

EXPERIMENTAL MODEL OF C6 BRAIN TUMORS IN ATHYMIC RATS

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Abstract – Malignant brain tumor experimental models tend to employ cells that are immunologically compatible with the receptor animal. In this study, we have proposed an experimental model of encephalic tumor development by injecting C6 cells into athymic Rowett rats, aiming at reaching a model which more closely resembles to the human glioma tumor. In our model, we observed micro-infiltration of tumor cell clusters in the vicinity of the main tumor mass, and of more distal isolated tumor cells immersed in normal encephalic parenchyma. This degree of infiltration is superior to that usually observed in other C6 models.

KEY WORDS: brain tumor, C6 cells, experimental model, glioma.

Modelo experimental de tumor cerebral C6 em ratos atímicos

Resumo – Modelos experimentais de tumores cerebrais malignos geralmente utilizam células imunologicamente compatíveis com o animal receptor. Neste estudo apresentamos um modelo experimental baseado na inoculação de células C6 em ratos atímicos Rowett, visando obter um tumor que se assemelhe mais àqueles observados nos seres humanos. Neste modelo observamos microinfiltração de ilhotas de células na periferia da massa tumoral principal e nas áreas mais distantes, células tumorais isoladas no tecido cerebral normal. Este grau de infiltração é superior àquele observado em outros modelos utilizando as células C6.

PALAVRAS-CHAVE: tumor cerebral, células C6, modelo experimental, glioma.

The need for therapeutic strategies for gliomas has led to the development of experimental models simulating the growth and invasive characteristics of these tumors. Experimental models tend to employ cells that are immunologically compatible with the receptor animal. One of the most classical multiform glioblastoma models is based on injecting C6 cells (murine glioma) into Wistar rats.

Recent studies have demonstrated that these cells induce a strong immunological response in Wistar rats which, in turn, may compromise interpretation of the results in therapeutic tests. Another approach is based on using immunosuppressed animals. Immunosuppressed (or athymic) rats are necessary when inoculating cell cultures derived from patient's tumors. Other models are either incompatible, or do not reproduce the invasive characteristics of gliomas¹⁻⁷.

The objective of the present work was to present the

technique, along with clinical and pathological results, for an experimental glioma model by injecting murine glioma cells (C6) in athymic rats.

METHOD

Cell culture

C6 murine glioma cells were provided by the Cell and Molecular Therapy Center (NUCEL) of the São Paulo University (USP). Cells were cultured in Dulbecco's modified medium (DMEM, Gibco BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil) and antibiotics (penicillin 200 UI/mL and streptomycin, 100 µg/mL, Invitrogen, USA), in 5% CO₂ atmosphere, at 37°C. Upon reaching confluence, the cultures were dissociated using trypsin/EDTA 250 mg% (Cultilab, Campinas, São Paulo) and resuspended in serum-free DMEM. The cells were centrifuged, counted in a Neubauer chamber (Optik Labor, Germany) and suspended at 1x10⁵ cells in 4 µL of serum-free DMEM.

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Subcutaneous inoculation

Female Rowett rats, (n=2) weighing 140g, were provided by the Animal Facility of the University of São Paulo Chemistry Institute. The animals were maintained in cages with individual ventilation systems (Tecniplast, Italy), with free access to water and autoclaved food. All animals were handled in accordance with the guidelines recommended by the USP Medical School's Laboratory Animal Care Committee. Following antisepsis treatment of the skin, using 70% ethanol, the C6 cell suspension (1×10^5 cells) in 4 μ L of serum-free DMEM was inoculated into the right flank of the animals.

The animals were observed daily for 21 days. At the end of this period, the tumor mass of both rats measured 2.5 cm across their widest diameter. Subsequently, the rats were sacrificed using a ketamine overdose (25 mg) and the tumor mass was surgically extracted using sterile techniques. The weight of the two masses was identical in both rats, namely 3.58g. These tumor masses were used to generate primary cultures and, repeating the culture process outlined, further aliquots of 1×10^5 C6 cells in 4 μ L of serum-free DMEM were prepared.

Intracerebral inoculation

Five female Rowett rats, weighing an average of 140g provided by the Animal Facility of the USP Chemistry Institute, were anesthetized using ketamine (90 mg/kg) and positioned in a stereotaxic device for small animals (Kopft, Germany). After antisepsis of the skin covering the cranium using 70% ethanol, a median incision of approximately 1 cm was made and access to

the cranial cavity was achieved by means of a right frontal trepanation, 2 mm in front of the coronal suture and 2 mm to the side of the sagittal suture (standard coordinates for brain tumor models). This procedure was carried out using a mini electric drill (Bosch).

An aliquot (1×10^5) of C6 cells in 4 μ L of serum-free DMEM was inoculated to a depth of 3 mm with a 25 μ L Hamilton syringe using the stereotaxic technique. Inoculation was performed slowly over a 5-minute period. The needle was left in position for 10 minutes to prevent from refluxing along the needle trajectory. After removing the needle, trepanation was occluded using wax for bone (Ethicon), and the skin was sutured using two separate 5-0 mononylon stitches (Ethicon). Following the surgical procedure, the rat was placed back into the cage with free access to water and food.

The rats were examined daily to check for any changes in gait, suggestive of motor deficit, or secretion build-up in the periorbital region. Three weeks later, despite slight signs of periorbital secretion build-up and no motor deficit symptoms, the rats were sacrificed using a thionembutal overdose. Cardiac perfusion was performed with 100 mL phosphate buffer saline (PBS) and 80 mL of 4% paraformaldehyde, followed by extraction of the brains. The whole brains were fixed in 4% paraformaldehyde for one week, then sliced in coronal preparations of 1 cm of thickness, mounted in paraffin blocks, followed by preparation of 5 μ m-thick serial sections. Relevant macroscopical slices of the tumor, together with surrounding cerebral tissue, were mounted on microscope slides and stained with eosin-hematoxylin for histological analysis.

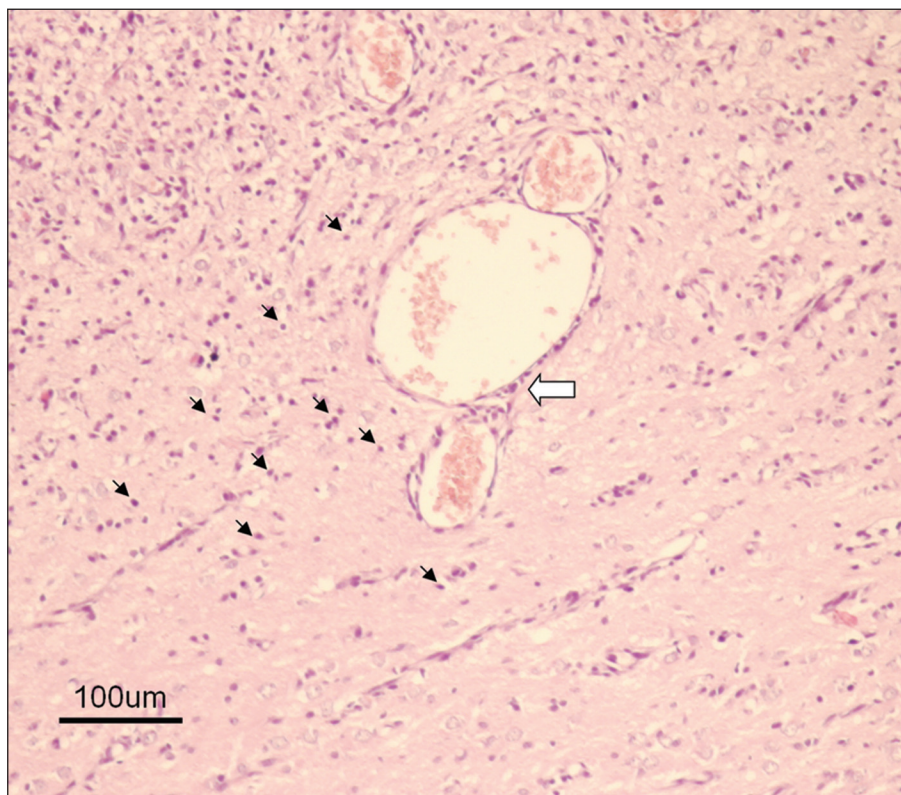


Figure. HE, 40x. Brain tumor transition area. No clear limit between tumor mass and neighboring brain parenchyma is observed. Numerous basophilic tumor cells clustering deeper in the brain parenchyma can be observed (arrows). The perivascular space is also infiltrated by tumor cells (arrowhead).

RESULTS

No peri-operative deaths occurred and none of the rats developed evident infectious complications, suggesting that basic aseptic care (asepsis of the skin and sterilization of surgical materials) was sufficient in handling athymic Rowett rats for the purposes stated.

Clinical assessment of the rats during the post-operative period revealed that the animals presented discrete periocular secretion build-up from the 17th day after inoculation. No motor deficits were manifest.

Macroscopic analysis of the brain fragments demonstrated a tumor mass, which was visible to the naked eye in 100% of the rats. The lesion spread to the cortical surface at the inoculation point in all animals, displacing structures from the median line.

Analysis of the slides at lower magnification showed that the tumors took a predominantly spheroid configuration, located at the right frontal lobe, compressing and infiltrating the adjacent structures.

Higher magnification revealed that tumors were formed by polygonal-shaped cells with basophilic nuclei and eosinophil cytoplasm. Hemorrhagic necrosis in small clusters was presented in all cases. The brain-tumor interface (Figure) demonstrated microscopic infiltration at the borders of the main lesion, where grouping of tumor cells were found beyond the edges of the main mass, even with some isolated tumor cells immersed in the cerebral parenchyma. This infiltration occurred not only in the cerebral parenchyma but also throughout the perivascular spaces. Infiltration of the leptomeningeal space was also observed. There was little inflammatory response adjacent to the tumor lesions.

DISCUSSION

Malignant brain gliomas, particularly glioblastomas have been the subject of intense study, mainly aiming at the discovery of more effective forms of treatment. The invasive pattern of gliomas and the migration of neoplastic cells along encephalic tracts and perivascular space renders such tumors very difficult to reproduce in experimental models.

Brain tumor cell culture models minimize the inflammatory and angiogenic responses which gliomas trigger in human brain tissue¹. Animal tumor models based on implantation of established cell lines complement studies carried out in cell cultures, allowing the *in vivo* study of tumor responses to various therapeutic strategies. In addition, animal models allow comparison between groups of control and treated rats harboring tumors with more homogeneous size and location characteristics². The ability to achieve accurate cell counts and homogeneously growing tumors was demonstrated by the two subcutaneously

inoculated rats in our experiment, since upon infection of the same number of cells (5×10^5), they developed tumors of identical weight.

One of the most classical multiform glioblastoma models is based on injecting C6 cells. These cells have been used in experimental models with Sprague-Dawley, Long-Evans and Wistar rats, which are immunocompetent, therefore growth is under influence of the immune system. C6 cells growth is strongly influenced by the breed of rat in which they are inoculated. Tumors formed in the Sprague-Dawley and Long-Evans breeds are encapsulated and have well-defined limits, resembling metastatic lesions³. Possibly the spheroid shape of the tumor masses represent more of a walling off and a degree of genetic incompatibility. However, when these cells are inoculated into the brain of Wistar rats, they exhibit invasive and pleomorphic GBM characteristics, including sites of necrosis and hemorrhage. Other aspects observed in Wistar rats include a high mitotic rate, cells forming palisades, and neovascularization⁴. These characteristics have led to the use of the C6/Wistar model in experiments due to its similarity to the human GBM⁵. Despite this similarity, other authors believe this model does not possess the same heterogeneity, immuno-histochemical characteristics or invasive capacity of the GBM⁶.

In vivo evidence shows that when C6 cells are inoculated into Wistar rats, they trigger a host-graft incompatibility immune response. When Wistar rats were simultaneously inoculated into the brain and subcutaneously into the flank, 100% survival was observed, compared to 11% survival when inoculated only into the brain. It was concluded that C6 cells inoculated subcutaneously induce a significant immune response in Wistar rats, mimicking an anti-tumoral immune response while preventing this tumor from growing in brain tissue⁷. Interestingly, spontaneous regression after 70 days of stereotaxic inoculation of C6 cell spheroids into brains of immunocompetent and thymectomized Sprague-Dawley rats has reported⁸.

The exact origin of C6 cells remains unclear. Some authors have reported that these cells in fact originated from Wistar rats⁶, and were chemically induced in Wistar-Furth rats by exposure to N,N'-nitrosomethylureia⁹. Other authors have described C6 cells as compatible with BDIX¹⁰ and Sprague-Dawley rats¹¹. More recent molecular studies have isolated the RT^U haplotype from C6 cells, indicating that it is consistent with the allogenicity in Wistar, BDIX, BDX and Sprague-Dawley rats¹². Some authors have stated that C6 cells are not genetically compatible with any rat breed, and hence the immune response represents a limitation for the use of this model, specially if the comparative index is survival^{7,13}. In fact, this C6 model is considered

to be unsuitable for studying inflammatory changes observed in human gliomas¹³.

The use of an athymic model is helpful when using a completely dissimilar human cell line in an animal model, therefore, athymic rats are more typically used in studies involving xenografts. To use an athymic animal for developing an animal model with a C6 cell derived from rat, as presented here, downregulates the inflammatory response which may enhance the invasive characteristics of the tumor.

Our findings are compatible with a low immune response characterized by the near absence of inflammatory cells surrounding the tumor masses. The variable degree of an inflammatory response observed in other models is possibly evidence of immune incompatibility rather than the nature of a high grade glioma. The presence of an intact immune system is more likely to the forming of more encircled lesions such as those described for Sprague-Dawley and Long-Evans rat breeds. A morphometric study of C6 cell implants in rats has reported that the brain-tumor interface was well defined in immunocompetent rats, when compared to New Zealand athymic rats¹⁴.

In our model, we observed micro-infiltration of tumor cell clusters in the vicinity of the main tumor mass, and of more distal isolated tumor cells immersed in normal encephalic parenchyma (Figure). This degree of infiltration is superior to that usually observed in other C6 models.

Notwithstanding results observed of tumor invasive being attributed to the low immunological response of Rowett rats, other complex interactions amongst tumor cells and brain structures should also be taken into account.

In conclusion, C6 cells have high infiltrative characteristics when inoculated into athymic rats, resembling those observed in human GBM.

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