussed in the literature [32] and cognitive impairment may be caused by direct infiltration and/or integration of tumor cells into the neuronal circuitry, compression or secretion of specific factors [32,33]. U87MG

tumors are encapsulated with a non-infiltrative pattern [34], indicating that cognitive deficits observed here were not caused by infiltration/integration of tumor cells into the neuronal circuitry. The compression of hippocampus is a strong candidate for altering cognitive function, since the OR task applied in this study is dependent on this brain structure [13,15]. Although any evident that hippocampal compression was observed in animals bearing tumors and presenting cognitive impairment, other brain structures also relevant for OR task may be

Fig. 4. Negative correlation between the discrimination index and tumor volume. Linear regression between tumor volume and index of discrimination $d2$ in the object recognition task measured (A) 90 min and (B) 24 h after training ($r = -0.3504$, $p < 0.05$; $r = -0.2753$, $p > 0.05$ respectively, Pearson's correlation test). Each symbol represents one animal.

compressed by the tumor mass [15]. Thus, we cannot discard that OR cognition impairment was caused by the compression of brain structures. Strikingly, OR cognition impairment observed here could be also modulated by soluble molecules, such as neurotransmitters, cytokines and growth factors released by tumor cells. In fact, glutamate, a neurotransmitter associated with excitotoxicity, is released in large amounts by tumors and contributes to neurodegeneration [35]. In addition, monocyte chemoattractant protein-1 (MCP-1), a cytokine involved in glioma infiltration [36], is also able to modulate hippocampal function and cognition [37]. Thus, further studies will be necessary to approach this intriguing question.

Our study also supports the concept that OR memory decline occurs before clinical signs appear. According to a five-point scale [18,38], the animals at D18 were grade 0 and started to develop the first clinical signs around day 22 after tumor implantation.

Together, our results indicate that tumor growth and the consequent behavioral changes in nude mice can be evaluated using the OR task, which is a simple and feasible test. Since the OR task allows for the identification of specific cognitive deficits before the appearance of clinical symptoms, it provides an important advantage for testing compounds with anti-tumor activity.

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