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Research Paper

The Regression of Glioblastoma Multiforme is Time Dependent in the Wild-type Rat Xenograft Model

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ABSTRACT

Introduction: Glioblastoma multiforme (GBM) is an aggressive case of primary brain cancer which remains among the most fatal tumors worldwide. Although, some *in vitro* and *in vivo* models have been developed for a better understanding of GBM behavior; a natural model of GBM would improve the efficiency of experimental models of human GBM tumors. We aimed the present study to examine the survival and durability of U87 cells in the brain of wild-type rats.

Methods: U87 cells were intracranially implanted in twenty-one wild-type rats. Tumor size and morphology as well as infiltration of immune cells were investigated at three-time points by H&E and immunohistochemistry (IHC).

Results: The results demonstrated that the inoculation of GBM cells led to the infiltration of host defense system cells which caused immunological regression of the tumor mass after six weeks. While the tumors successfully developed without any sign of host defense invasion in the second week of GBM inoculation. Also, a decrease in tumor size and infiltration of immune system cells were observed in the fourth week.

Conclusion: These data remarkably suggest that time plays a crucial role in activating the immune system against human GBM tumors in rats; it shows that the regression of tumor mass depends on a time slope.

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Highlights

- A noticeable proliferation of tumor cells was observed in the rat's brain by the second week.
- The distant metastatic masses of cancer infiltrated into the adjacent normal tissue by the second week.
- Tumor mass underwent a noticeable diminution in the size by the fourth week.
- Cancer cells completely regressed by the sixth week due to immunological reactions.
- In tumor rejection, the effective mechanism depends on immune system activity and the slope of time.

Plain Language Summary

One of the most malignant tumors is the brain tumor in the world. Unfortunately, no effective treatment has yet been found for it. Of course, researchers need efficient animal models to find the appropriate treatment. The xenograft model is one of the tumor models in the laboratory. However, the main challenge is the interaction of the animal's immune system with induced-cancer cells so that the immune system finally rejects the tumor. In this study, we investigated how long the immune system needs to reject induced tumors in the xenograft model completely. For this purpose, we studied the animals in three periods (second week, fourth week, and sixth week). We concluded that the immune system does not recognize the induced cancer cells until the second week of the experiment. It results in the growth of cancer cells and the formation of tumors in the animal brain. However, the immune system begins to recognize the tumor mass after the fourth week which leads to a reduction in metastasis and tumor size. Eventually, the immune system completely rejects the formed tumor in the sixth week.

1. Introduction

Glioblastoma multiform (GBM) is the most aggressive case of primary brain cancer (Holland, 2001a). Despite the development of new strategies, improvement in treatment techniques, and new medical therapies, the survival rate is unfortunately about 12-15 months post-diagnosis (Perry et al., 2017).

GBM has been indicated by several hallmarks during growth: producing proliferative signaling, angiogenesis, invasion, metastasis, escaping growth suppressors, evading immune destruction, and also standing up to cell death (Hanahan & Weinberg, 2011; Holland, 2001b; Smith & Ironside, 2007). For a better understanding of GBM causative factors, its behavior, and the development of new treatment strategies, *in vitro* and *in vivo* models were most widely employed.

Over the past several decades, some *in vivo* tumor models including xenografts, and genetically and chemically induced tumors were established to understand tumor features and behavior. Of course, the xenograft model is one of the most common approaches in the pharmaceutical industry. In this model, human tumor biopsies or cancer cells are transplanted into rodents. Rodent cancer models especially mouse and rat cancer models are the most frequent models and remain the best choice to study tumors in *in vivo* conditions due to various features such as the physiological, histological, and genetic similarities to humans, small size, gestation times, and their lifespan. However, there are some limitations; a serious limitation is a complex interaction between the immune system and heterotransplantation cancer cells which eventually leads to the suppression of formed tumors. This reaction induces a pattern of biological events including activation of the cellular immune system, activation of the humoral immune system, activation of endothelial cells, loss of vascular structure, hemorrhage, and edema. However, in the heterotransplantation model, the role and duration of immune system response in the process of tumor suppression are still unclear (Halldén et al., 2003; Khalfoun et al., 2000; Ruggeri et al., 2014; Baklaushev et al., 2012).

In our previous work, we could show that transplantation of U87 cells into the brains of male rats resulted in the formation of tumor mass after two weeks (Abdi et al., 2017). However, the long-term fate of GBM inoculation in the wild-type male rats is an open question, in the present study, we investigated tumor growth at three-time points, using H&E and IHC techniques.

2. Materials and Methods

The reagents were purchased from Sigma Company (Sigma-Aldrich, Mo, USA) unless stated otherwise.

Cell culture

The U87 cell line (human glioblastoma multiforme cell line) was purchased from the Pasteur Institute (Tehran, Iran). They were grown in Dulbecco's modified eagle's medium (DMEM-F12), plus 1% antibiotics (penicillin/streptomycin) and 10% fetal bovine serum (FBS) (Gibco, USA). U87 cells were harvested by trypsin–EDTA digestion, washed with PBS and counted with a hemocytometer to mix 10 µL of U87 cells gently with 10 µL of 4% trypan blue. Then, 10 µL of the cell suspension was put on a hemocytometer, and living cells were evaluated by trypan blue (live cell without dye although dead cell was blue). Then cells were diluted in phosphate-buffered saline (PBS) for injection into animals.

Animals and grouping

In this study, twenty-one male Sprague-Dawley rats weighing 220-250 g were used (lack of these criteria was the exclusion criteria of the experiment). Animals were housed in an animal room with 12/12 h light/dark, 22-26°C temperature, and free access to rodent food and healthy water. Following tumor inoculation, the animals were randomly allocated into 3 groups; second weeks (2-w), fourth weeks (4-w), and sixth weeks (6-w) (n=7).

Tumor inoculation

The rats were anesthetized by injection of a mixture of 50 mg/kg %10 ketamine and 10 mg/kg %2 xylazine (Alfasan Co) intraperitoneally. On the first day of the experiment, the scalp was shaved, the rat was fixed in a stereotaxic frame (Narishige Co, SR-6R Model, Japan), the skin was gently incised in the central longitudinal line of the skull, and a hole with the following coordinate; anterior=1 mm and lateral=2 mm of the Bregma point was drilled in the right half of central longitudinal line (X, Y, and Z coordinates of 2 mm, 1 mm, and 5 mm concerning the bregma). Five µL of cell suspension (5×10^5 U87 cells in PBS) was slowly injected (2 µL/minute) 5 mm deep in the brain by a Hamilton syringe (Hamilton Co, 701 NSYR Model, USA) located on the stereotaxic device. The needle was then gently withdrawn, and the skin was sutured. At specified intervals, the animals were anesthetized and heart perfusion was performed; then the animal's brain was removed and fixed in formalin buffer.

Histologic evaluation

Hematoxylin and eosin (H&E) and immunohistochemical (IHC) studies were performed on paraffin-embedded slides as described previously (Abdi, Eskandary, & Nematollahi-Mahani, 2018). For IHC studies, briefly, tissue blocks were cut into 3 µm sections by rotary microtome. The sections were then transferred onto histological slides. The slides were deparaffinized, and also they were rehydrated. They were incubated in 3% H_2O_2 solution in methanol for 10 minutes to block endogenous peroxidase activity. The slides were then added blocking buffer for 1 hour and after washing them, primary antibodies (appropriately diluted of each primary antibody mentioned in Table 1) were applied to them. The slides were eventually incubated with secondary antibodies with streptavidin/biotinylated peroxidase complex. Cell software was used to count positive cells (cells with brown nuclei). At first, ten images at 40 magnifications were taken (by digital camera Olympus-DP72-Japan) from each of the prepared sections and then the number of positive cells in each field was counted by the software. The percentage of positive cells with GFAP, p53, Nestin, Ki-67, and CD31 antibodies was calculated.

Table 1. List of primary antibodies was used in this study

Product name	Product type	Company	Country	Cat No	Sourc	Dilution
K1-67 antigen	Monoclonal antibody	Dako	USA	M7240	Mouse	1:100
CD31 antigen	Monoclonal antibody	Bio.Rad	USA	MCA 1334G	Rat	1:100
GFAP (GA.5)	Monoclonal antibody	Santa Cruz Biotechnology	USA	sc.58766	Mouse	1:50.1:500
Nestin (2Q178)	Monoclonal antibody	Santa Cruz Biotechnology	USA	sc.58813	Rat	1:50.1:500
p53 (A.1)	Monoclonal antibody	Santa.Cruz Biotechnology	USA	sc.393031	Rat	1:100

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Tumor volume

Serial sections were used to measure the tumor volume. The thickness of each section was 3 µm with a 30 µm interval. H&E stained serial sections were photographed by a light microscope (Olympus- BX51 model- Japan) equipped with a digital camera (Olympus-DP72-Japan). Afterward, the areas (A) of the tumor in each image were obtained through A Cell software (Germany). Tumor volume was calculated by the following formula (Equation 1):

1. $V=(A\ (\mu m^2)+A_1+A_2+...+A_n)\times 30\ n\ +3\ n.$

Here, "n" is the number of sections containing the tumor (Abdi et al., 2018).

Statistical analysis

Data were expressed as Mean±SEM. The Kolmogorov-Smirnov test was used for normality determination. Data were analyzed by the one-way ANOVA by using Tukey's post hoc test for multiple comparisons (SPSS software, version 16) and a $P<0.05$ was considered significant.

3. Results

Histological findings

H&E specimens were used for the evaluation of angiogenesis, migration of cancer cells, hemorrhage, and other behaviors of GBM cells as well (Figures 1 and 2).

The neoplastic changes in the brain of rats were seen at the site of tumor inoculation, two weeks after induction of the tumor. At this time a noticeable proliferation of tumor cells was observed in the brain with penetration of the cancer cells into the normal surrounding tissue (Figure 1 A-C). Tumor mass successfully grew in size in all rats of the 2-W group with abundant angiogenesis in the tumor mass (Figure 1 D, F and 3 D), and also the distant metastatic masses of cancer cells infiltrated into the adjacent normal tissue were frequently observed (Figure 1 E and F). Five rats developed necrosis at the center of the tumor mass but two rats were free from necrosis (Figure 1 C). Hemorrhage was also frequent in the tumor masses (Figure 1 D).

In the 4-W group, the tumor mass was detectable in all animals but the induced tumors were different from the tumor masses in the 2-W group (Figures 1 and 2). Tumor masses were formed in both 2-W and 4-W rats with bizarre and normal mitotic cancer cells, but tumors of the 4-W group were free of necrosis (Figure 2 A and B).

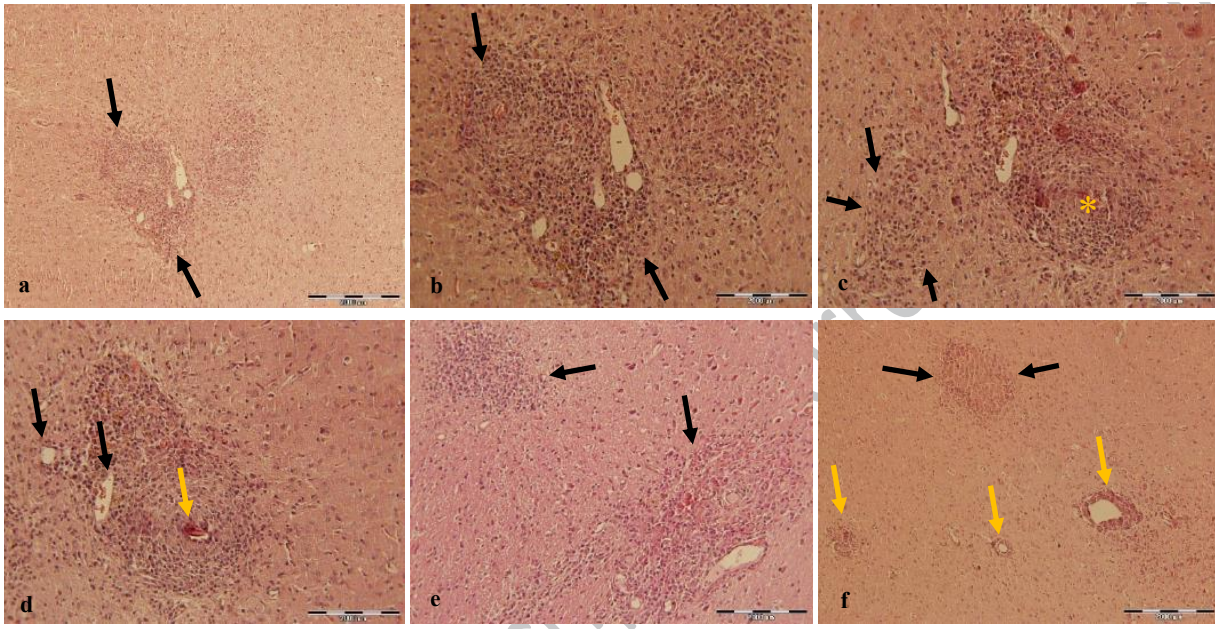

Figure 1. The light microscopy of H&E staining iin different groups.

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In the 2-W group a and b: the tumor mass was formed. C) Penetration of the cancer cells into the normal surrounding tissue (black arrows) and formation of necrosis at tumor mass (yellow star), D) Angiogenesis (black arrows) and hemorrhage (yellow arrows) in the tumor mass. E and F) Distant metastatic masses of cancer cells infiltrated into the adjacent normal tissue (black arrows) and neoangiogenesis in normal tissue along with metastasis of cancer cells into vessels (yellow arrows).

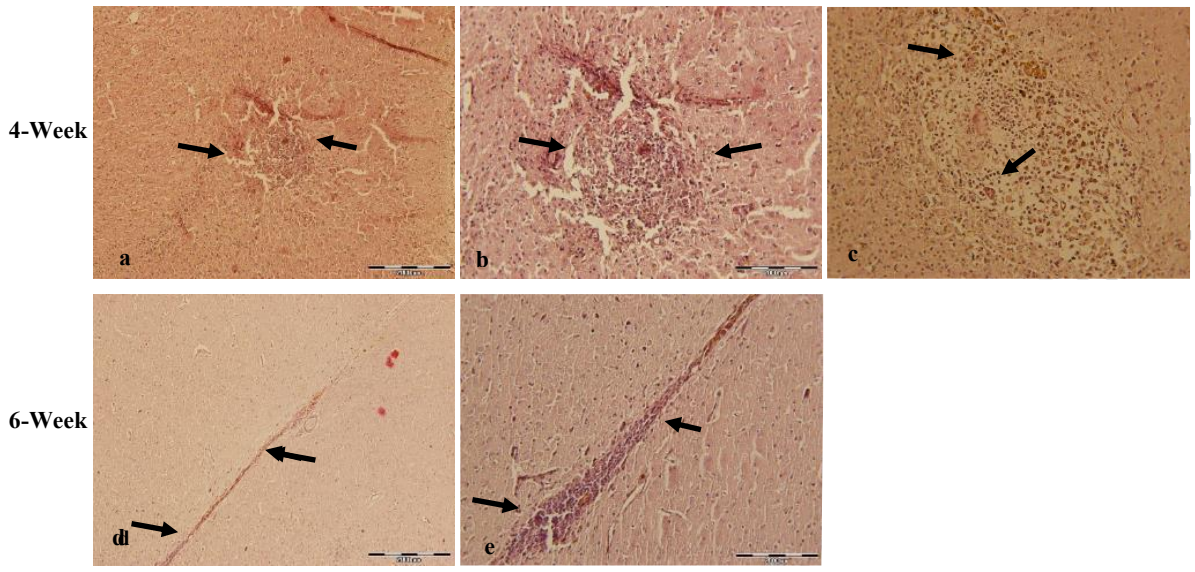

Figure 2. In the 4-W group

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A and B) The tumor mass was formed (black arrows). C) Infiltration of inflammatory cells (black arrows).

In the 6-w group D and E) regression of tumor mass along with injection trace lined with rows of cancer and immune cells (black arrows). Scale bar=2000 µm.

Besides, small vessels in the tumor mass were significantly reduced (P<0.001). Animals of the 4-W group underwent a noticeable diminution in the size of the tumor and the changes were accompanied by infiltration of inflammatory cells (Figure 2 C). Besides, the reduction of cancer mass exhibited a decrease in the immigration of cancer cells, which was similar in all rats of this group. In two rats of the 4-W group tiny metastatic masses were found; while other rats were free of metastatic masses. Indeed, in 6 animals the hemorrhage considerably decreased while in one rat the hemorrhage was substantial. In rats of the 6-W group, the cancer mass intensely reduced in size, and a noticeable regression of tumor mass occurred in all animals of this group (Figure 2 D and E). Some of the injection traces (in 3 rats) were lined with rows of cancer and immune cells whilst injection traces (Figure 2 E) in other animals (4 rats) were lined with a cell row or were greatly free of infiltration of macrophages and neutrophils (Figure 2 D).

Tumor volume

As shown in Figure 3 (A-C and E), the tumor volume was significantly higher in 2-W rats (Figure 3 E). In contrast, a significant decrease in tumor size was seen by four weeks after tumor induction (Figure 3 B) and complete regression of tumor mass was observed in the 6-W group (Figure 3 C).

IHC findings

The expression pattern of Ki-67, GFAP, Nestin, p53, and CD31 markers was compared in the different groups (Figures 4 and 5). Tumor masses in the 2-W group were highly immunoreactivity to Ki67 monoclonal antibody, were weakly reactive in the 4-W group, and had very few immunoreactivities in the 6-W group (Figure 4 A-C and Figure 5 G).

Strong reaction to GFAP antibody was detected in the tumor masses of the 2-W group; especially it was noticeable at the borders of tumor masses in this group, although cancer cells in tumor mass were without staining to GFAP. Besides, tumor masses in the 4-W group considerably showed a high reaction to GFAP at the border of the tumor masses but not in the center. In the traces of U87 cells that were present at the injection site of the 6-W group, no GFAP-positive cell was detected but some GFAP-positive cells were detected at the borders (Figure 4 D-F and Figure 5 G). Interestingly, Nestin was highly expressed in the tumor mass of the 2-W group, was moderately expressed in the 4-W group, and was weakly expressed in the 6-W group (Figure 4 G-I and Figure 5 G). CD31 expression as the marker of angiogenesis was weakly positive in the tumor mass of the 2-W group; also small vessels formed around the tumor mass were highly positive. Whereas in the 4-W group, the sections were diffusely positive for CD31, and also there was a statistically significant difference between this group with the other groups. Besides, there were moderately or weakly

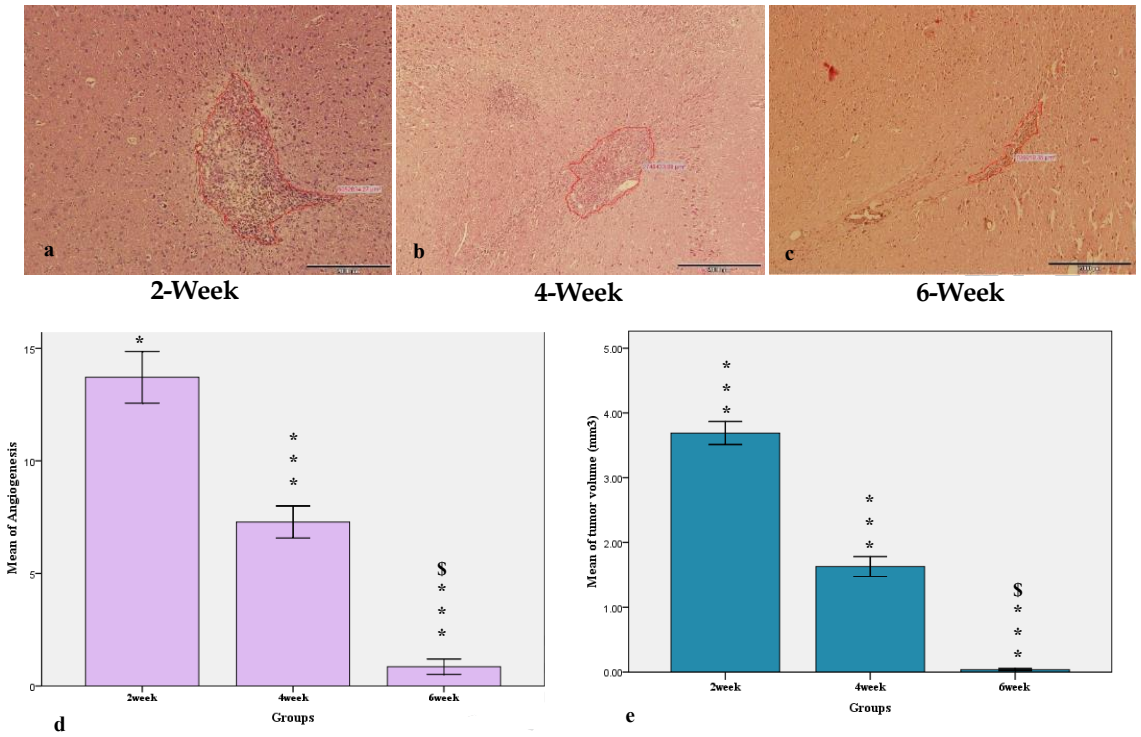

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Figure 3. A) Area of tumor mass in the 2-w group. B) Area of tumor mass in the 4-w group; C) Area of tumor mass in the 6-w group. Scale bar=2000 µm.

D) Analysis of angiogenesis among the groups. All data are presented as Mean±SEM. ***P<0.001 (as compared to 2-w group); $P<0.001 (as compared to 4-w group); E) Analysis of the tumor volume among the groups.

All data are presented as Mean±SEM. ***P<0.001 (as compared to 2-w group); $P<0.001 (as compared to 4-w group).

positive CD31 cells in the needle trace of the 6-W group which was significantly different compared with the angiogenesis in the 4-W group (Figure 5 A-C and G). In addition, p53 positive cells were weakly present inside the cancer mass in the 2-W group. The same pattern was observed in the 4-W group and to a lesser extent in the 6-W group, with no statistically significant difference among the group (Figure 5 D-F and G).

4. Discussion

The results of this experiment demonstrated that human glioblastoma multiform cells induced as a xenograft in the brain of wild-type rats could successfully infiltrate in the host animal, considerably grew in size for two weeks, regress to nearly half the size of two weeks after four weeks, and completely regress after six weeks due to immunological reactions. Our observations are in agreement with the studies that reported the rejection of neural cells or brain tumor cells in xenograft models of animals (Baklaushev et al., 2012; Barth & Kaur, 2009; Englund et al., 2002; Strojnik et al., 2006).

This study has also described the significant development of human tumor cells in the brain of wild-type rats for two weeks. It appears that human GBM tumor mimics some of the features of GBM such as migration, metastatic mass, angiogenesis, and hemorrhage which are characteristic of GBM tumors in humans (Adamson et al., 2009). Higher expression of Ki67 and Nestin inside and at the margin of tumor mass also confirmed the survival and growth of tumor mass by two weeks (Calabrese et al., 2007; Li et al., 2015; Singh et al., 2004). Also, Ki67 is a nuclear protein that is associated with cellular proliferation and ribosomal RNA transcription which is active during all phases of cycle cell (G_1, S, G_2, and M) except G_0 (Kee et al., 2002). In addition, Nestin, a type VI intermediate filament associated with the proliferation and function of neuronal cells is especially present in neural stem cells (Park et al., 2010). Ki67 and Nestin expression are significantly associated with proliferative activity and tumor outgrowth, therefore a high labeling index of Ki67 and Nestin in the 2-W group suggested a high rate of cancer cell proliferation. These findings are in agreement with other reports (Calabrese et al., 2007; Li et al., 2015; Singh et al., 2004).

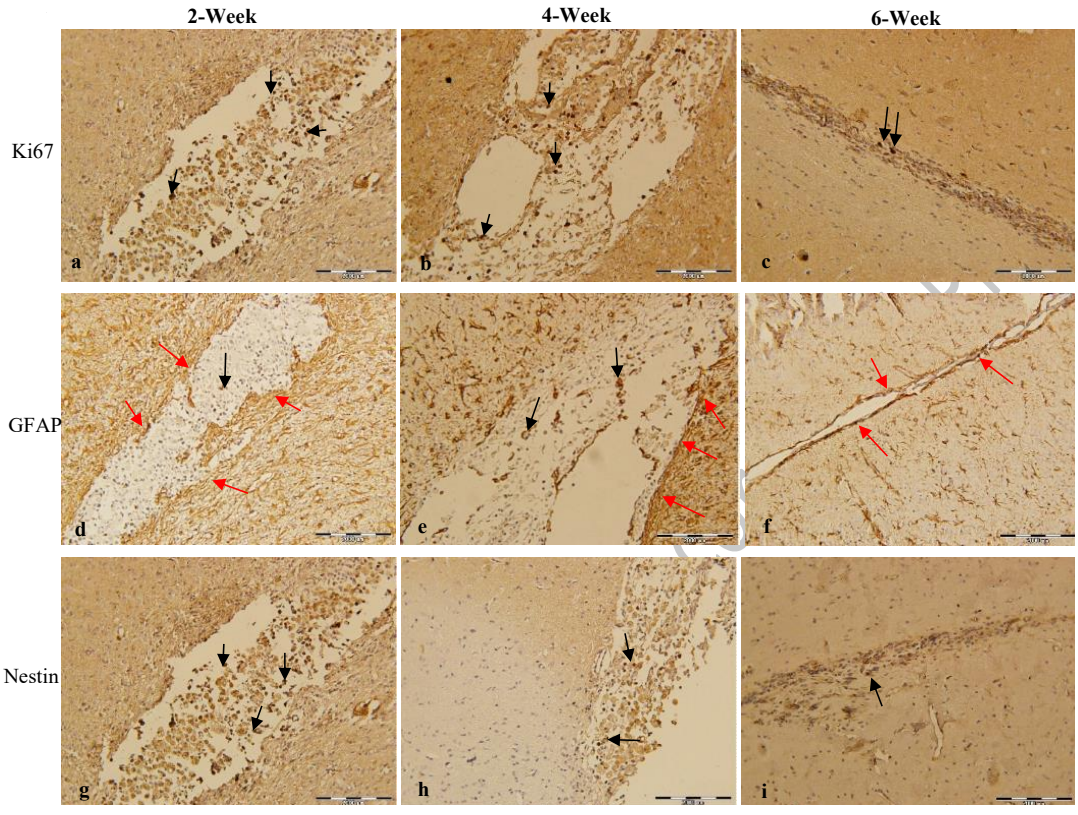

Figure 4. Photomicrographs of brain section stained with above Ki67, GFAP, and nestin aantibodies. NEUROSCIENCE

A-C) Anti-Ki67 sstained ssections. A) In the 2-W group, the cancer cells were highly positive to for Ki67 (black arrows); B) In the 4-W group, the tumor mass was weakly reactive to Ki67 (black arrows); C) The cancer cells were little reactive immunoreactivity in the 6-W group (black arrows); D-F) Anti-GFAP-stained sections. D) In the 2-w group, a strong reaction to the GFAP antibody was detected in the borders of the tumor mass (red arrows) cancer cells inside the tumor mass were negative to GFAP (black arrows); E) in the 4-W group, high reactions were showed shown to GFAP at the border of the tumor mass (red arrows) but not in the center (black arrows); F) GFAP-positive cells were detected at the borders in the 6-w group (red arrows). G-I) anti-nestin stained sections; G) In the 2-w group, nestin was highly expressed in the tumor mass (black arrows); H) In the 4-w group, nestin was moderately expressed (black arrows); I) Nestin was weakly expressed in the 6-W group (black arrows). Scale bar=2000 µm.

Although the cytological appearance of tumor masses was almost similar in rats of the 2-W and the 4-W groups, cancer masses were considerably infiltrated by host defense cells by four weeks after induction of the tumor which prevented tumor growth. While the intense staining of CD31 (PECAM-1) as a marker of angiogenesis, in the 4-W group, indicates the invasion of tumor mass by the host immune system cells to the tumor mass resulting in higher angiogenesis activities in the tumor mass (Basilio-de-Oliveira & Pannain, 2015; Khalfoun et al., 2000; Khattab et al., 2009)

These observations suggested that the effector mechanism in tumor rejection is dependent on host immune system activity; and in fact, host humoral and cellular immune systems are involved in cancer cell killing. We detected noticeable infiltration of host defense system cells in the cancer mass, which caused the gradual incomplete regression of the tumor mass during the fourth week. The immune cells attack cancer mass which leads to the production of interleukin 12 (IL-12). IL-12 plays a major role in the stimulation, development, and function of T-cells and natural killer cells (NK cells) (Benatar et al., 2010; Trinchieri, 2003). It consequently stimulates the production of tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-λ) from T cells and NK-cells (Benatar et al., 2010; Igney & Krammer, 2002; Khalfoun et al., 2000; Trinchieri, 2003). TNF-α, the most important cytokine, which is secreted during the infiltration of defense cells, (Blankenstein, 2005; Clark et al., 2005; Goetz et al., 2004) has a dual role in tumor growth. In fact, at high concentrations, TNF-α acts as an anti-tumoral factor and it disturbs tumor angiogenesis and prevents tumor progression while at low concentrations, TNF-α performs as a tumor progression factor. Therefore,

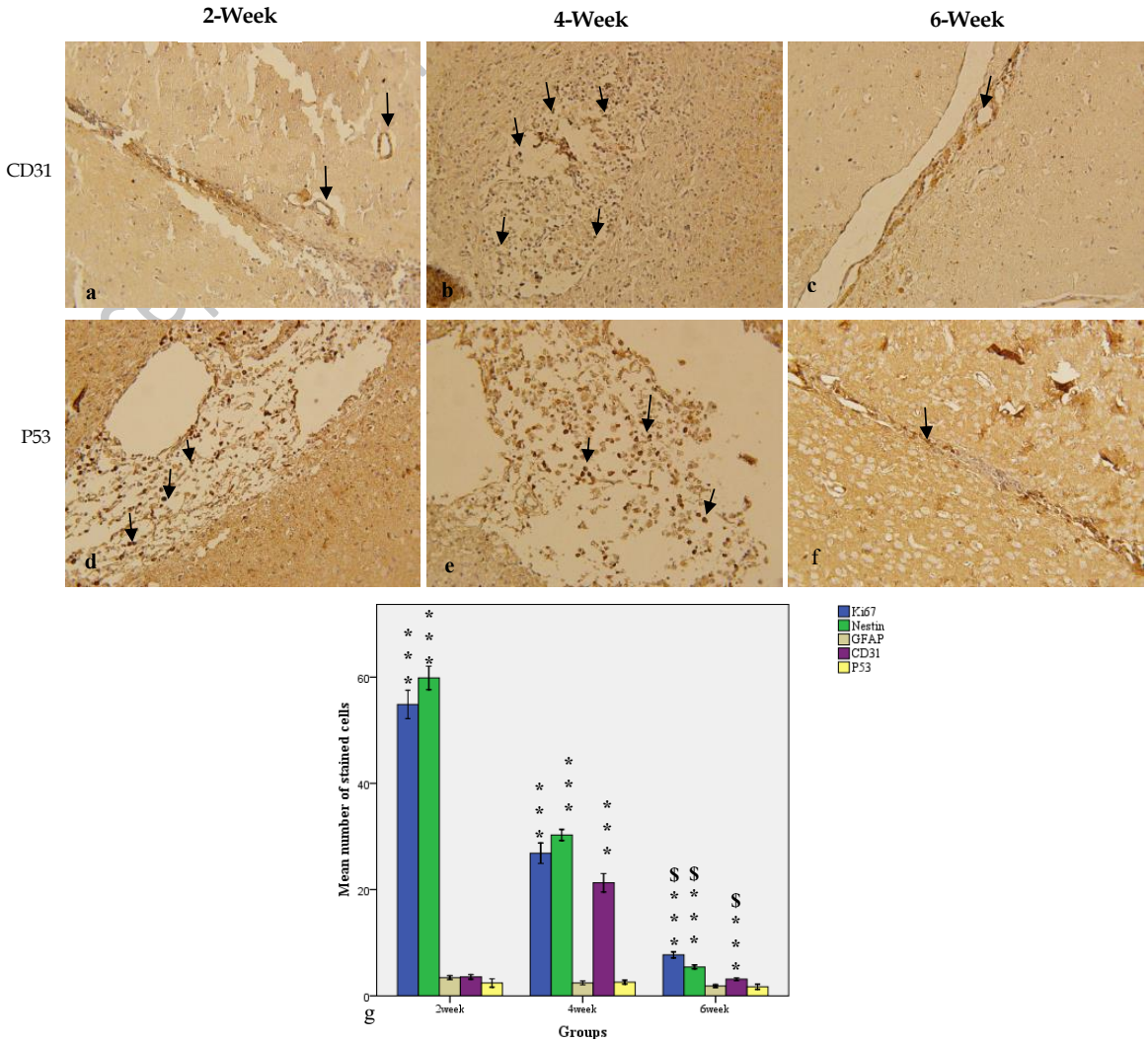

Figure 5. Photomicrographs of brain section stained with above CD31 and P53 antibodies.

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a-b: Anti-CD31 stained sections. a: In the 2-w group, the CD31 expression was weak in the tumor mass; but vessels formed around the tumor mass were highly positive (black arrows). b: In the 4-w group, CD31 expression was diffusely positive (black arrows). c: The CD31 expression was moderately or weakly positive in the 6-W group (black arrows). d-f: Anti- CD31 stained sections. d: In the 2-w group, the P53 expression was weak inside the tumor (black arrows). e: In the 4-w group, the P53 expression was weak in the tumor (black arrows) f: The P53 expression was weaker in the 6-W group (black arrows). Magnification 200× g: Analysis of positive cells to different antibodies among the groups. All data presented as Mean±SEM.

***P<0.001(as compared to 2-w group); $^{\$}$P<0.001 (as compared to 4-w group).

TNF-α has been proposed to play a key role in the early stages of tumor growth. Accordingly, the main reason for tumor growth during the two weeks after induction may be due to the low activity of the immune system in the tumor mass that resulted in the low concentration of TNF-α. Also, at lower concentrations, TNF-α can induce some chemokines such as vascular endothelial growth factor (VEGF) as an angiogenesis factor, and concurrently destroy Extra Cellular Matrix (ECM) (Brigati et al., 2002; Keibel et al., 2009; Moore et al., 1999). While, it is likely that increased TNF-α concentration due to the strong activity of the rat defense system, led to the destruction of metastatic masses, and inhibition of angiogenesis, migration, and tumor progression in the fourth and sixth week.

These data remarkably described the role of time in the development, metastasis, angiogenesis, and hemorrhage of GBM cancer masses in a wild animal model of tumor inoculation. This issue is important when tumor inoculation is used as an experimental model for brain tumor investigations.

5. Conclusion

A novel and major observation of this study is the transformation of immune system behavior against cancer mass as time goes on, during which the regression of tumor exhibited a sloping dependence on time. In the case of utilizing a wild-type model of rats with GBM inoculation, the time restriction in GBM tumor behavior should be considered precisely. However, our knowledge of how immune system cells control tumor fate for development or regression is still incomplete.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by Kerman University of Medical Sciences Ethics Committee (Code: EC/KNRC/93/23)

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Authors' contributions

Conceptualisation and study design: Seyed Noureddin Nematollahi-Mahani and Zahra Abdi. Methodology: Zahra Abdi and Sepideh Ganjalikhan-hakemi; Data collection: Sepideh Ganjalikhan-hakemi; Data analysis and writing of the original draft: Zahra Abdi; Editing and reviewing by Seyed Noureddin Nematollahi-Mahani.

Conflict of interest

The authors declared no conflict of interest.

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