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The 9L^{LUC}/Wistar rat glioma model is not suitable for immunotherapy☆

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Abstract

The availability of a well-characterized animal brain tumor model will play an important role in identifying treatments for human brain tumors. Wistar rats bearing 9L glioma cells can develop solid, well-circumcised tumors, and may be a useful animal model for the evaluation of various therapeutic approaches for gliosarcomas. In this study, the 9L/Wistar rat glioma model was produced by intracerebral implantation of 9L^{LUC} glioma cells syngenic to Fischer 344 (F344) rats. Bioluminescence imaging showed that tumors progressively grew from day 7 to day 21 in 9L^{LUC}/F344 rats, and tumor regression was found in some 9L^{LUC}/Wistar rats. Hematoxylin-eosin staining verified that intracranial tumors were gliomas. Immunohistochemistry results demonstrated that no CD_4- and CD_8-positive cells were found in the syngeneic 9L^{LUC}/F344 model. However, many infiltrating CD_4- and CD_8-positive cells were observed within the tumors of the 9L^{LUC}/Wistar model. Our data suggests that compared with 9L/F344 rats, 9L glioma Wistar rats may not be suitable for evaluating brain glioma immunotherapies, even though the model induced an immune response and exhibited tumor regression.

Key Words

9L cells; glioma; F344 rats; Wistar rats; animal model; bioluminescence imaging; immune response; neural regeneration

Abbreviations

F344, Fischer 344; LUC, luciferase

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INTRODUCTION

Glioblastomas are the most frequent human brain tumor, and generally carry a poor prognosis. Therapeutic options, such as surgery, radiation therapy[1-2], chemotherapy[3-5], or any other available treatment modality[6-7] have been explored in experimental glioma models. The immunologic properties of glioblastomas have important clinical implications because immune-gene therapy and immunotherapy have shown promising therapeutic results in animal models, which may potentially provide a theoretically attractive new approach for treatment of this malignancy[8-11]. Because laboratory investigations of primary human brain tumors have largely depended on the use of animal models, a well-characterized animal brain tumor model will play an important role in approaching treatments for human brain tumors. The C6/Wistar model and 9L/Fischer 344 (F344) model are the most frequently used animal models. The C6/Wistar model has been reported to induce a vigorous immune reaction that may mimic a specific anti-tumor response in Wistar rats. However, utility of the C6 rat

glioma model for evaluating immunotherapy is controversial[12-13].

The 9L gliosarcoma was derived from a line of brain tumors produced in F344 rats by weekly injections of 5 mg/kg methyl-nitroso-urea[14]. The 9L/F344 glioma model is probably the only brain tumor model that has been developed for both *in vivo* and *in vitro* study[15-16]. Stojiljkovic *et al* [17] described that, allogeneic Wistar rats bearing 9L glioma cells syngenic to F344 could develop solid, well-circumcised tumors. Their findings suggest that Wistar rats may be used as an animal model for the evaluation of various therapeutic approaches for gliosarcomas.

The present study aimed to define whether 9L glioma tumors in Wistar rats is an appropriate animal model for evaluation of various therapeutic approaches by comparing the characteristics of 9L glioma tumors in Wistar rats and F344 rats.

RESULTS

Bioluminescence correlates to tumor cell number and is stable over time *in vitro*

9L tumor cells transfected with the Lenti-hPGK-LUC were selected. Positive bioluminescent tumor cells were serially diluted, imaged and evaluated *in vitro*. Photon emission from suspensions of $9L^{LUC}$ glioma tumor cell lines expressing luciferase (LUC) was dependent on the number of cells. The minimum number of detectable cells in suspension was approximately 100 cells per well (Figure 1).

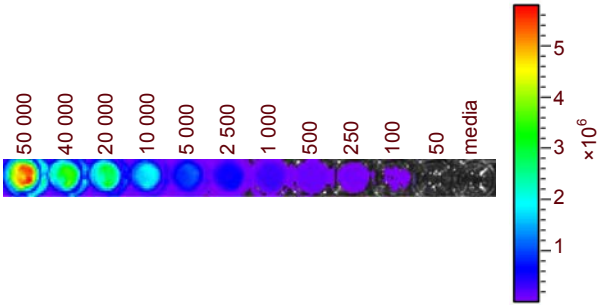

Figure 1 Correlation between $9L^{LUC}$ cell number and *in vitro* bioluminescence.

The $9L^{LUC}$ cell line was serially diluted and photon emission from suspensions of the $9L^{LUC}$ glioma tumor cell line expressing luciferase was observed. The minimum number of detectable cells in suspension was approximately 100 cells per well.

Monitoring intracranial tumor growth *in vivo*

$9L^{LUC}$ glioma cells were injected into the cranium of Wistar or F344 rats to monitor tumor growth. Tumor growth was measured at the beginning on day 0 and weekly thereafter using bioluminescence imaging. By day 7, all animals showed successful tumor development following bioluminescence imaging. Tumor growth after intracranial injection of $9L^{LUC}$ cells at varying doses into the right caudate nucleus of Wistar or F344 rats is shown in Figure 2. All $9L^{LUC}$/F344 rats yielded tumors with an injection of 10^5 cells (Figures 2A–D); in contrast, Wistar rats injected with 10^6 cells showed successful tumor development (Figures 2I–L), yet Wistar rats injected with 10^5 cells developed tumors only on day 7 and regressed thereafter (Figures 2E–H). Compared with 9L/Fischer rats, Wistar rats implanted with 9L glioma cells (10^6) showed a significant ($P < 0.01$) increase in photon counts at day 7, however, there was no difference in photon counts between 9L/Fischer rats and Wistar rats at day 21 ($P > 0.05$; Figure 3).

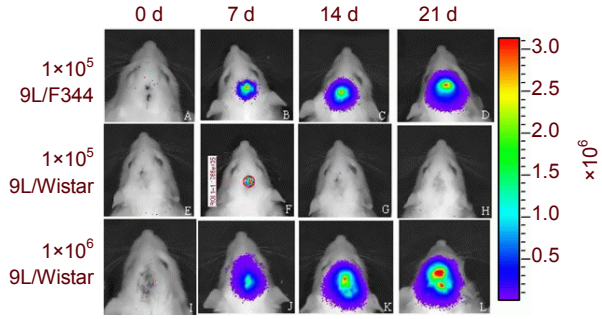

Figure 2 Tumor growth was detected by bioluminescence imaging *in vivo*.

$9L^{LUC}$ cells were implanted intracerebrally into Fischer 344 or Wistar rats (n=4 per group).

Tumor growth in F344 rats is presented (A–D) from day 7 to day 21 after cell implantation. No continued growth of tumors in Wistar rats receiving simultaneous intracranial injections of $9L^{LUC}$ (10^5) from day 7 to day 21 was observed (E–H), while globular brain tumor growth occurred constantly after implantation in 9L/Wistar rats (I–L) receiving 10^6 $9L^{LUC}$ cells, without evidence of regression.

Right scale is used for photon counting, and tumor size, and tumor cell number are calculated according to the right scale and colors.

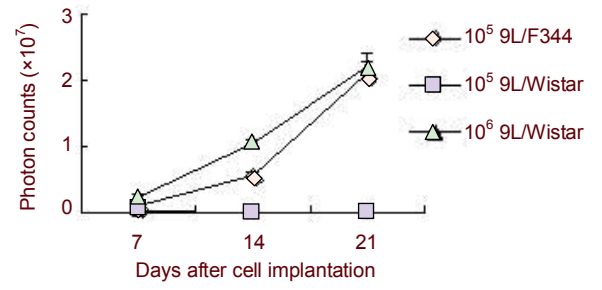

Figure 3 Photon counts of 9L/F344 and 9L/Wistar rats implanted with $9L^{LUC}$ cells at day 7 to day 21.

There were no obvious differences among groups at day 7 after cell implantation. Tumors disappeared gradually in 9L/Wistar rats receiving 10^5 $9L^{LUC}$ cells, while a significant increase was observed between 9L/Wistar rats receiving 10^6 $9L^{LUC}$ cells and 9L/F344 rats receiving 10^5 $9L^{LUC}$ cells at day 14. Compared with 9L/Wistar rats, the glioma in 9L/F344 rats continued to develop. The data are expressed as mean ± SEM and were analyzed with Student's *t*-test.

Survival after intracranial tumor implantation

All F344 rats receiving intracranial doses of 10^5 $9L^{LUC}$ cells died during 20–30 days. In contrast, all Wistar rats receiving simultaneous intracranial injections of $9L^{LUC}$ (10^5) survived beyond 30 days, while 80% of 9L/Wistar rats receiving 10^6 $9L^{LUC}$ cells also died within 30 days.

Histological characterization of tumors in glioma-bearing rats

At 7 days after implantation, all experimental animals including F344 and Wistar rats developed solid tumors. Margins at the tumor-brain interface were macroscopically demarcated. Histologically, tumors were hypercellular with neovascularization and hemorrhaging. Light microscopy revealed that tumors exhibited malignant characteristics, including pleomorphic growth, more than one nucleus per cell, prominent nucleoli or more than three distinct nucleoli, and a nuclear to cytoplasm ratio of > 0.5 (Figure 4).

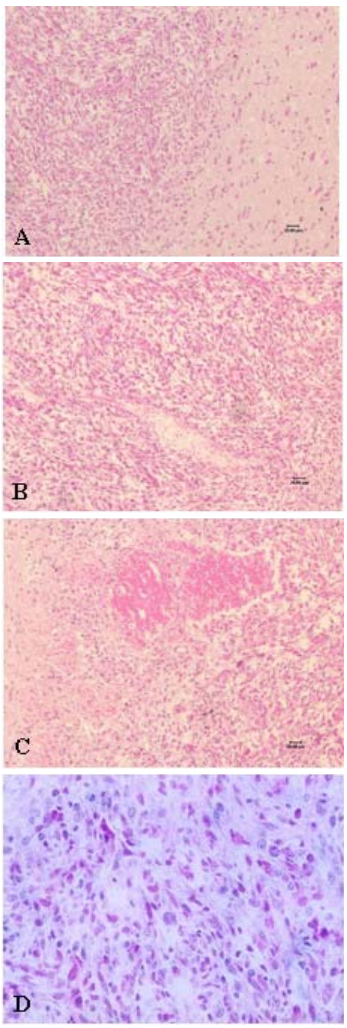

Figure 4 Histopathological changes in the 9L/Wistar rat model, which expressed well-circumscribed gliomas (A), neovascularization (B), hemorrhaging (C) and pleomorphic growth (D) (Scale bars: 20 μm in A–C; × 400 in D).

Cellular and humoral immune response

The syngeneic $9L^{LUC}$ tumors in F344 rats had fewer, diffusely present CD_4 or CD_8 cells. Wistar rats bearing $9L^{LUC}$ tumors developed many tumor-infiltrated lymphocytes, including CD_4- and CD_8-positive cells (Figure 5).

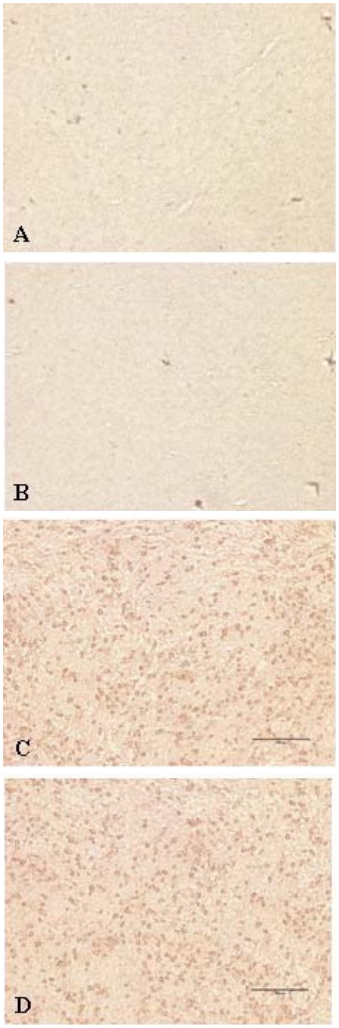

Figure 5 Immunohistochemical detection of CD_4 and CD_8 positive-macrophages/microglia in the brains of Wistar and F344 rats after cell implantation.

Very few CD_4 (A) or CD_8 (B) cells can be found in the tumors of 9L/F344 rats, while lots of lymphocytes including CD_4 (C) and CD_8 (D) cells infiltrated gliomas in Wistar rats. Scale bars: 100 μm.

DISCUSSION

Several excellent syngeneic brain tumor models have been widely used in experimental neuro-oncology research[18]. Recently Stojiljkovic *et al* [17] developed the non-syngeneic glioma model 9L/Wistar, which may be used for the evaluation of therapeutic approaches. Our data demonstrates that Wistar rats, after receiving $9L^{LUC}$ cells, developed solid, well-circumscribed tumors, which were clearly separated from the surrounding brain

tissue at 7 days after implantation. This is consistent with previous findings[17, 19]. Wistar rats bearing $9L^{LUC}$ intracranial glioma cells exhibited malignant characteristics with pleomorphic growth, prominent nucleoli, more than three distinct nucleoli and reactive astrogliosis, which is similar to characteristics of tumor cells in 9L/F344 rats. However, compared with 9L/F344 rats, the development of the tumor in Wistar rats was slower. The photon counts were higher in 9L/Wistar rats implanted with 10^6 glioma cells than in 9L/F344 rats injected with 10^5 cells at day 7, but there was no significant difference at day 21. Tumor growth was constant after implantation in syngeneic F344 rats, and did not show any infiltration of immune cells, which ultimately resulted in animal death. However, tumor growth was slow after glioma cell injection in Wistar rats, which was probably due to significant infiltration of immune cells, as found in the present study. These observations suggest that peripheral immunization stimulates a potent humoral and cellular immune response, resulting in rejection of the brain tumor and anti-9L allogeneic graft rejection.

In conclusion, our study demonstrates that the 9L/Wistar glioma model induces a potent immune response *in vivo*. This response prevents tumor growth, allowing Wistar rats to survive longer. This should be kept in mind when using the 9L/Wistar model for the evaluation of therapeutic efficacy for brain gliomas, especially when tumor growth and survival are utilized as an endpoint.

MATERIALS AND METHODS

Design

An *in vitro* cell biological experiment and an *in vivo* controlled animal experiment.

Time and setting

This study was performed at the Academy of Military Medical Science of Chinese PLA, China from January 2007 to May 2008.

Materials

A total of 40 adult male Wistar rats aged 4–5 weeks, weighing 125–150 g, were provided by the Experimental Animal Center of Academy of Military Medical Science, China. Fifteen adult male F344 rats aged 4–5 weeks, weighing 125–150 g, were provided by the Experimental Animal Center of Beijing (license No. SCXK (Jing) 2006-0009).

Methods

Production of $9L^{LUC}$ cell line

The lentivirus vector (Naldnini L N, Vita Salute San Raffaele University, Italy) carrying LUC was prepared as described previously[19-21]. Briefly, the PGL3-Basic plasmid (Promega, Madison, WI, USA) was digested with NheI and HpaI and a 1881 bp cDNA fragment encoding firefly LUC (F-LUC) was isolated. The gene for F-LUC was cloned into the NheI and AorVIH digested Lentivirus expression plasmid under the control of the hPGK promoter. Lenti-hPGK-LUC was produced by cotransfecting the expressing plasmid with the three packaging plasmids (Invitrogen, Carlsbad, CA, USA), pLP1, pLP2, pLP/VSVG, into 293FT cells. The transfected cells were maintained in a humidified atmosphere containing 5% CO_2 for 12 hours, and then the medium was replaced with fresh medium containing sodium pyruvate. Forty-eight hours after transfection, virus-containing medium was harvested, centrifuged at 900 × *g* for 15 minutes and passed through a 0.45-μm filter. Lentivirus titers were determined by infecting 293FT cells with Lenti-hPGK-LUC. For lentiviral infection, the viral vector Lenti-hPGK-LUC was added to the medium overnight, and the medium of 9L cells (American type culture collection, Manassas, Virginia, USA) was renewed thereafter. $9L^{LUC}$ cells were selected using a flow cytometer sorter and the FACSVantage SE.

Cell culture and lentiviral infection

The $9L^{LUC}$ rat glioma cell line (syngenic with the F344 rats) was maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, Utah, USA), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Hyclone, Logan, Utah, USA), 2 mM L-glutamine (Hyclone, Logan, Utah, USA), 50 μg streptomycin, and 50 U/mL penicillin. The cells were incubated with 5% CO_2 at 37°C in a humidified chamber (Heraeus, Wehrheim, Germany). Once a monolayer of cells had reached 90% confluency, the cell line was subcultured. The cells were washed twice using PBS and incubated at 37°C for 1 minute with 1.0 mL trypsin (Gibco, Carlsbad, CA, USA), EDTA solution (0.25% (w/v)) and trypsin (0.02% (v/v)). The resulting cell suspension was then diluted and divided into two flasks.

Intracerebral tumor cell implantation

Animals were anesthetized with 3.5% (w/v) chloral hydrate (10 mL/kg) and placed in a stereotaxic apparatus (China)[19]. Aseptic surgical techniques were used to make a midline incision and open the scalp to expose the frontal and temporalis bones. A burr hole was made at an appropriate location (1 mm posterior to bregma and 3 mm right to midline) through the skull without breaking the dura. A 26-gauge needle was inserted 5 mm ventral to the dura, retreated 0.5 mm (for injection), and the $9L^{LUC}$ cells were implanted stereotaxically using a 10-μL microsyringe at an infusion rate of 1 μL/min. A total of

1×10^5 cells per animal were administered into F344 rats and some of the Wistar rats, and 1×10^6 cells were injected into the remaining Wistar rats. After infusion of cells, the needle was kept in place for approximately 5 minutes to allow for equilibration of pressure within the cranial vault. The needle was removed slowly and the hole was immediately sealed with a sterile bone-wax to prevent leakage of the solution. The animals recovered from the anesthesia and were returned to the animal care facilities.

Bioluminescence imaging

Fluc-expressing $9L^{LUC}$ imaging was performed with a cryogenically cooled high-efficiency CCD camera system (Xenogen Corp., Alameda, CA, USA). For *in vitro* imaging, bioluminescent cells were diluted from 50 000 to 25 cells per well into appropriate cell culture media in 96-well plates (Costar, New York, NY, USA). D-luciferin (150 μg/mL; Xenogen Corp., Alameda, CA, USA) was added to each well 2–5 minutes prior to imaging. Imaging times were 1–2 minutes per plate. *In vivo* imaging was performed as previously described[19, 21-22]. Animals were injected intraperitoneally with 150 mg/kg D-luciferin at 0, 7, 14 and 21 days after implantation. Seven minutes after LUC injection, animals were anesthetized with 3.5% (w/v) chloral hydrate and placed into the light-tight chamber of the CCD camera system, and a grayscale body surface reference image (digital photograph) was taken under weak illumination. Photons emitted from specific regions were quantified over a defined period of time ranging up to 3 minutes using the software program “Living Image” (Xenogen Corp., Alameda, CA, USA). *In vivo* LUC activity is presented in photons per second.

Tissue processing and hematoxylin-eosin staining

After the last bioluminescence imaging measurements, rats were sacrificed. Animals were anesthetized with 3.5% (w/v) chloral hydrate (10 mL/kg). Each rat was transcardially perfused with saline containing 1 000 U/L heparin followed by 4% (w/v) paraformaldehyde. The brains were rapidly removed and placed in 4% (w/v) paraformaldehyde at 4°C for 48 hours. Tissue was sectioned (6–8 μm) and processed for hematoxylin-eosin staining, or immunohistochemistry. Imaging was performed using a microscope (Olympus, Tokyo, Japan) equipped with a cooler CCD digital imaging system software (Spot).

Immunohistochemistry for CD_4 and CD_8

Sections at 6–8 μm thickness were used for immunohistochemical detection according to established methods[23]. Sections were preincubated for 30 minutes with 0.3% (v/v) H_2O_2 to inhibit endogenous peroxidase activity, and placed in a solution containing normal goat serum for 30 minutes at 37°C to block nonspecific binding sites of protein. The primary antibodies of monoclonal mouse anti-rat CD_4 (OBT Inc., Bronx, NY, USA; 1:1 000), or monoclonal mouse anti-rat CD_8 (OBT; 1:1 000) were incubated with the tissue overnight at 4°C in a refrigerator in a humidified chamber. The next day, sections were rinsed three times in PBS for 10 minutes each before being incubated with biotinylated-goat anti-mouse (Beijing Zhongshan Co., Ltd., Beijing, China) solution for 30 minutes at 37°C. Subsequently, sections were rinsed three times in PBS and incubated with horseradish peroxidase for 30 minutes at 37°C. Antibody binding was detected using diaminobenzidine. For negative controls, the primary antibody was omitted. Reacted sections were dried, dehydrated and coverslipped.

Statistical analysis

SPSS 13.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis of data, and data were expressed as mean ± SEM. The unpaired Student’s *t*-test was used to determine statistical significance. The level of significance for results is indicated as $P < 0.05$.

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Author contributions: Liping Yang and Jingxiang Zhao were responsible for the study. Guihong Zhou provided information. Yunfang Wang provided intellectual help. Lusi Li coordinated the study. Hongfeng Yuan, Xue Nan and Lidong Guan were responsible for data acquisition. Xuetao Pei designed and supervised this study.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee of Academy of Military Medical Science of Chinese PLA, China.

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