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New Experimental Model of Brain Tumors in Brains of Adult Immunocompetent Rats

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Research Article

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ABSTRACT

Aims: Xenograft models, namely heterotransplantation of human cancer cells or tumor biopsies into immunodeficient rodents are the major preclinical approach for the development of novel cancer therapeutics. However, in these models the animals must be used only after the severe systemic immune suppression in order to ensure graft survival. Thus, additional new human brain tumor models without immune suppression of the recipient rodent may be required.
Place and Duration of Study: Laboratory of Immunochemistry, V.P. Serbsky National Research Centre for Social and Forensic Psychiatry and Department of Nanobiotechnology, N.I. Pirogov Russian State Medical University and Department of Biosynthesis of Nucleic Acids, Institute of Molecular Biology and Genetics between June 2009 and July 2010.
Methodology: Brain tumor modeling was performed by intracerebral stereotactic implantation of cells to the healthy adult rats without any artificial immunodepression. Cells were implanted to the striatum region of ketamine-anesthetized rats at specific coordinates according to Swanson's rat brain atlas. Tumor growth was monitored

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weekly *via* registration of neurological signs and *in vivo* Bruker MRI system.
Results: On the 21st day after implantation of C6 glioma, U251 or 293_*CHI3L1* cells severe neurological deficit appeared in rats. Huge intracerebral tumors were found in each animal under investigation while no tumor growth was observed for at least 8 weeks in rats injected with empty vector-transfected 293 cells. Tumors contained the dense superficial cell layer and prominent lobules with central newly ingrowing blood vessels. Histological assay revealed displacement of median cerebral structures and hydrocephalus in contralateral hemisphere. All tumors were surrounded by numerous GFAP-positive reactive astrocytes.
Conclusion: Positive results with transplantation of 293_*CHI3L1* cells into adult rat brains without any immunosupression show the validity of this animal model. In all experiments such implantations provoked malignant tumor formation while there were no visible tumors in control rats. We believe this to be the first animal model of human brain tumor that displays the possibility to study various biologic features of and host therapeutic response to brain tumor in an immunocompetent host.

Keywords: Brain tumors; immunocompetent; heterotransplantation; xenograft models.

1. INTRODUCTION

There is a substantial immune privilege found in the brain due to the blood brain barrier (BBB) exhibited by the cerebral capillaries, a mechanism by which high molecular weight preparations and immunological cells are prevented from passing from the blood to the brain tissue. The lymph system in the brain is also very different from the lymph system of the body; it does not drain directly into the blood. Besides, levels of MHC antigens which recognize non-self cells exist here at much lower level. Nevertheless, it is thought that microglial and astroglial cells may have this capability. There is also evidence that CNS neurons are refractory to MHC antigen induction (Sloan et al., 1991).

Thus, while there is a substantial immune privilege found in the brain, it is not absolute. Moreover, the neurotransplantation surgery breaks the BBB and introduces foreign antigens into the brain. Since it takes 12 days for the BBB to be reestablished after transplantation (Borlongan et al., 1996), the transplanted cells are exposed to the immune system via the blood during that time. As a result, the amount of time that the BBB remains open after surgery may affect immune response and graft survival (Sloan et al., 1991). In addition, xenographic antigens can reach the deep cervical lymph nodes and then elicit an immune response. So, the primary cause of tissue transplant to the human brain failure is rejection of the grafts by the host. Accordingly, different approaches have been developed to circumvent the immune response brought about by transplantation of neural tissue.

On the other hand, despite decades of study, the etiology of brain cancer remains elusive and so there is a substantial need for increasing preclinical testing throughout using clinically relevant models. Xenograft models, namely heterotransplantation of human cancer cells or tumor biopsies into immunodeficient rodents are the major preclinical approach for the development of novel cancer therapeutics. Although there are serious limitations, these models have identified clinically efficacious agents, and remain the very important tool in the pharmaceutical industry. However, in these models the recipient animals must be used only after the severe systemic immune suppression in order to ensure graft survival. Several

methods have been suggested and in the first turn those including the use of immune deficient (nude, SCID, athymic) or pharmacologically immune suppressed rodents. Thus, already in the beginning of 80's human tumor xenograft models were reported in nude mice (Houchens et al., 1983) and continue to be used for different purposes. However, immune deficient animals are quite expensive, need special facilities for their maintenance and have some other disadvantages.

At present, pharmacological immunosuppression is the most widely used method for xenotransplantation. The most common immunosuppressive drug used for treatment is Cyclosporin-A. Cyclosporin-A (CsA) works by blocking T cell mediated immune response, directly affecting the immune system (Borlongan et al., 1996). Thus, pieces of brainstem tissue from mouse embryos were transplanted into the cerebellar vermis of adult rats (Nakashima et al., 1988), cultured human GOS-3 and MRC-5 cells were injected into the right striatum of adult female albino Sprague_Dawley rats (Just et al., 2003), U87 cells which are considered to be a rapidly proliferating cell line and can be grown in culture as monolayers were implanted into brains of Wistar rats (Strojnik et al. 2006), which had been treated with Cyclosporin-A. The xenografts in immunosuppressed rodents were also investigated in other works. While CsA has been shown very effective at preventing graft rejection, it has the disadvantage of harboring numerous side effects (Borlongan et al., 1996). Unfortunately, short-term treatment with CsA does not support long-term survival. Nevertheless, if treatment time surpasses the time required for BBB reformation, long-term survival is supported. Immunosuppressive effect of 15-deoxy-spergualin (DSG) was investigated also on the survivability of rat embryonic dopaminergic neurons grafted into the lateral ventricle of adult mice as alternatives to CsA (Zhou et al., 1993). Recently drug combinations such as lower doses of CsA with steroids and azothiaprone have come into use instead of high CsA doses. Since CsA is very toxic, it did not obtain FDA approval, and no longer be the drug of choice for immunosuppression. There are many other immunosuppressants under investigation now. The aim of this study was elaboration of new brain tumor model without immune suppression of recipient animal that will display the possibility to study various biologic features of and host therapeutic response to brain tumor in an immune-competent host. Rodent models of human cancers have been instructional in understanding the basic principles of cancer biology, that’s why creating a model of brain tumors in rats by orthotopic implantation of human xenogenic transformed cells seems to be very timely.

2. MATERIALS AND METHODS

C6 glioma cells and U251 human glioblastoma cell line were a generous gift of Dr. A.S. Khalansky (Research Institute of Human Morphology, Russian Academy of Medical Sciences). 293_*CHI3L1* cells stably producing CHI3L1 oncoprotein and 293 cells transfected with empty vector as negative control were obtained as described earlier (Kavsan et al., 2011). Previously, it was demonstrated that the widely used 293 cells have a neural origin or at least dispose a lot of neural markers (Show et al., 2002). Primary lung tumor cells were obtained from Dr. A.S. Brukhovetcky (Collection of Russian Oncology Centre). All samples were collected with approval of Ethical Committee of ROC and studies were conducted in accordance with the institutional regulations for use of laboratory animals. Brain tumor modeling was performed by means of intracerebral stereotactic implantation of cells to adult female Wistar rats weighing 200-220 g at the beginning of the experiment. There were 10 rats in the group with implanted 293_*CHI3L1* cells and 5 rats in each group of animals with implantation of wild type 293 cells, 293 cells transfected by empty vector (293_pcDNA3), U251 or C6 glioma cells. Cells were implanted to the striatum region of ketamine-

anesthetized (100 mg/kg) rats using a Narishige stereotactic device 99 (Japan) at coordinates Ap –1, L 3.0, V 4.5, TBS –2.4 mm according to Swanson's rat brain atlas. Cell suspension (5×10^5 cells per animal in 5-7 µl) was injected using a Hamilton syringe connected to infusomat at the rate of 3 µl/min. Tumor growth was monitored weekly via MRI (Clinscan Bruker in vivo MRI system) and via registration of neurological signs for up to 6 weeks. In three weaks after implantation severe neurological deficit appeared in rats, which were killed and histological or immunohistochemical studies were performed.

3. RESULTS AND DISCUSSION

Allogenic transplantations (transplantation of cells, sourced from a genetically non-identical member of the same species as the recipient) by implantation of 9L, C6, T9, F98, RG2 (D74), RT-2, and CNS-1 rat glioma cells into normal rat brains were reviewed in details by Barth (1998). Several years ago we tried such model for the stereotactic intracerebral implantation of C6 cells into the brains of intact adult Wistar rats (Chekhonin et al., 2007). The glial cell strain C6, often referred as the glioblastoma C6 cell line, originally was cloned from a rat glial tumor induced by N-nitrosomethylurea after a series of alternate culture and animal passages (Benda et al., 1968). So, the first our success was really allogenic orthotopic transplantation (transplantation of cells, sourced from a genetically non-identical member of the same species as the recipient and grafted in a natural position). Analysis of histological preparations showed similar morphology of rat C6 glioma and glioblastoma, the most common high-grade human glioma characterized by strikingly poor therapeutic outcome with survival time of about a year. This makes a search for new therapeutic approaches to glioblastoma treatment an area of great clinical importance. A reproducible C6 glioma was developed in Wistar rats. The formation of a glial border at the periphery of the glioma, consisting of GFAP-positive reactive astrocytes (giant cells with an enlarged nucleus, first described by Fedoroff et al., 1984) was shown by the immunohistochemical method on day 8 after implantation, astrogliosis was observed until animal death (day 28). Reactive astrocytes with branched processes surrounded not only the primary glioma focus, but also all sites of tumor invasion in the nervous tissue. Expression of EBA (blood-brain barrier marker) was disturbed and synthesis of AMVB1 (endothelial antigen) increased in neoplastic endotheliocytes, which suggested pronounced functional restructuring of the blood-tumor barrier in comparison with the blood-brain barrier (Chekhonin et al., 2008). The phenomenon of predominant expression of GFAP and AMVB1 in the tumor tissue can be used for the development of systems for targeted drug transport into the tumor by means of appropriate antibodies as a strategy for in vivo tumor targeting (Chekhonin et al., 2007, 2011; Baklaushev et al., 2011).

However, as to our knowledge nobody yet tried to use the normal adult rat brain for the transplantation of human tumor tissue without any immunodepressant administration. It was successfully performed with xenograft, U251 human glioblastoma cell line and then with another xenograft, human embryonic kidney cells 293 transfected with oncogene *CHI3L1* (293_*CHI3L1* cell line) (Kavsan et al., 2011). Thus 293_*CHI3L1* cell line xenograft seems to be the first published xenogenic brain tumors in non-immunodeficient adult normal rats. The protocol details a method for establishing allografts or xenografts from cells grown in culture that relies on healthy adult rats.

In the next series of experiments described here, tumor growth was monitored weekly via registration of neurological signs for up to 8 weeks. On the 21st day after implantation severe neurological deficit appeared in 7 of 10 rats with implanted 293 cells, which stably expressed *CHI3L1*. While in vivo MRI study did not reveal any tumor in cases of primary

lung cancer cells, wild 293 cells and 293 cells transfected with empty pcDNA3 vector (Figs 1 A-C), huge tumors with approximately similar tumor volume were visible on day 21 both in allogenic (C6 glioma) (Fig. 1 D) and xenogenic models (U251 intrastriatal xenograft and xenograft of 293_*CHI3L1* cells) (Figs 1 D and 1 F). It is possible to see the track after stereotactic injections (Figs 1 A-C), a little brain cyst in the region of implantation (Fig. C), intracerebral dislocation, and central necrosis (Fig. 1 F). Red arrows mark the tumor border (Figs 1 D, 1 E, and 1 F).

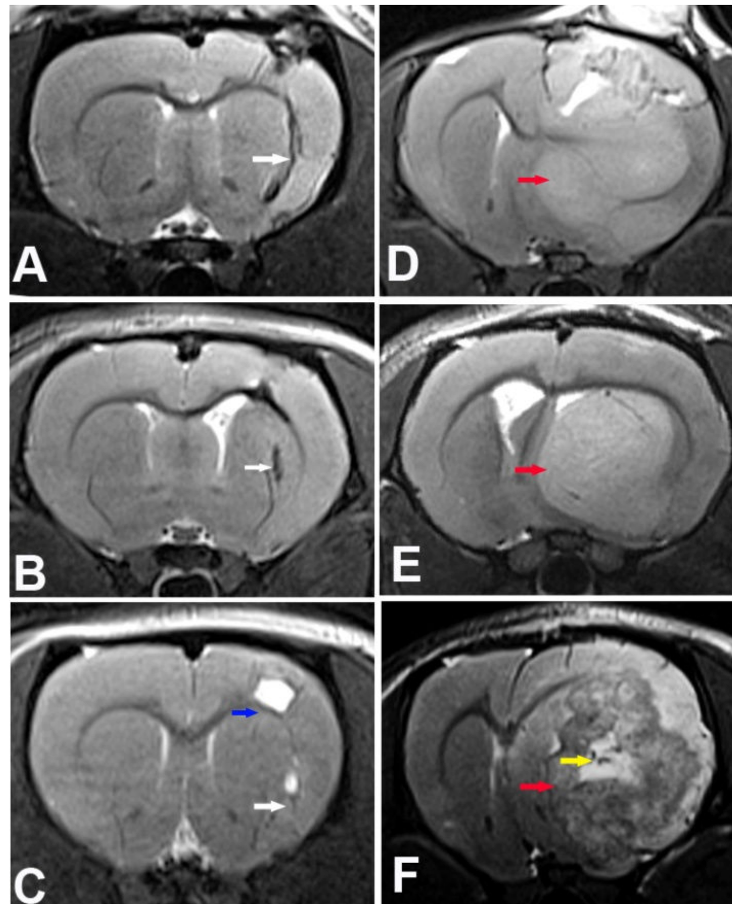

Fig. 1. In vivo MRI of brain allogenic and xenogenic tumors 21 days after stereotactic intracerebral implantation to immunocompetent rats.

A. Implantation of tumor cell culture obtained from the primary human lung cancer.
B. Implantation of wild 293 cells.
C. Implantation of 293 cells transfected with empty vector (293_pcDNA3). White arrows mark the track after stereotactic injections. Blue arrow marks a little brain cyst in the region of implantation.
D. Allogenic C6 glioma tumor.
E. U251 intrastriatal xenograft.
F. The xenograft of 293_CHI3L1 cells with intracerebral dislocation and central necrosis (yellow arrow). Red arrows mark the tumor border.

The rats were killed and intracerebral tumors were observed in each of these rats. The average volume estimated on serial tumor slices using the ellipsoid volume formula was 514.4± 31.3 mm^3 (about 25% of the whole rat brain). This volume was approximately same as for C6 allografts in the same period after transplantation. Histological assay found displacement of median cerebral structures and hydrocephalus in contralateral hemisphere. Tumors contained the dense superficial cell layer and prominent lobules (100-150 μm) with central newly ingrowing blood vessels (Fig. 2 D and 2 E). Immunocytochemical analysis of CHI3L1 production in 293 cells before intracerebral implantation is shown in Figs. 2 A-C. Immunostaining with anti-CHI3L1 antibodies revealed CHI3L1-positive cells both in tumor tissue and in the border of tumor (an invasion zone) (Fig. 2 H). All tumors were surrounded by numerous GFAP-positive reactive astrocytes (Fig. 2 G). The immunohistochemical analysis of brain sections showed a significant increase in the GFAP expression compared to the normal hemisphere, same as it was observed in tumors, initiated by high grade glioma cultured cells (Chekhonin et al., 2006). Tumors contained central necrosis as other quickly progressing tumors. However, histological examination of paraffin sections did not reveal large-scale necrosis zones (Fig. 2 D). No tumor growth was observed for at least 8 weeks in 5 rats injected with empty vector-transfected 293 cells (negative control). As it concerns primary lung tumor cells, no tumor growth was observed in 3 injected rats after 21 day since implantation via in vivo MRI and finally via histological examination after 2 months obsrevation.

What could be a reason of such surviving of transplanted cells in the brains of adult immunocompetent animals? There are only few reports about transplantation of adult neural tissue into the brains of adult or immature mammals without immune suppression. Thus, it was shown that adult serotogenic neurons survive about 1 month in an aged brain when transplanted into the hippocampus (Azmitia, 1987). Rejection of neural mouse xenografts in the adult recipient rat brain occurred within 35 days (Finsen et al., 1991). Fetal human brain cells implanted into the cerebral ventricles of embryonic rats incorporate individually into all major compartments of the brain, differentiated into neurons, astrocytes, and oligodendrocytes, which populate the host fore-, mid-, and hindbrain (Brüstle et al., 1998). It was shown also that glial cell population (including any possible stem cells) from a mature animal could survive transplantation and integrate into an immature host tissue. It was reported that cells of the astroglial lineage from an adult transplant were able to survive after implantation into the brain of an immature host. Surviving xenogenic cells were found outside the graft, forming colonies in the host brain parenchyma. However, after 3 months, transplant-derived astrocytes were no longer detected (Ignacio et al., 1989). The differentiated astrocytes present in the adult transplant were shown to degenerate and die within 2 days after the implantation. Therefore, it was suggested that stem cells present in adult tissue would account for the surviving population of transplant-derived glial cells (Ignacio et al., 1990).

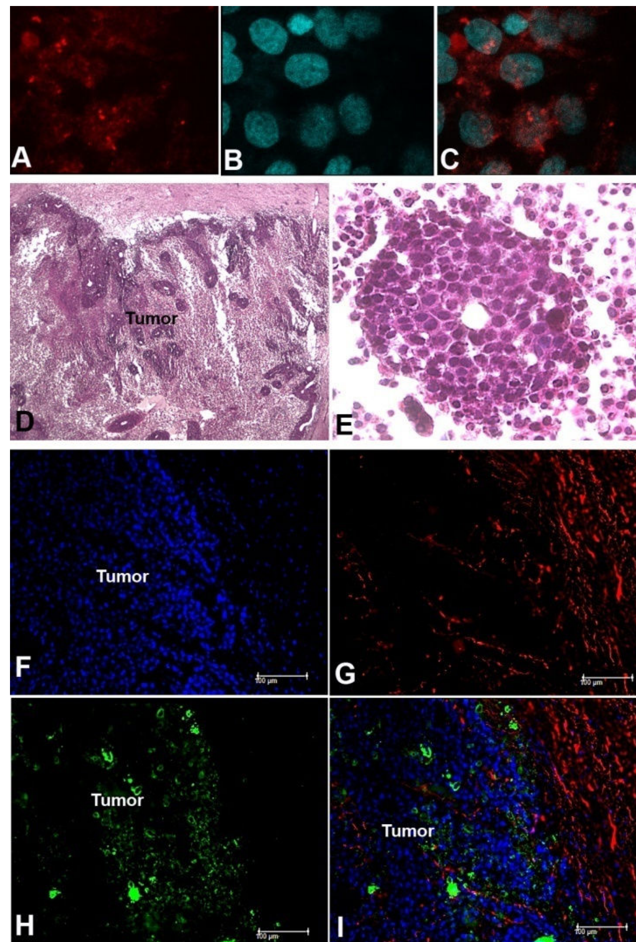

Fig. 2. Immunocytochemical analysis of CHI3L1 productiion in 293 cells before intracerebral implantation (A-C), Histological (D,E) and Immunohistochemical (H-I) study of CHI3L1-positive brain xenografts.

A. CHI3L1-positive cells stained with primary goat anti-CHI3L1 antibodies (Santa Cruz Biotech) and secondary Alexa 633 labeled antibodies (Invitrogen).
B. Nuclear staining with DAPI (Invitrogen).
C. Merged A and B, Magnification ×1000.
D-E Hematoxylin staining on paraffin sections. Magnification ×50 (D), ×400 (E).
F. Nuclear staining of frozen brain slice with DAPI (Invitrogen).
G. Immunostaining of GFAP with polyclonal rabbit antibodies (Serbsky Centre) and donkey antirabbit antibodies labelled with Alexa 633.
H. Immunostaining of CHI3L1 with polyclonal goat antibodies (Santa Cruz Biotech) and donkey antigoat antibodies labelled with Alexa 488.
I. Merged image. Bar 100 µm.

It was also shown that transplanted neural stem/progenitor cells from human brains survived well for one month in all areas of adult rat brain without immunosuppression. Cells from suspension transplants migrated intensely and differentiated into neurons and gliocytes. At the same time, transplants of whole neurospheres showed limited or no migration because of the development of a glial barrier (Aleksandrova et al., 2004). This was in agreement with

previously obtained results (Fricker et al., 1999; Aleksandrova et al., 2002; Englund et al., 2002). Later in the same laboratory the human neural stem cells were efficiently transplanted to rats with spinal trauma (Lebedev et al., 2010) and studied the influence of the neural stem cell transplantation on the restoration of CNS functions in rats with cortical stroke (Volkov et al., 2010). So, if to accept the data that the implanted astrocytes could survive some time as it is described above, the tumor will be already developed and will kill the animal itself or animal will be killed due to the terms of the experiment according to specific signs in behavior of the brain tumor bearing animal. Xenogenic cells in animals under control which were implanted with cells stably transfected by empty vector will not survive too long and will be rejected. The experiments with failed transplantation of small cell lung cancer tumor to the brains of adult immune-competent rats confirm that the tumor cells of neural origin have some affinity to the host brain and can survive in the bran without immediate rejection longer than any other tissue or cells.

4. CONCLUSION

In summary, we believe this to be the first animal model of human brain tumor that displays the possibility to study various biologic features of and host therapeutic response to brain tumor in an immunocompetent host. Presented model of human brain tumors in brains of adult immunocompetent rats is quite reproducible, the procedure can be completed in 1-2 h with results being obtained in about 1 month. Newly ingrowing blood vessels in tumors may signal angiogenesis which is a major process in carcinogenesis and this should be further characterised in future studies. Heterotransplantation of human cancer cells or tumor biopsies into immunocompetent rodents can be used as an alternative preclinical approach for the development of novel cancer therapeutics.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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