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Original Research

Conditioned medium from neural stem cells inhibits glioma cell growth

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Abstract: Malignant glioma is one of the most common brain tumors in the central nervous system. Although the significant progress has been made in recent years, the mortality is still high and 5-year survival rate is still very low. One of the leading causes to the high mortality for glioma patients is metastasis and invasion. An efficient method to control the tumor metastasis is a promising way to treat the glioma. Previous reports indicated that neural stem cells (NSCs) were served as a delivery vector to the anti-glioma therapy. Here, we used the conditioned medium from rat NSCs (NSC-CM) to culture the human glioblastoma cell lines. We found that NSC-CM could inhibit the glioma cell growth, invasion and migration in vitro and attenuate the tumor growth in vivo. Furthermore, this anti-glioma effect was mediated by the inactivation of mitogen activated protein kinase (MAPK) pathway. Above all, this study provided the direct evidence to put forward a simple and efficient method in the inhibition of glioma cells/tumor growth, potentially advancing the anti-glioma therapy.

Key words: Glioma, conditioned medium, neural stem cell, MAPK, metastasis.

Introduction

Malignant gliomas including grade III/IV and grade IV/IV tumors, are the most common subtype of primary brain tumors in the central nervous system (1). Glioblastoma belongs to the grade IV/IV tumor and is the most common malignant primary brain tumor, with an incidence of 3.19 cases per 100,000 persons and a 5-year survival rate of less than 5% postdiagnosis (2). The main treatments for high grade gliomas have remained consistent for the past 3 decades, including maximal surgical resection, external beam radiation therapy, and chemotherapy (3). However, the efficiency of treatment for glioblastoma is still not satisfactory yet. This is probably due to fact that the precise histogenesis of glioblastoma remains elusive (4). Therefore, despite of considerable advancements in the development of glioma treatments, long-term remission remains elusive (5).

Recently, significant progress has been reported in the application of neural stem cells (NSCs) as a novel gene delivery vector for the glioma treatment (6). Luo and colleagues found some miRNAs highly expressed in NSCs but lowly expressed in glioma cells and established a cell fusion expression system for NSCs-based antiglioma gene therapy (7). Chen and his colleagues found that glioma cells and NSCs can interact at the niche or micro-environment level (8). Internalized NSCs with magnetic discs could trigger mechanically induced apoptotic cell death of the glioma cells (9). Besides, NSCs mediated delivery of irinotecan-activating carboxylesterases to treat the malignant glioma (10). All these studies provided evidences to support the potential clinical application of NSCs in the treatment of malignant brain tumors. However, how these two types of cells interact with each other and the molecular mechanisms associated with glioma pathogenesis in the presence of NSCs were still poorly understood.

Liu and colleagues elucidated the transformation of NSCs to glioma stem cells by the differentially expressed miRNAs, providing novel therapeutic strategies to glioblastoma (11). This work put forward a potential interaction between the origin of glioma and NSCs. In the current study, we made use of a relatively simple method to explain the potential clinical role of NSCs in the treatment of glioma. Both in vitro and in vivo studies indicated that NSC-CM could inhibit the growth, migration and invasion of glioma cells. Besides, this inhibitory effect was mediated by the MAPK signaling. Taken together, these data suggested that the factors in the conditioned medium may mediate interactions between glioma cells and NSCs.

Materials and Methods

Cell culture

Human malignant glioma cell lines U87 and U251 derived were purchased from the Institutes for Biological Science, Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Rat neural stem cell culture was described previously (12). Briefly, NSCs were obtained from hippocampus of neonatal rats. The cells were cultured in DMEM/F12 medium, supplemented by 1% penicillin/streptomycin, 1% N2, 20 ng/ml EGF, 10 ng/ml bFGF, and 1% B27 (All were purchased from Life Technologies). A half-volume of medium was replaced with fresh medium every 3 days.

Collection of conditioned medium

Seven days after culture, neurospheres were separated by centrifugation and grown in the medium for ano-

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ther 2–3 days. The supernatant serum-free medium collected from NSC culture was filtered by 0.22μm syringe filter and store at 4°C. This medium was described as 'NSC-conditioned medium' (NSC-CM). We also used the fresh NSC culture medium as control medium.

BrdU analysis

BrdU reagent was added to the glioma cells and incubated for 2 or 24 hours. Then, cells were fixed in 2% paraformaldehyde for 20 minutes. After washing three times with phosphate buffered saline (PBS), cells were incubated with primary anti-BrdU antibody (1:1000, purchased from Sigma) overnight at 4°C, and goat anti-mouse immunoglobulin G peroxidase conjugate (1:2000) for 30 minutes at room temperature, and then 3,3',5,5'-tetramethyl benzidine peroxidase substrate in the dark for another 30 minutes. BrdU-positive cells could be visualized by a blue to purple color.

MTT assay

MTT was used to detect effect of NSC-CM on glioma cell viability at different time points (1, 2, 4, 8, 12, 24, 48, and 96 hours after NSC-CM treatment). Briefly, MTT was incubated with cells for at least 4 hours to produce formazan. When formazan was completely dissolved by SDS-HCl, the absorbance at 570 nm was measured with a Universal Microplate Reader (Bio-Tek instruments), and OD (NSC-CM-treated group)/OD (blank control group) was calculated.

Real time PCR

Total RNA was isolated using TRIZOL reagent (Life Technologies). Synthesis of cDNA was performed by using one-step RT-PCR kit from Takara. SYBR Green (Toyobo) RT-PCR amplification and real time fluorescence detection were performed using the PRISM 7300 sequence detection system (Applied Biosystems). Relative gene expression was calculated by the ΔΔCt method. The primer sequences were as follows: GFAP, sense 5'-CCGACAGCAGGTCCATGTG-3' and antisense, 5'-GTTGCTGGACGCCATTGC-3'; S100B, sense, 5'-CTGGAGAAGGCCATGGTTGC-3'; antisense, 5'-CTCCAGGAAGTGAGAGAGCT-3'. MMP2, sense, 5'-CAGGGAATGAGTACTGGGTCTATT-3'; antisense, 5'-ACTCCAGTTAAAGGCAGCATCTAC-3', Ki67, sense, 5'-GAGGTGTGCAGAAAATCCAAA-3'; antisense, 5'-CTGTCCCTATGACTTCTGGTTGT-3'; VEGF sense 5'-ATCTGCATGGTGATGTTGGA-3'; antisense 5'-GGGCAGAATCATCACGAAGT-3'. CDKN2A, sense, GTGGACCTGGCTGAGGAG; antisense, CTTTCAATCGGGGATGTCTG. p53, sense 5'-ATG GAG GAG CCG CAG TCA GAT-3'; antisense, 5'-GCA GCG CCT CAC AAC CTC CGT C-3'. GAPDH, sense, 5'-GGTATCGTGGAAGGACTCATGAC-3' and antisense, 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3'.

Western blotting

2 μg cell lysates were loaded on each lane of 10% polyacrylamidegel, and then blotted onto a polyvinylidene difluoride (PVDF) membrane. After blocking with a PBST containing 5% nonfat dry milk, the blots were incubated with antibodies against phospho-p44/42 MAPK, total MAPK, and GAPDH (Cell Signaling Technologies). Peroxidase-linked anti rabbit IgG (Life Technologies) were used as secondary antibodies. These proteins were visualized by using an ECL western blotting detection kit (Amersham Bio-sciences).

Analysis of cell cycle phase by flow cytometry

Four or twenty-four hours following the treatment of conditioned medium, the glioma cells were resuspended in PBS twice before fixation by adding dropwise into to 95% precooled ethanol. Prior to analysis, the cells were warmed, centrifuged at 450 g for 5 min and resuspended twice in PBS, then stained with PI (containing RNase A at 50 μg/ml) at room temperature in the dark for 30 min. The DNA content was analyzed by flow cytometry using the CellQuest program (Becton-Dickinson and Co., USA).

Scratch wound assay

Glioma cells were seeded on 60 cm^2 tissue-culture plastic dishes at 80% cell confluence. A scratch wound was performed using a sterile 200 μl pipette tip. The number of cells crossed the scratch line was calculated at 48 hours after scratching in NSC-CM-treated group and untreated group.

Cell invasion assay

Cell invasion assay was performed using a 24-well transwell chamber (Corning Costar). The inserts were coated with Matrigel (BD Bioscience). Cells were treated in NSC-CM for 24 hours before transferred to the upper Matrigel chamber. The lower chamber was filled with culture medium containing 10% FBS. After 24 h incubation, the cells that passed through the filter were stained by crystal violet, and the number of invading cells was counted in six randomly selected fields.

Intracranial implanted models

To explore the effects of NSC-CM on tumor growth and invasion in vivo, an intracranial nude mouse model was established using 1×10^6 treated and untreated glioma cells (three mice in each group and biological triplicate was conducted). On day 10, 20, 30, 40 and 50 after implantation, animals were sacrificed and tumor size was measured. Tumor size (V) was calculated using the following equation: $V=(a^2 \times b)/2$, where a is the width of the tumor (small diameter) and b is the length (large diameter) (mm).

Statistical analysis

All the experiments were in biological triplicate. Data were presented as mean ± SEM. Paired Student's t-test and one-way ANOVA were used to determine significant differences by Graphpad Prism 6.0. The intensity for blots was determined by Image J software. A p value less than 0.05 is considered as significantly different, and less than 0.01 is considered as very significantly different.

Results

Conditioned medium from NSCs inhibited the proliferation of glioma cells

Firstly, we used BrdU staining to analyze the glioma cell proliferation after the conditioned medium treat-

ment. Two hours after BrdU incorporation, we found the percentage of BrdU positive cells was significantly decreased in glioma cells U251 and U87 pretreated with NSC-CM for 12 hours (Fig.1A, $p<0.05$ in U87 cells; Fig.1B, $p<0.01$ in U251 cells). Similar results were obtained when the BrdU incorporation was extended to 24 hours (Fig.1, $p<0.01$ in both U87 and U251 cells). Next, MTT assay was used to further confirm the specific effect of NSC-CM on the glioma cell growth. Glioma cells were incubated in NSC-CM and the cell growth was detected at different time points. The results indicated that inhibition of glioma cell growth was initiated at 4 hours after treatment of NSC-CM (Fig. 2, $p<0.05$ in both U87 and U251 cells) and reach the maximum when incubated in NSC-CM for (more than) 24 hours (Fig.2, $p<0.001$ in both U87 and U251 cells). Above all, these data demonstrated that proliferation of glioma cells was inhibited by culturing in the conditioned medium from NSCs.

NSC-CM altered the cell cycle in glioma cells

Then, we determined whether NSC-CM treatment could alter the cell cycle, tested by flow cytometry. In the presence of NSC-CM for 4 hours, ratio of cells at the G_0/G1 and S phases was increased, while the ratio of cells at G2/M phase was decreased compared to the untreated cells (Fig.3A). With the extension of treatment, we observed more significantly increasing ratio of cells at G_0/G1 and S phases, and further decreasing ratio of cells at G2/M phase (Fig.3B). This data showed NSC-CM led to the more cells arrested in the cell cycle staying at G_0/G1 and S phases, and decreased the cell ratio at pre-dividing and dividing (G2/M) phases.

NSC-CM weakened the migration and invasion ability of glioma cells

Migration is the typical feature of the glioma at the advanced stage, which leads to the high mortality. Therefore, we determined whether incubation with NSC-CM could affect migration of glioma cells. Firstly, the scratch wound assay indicated glioma cell migration

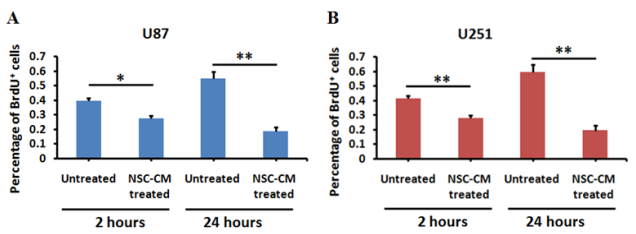

Figure 1. NSC-CM treatment reduced the number of BrdU$^+$ cells in both U87 (A) and U251 cells. The significance was determined by paired student t test. *p<0.05, **p<0.01.

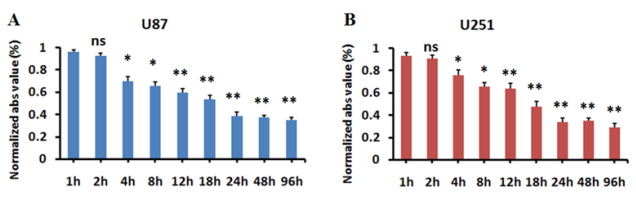

Figure 2. MTT assay showed NSC-CM negatively regulated U87 (A) and U251 (B) cell viability. The significance was determined by one-way ANOVA compared to the group with NSC-CM treatment for 1h. 'ns' represented not significant, *p<0.05, **p<0.01.

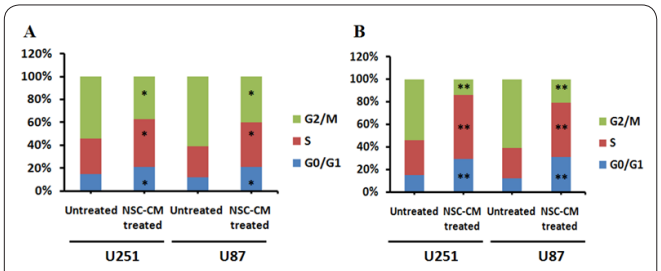

Figure 3. The cell cycle of glioma cells was altered with NSC-CM treatment for 4 hours (A) and 24 hours (B). The significance was determined by paired student t test. *p<0.05, **p<0.01.

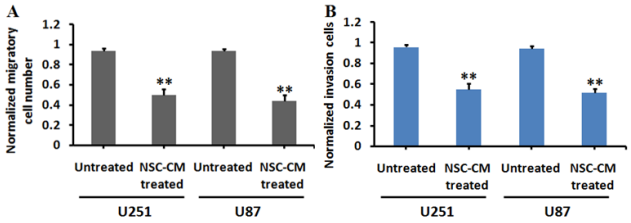

Figure 4. The number of migratory cells (A) and invaded cells (B) was decreased when glioma cells were cultured in NSC-CM. The significance was determined by paired student t test. **p<0.01.

was negatively affected in the presence of NSC-CM. 48 h after scratch, the number of cells crossed the scratch line deceased significantly by 47% in U251 and by 53% in U87 cells (Fig.4A, $p<0.01$). Furthermore, transwell invasion assay showed that NSC-CM drastically suppressed the invasion of glioma cells. Invasiveness of glioma cells with the treatment of NSC-CM for 12 hours was decreased by 43% in U251 cells and by 46% in U87 cells (Fig.4B, $p<0.01$). This indicated that the migration and invasion of glioma cells became weaker when cultured in NSC-CM.

NSC-CM decreased the expression level of glioma tumor biomarkers

Next, we quantitatively determined how the NSC-CM treatment altered the expression level of glioma cell biomarkers, including GFAP, S100, VEGF, Ki67, MMP2, p53, and CDKN2A. The quantitative RT-PCR results showed that the mRNA expression level of GFAP, S100, VEGF, Ki67 and MMP2 was decreased in the NSC-CM-treated U87 and U251 glioma cells (Fig.5A, 5B). By contrast, those tumor suppressor gene including p53 and DKN2A was more strongly expressed in glioma cells when cultured in NSC-CM (Fig.5A, 5B). This indicated that NSC-CM treatment decreased the expression level of glioma biomarkers as well as increased the expression of anti-tumor genes.

Inhibition of glioma cell growth and migration was mediated by MAPK signaling pathway

In many cancers, a defect in the MAPK pathway leads to that uncontrolled growth (13). Thus, we elucidated if the inhibition of glioma cell growth and migration induced by NSC-CM treatment was associated with MAPK signaling pathway. The expression level of phosphorylated p44/42 MAPK (ERK1/2) was decreased in both U251 and U87 cells with the treatment of NSC-CM (Fig.6 and Supplementary Fig. 1A). By contrast, we did not observe the similar expression change in the presence of fresh NSC culture medium (Supplementary

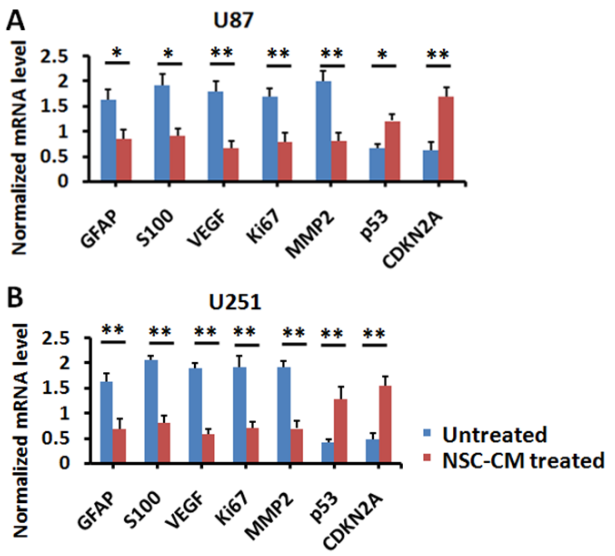

Figure 5. The real time PCR result indicated the expression level of GFAP, S100, VEGF, Ki67 and MMP-2 was decreased in U87 (A) and U251 cells treated with NSC-CM. By contrast, the expression of p53 and CDKN2A was increased after NSC-CM treatment. The significance was determined by paired student t test. *p<0.05, **p<0.01.

Fig.2A). Furthermore, in the presence of Anisomycin (a MAPK activator), the expression level of ERK1/2 was not significantly decreased when the glioma cells were incubated with NSC-CM (Fig.6). By contrast, the expression of ERK1/2 was significantly decreased in the presence of both U0126 (a MAPK inhibitor) and NSC-CM compared to that in cells treated with NSC-CM only (Fig.6B). However, no significant difference was found in the DMSO control compared to the NSC-CM treated cells (Supplementary Figure 2B). Collectively, these data showed that inhibition of glioma cell growth and migration by NSC-CM was correlated with dephosphorylation of p44/42 MAPK.

NSC-CM suppressed glioma cell proliferation in vivo

To determine the effect of NSC-CM on glioma growth in vivo, NSC-CM-treated and untreated glioma cells were transplanted into nude mice. As shown in the Fig.7A, the growth of tumor from NSC-CM-treated U87 cells was much slower than the tumors from untreated cells. The size of tumor pretreated with NSC-CM was only 49% and 53% of the untreated one at 40 and 50 days after cell graft ($p<0.01$). Similarly, The size of tumor formed by NSC-CM-treated U251 was only 52% and 47% of the untreated cells-formed tumors at 40 and 50 days after cell graft (Fig.7B, $p<0.05$). The image of tumors at 50 days after xenograft was shown in Supplementary Figure 3. Furthermore, the mRNA expression level of Ki67 and MMP2 was significant decreased in NSC-CM-treated tumor compared to the untreated cells-formed tumor (Fig.7C, 7D, $p<0.01$). By contrast, the expression level of p53 and BAD was significant increased (Fig.7C, 7D, $p<0.01$). Besides, the expression level of ERK1/2 was significantly decreased in tumor formed by NSC-CM-treated U87 or U251 cells compared to the tumor formed by untreated cells (Fig.7E, $p<0.01$, Supplementary Fig.1B). These in vivo studies further supported the conclusion that NSC-CM

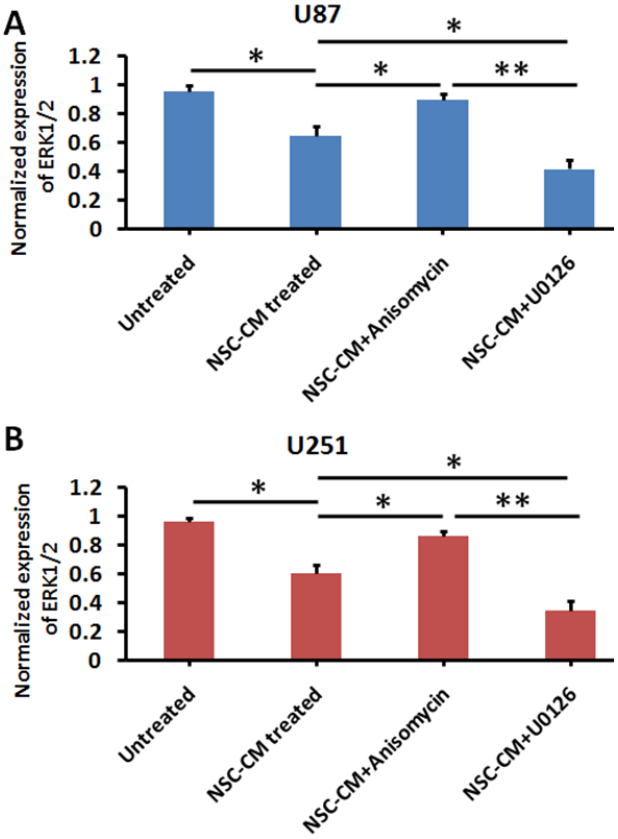

Figure 6. The protein expression level of p44/42 MAPK (ERK1/2) was decreased when the U87 (A) and U251 (B) cells were treated with NSC-CM. Anisomycin, a MAPK activator alleviated NSC-CM-induced p44/42 MAPK expression increase in the U87 (A) and U251 (B) cells. By contrast, application of MAPK inhibitor U0126 could further decreased the p44/42 MAPK expression when treated with NSC-CM. The significance was determined by paired student t test. *p<0.05, **p<0.01.

treatment suppressed the glioma tumor growth through inactivation of MAPK pathway.

Discussion

In this study, we found that conditioned medium from NSCs could inhibit the glioma cell proliferation, invasion and migration. Besides, the conditioned medium treated cells showed attenuating tumor formation in vivo. This study provided a new potential candidate for delivering the medicine to treat glioma as well as showed a previously unidentified signaling mechanism involved in the anti-glioma therapy.

The mechanism of the NSC-CM's inhibitory effect on glioma is still unclear. Recently, it has been reported that extracellular vesicles (EVs) are important factors in regulating brain tumor progression (14, 15). EVs are membrane surrounded structures released by cells, serving as unique carriers of biological molecules. EVs secreted by neural stem/progenitor cells include a complex array of homeostatic molecules called stem cell secretome with immune regulatory and tissue trophic functions (16). Until now, there is no direct study showing the role of stem cell secretome in glioma progression. Conversely, when NSCs were cultured in conditioned medium from glioma cells, they were more proliferative and the capacity of self-renewal was promoted (17). This work put forward a potential correla-

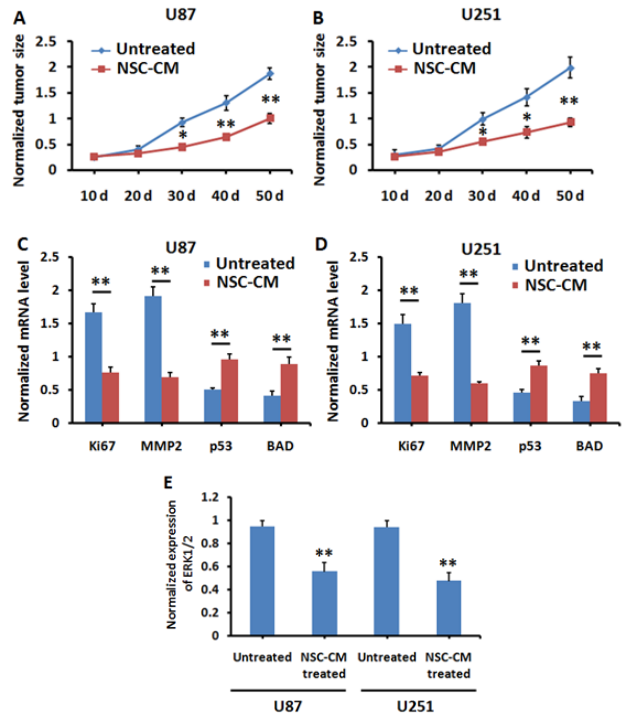

Figure 7. NSC-CM treatment attenuated tumor growth in vivo formed by U87 (A) and U251 (B) cells. (C, D) The mRNA expression level of Ki67 and MMP-2 was decreased in NSC-CM-treated glioma cells formed tumor compared to the tumors formed by untreated cells. By contrast, the expression level of p53 and BAD was increased in NSC-CM-treated glioma cells formed tumor compared to the tumors formed by untreated cells. (E) The protein expression of ERK1/2 was decreased in NSC-CM-treated glioma cells formed tumor compared to the tumors formed by untreated cells. The significance was determined by paired student t test. **p<0.01.

tion between NSCs and glioma cells through EVs. In U87 glioma cells, the secretome signature of high invasiveness was identified, such as the high expression of ADAM9, ADAM10, cathepsin B, cathepsin L1, osteopontin, neuropilin-1, semaphorin-7A, suprabasin and chitinase-3-like protein 1 as well as low levels of cell adhesion proteins such as periostin and EMILIN-1 (18). Based on these work, the future work may focus on the effect of NSC-CM on the expression level alteration of above proteins, providing a comprehensive view of the mechanism underlying the glioma growth inhibition by NSC-CM.

The mitogen activated protein kinase (MAPK) signaling pathway has been widely studied and is reported to be significantly altered or activated in glioma (19). Mutation in the MAPK in the leading mediators caused the genesis and progression of glioma. In this process, several key factors were involved in it, including RTKs (20), RAS, RAF, ERK, JNK, and p38 (21). Our study showed that the NSC-CM-induced glioma growth inhibition was accompanied with the inactivation of p44/p42 MAPK. This is compatible with the reported role of MAPK in the glioma cell growth (22). Future work will be focused on the expression level alteration of those key downstream factors in the MAPK signaling pathway in the conditioned medium treated glioma cells. This will provide a more comprehensive understanding of the molecular mechanism of the NSC-CM-induced inhibition of glioma tumor growth.

Collectively, we established a simple and efficient method to inhibit the glioma growth by using the conditioned medium from neural stem cells. However, there are still some limitations regarding this study. For example, we only elucidated possible involvement of MAPK signaling in this NSC-CM-induced inhibitory effect. Apparently, other signals must be critical in this process. Secondly, we collected the conditioned medium from rat NSCs and used it in the human glioma cells. From the point of view for future clinical application, it will be preferential if the conditioned medium is collected from human neural stem cell culture. Finally, the conditioned medium contains complex components, some of which may have inhibitory effect on glioma cells. The complexity of NSC-CM made it different to apply this as a specific medicine in the treatment of glioma. Therefore, it is more applicable if the effective components could be identified through HPLC, for example. All the above points may serve as the future direction of the ongoing research.

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