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Effects of INF- γ -Modified Bone Marrow Mesenchymal Stem Cells on the Distribution and Inhibition of Tumours *In-vivo* in a Glioma Nude Mouse Model

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Abstract

Background: We explored the effects of INF-γ-modified Bone Marrow Mesenchymal Stem Cells (BMSCs) on the distribution and inhibition of tumour tissues.

Methodology: BALB/c mouse BMSCs were cultured, isolated and identified by flow cytometry. Constructed a lent viral expression vector for the INF-γ gene; BMSCs transfected *in vitro* were labelled with SYBR Green I fluorescent dye and detected by fluorescent quantitative Polymerase Chain Reaction (qPCR). A blank BALB/c nude mouse control group was randomly selected and the remaining mice were inoculated with glioma cells in the armpit for modelling and the nude mice were randomly divided into model, BMSC and INF-γ+BMSC groups. After 14 days of treatment, during which the long and short diameters of tumours were measured every other day, calculated the tumour volume in tumour-bearing nude mice for each group. Then, observed the distribution of fluorescent BMSCs in nude mice transfected with INF-γ through laser confocal microscopy. Tumour tissues of nude mice were subjected to Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labelling (TUNEL) staining and the number of positive cells was determined.

Results: BMSCs were cultured *in-vivo* and exhibited adherent growth. Flow cytometry indicated that CD44 (98.01%) and CD105 (96.17%) were overexpressed, whereas the CD34 (1.46%), CD45 (1.32%) and CD11b (1.48%) expression levels were low, indicating that the latter were BMSCs. Fluorescence analysis and PCR were applied to confirm INF-γ transfection into the BMSCs. Immunofluorescence staining showed clear accumulation of BMSCs in nude mouse tissues, with no fluorescence observed in the model group. TUNEL staining showed a higher apoptosis rate in the INF-γ+BMSC group than in the model group ($P<0.05$). On day 9, the tumour volume differed significantly between the INF-γ+BMSC group and the other groups ($P<0.05$).

Conclusion: A lentiviral vector effectively transfected the INF-γ gene into BMSCs, where it was homed and distributed to tumour tissues, significantly inhibiting tumour growth.

Keywords: Bone marrow mesenchymal stem cells; Gene modification; Glioma; Interferon gamma (INF-γ); Tumour-bearing nude mice

Introduction

Glioma is the most common and fatal type of intracranial tumour; it has an aggressive malignant progression characterised by rapid growth, invasiveness, and a poor prognosis throughout the brain due to many factors [1,2]. Among the many new medicines currently under investigation to target the cell function and signalling pathways of various cancers [3] are Bone Marrow Mesenchymal Stem Cells (BMSCs), which exhibit multidirectional differentiation potential, easy replication and homing, low immunogenicity, easy gene modification, and good immune regulation [4-10]. Studies of BMSCs have attracted great attention [11,12], especially in the development of tumour therapy [13].

BMSCs exhibit inhibitory effects on tumours [14,15], and those carrying exogenous genes strongly inhibit solid tumours [9,16]. Researchers have speculated that BMSCs promote tumour growth and invasion through the multidirectional differentiation of mesenchymal cells and the secretion of cytokines into the tumour microenvironment [10,17]. The self-renewal and *in vitro* transformation properties of BMSCs are similar to those of tumour cells, and it has been proposed that tumours can be transformed from mesenchymal stem cells [18]. However, BMSCs and cancer cells show significantly different expression of proliferation-associated genes [19], and the bidirectional effects and mechanisms of BMSCs remain controversial [20]. Therefore, it is important to address these issues before widely promoting the clinical use of BMSCs. In this study, we explored whether BMSCs modified with the INF-γ gene could stably express INF-γ in animals with solid tumours and inhibit tumour growth through targeted distribution.

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Materials and Methods

Human glioma U251 cell culture

U251 cells were cultured as described previously [21] at saturated humidity in Roswell Park Memorial Institute (RPMI) 1640 medium, which was changed every 2–3 days.

Primary culture, isolation and identification of BMSCs

Five 8-week-old specific pathogen-free BALB/c mice were purchased from the Experimental Animal Center of the Third Military Medical University, Chongqing, China. The mouse feed was not supplemented with foetal bovine serum in this study. Mice were handled post mortem as described previously [21]. The cell surface molecular markers CD44, CD105, CD34, CD45 and CD11b were detected by flow cytometry [5,22,23].

Development of BALB/c nude mouse models

We obtained and fed 36 BALB/c nude mice (weight, 15 g-20 g; aged 6-7 weeks) as described previously [21]. The experiment was conducted after 1 week of adaptive feeding. All animals were fed according to the feed management specifications for experimental animals using standard pellets provided by the Experimental Animal Center of the Third Military Medical University.

Three mice were randomly selected as a blank group; the remaining mice were used to establish the glioma model as described previously [21]. The weight and growth of the nude mice were observed on the day after tumour inoculation; the tumour dimensions were measured and the volume calculated. Once the subcutaneous tumour had grown to approximately 1 cm in diameter, 30 mice were selected and randomly divided into three groups.

Experimental groups and treatment

The mice were randomly divided into four groups: Negatively control, nude mouse tumour model (model group), lentiviral BMSC transfection with empty vector (BMSC group), and lentiviral BMSC transfection with INF-γ (INF-γ+BMSC group). Prior treatment and screening procedures were performed as described previously [21]. After 1 day, BMSCs transfected with and without INF-γ were injected into mice in the INF-γ+BMSC and BMSC groups, respectively *via* the tail vein.

INF-γ expression lentiviral vector construction

Target gene fishing, synthesis and purification: The primers used in this study were described previously [21]. The INF-γ overexpression sequence was amplified by Polymerase Chain Reaction (PCR). The target vector was subjected to enzyme digestion and purification.

Target gene and vector-directed connection: The purified PCR product was connected with the linearised vector, and the linked product was transformed into competent bacterial cells. The receptive cells were removed from -80°C, the centrifuge tube containing the receptive cells was thawed on ice, 10 μL of the connection product was added, and the contents were gently mixed and placed in ice for 30 min. Place centrifuge tube on test tube stand in water bath pre-heated to 42°C for 60 sec, do not shake centrifuge tube. The centrifuge tube was quickly transferred to the ice bath and the cells were cooled for 5 min. 300 μL LB medium (without antibiotics) was added to each tube, and the tubes were transferred to a 37°C shaker at 220 rpm for 60 sec to resuscitate bacteria. 100 μL cultured cells were uniformly coated on LB plate containing 50 μg/mL Ampicillin. After the liquid on the plate was absorbed, the plate was inverted into an incubator at 37°C and cultured for 16 hours. The clonal colonies were selected from the plate, and the positive clones were identified by small plasmid extraction. Four individual, plump colonies were selected from a cultured plate and placed in a test tube containing 5 mL LB medium containing 50 μg/mL Ampicillin. The test tubes were cultured in a bacterial shaker at 37°C at 250 rpm for 16 h. The cultured bacterial solution was extracted with plasmid small extraction kit. The clones were first identified by enzyme digestion to confirm that the target gene had been directionally linked into the target vector.

Transformation of linker products, positive clone identification, and sequencing: The corresponding bacterial solution of the positive clone was sequenced, and the sequencing result was compared with the target gene sequence. The result indicated successful construction of the target gene expression plasmid vector.

Gene lentivirus packaging and purification: The lentivirus shuttle plasmid and its auxiliary plasmids were prepared and co-transfected into 293T cells, which were cultured in complete culture medium. The medium was replaced at 8 h after transfection, and incubation continued for another 48 h. Cells with a supernatant rich in lentiviral particles were collected. According to the requirements for lentivirus titre in *in-vivo* and *in-vitro* experiments, the cells were concentrated by density gradient centrifugation to obtain high-titre lentivirus particles, sub packaged for standby, and stored at −80°C. Fluorescence microscopy or fluorescence-activated cell sorting was used to count the fluorescent cells and calculate the virus titre and dilution ratio.

BMSC gene modification through INF-γ-loaded lentivirus: Once the BMSCs showed adequate growth, we inoculated 104 BMSCs into 24-well plates for transfection. The virus particles were successively warmed to room temperature on a gradient from −80°C to −20°C and 4°C on ice. A pipette was used to transfer exact volumes of the virus solution to the 24-well plate containing prepared culture medium. The virus solution was thus transferred to the target cells and control cells, which were incubated overnight under 5% CO_2 at 37°C after mixing. After 10 h, we replaced the fresh culture medium and continued to culture the transfected BMSCs.

Verification of INF-γ overexpression in BMSCs by quantitative PCR (qPCR): The BMSC and INF-γ+BMSC groups were used to verify the INF-γ transfection effect. After transfection, the media from both groups were discarded and the cells were dissolved with TRIzol reagent. RNA was separated by shaking, followed by standing and centrifugation at 10,000 rpm (all steps at 4°C). INF-γ was detected by fluorescence qPCR after the separated RNA was dried at room temperature and dissolved in RNase-free water.

All primers were designed using Primer v5.0 software and the gene sequences of IFN-γ and β-actin were obtained from GenBank. No amplification of non-target genes was observed with any of the primers after BLAST verification and none formed a dimer. PCR was performed, the products were electrophoretically separated in 2% agarose gels, and the amplified fragments were observed by gel imaging analysis. The IFN-γ and β-actin genes were amplified by qPCR using a Western Biotechnology fluorescent dye kit under optimised reaction conditions. Following amplification, the fusion and amplification curves were analysed and IFN-γ expression was quantified using the $2^{-\Delta Ct}$ method.

Tumour tissue apoptosis assay

A tumour tissue apoptosis assay was performed as described previously [21]. Cells were stained using a terminal Deoxynucleotidyl

Transferase dUTP Nick-End Labelling (TUNEL) apoptosis detection kit, according to the manufacturer's instructions.

Tumour volume measurement

Tumour volume was measured as described previously [21] using the following formula: Tumour volume = Long diameter × (Short diameter^2)/2.

Experimental reagents

RPMI 1640 medium, Foetal Bovine Serum, and Dulbecco's Modified Eagle Medium were purchased from Gibco (Billings, MT, USA). Dimethyl sulfoxide was purchased from Amresco (Dallas, TX, USA). Reverse transcription reagent, fluorescent qPCR reagent, and SYBR Green I were purchased from Beyotime Biotechnology (Shanghai, China). Other reagents of analytical purity were purchased from domestic reagent suppliers.

Analytical methods

Differences were evaluated using a one-way Analysis of Variance (ANOVA). Data are expressed as means ± Standard Deviation (SD). Student's t-test was used to evaluate differences between two groups. A repeated measures ANOVA was performed to evaluate differences in multiple factors at different time points between different groups, with time as the dominant effect. Differences were considered significant at $P<0.05$ and highly significant at $P<0.01$.

Results

Primary culture, isolation and identification of BMSCs

BMSCs were cultured *in vitro* and exhibited adherent growth as described previously [21]. After fluid change, the cells showed clear proliferation and several types of fusiform morphology were observed (Figure 1). Flow cytometry indicated that CD44 (98.01%) and CD105 (96.17%) were overexpressed, whereas CD34 (1.46%), CD45 (1.32%) and CD11b (1.48%) were weakly expressed. Cell morphology and the expression of immune markers on the cell surface were consistent with the literature [5,6,22,23], indicating BMSCs.

Detection of INF-γ overexpression in BMSCs cells by qPCR

Gel electrophoresis of the qPCR products showed clear INF-γ mRNA bands in the INF-γ+BMSC group, but no bands in the BMSC group (Figure 2). Relative INF-γ relative expression was significantly lower in the BMSC group than in the INF-γ+BMSCs group ($P<0.05$) (Table 1).

Fluorescent INF-γ-transfected BMSC distribution in nude mouse tumours

After immunofluorescence staining, no fluorescent signals were observed in the tumour tissues of mice in the model group under confocal microscopy, whereas fluorescent signals were observed in those of mice in the BMSC and INF-γ+BMSC groups. BMSCs transfected with INF-γ showed a significant homing effect and were concentrated in tumour tissues (Figure 3).

Detection of apoptotic cells by TUNEL staining

TUNEL staining indicated that apoptosis was more prevalent in the BMSC group and the INF-γ+BMSC group than in the model group, whereas the INF-γ+BMSC group showed a clearer distribution of apoptotic cells with brown nuclei (non-apoptotic cell nuclei were counterstained blue with haematoxylin) (Figure 4). For each group, we randomly selected six visual fields in which we counted positive cells (with apoptotic nuclei) and the total number of cells and calculated the ratio of positive cells. Apoptotic cells had higher relative expression levels in the INF-γ+ BMSC group than in the model group ($P<0.05$) (Table 2).

Tumour volume changes in tumour-bearing nude mice

The tumour volume of tumour-bearing nude mice was measured every other day during the 14-day period of intragastric administration, and changes in the average tumour volume over time were calculated for each group (Table 3). The model and INF-γ+BMSC groups showed a significant difference in tumour volume on day 9, day 11 and day 14 ($P<0.05$). Tumour volume changes for tumour-bearing nude mice in the treatment groups were plotted as growth curves (Figure 5), which showed significantly less tumour growth in the INF-γ+BMSC group than in the model and BMSC groups.

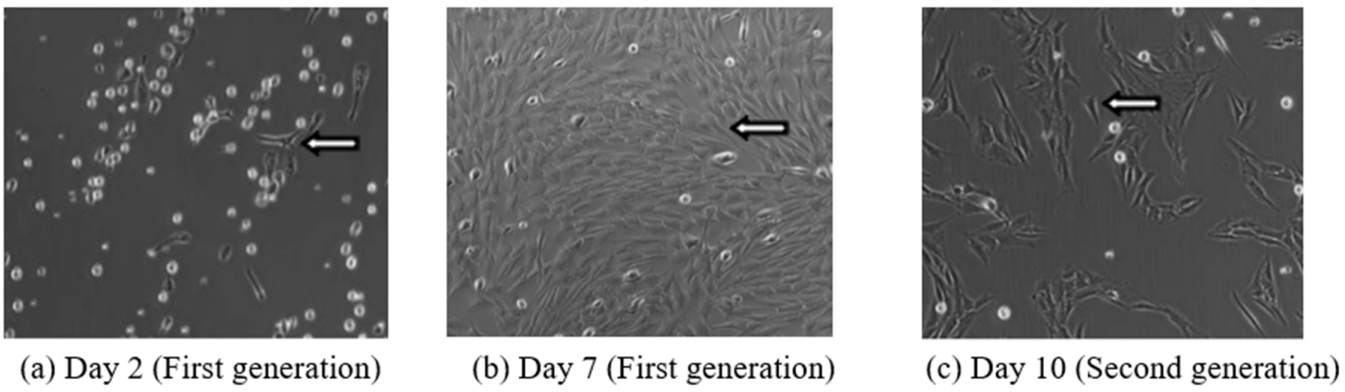

Figure 1: Stages of Bone Marrow Mesenchymal Stem Cell (BMSC) primary culture at 2 and 7 days and after passage (10 days). **Note:** Images were taken under white light (× 100).

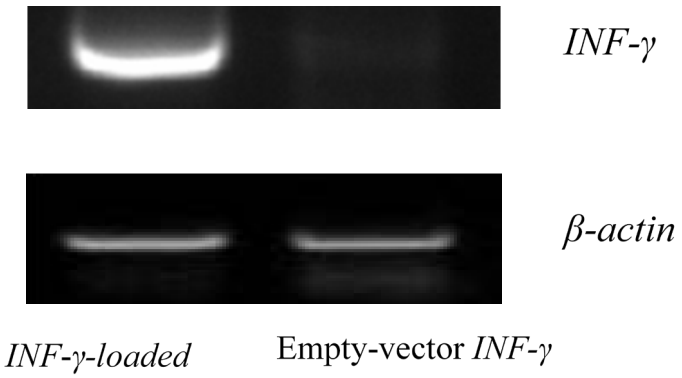

Figure 2: INF-γ mRNA bands on gel electrophoresis following fluorescence quantitative Polymerase Chain Reaction (qPCR).

Group	Relative INF-γ expression (%)
INF-γ+BMSC	96.34 ± 13.17[a]
BMSC	1.96 ± 0.22
Note: [a]: P<0.05 compared with the model group	

Table 1: Relative expression of INF-γ in Bone Marrow Mesenchymal Stem Cells (BMSCs) after transfection.

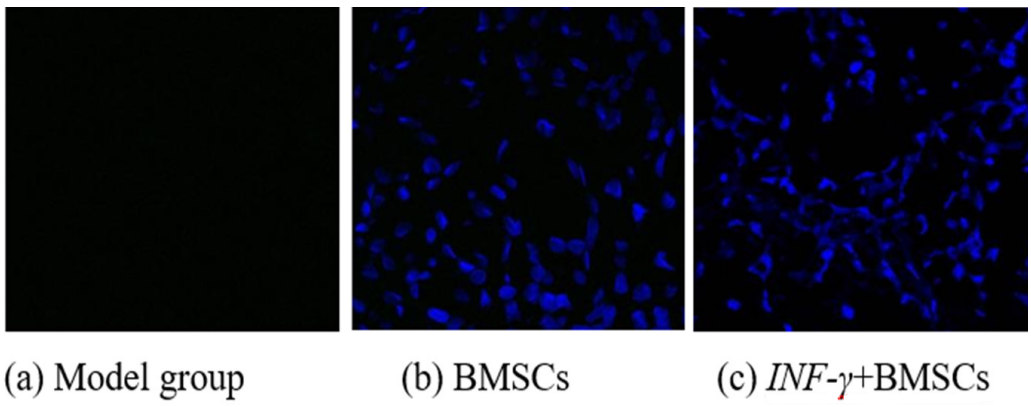

Figure 3: Fluorescence distribution of BMSCs in nude mice observed by confocal microscopy (× 400); (a) Nude mouse tumour model (model group) Lentiviral BMSC transfection groups with (b)empty vector (BMSC group) and (c)INF-γ (INF- γ +BMSC group).

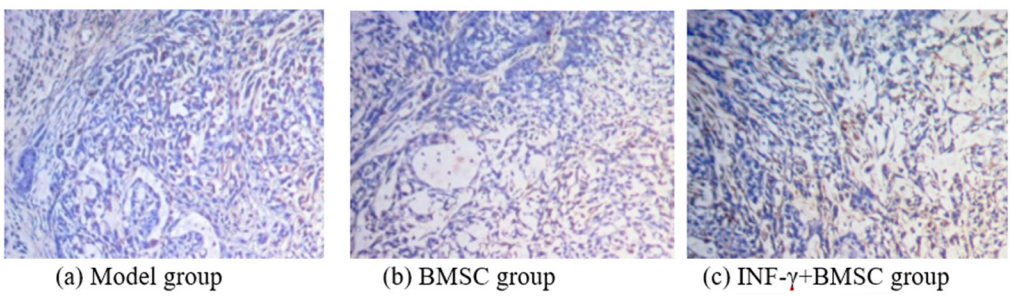

Figure 4: Apoptosis of tumour cells in nude mice determined by Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labelling (TUNEL) staining (× 100); (a) Model group (b) BMSC group (c) INF-γ+BMSC group.

Group	N	Relative abundance of positive cells (%)
Model	6	16.57 ± 6.86
BMSC	6	20.33 ± 9.21
INF-γ +BMSC	6	44.14 ± 8.55[a]
Note: [a]: P<0.05 compared with the model group.		

Table 2: Relative expression of tumour cells in nude mice by Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labelling (TUNEL) staining ($\bar{x} \pm s$).

Groups	Tumour volume (mm^3)						
	1 day	3 days	5 days	7 days	9 days	11 days	14 days
Model	90.6 ± 0.5	131.2 ± 25.5	216.8 ± 4.0	279.1 ± 11.9	426.5 ± 56.7	877.1 ± 61.8	1865.0 ± 415.4
BMSCs	90.8 ± 8.3	123.2 ± 22.5	219.7 ± 4.7	273.3 ± 13.9	391.6 ± 37.2	894.4 ± 89.0	1908.1 ± 15.5
INF-γ +BMSCs	78.4 ± 5.7	117.5 ± 20.4	195.6 ± 2.6	211.2 ± 15.5	298.5 ± 11.4a	638.2 ± 63.5[a]	1425.3 ± 89.2[a]
Note: [a]: P<0.05 compared with each group. There was no interaction between time and group.							

Table 3: Tumour volume changes in tumour-bearing nude mice in the treatment groups over time (mean ± standard deviation).

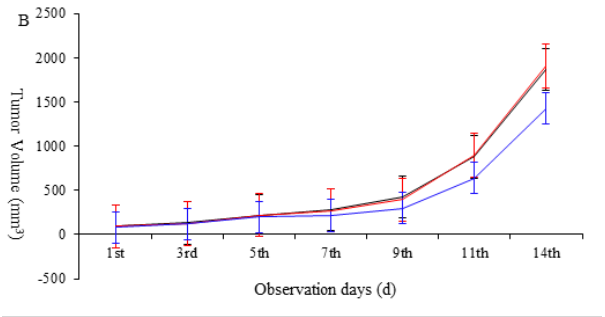

Figure 5: Tumour volume changes over time in each treatment group. **Note:** (—) model groups, (—) BMSCs, (—) INF-γ+BMSCs.

Discussion

Glioma is the most common type of brain tumour. Due to its high degree of malignancy, strong invasiveness and difficulty of eradication through surgery, glioma has a poor prognosis after surgery, radiotherapy, and chemotherapy, and is therefore considered to be one of the most difficult tumours to treat with neurosurgery [24,25]. Therefore, it is important to find new therapeutic methods for the clinical treatment of glioma. Conventional tumour therapy combined with targeted biological therapy [25-27] and immunotherapy [28] have become research hotspots in this field. BMSCs have attracted attention in biological therapy and immunotherapy because they facilitate the transfection of exogenous genes and promote homeostasis, among other biological benefits [9,28]. Transgenic BMSCs can provide targeted delivery of tumour inhibitory drugs and have exerted strong tumour inhibitory effects in immunodeficient nude mouse models [9,16,27]. In this study, qPCR revealed that BMSCs stably expressed the INF-γ gene, and confocal microscopy showed that the BMSCs exhibited a strong homing effect in tumour tissues, confirming their viability as tumour biologic therapy vectors.

As an important part of the tumour matrix, BMSCs are involved in both the occurrence and development of tumours. However, controversial results have been reported in different studies, perhaps due to differences in the methods and doses used to inject BMSCs into tumour models and differences in the tumour microenvironment [9]. Aggregated BMSCs can secrete up to 20 cytokines, including angiopoietin, VEGF, FGF-b, and interleukin (IL)-11, leading to interactions between cells and between cells and molecules to enhance or inhibit tumour growth [29]. Although uncertainty about the roles of BMSCs and even their potential tumourigenicity have led to limited clinical application, the prospect of applying BMSCs as vectors to carry exogenous genes in the treatment of tumours has been unanimously recognised.

INF-γ is a multi-effect cytokine that is important for regulating cellular and humoral immunity. INF-γ can interfere with the DNA replication of exogenous antigen cells such as tumour cells, promoting apoptosis, which induces BMSCs to activate non-specific immune

functions by antigen-presenting cells *via* the expression of major histocompatibility complex class II molecules. INF-γ also stimulates the immunoregulatory activity of BMSCs by activating the synthesis of indoleamine 2,3-dioxygenase. Therefore, it is critical to investigate the potential roles of INF-γ in immune cell differentiation and tumour death.

Because nude mice exhibit a T cell immunodeficiency, nude mice can maintain their original biological characteristics following tumour transplantation, in a manner very similar to clinical patients; therefore, this experimental model has been widely used in immune and tumour studies [21]. Adult nude mice (6–8 weeks old) have a higher level of non-specific immune function than ordinary mice, and their Natural Killer (NK) cells and macrophages exhibit stronger immune activity [30]. Exogenous antigens induce the recruitment of NK cells and macrophages to the tumour site, promoting cytokines such as IFN-γ and Tumour Necrosis Factor-α (TNF-α), and show tumour inhibitory effects [31-33]. The immune function of tumour-bearing nude mice may not be regulated through the injection of BMSCs alone; however, it remains to be determined whether continuous expression of INF-γ in tumour tissues using BMSCs transfected with INF-γ could activate NK cells and macrophages to play non-specific immune roles. BMSCs have immune plasticity, as demonstrated by immunosuppression or IFN-γ activation, and antigen-presenting cells can present tumour-related antigens to activate effective tumour-inhibitory immunity under IFN-γ conditions [34]; this mechanism may be related to interactions between cytokines such as IL-6, IL-12, and INF-γ and between BMSCs and tumour cells [9,35]. In this study, although the INF-γ+BMSC group showed a significant inhibitory effect on tumour growth compared with the model and BMSC groups on day 9, no significant effect was observed on subsequent days, perhaps due to our small sample sizes. However, the tumour growth curves of each group showed that BMSC-targeted delivery of INF-γ had a stronger tumour inhibitory effect than observed in the model and BMSC groups.

TUNEL staining is used to detect the breakage of nuclear DNA in tumour tissue cells during the early phase of apoptosis. Apoptotic nuclear DNA is stained dark brown, whereas normal or proliferating cells are mainly stained blue, without DNA breakage. In this study, TUNEL staining of tumour tissues showed clear dark brown particles in the BMSC and INF-γ+BMSC groups compared with the model group, which indicated tumour cell apoptosis. Quantitative analysis revealed higher apoptosis rates in the INF-γ+BMSC group than in the model and BMSC groups ($P<0.05$), which further suggests that INF-γ and other humoral immune molecules promote glioma cell apoptosis.

Conclusion

Lentiviral vectors can be used to effectively transfect the INF-γ gene into BMSCs, which then home and distribute the gene in the tumour tissues of glioma model mice. Although INF-γ-transfected BMSCs demonstrated a clear targeted tumour inhibitory effect, we found no direct evidence that INF-γ-transfected BMSCs could activate the non-specific immunity of tumour-bearing nude mice in this study. In the clinical treatment of refractory tumours, conventional surgery and radiotherapy combined with targeted chemotherapy promise broad application. However, BMSC-based glioma therapy is still in its early stages, and the interaction between cytokines such as INF-γ, antigen-presenting cells, and BMSCs requires further investigation. A better understanding of the mechanisms influencing the potential biological consequences of BMSC therapy is required before it can be widely used in the treatment of glioblastoma.

Limitations

The main limitations of the study were as follows: (i) The study hasn't verified the presence of BMSCs also in peritumoral and non-tumor tissues in order to control the effective and exclusive tumor homing of BMSCs transfected with INF-γ. (ii) BMSCs that have been transduced with a lentivirus carrying an INF-γ sequence overexpress this sequence, compared to non-transduced BMSCs, and the study have no evidence to show the presence of the exogenous cells in tumour tissue.

Authors' Contributions

TX, YS, ZY and WY designed and supervised the study. LZ, XY, XX, TY and WJ performed laboratory protocols. WJ and YS performed statistical analysis on the results. All authors contributed to the writing and review of the manuscript. All authors read and approved the final manuscript.

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Availability of Data and Materials

All data and materials generated and analyzed during the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The animal experiments in this study were authorized and approved by the Institutional Animal Care and Use Committee of Gansu University of Chinese Medicine (approving No: 2016-269).

Consent publication

The authors all agreed for publication of this paper.

Competing interests

The authors report no 'conflict of interest' in connection with this manuscript.

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