Apoptosis in glioma-bearing rats after neural stem cell transplantation

Hua Li[1], Zhenjun Chen[1], Shaopeng Zhou[2]

1 Department of Neurology, the 476 Hospital of Chinese PLA, Fuzhou 350002, Fujian Province, China
2 Department of Anesthesiology, the Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai 519000, Guangdong Province, China

Research Highlights

(1) Neural stem cells transplanted into tumor-bearing rats can hinder tumor cell growth and proliferation; however, the mechanism remains unclear.

(2) Glioma development and malignant biological characteristics are associated with abnormal signal transduction networks in tumor cells.

(3) This study aimed to explore neural stem cell therapy for glioma from the viewpoint of the Ras/Raf/Mek/Erk pathway. The results showed that transplantation of neural stem cells could inhibit the abnormal activation of Ras/Raf/Mek/Erk signaling, thus promoting apoptosis and potentially treating glioma.

Abstract

Abnormal activation of the Ras/Raf/Mek/Erk signaling cascade plays an important role in glioma. Inhibition of this aberrant activity could effectively hinder glioma cell proliferation and promote cell apoptosis. To investigate the mechanism of glioblastoma treatment by neural stem cell transplantation with respect to the Ras/Raf/Mek/Erk pathway, C6 glioma cells were prepared in suspension and then infused into the rat brain to establish a glioblastoma model. Neural stem cells isolated from fetal rats were then injected into the brain of this glioblastoma model. Results showed that Raf-1, Erk and Bcl-2 protein expression significantly increased, while Caspase-3 protein expression decreased. After transplantation of neural stem cells, Raf-1, Erk and Bcl-2 protein expression significantly decreased, while Caspase-3 protein expression significantly increased. Our findings indicate that transplantation of neural stem cells may promote apoptosis of glioma cells by inhibiting Ras/Raf/Mek/Erk signaling, and thus may represent a novel treatment approach for glioblastoma.

Key Words

neural regeneration; stem cells; Ras/Raf/Mek/Erk signaling pathway; neural stem cells; glioblastoma; C6 glioma cells; Caspase-3; Bcl-2; apoptosis; brain tumor; neuroregeneration

INTRODUCTION

Glioblastoma is the most common intracranial tumor derived from the neuroectoderm, and has high heterogeneity[1]. The present recommended treatment plan is radical surgery combined with radiotherapy and chemotherapy; however, prognosis is poor and patients only survive on average for 18 months[2]. Neural stem cells have pluripotent differentiation potentials, and can restore the normal structure and function of the host central nervous system[3]. After neural stem cells are transplanted into tumor-bearing rats, they can prevent tumor

cell growth and proliferation[4]; however, the underlying mechanism remains unclear.

The Ras/Raf/Mek/Erk signaling cascade is a widely activated mitogen-activated protein kinase pathway present in all eukaryotes, and can control cell proliferation, differentiation, survival and apoptosis[5]. Abnormal activation of this cascade is crucial for glioma, and its suppression can effectively inhibit glioma cell proliferation and promote cell apoptosis[6]. However, it is unclear if neural stem cells can inhibit Ras/Raf/Mek/Erk signaling in the treatment of glioma.

Ras/Raf/Mek/Erk signaling is closely associated with apoptosis at the molecular level[7-8]. Bcl-2 is a downstream substrate of Erk1/2[9], and allows the survival of poorly differentiated and metaplastic cells to promote tumor growth, ultimately leading to invasive malignant cancer[10]. Increases in Bcl-2 expression are associated with glioma[11-13]. Bcl-2 acts on Caspase-3 upstream and can inhibit its activation[14]. Caspase-3 expression decreases during the glioma cell apoptosis process[15].

In this study, we observed Raf-1, Erk, Caspase-3 and Bcl-2 protein expression following neural stem cell transplantation, in a broader attempt to explore the correlation between neural stem cell transplantation, the Ras/Raf/Mek/Erk pathway and apoptosis in glioma cells.

RESULTS

Quantitative analysis of experimental animals

One hundred and twenty Sprague-Dawley rats were randomly divided into three groups, with 40 rats in each group as follows: normal group, no intervention was given; model group, glioblastoma models were established and injected with normal saline; cell transplantation group, glioblastoma models were established and injected with neural stem cells from fetal rats. During the modeling process, one rat in the model group and one rat in the cell transplantation group died, resulting in 118 rats used in the result analysis.

Brain imaging observations of glioblastoma rats

One week after modeling, MRI examination showed that the midline structure, sulci, encephalocoele and cisterns were normal in the normal rats, and there were no abnormal brain signals. Tumor lesions were visible in the model rats, with long T1 and T2 signals, and the boundary between lesions was blurry with mild edema present around the lesions. Furthermore, midline structures had

shifted, and the ventricular systems were compressed and deformed, which confirmed successful establishment of the model (Figure 1).
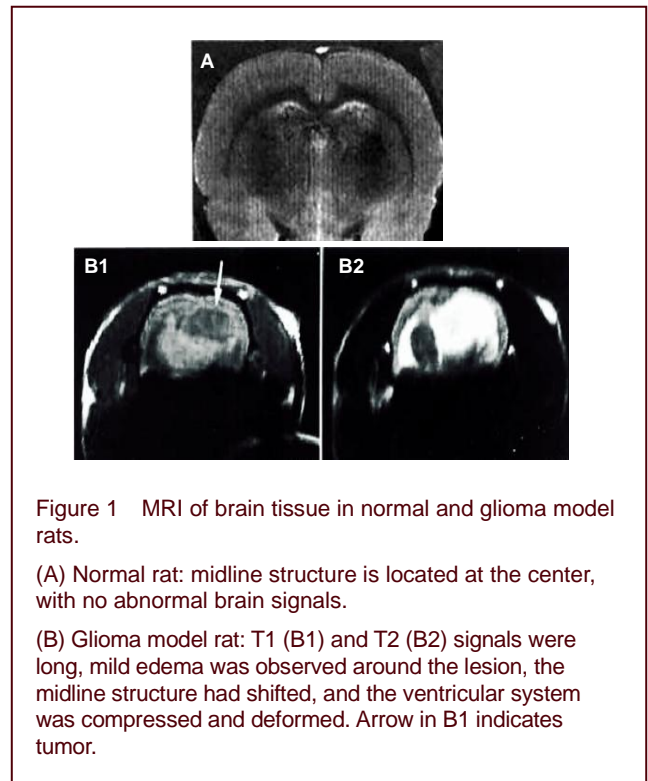

Figure 1   MRI of brain tissue in normal and glioma model rats.

(A) Normal rat: midline structure is located at the center, with no abnormal brain signals.

(B) Glioma model rat: T1 (B1) and T2 (B2) signals were long, mild edema was observed around the lesion, the midline structure had shifted, and the ventricular system was compressed and deformed. Arrow in B1 indicates tumor.

Morphology and identification of neural stem cells isolated from fetal rats

Primary neural stem cells were round and in suspension. After 24 hours of culture, cells began to adhere; at 7–9 days cell spheres formed, showing a rounded shape, uniform size, and suspended proliferation. Adherent cells were detected with anti-nestin immunofluorescence staining, and results showed that cell spheres were positive for nestin and elicited strong yellow-green fluorescence. At 10 days, anti-glial fibrillary acidic protein immunofluorescence staining revealed that there were a large amount of protruding glial fibrillary acidic protein-positive cells, emitting yellow-green fluorescence. This evidence indicates that the cultured and isolated cells had the characteristics of neural stem cells and differentiation potentials (Figure 2).

Effect of neural stem cell transplantation on Raf-1, Erk, Bcl-2 and Caspase-3 protein expression in tumor tissue from glioma model rats

One week after stem cell transplantation, western blot analysis showed that Raf-1, Erk and Bcl-2 protein expression in the model group was significantly increased ($P < 0.05$), while Caspase-3 protein expression was significantly decreased ($P < 0.05$) compared with the normal group. In the cell transplantation group, Raf-1, Erk and

Bcl-2 protein expression was significantly decreased ($P <$ 0.05), while Caspase-3 protein expression was significantly increased ($P < 0.05$) compared with the model group (Figure 3, Table 1).
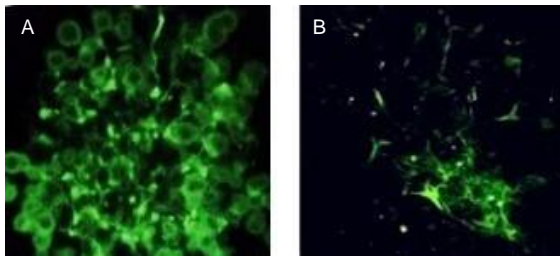

Figure 2   Nestin and glial fibrillary acidic protein (GFAP) expression in cultured neural stem cells (immunofluorescence staining, × 400).

Under fluorescence microscopy, subcultured cells expressed nestin, a marker of neural stem cells (A); while some cells also expressed an astrocyte marker, GFAP (B). Yellow-green fluorescence indicates FITC-labeled nestin and GFAP expression.
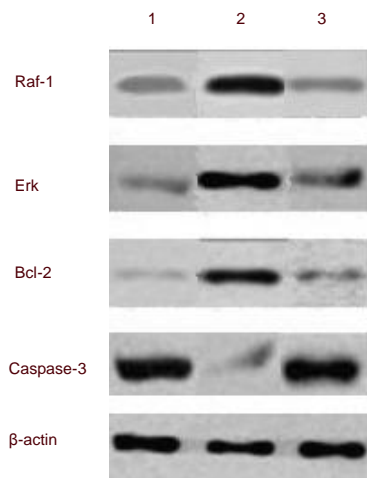

Figure 3   Raf-1, Erk, Bcl-2 and Caspase-3 protein expression in the tumor tissue of glioma model rats after stem cell transplantation.

1: Normal group; 2: model group; 3: cell transplantation group.

Western blot analysis showed that Raf-1, Erk and Bcl-2 expression was low in normal rat brains and upregulated in the tumor tissue of glioma rats, while Caspase-3 expression was reduced in glioma rats; 1 week after stem cell transplantation, Raf-1, Erk and Bcl-2 expression had decreased, and Caspase-3 expression had increased in the tumor tissue of glioma rats.

Effect of neural stem cell transplantation on Raf-1, Erk, Bcl-2 and Caspase-3 immunopositive expression in tumor tissue of glioma model rats
Immunohistochemical assay showed that Raf-1, Erk and

Bcl-2 were expressed in the cytoplasm and cell membrane, and Caspase-3 expression was observed in the nucleus. In the normal group, only a small number of Raf-1-, Erk-, Bcl-2-positive cells were visible, while Caspase-3-positive cells increased; in the model group, there were a large number of deeply stained Raf-1-, Erk- and Bcl-2-positive cells, and more weakly stained Caspase-3-positive cells; in the cell transplantation group, there was a reduction in Raf-1-, Erk- and Bcl-2-positive cells, while Caspase-3-positive cells had increased compared with the model group (Figure 4).

Table 1   Raf-1, Erk, Bcl-2 and Caspase-3 protein expression (absorbance ratio to β-actin) in the tumor tissue of glioma model rats at 1 week after stem cell

Group	Raf-1	Erk
Normal	0.527±0.036	0.432±0.037
Model	0.904±0.028[a]	0.728±0.103[a]
Cell transplantation	0.614±0.035[ab]	0.516±0.051[ab]

Group	Bcl-2	Caspase-3
Normal	0.062±0.015	0.324±0.017
Model	0.183±0.021[a]	0.152±0.018[a]
Cell transplantation	0.074±0.011[ab]	0.309±0.012[ab]

Data are expressed as mean ± SD, with 20 rats per group. [a]$P <$ 0.05, *vs.* normal group; [b]$P < 0.05$, *vs.* model group (one-way analysis of variance followed by least significant difference test).

Quantitative analysis showed that the number of Raf-1-, Erk- and Bcl-2-positive cells and their expression levels in the model group were significantly higher than the normal group ($P < 0.05$), and those in the cell transplantation group were significantly lower than the model group ($P < 0.05$), which was still slightly higher than the normal group ($P < 0.05$). Conversely, Caspase-3 positive cells and its positive expression rate in the model group was significantly lower than the normal group ($P < 0.05$), and those in the cell transplantation group was significantly higher than the model group ($P < 0.05$), which was lower than the normal group ($P < 0.05$; Tables 2–5).

DISCUSSION

Glioma is the most common primary intracerebral tumor, accounting for 2% of all malignant tumors in adults. It is characteristics consist of invasive growth, high relapse rate, strong aggression, and abundance in blood vessels[16]. According to WHO classification, patients with grade-3 glioma survive on average for 3–5 years[17]. Glioma occurrence, development and malignant biological characteristics are associated with abnormal signal transduction in tumor cells[18].
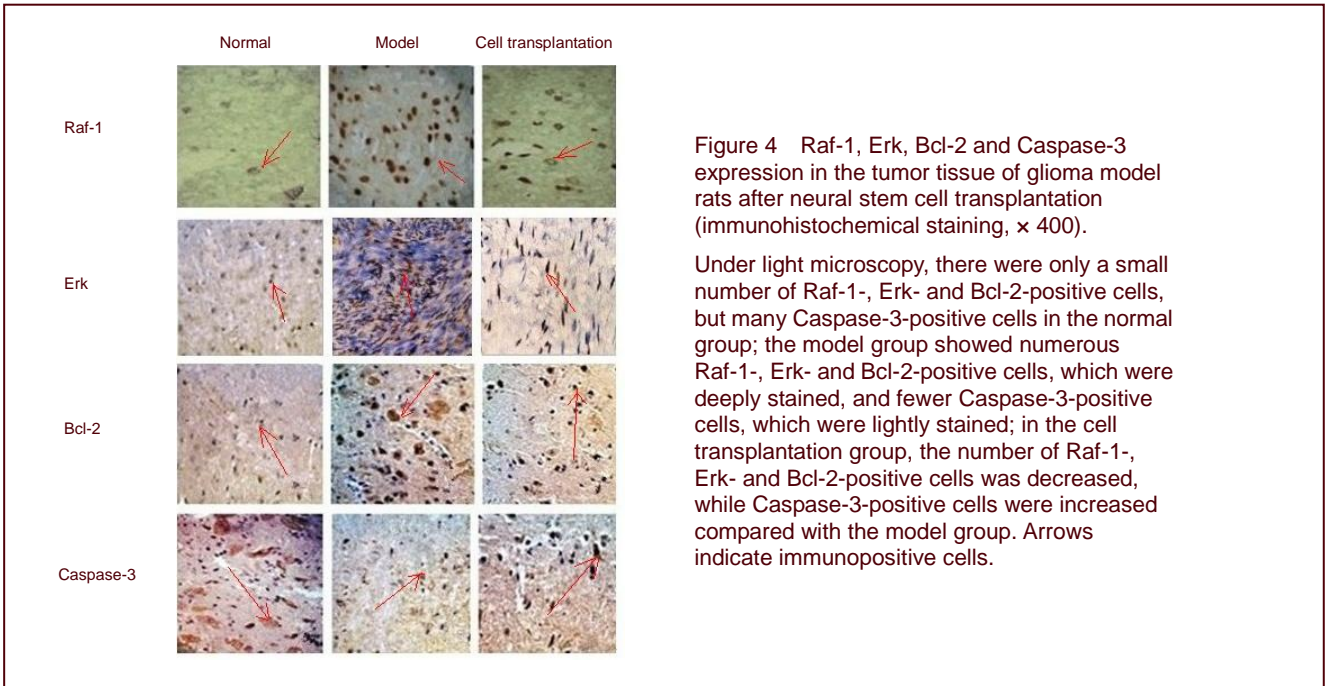
Figure 4   Raf-1, Erk, Bcl-2 and Caspase-3 expression in the tumor tissue of glioma model rats after neural stem cell transplantation (immunohistochemical staining, × 400).

Under light microscopy, there were only a small number of Raf-1-, Erk- and Bcl-2-positive cells, but many Caspase-3-positive cells in the normal group; the model group showed numerous Raf-1-, Erk- and Bcl-2-positive cells, which were deeply stained, and fewer Caspase-3-positive cells, which were lightly stained; in the cell transplantation group, the number of Raf-1-, Erk- and Bcl-2-positive cells was decreased, while Caspase-3-positive cells were increased compared with the model group. Arrows indicate immunopositive cells.

Table 2   Effect of neural stem cell transplantation on Raf-1 expression in tumor tissue of glioma model rats

Group	Positive cells (/× 400 field)	Positive expression				Positive rate (%)
		(−)	(+)	(++)	(+++)	
Normal	23.18±1.49	14	6	0	0	30.00
Model	68.14±2.34[a]	2	2	9	6	89.47[a]
Cell transplantation	34.03±1.35[ab]	10	3	5	1	47.36[ab]

Data are expressed as mean ± SD, and there were 20, 19, 19 rats in the normal, model, cell transplantation groups, respectively. [a]$P < 0.05$, vs. normal group; [b]$P < 0.05$, vs. model group (measurement data were compared using one-way analysis of variance, paired comparisons were performed using least significant difference test, and the positive rate was compared using chi-square test).

Table 3   Effect of neural stem cell transplantation on Erk expression in tumor tissue of glioma model rats

Group	Positive cells (/× 400 field)	Positive expression				Positive rate (%)
		(−)	(+)	(++)	(+++)	
Normal	20.07±1.28	15	5	0	0	25.00
Model	63.52±1.24[a]	3	3	8	5	84.21[a]
Cell transplantation	29.84±1.12[ab]	11	3	5	1	42.10[ab]

Data are expressed as mean ± SD, and there were 20, 19, and 19 rats in the normal, model, cell transplantation groups, respectively. [a]$P < 0.05$, vs. normal group; [b]$P < 0.05$, vs. model group (measurement data were compared using one-way analysis of variance, paired comparisons were performed using least significant difference test, and the positive rate was compared using chi-square test).

Table 4   Effect of neural stem cell transplantation on Bcl-2 expression in tumor tissue of glioma model rats

Group	Positive cells (/× 400 field)	Positive expression				Positive rate (%)
		(−)	(+)	(++)	(+++)	
Normal	21.22±1.15	15	5	0	0	25.00
Model	75.11±1.34[a]	1	4	7	7	94.74[a]
Cell transplantation	38.06±1.97[ab]	9	3	5	2	58.82[ab]

Data are expressed as mean ± SD, and there were 20, 19, and 19 rats in the normal, model, cell transplantation groups, respectively. [a]$P < 0.05$, vs. normal group; [b]$P < 0.05$, vs. model group (measurement data were compared using one-way analysis of variance, paired comparisons were performed using least significant difference test, and the positive rate was compared using chi-square test).

Table 5 Effect of neural stem cell transplantation on Caspase-3 expression in tumor tissue of glioma model rats

Group	Positive cells (/× 400 field)	Positive expression				Positive rate (%)
		(−)	(+)	(++)	(+++)	
Normal	58.26±1.32	5	3	6	6	75.00
Model	26.18±1.46[a]	13	4	2	0	31.58[a]
Cell transplantation	51.27±1.55[ab]	6	6	5	2	68.42[ab]

Data are expressed as mean ± SD, and there were 20, 19, and 19 rats in the normal, model, cell transplantation group, respectively. [a]$P < 0.05$, *vs.* normal group; [b]$P < 0.05$, *vs.* model group (measurement data were compared using one-way analysis of variance, paired comparisons were performed using least significant difference test, and the positive rate was compared using chi-square test).

Activation of the Erk1/2 signaling pathway, also called the Raf/Mek/Erk signaling pathway, can cause multiple protein kinase cascade reactions, and transmit extracellular signals into the cells[19]. Under the stimulation of extracellular signals, Ras can be activated by binding with guanosine triphosphate, thereby activating phosphorylation of Raf, Mek and Erk. Then phosphorylated Erk enters the nuclei and triggers activity of transcription factors[20]. *Via* this signal pathway, extracellular growth and neurotrophic signals are transferred to the cells, causing a series of cellular reactions that can regulate cell proliferation and differentiation[21-22]. Ras/Raf/Mek/Erk signaling pathway disorders are also crucial for tumor occurrence and development[23]. Ras and Raf oncogenic mutations can be detected in many tumors, leading to excess activation of Ras/Raf/Mek/Erk signaling, which contributes to tumor development and malignancy[24]. This protein kinase cascade is the key signaling pathway affecting the abnormal biological characteristics of glioma[25]. Glioma development and malignancy may be the results of abnormal activation of Ras/Raf/Mek/Erk signaling[26]. This signaling pathway exerts the most apparent effect on the promotion of glioma cell survival and proliferation[27]. Moreover, the disorder is also associated with glioma angiogenesis, invasion and metastasis[28]. In glioma, growth factors and their receptors upstream of the Ras/Raf/Mek/Erk signaling pathway, such as epidermal growth factor receptor and platelet-derived growth factor receptor, may be overexpressed as a result of gene amplification or mutation[29-30]. The epidermal growth factor receptor binds with vascular endothelial growth factor-A on the glioma cell surface, thus activating downstream Ras/Raf/Mek/Erk signaling and promoting vascular endothelial cell proliferation, inhibiting vascular endothelial cell apoptosis, and playing a catalytic role in glioma angiogenesis[31]. Excessive activation of this pathway also induces vascular endothelial growth factor expression. Raf-1 and Mek activation can induce the formation of high-level malignant glioma[32-33]. Therefore, suppressing excessive activation of this signaling pathway may kill glioma cells. Raf kinase inhibitors

have a positive effect in the treatment of gliomas[34]. Our immunohistochemical assay results showed that Raf-1 and Erk were weakly expressed in normal brain tissue, while a large number of positive granules were observed in the model group. Furthermore, western blot analysis showed that Raf-1 and Erk protein expression in the tumor tissue of model rats was significantly higher than the normal rat brain tissue. This evidence also supports the involvement of abnormal activation of Ras/Raf/Mek/Erk signaling in the pathogenesis of glioma.

Neural stem cells are a type of precursor cells that have high self-renewal capacity and can differentiate into neurons and glial cells, and also generate a variety of nervous tissue cells through unequal division; thus, their differentiation and regulatory mechanisms are crucial in the treatment of nervous system[35-36]. Transplanted neural stem cells may differentiate into new neurons to replace defective or dead neurons, and connect with the surrounding nervous system to establish correct synaptic connection for the recovery of neurological functions[37-38]. Neural stem cells *in vivo* and *in vitro* have a strong ability to track glioma[39]. In addition, neural stem cells also exert transdifferentiation[40] and cell fusion[41-42] phenomena. Neural stem cells can specifically target and accumulate around glioma cells, and even track single glioma cells for a long distance[43]. Aboody *et al* [44] have found that when neural stem cells were transplanted into tumor-bearing rats, they spread throughout the tumor and migrate to other organs, surrounding the tumor to stimulate its growth and development and secreting various factors to reduce tumor size. Neural stem cells also can differentiate into neurons, thus promoting endogenous neurogenesis, generating glial cells, and promoting nerve fibers formation[45]; in addition, neural stem cells also secrete neurotrophic factors, which can promote proliferation, migration and neurogenesis of endogenous neural stem cells, and they play a key role in the reconstruction and functional recovery of neural pathways[46-47]. Scholars[48] showed that transplanted neural stem cells

could express neurotrophic factors *in vivo* and promote host axonal growth; our findings suggested that neural stem cells have the potential to replace missing tissue after nervous system injury. In this study, fetal rat hippocampal cells showed the morphology of neural stem cells, and were induced to differentiate into neurons and glial cells, indicating that these cells possessed the characteristics and differentiation potential of neural stem cells. Immunohistochemical staining showed that Raf-1 and Erk expression in the cell transplantation group was significantly lower than in model group; however, Raf-1 and Erk protein expression significantly reduced after transplantation of neural stem cells. We speculate that neural stem cells may inhibit abnormal activation of Ras/Raf/Mek/Erk signaling, thus inhibiting glioma development as consistent with previous findings.

Apoptosis, also known as programmed cell death, is a complex protease cascade reaction involved in multiple protein molecules. The mitochondrial inner membrane potentials depolarize, and mitochondria release apoptosis-inducing factors, which trigger the activation of proteases and induce changes in cell morphology; nuclear condensation is observed, DNA is sheared and degrades, cell membrane ruptures, apoptotic bodies form, and ultimately cell death occurs[49]. The Bcl-2 proteins are important regulatory molecules in the activation of apoptotic proteins[50], and Bcl-2 plays a crucial role in the inhibition of tumor cell apoptosis[51]. Bcl-2 is expressed during normal cell development, and is scarcely or poorly expressed in the maturation or apoptosis process[52]. Bcl-2, P53 and Bax expression levels may affect tumor cell apoptosis and are regarded as the key evidence for evaluating the severity and pathological grade of malignant tumors[53]. Lyustikman *et al* [54] have reported that Ras protein activity increased in the majority of glioma patients, triggering the activation of downstream Raf/Mek/Erk signaling. Sustained activation can upregulate Bcl-2 expression and ultimately immortalize tumor cells. Our findings indicate that Bcl-2 was poorly expressed in normal brain tissue, and greatly expressed in the model group. In addition, Bcl-2 protein expression in the tumor tissue of model rats was significantly higher than that in normal brain tissue. It is reasoned that abnormal activation of Ras/Raf/Mek/Erk signaling pathways in glioma rats may increase Bcl-2 expression. After transplantation of neural stem cells, Bcl-2 protein expression levels were significantly decreased compared with the model group. This indicates that transplanted neural stem cells may inhibit abnormal activation of Ras/Raf/Mek/Erk signaling and decrease Bcl-2 expression, thereby promoting tumor cell apoptosis.

The Caspases are a group of cysteine proteases that contribute to cytokine maturation, cell proliferation and differentiation, as well as cell apoptosis. Caspase activation is necessary in all apoptotic pathways[55]. Caspase-3 is a key protease in downstream pathways triggered by cell apoptosis[56] and is an effector molecule of apoptosis. It is also important in the Caspase cascade and at the final apoptotic execution stage[57-58]. When Caspase-3 activity is inhibited, apoptotic disorders occur and regulation of cell proliferation and apoptosis are disrupted, which possibly affects tumor occurrence and development as well as biological behavior and prognosis[59]. It is widely acknowledged that suppression of apoptosis is associated with P53 and Caspase-3 expression, and apoptosis mechanisms inevitably lead to tumor and immune system abnormalities[60]. Caspase-3 can inactivate apoptosis inhibitors, degrade intracellular protein substrates, and promote the formation of apoptotic bodies[61], and its expression is low or absent in tumor cells with low apoptotic rates[62]. Inhibition of Caspase-3 enzyme activity or antagonizing of Caspase-3 functions also suppresses cell apoptosis[63]. Dai and colleagues[64] activated hematoporphyrin monomethyl ether in C6 glioma cells using ultrasound and found Bcl-2 expression decreased, Caspase-3 expression increased, and the apoptotic rate significantly increased. Inhibition of Raf/Mek/Erk signaling can downregulate Bcl-2 expression and promote the apoptosis of human K562 leukemia cells[65]. After the Mek/Erk pathway was activated, Caspase-3 activity decreased, and the anti-apoptotic ability of chlamydia increased[66]. Immunohistochemical staining results in this study showed that Caspase-3 was highly expressed in normal brain tissue, but poorly expressed in the model group; western blot analysis showed that its protein expression was also significantly lower than that the normal group. This evidence indicates that activation of the Ras/Raf/Mek/Erk signaling pathway may reduce Caspase-3 activity and enhance the anti-apoptotic ability of glioma cells. Furthermore, high Bcl-2 expression and low Caspase-3 expression contributed to inhibition of glioma tumor cell apoptosis and promoted tumor growth. Caspase-3 protein expression levels in the stem cell transplantation group were higher than in the model group, indicating that neural stem cell transplantation enhanced Caspase-3 activity and promoted tumor cell apoptosis. Due to the physiological index vary between human and animals, further studies will focus on the potential effect of neural stem cell transplantation in human. The experimental objects of this study are the rats, which is a different species compared with the human. Further research of the neural stem cells and glioma is required to provide evidence for neural stem cells treatment of the glioma.

In summary, neural stem cell transplantation could inhibit abnormal activation of the Ras/Raf/Mek/Erk pathway, decrease Bcl-2 expression, and promote Caspase-3 activation and glioma cell apoptosis. Therefore, it may represent a novel method of treating glioma.

MATERIALS AND METHODS

Design
A randomized, controlled animal experiment.

Time and setting
Experiments were performed in the Experimental Center, Hubei University of Chinese Medicine, China from January to December 2012.

Materials
One hundred and twenty adult male Sprague-Dawley rats, aged 4–6 months and weighing 120 ± 30 g, were used to establish glioma models. One Wistar rat at 14 days of pregnancy was used to prepare fetal rat neural stem cells. All animals were provided by the Experimental Animal Center of Wuhan University, China (license No. SCXK (Hubei) 2008-0005). Experimental protocols were performed according to the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[67].

Methods
Isolation, culture and identification of neural stem cells
Embryonic hippocampal tissue isolated from Wistar rats at 14 days of pregnancy was cut into pieces, digested with Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F12 (Gibco, Carlsbad, CA, USA) and prepared into a suspension. After centrifugation and supernatant removal, cell suspension was dissolved in DMEM/ Nutrient Mixture F12 containing 20 µg/L epidermal growth factor (Sigma, St. Louis, MO, USA), 20 µg/L basic fibroblast growth factor (Sigma) and 2% B27 (Gibco) at 1×10^6 cells/mL. Then cells were cultured in medium supplemented with 10% fetal bovine serum (Gibco) in 5% CO_2 at 37°C for 7 days, and incubated with mouse anti-rat nestin monoclonal antibody (1:400; Santa Cruz Technology, Santa Cruz, CA, USA) and rabbit anti-rat glial fibrillary acidic protein polyclonal antibody (1:400; Chemicon) for identification.

Establishment of glioma models
C6 glioma cells were cultured in DMEM containing 10% fetal bovine serum (Shanghai Ai Biological Research Co., Ltd., Shanghai, China), and a cell suspension was prepared. Rats in the model group and cell transplantation group were anesthetized intraperitoneally, and the scalp was excised to expose the skull. A hole of 1.2 mm diameter was drilled at 3 mm to the right of sagittal suture and 1 mm advanced to the midpoint of anterior and posterior fontanelle[68], and injected with 50 µL cell suspension containing 3×10^6 C6 glioma cells, then the scalp was sutured, and 1×10^4 units penicillin was given intramuscularly. One week after modeling, rats in the cell transplantation group and model group were detected with MRI (Philips Gyroscan T5-NT 0.5 T MRI machine; Amsterdam, the Netherlands), to observe glioma growth.

Transplantation of neural stem cells
One week after modeling, rats were anesthetized and fixed in a prone position on the stereotaxic instrument. The transplantation site was located at 1 mm to the bregma and 2 mm lateral[68], and the penetration depth was 5 mm away from the skull surface. A 5 µL suspension containing 1×10^5/µL fetal rat neural stem cells was injected into the brain tissue of tumor-bearing rats, then bone hole was closed with bone wax and the wounds were sutured. Three days before stem cell transplantation, rats in the cell transplantation group (*n* = 39) were intraperitoneally injected with immunosuppressant cyclosporine (10 mg/kg; North China Pharmaceutical Group Corporation, Shijiazhuang, Hebei Province, China), once per day, until 1 week after cell transplantation. Rats in the model group (*n* = 39) were injected with cyclosporine and saline. The normal group (*n* = 40) received no treatment.

Western blot analysis of Raf-1, Erk, Caspase-3 and Bcl-2 protein expression in tumor tissue
One week after stem cell transplantation, rats were killed, and brain tissue was harvested, homogenized and triturated into a cell suspension, followed by cell lysis, denaturation and centrifugation at 4°C. After the supernatant was removed, the total protein quantity was determined using the Bradford assay[69]. Then 10 µg protein was electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membrane, and then blocked in T-TBS containing 50 g/L skimmed milk at room temperature for 2 hours, rinsed with PBS, and incubated with rabbit anti-rat Raf-1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-rat Erk monoclonal antibody (Life Technologies Inc, Carlsbad, CA, USA), rabbit anti-Caspase-3 polyclonal antibody (Sinovac Biotech Co., Ltd., Shanghai, China), and mouse anti-rat Bcl-2 monoclonal antibody (Seitz Biotechnology

Co., Ltd., Beijing, China) at 4°C overnight. All antibodies were diluted to 1:1 000. After PBS washing, specimens were incubated with horseradish peroxidase-conjugated goat anti-rabbit/ mouse IgG (1:2 000; Santa Cruz Biotechnology) and reacted with chemiluminescence reagent for 5 minutes, then scanned with an OmniMet 9.5 image analysis system (Buehler Biotechnology, Chicago, IL, USA). The absorbance values were analyzed using Bandscan software (Bio-Rad, Hercules, CA, USA) with β-actin as a reference.

Immunohistochemical staining of Raf-1, Erk, Caspase-3 and Bcl-2 expression in tumor tissue

Rats were killed at 1 week after cell transplantation, and brain tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and serially sectioned into slices of 5 μm thickness. After endogenous peroxidase was blocked with 3% hydrogen peroxide, brain tissue was retrieved by microwave heating, blocked with 1% bovine serum for 30 minutes, and incubated at 4°C overnight with the following antibodies diluted at 1:100: rabbit anti-rat Raf-1 monoclonal antibody, rabbit anti-rat Erk monoclonal antibody, rabbit anti-Caspase-3 polyclonal antibody, and mouse anti-rat Bcl-2 monoclonal antibody. Then specimens were incubated with horseradish peroxidase donkey anti-rabbit/ mouse IgG (1:100; Amyjet Scientific Inc, Wuhan, Hubei Province, China) at 37°C for 30 minutes and at room temperature for 1 hour. Subsequently, specimens were rinsed with PBS three times, stained with 3,3'-diaminobenzidine (Boster, Wuhan, Hubei Province, China), counterstained with hematoxylin, dehydrated, developed, and mounted using gum. Finally brain tissue was observed by optical microscopy (Olympus, Shibuya, Japan).

Result determination

Positive cells presented brown-yellow particles in the nucleus or cytoplasm, and the number of positive cells was measured using Image-Pro Plus analysis software (Media Cybernetics Inc., Bethesda, MD, USA) on five high-power fields (400 × magnification) of six pathological slices randomly selected from each rat. The number of blue-stained nuclei cells were counted, and the percentage of positive cells was calculated according to the following formula: number of positive cells/(number of positive cells + number of blue-stained cells) × 100%. ≤ 5%: 0 point, 6–25%: 1 point, 26–50%: 2 points, ≥ 51%: 3 points[70]. Cells were also evaluated according to the degree of staining (no staining: 1 point, weak staining: 2 points, moderate staining: 3 points, strong staining: 4 points). The final scores = degree of staining × percentage of stained cells. 0 is negative (–), 1 is weak positive (+),

2–3 is positive (++), 4–6 is strongly positive (+++)[71].

Statistical analysis

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and measurement data were expressed as mean ± SD. Differences between groups were compared using analysis of variance, and paired comparisons were performed using least significant difference test; count data were expressed as a percentage, and differences between two groups were compared using chi-square test, with P values of < 0.05 considered significant.

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