NEURAL REGENERATION RESEARCH
Volume 8, Issue 22, August 2013

www.nrronline.org

doi:10.3969/j.issn.1673-5374.2013.22.009 [http://www.nrronline.org; http://www.sjzsyj.org]
Fan CG, Wang DL, Zhang QJ, Zhou JR. Migration capacity of human umbilical cord mesenchymal stem cells towards glioma in vivo. Neural Regen Res. 2013;8(22):2093-2102.

Migration capacity of human umbilical cord mesenchymal stem cells towards glioma *in vivo*

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Research Highlights

(1) Human umbilical cord mesenchymal stem cells displayed potent glioma-specific targeting capability and extensive distribution in the tumor bed, indicating that ventricular administration may serve as a less invasive administration route compared with intratumoral injection.
(2) The inherent glioma-targeting tropism of human umbilical cord mesenchymal stem cells implies their potential application as a cellular vehicle for the delivery of therapeutic agents in glioma therapy.

Abstract

High-grade glioma is the most common malignant primary brain tumor in adults. The poor prognosis of glioma, combined with a resistance to currently available treatments, necessitates the ment of more effective tumor-selective therapies. Stem cell-based therapies are emerging as novel cell-based delivery vehicle for therapeutic agents. In the present study, we successfully isolated human umbilical cord mesenchymal stem cells by explant culture. The human umbilical cord senchymal stem cells were adherent to plastic surfaces, expressed specific surface phenotypes of mesenchymal stem cells as demonstrated by flow cytometry, and possessed multi-differentiation potentials in permissive induction media *in vitro*. Furthermore, human umbilical cord mesenchymal stem cells demonstrated excellent glioma-specific targeting capacity in established rat glioma models after intratumoral injection or contralateral ventricular administration *in vivo*. The excellent glioma-specific targeting ability and extensive intratumoral distribution of human umbilical cord mesenchymal stem cells indicate that they may serve as a novel cellular vehicle for delivering therapeutic molecules in glioma therapy.

Key Words

neural regeneration; umbilical cord; mesenchymal stem cell; glioma; migration; cell-based therapy; grants-supported paper; neuroregeneration

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Received: 2013-04-25
Accepted: 2013-06-30
(N201302052)

Funding: This work is supported by the National Natural Science Foundation of China, No. 81001009.

Author contributions: Fan CG designed and conducted experiments, provided and integrated experimental data, wrote the manuscript, and was responsible for the funding. Wang DL and Zhou JR contributed to the cell culture and animal experiment, respectively. Zhang QJ contributed to the design and guidance of the study. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: This study was approved by the Animal Ethics Committee, Peking University People's Hospital in China.

INTRODUCTION

Glioblastoma, the most common malignant primary brain tumor in adults, represents an important cause of cancer-related mortality in patients[1]. Although its incidence rate is only 3.19 per 100 000 people[2], it remains the cause of approximately 13 000 cancer-related deaths in the United States annually[3]. The aggressive growth manner, terized by marked angiogenesis and sive tumor cell invasion into normal brain renchyma with frequent formation of tumor microsatellites at distal sites, makes eradication impossible even after extensive microsurgical resection combined with current standard chemoradiation and adjuvant temozolomide[4]. Without treatment, most patients will die within 3 months after diagnosis[5].

Author statements: The manuscript is original, has not been submitted to and is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

Even with the above-mentioned standard therapy, the median survival of newly-diagnosed glioblastoma patients is 14.6 months[4] and the overall survival rate is 9.8% at 5 years, while with radiotherapy alone it is only 1.9%[6]. Moreover, almost all glioblastomas will eventually relapse and survival following disease progression is estimated to be 25–30 weeks[7-8]. Thus, novel therapeutic strategies must to be investigated for the development of a more effective treatment strategy.

In seeking new therapeutic options, current efforts have focused on exploiting the fact that glioblastomas are highly vascularized tumors characterized by activation of multiple proangiogenic signaling pathways and overexpression of a variety of proangiogenic factors[9]. Consequently, new drug regimens are being developed to target angiogenesis in an attempt to arrest tumor growth. In particular, the novel antiangiogenic agent bevacizumab (Avastin), which targets vascular endothelial growth factor, has been used for glioblastoma treatment[10] and received accelerated Food and Drug Administration approval for use in patients with recurrent glioblastoma in 2009[11]. Bevacizumab has shown promising results with prolonged progression-free survival and favorable consequences for patient quality of life by alleviating peritumoral vasogenic cerebral edema and decreasing corticosteroid dependence[12]. However, in addition to frequently occurred treatment-associated toxicities[13], the response to anti-vascular endothelial growth factor therapy is always transient and the majority of patients will eventually relapse[14]. Moreover, several other novel strategies, including carmustine wafer[15], passive and active immunotherapy[16], oncolytic virus therapy[17], gene therapy[18] and novel delivery systems, such as the controlled release system[19] and convection-enhanced delivery system[20], have failed to achieve significant therapeutic effect due to either lack of selectivity for glioma or inability to effectively deliver therapeutic agents to the invasive tumor cells infiltrating into normal brain parenchyma. Therefore, innovative treatments that effectively and specifically target glioblastoma tumor cells while sparing normal brain tissue are critically needed to substantially improve the outcome of glioblastoma patients.

In the past decade, a variety of *in vitro* and *in vivo* studies have demonstrated that stem cells possess intrinsic tumor-inhibitory effects as well as inherent tropism toward primary[21] and metastatic[22] invasive malignancies within the brain, including glioblastoma[23]. These observations promoted investigators to design targeted therapeutic delivery modalities employing stem cells as vehicles to track invasive tumor burden and selectively distribute anticancer agents to glioblastoma tissue, in which stem cells demonstrated unprecedented specificity and coverage of the whole tumor, including tumor microsatellites[24]. Among various types of stem cells, neural stem cells and mesenchymal stem cells are the two main sources that have been extensively investigated as potential vehicles for the delivery of numerous tumoricidal agents, such as immunomodulatory, anti-mitotic, pro-apoptotic, pro-necrotic, and viral oncolytic therapies, with promising results, including a significant decrease of tumor burden as well as prolongation of survival in numerous animal models[25-26]. Because of the ethical problems and technical difficulties associated with neural stem cells, mesenchymal stem cells are increasingly regarded as an alternative cell source for such applications as they can be easily isolated and expanded in culture and can overcome immunological incompatibilities *via* autologous transplantation[26]. As one of the most intensively investigated sources of mesenchymal stem cells, human bone marrow mesenchymal stem cells have shown strong migratory capacity towards glioma, which is mediated by the interaction of cytokines secreted by glioma cells and their related receptors expressed by human bone marrow mesenchymal stem cells[27]. In addition, human bone marrow mesenchymal stem cells carrying proapoptotic genes, immune genes, tumor-inducing death ligands and oncolytic viruses have exerted potent antitumor effects on glioma[28]. As an alternative source of mesenchymal

stem cells, human umbilical cord blood mesenchymal stem cells have also shown similar glioma-trophic capacity and inhibition of tumor growth[29].

Although both human bone marrow mesenchymal stem cells and human umbilical cord blood mesenchymal stem cells have been studied as potential vehicles for delivery of therapeutic agents, there are few reports that have focused on umbilical cord mesenchymal stem cells, even though they have many advantages over their counterparts, such as easy isolation, enhanced proliferation potency, and low risk of infection. Based on our previous studies demonstrating the migratory capacity of umbilical cord mesenchymal stem cells toward lesions of spinal cord injury[30], we hypothesized that umbilical cord mesenchymal stem cells may have similar targeting capabilities for glioma.

Thus, we investigated the glioma-targeting behavior of umbilical cord mesenchymal stem cells in Sprague-Dawley rat glioma models *in vivo* by intratumoral injection and contralateral intraventricular administration of umbilical cord mesenchymal stem cells.

RESULTS

Morphological characteristics of umbilical cord mesenchymal stem cells

At 3 to 5 days after primary culture, a few of the adherent cells grew out from the small umbilical fragments. These cells initially appeared to be small, polygonal or irregular, refractive shaped (Figure 1A), and subsequently grew into homogenous spindle-like cells, which were similar to fibroblasts in morphology. When more adherent cells grew out from the umbilical fragments, they formed radial clusters around the fragments (Figure 1B). Within approximately 2 weeks of cultivation, the fibroblast-like cells reached 80–90% confluence. They maintained homogenous fibroblastic appearance without obvious difference in morphology and growth characteristics after several passages (Figure 1C, D).

Immunophenotype of umbilical cord mesenchymal stem cells

Fluorescence activated cell sorting analysis revealed that cells of passage 3 expressed high levels of putative mesenchymal stem cell markers, such as CD13, CD29, CD44, and CD90. However, they failed to express the surface markers of hematopoietic lineages, including CD14, CD34, CD45 and the endothelial-related antigen CD31 (Figure 2).

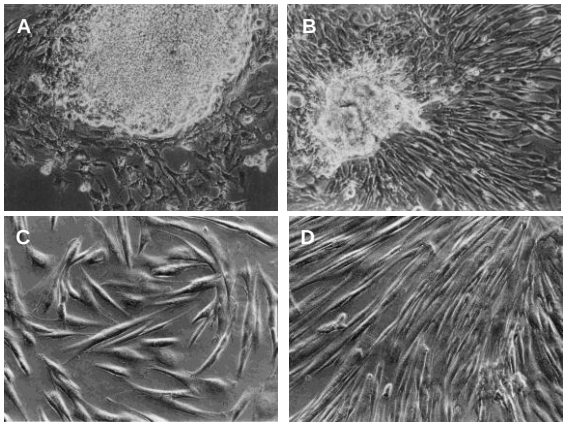

Figure 1 Primary and secondary culture of human umbilical cord mesenchymal stem cells.

(A, B) Primary culture on days 5 and 10 of the first passage (× 10). (C) Cell culture on day 3 of passage 5 (× 20). (D) Cell culture on day 5 of passage 9. Photos were obtained periodically by phase-contrast microscopy (× 20). On day 5 of passage 9, the fibroblast-like cells maintained homogenous fibroblastic appearance without obvious differences in morphology and growth characteristics.

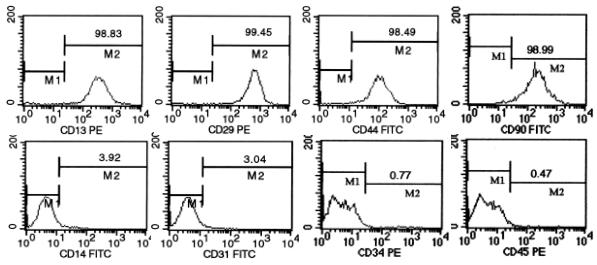

Figure 2 Immunophenotypes of human umbilical cord mesenchymal stem cells.

X-axis: Fluorescence content; Y-axis: relative cell/particle number. Cells of passage 3 express high levels of CD13, CD29, CD44, and CD90. However, they failed to express CD14, CD34, CD45 and CD31.

Differentiation capacities of umbilical cord mesenchymal stem cells *in vitro*

After being exposed to adipogenic differentiation medium for 2 weeks, most fibroblastic cells changed into large, flattened morphology with accumulated lipid vacuoles, which stained red with oil red O (Figure 3A). After being exposed to osteogenic differentiation medium for 2 weeks, osteogenic differentiation was observed as the calcium deposition in differentiated multi-angular or irregular cells stained red by alizarin red (Figure 3B). In neural differentiation medium, some of these cells were transformed into typical morphology of neurons, astrocytes or oligodendrocytes, which were strongly stained with neuron-specific enolase, glial fibrillary acidic protein and myelin basic protein, respectively (Figure 3C–E).

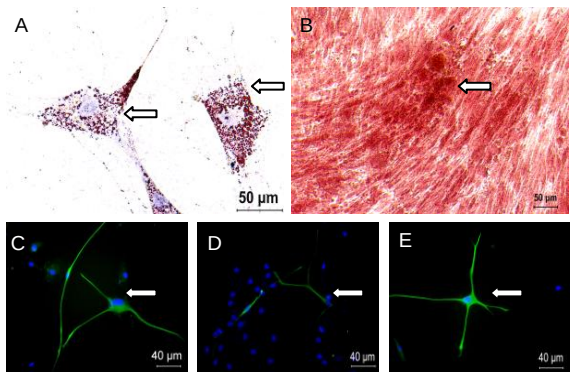

Figure 3 Adipogenic, osteogenic, and neural differentiation potentials of human umbilical cord mesenchymal stem cells *in vitro*.

All induction media were changed twice weekly and the cells were observed with light or fluorescent microscopy. (A) Large, flattened cells with accumulated lipid droplets stained red with oil red O after adipogenic induction under a light microscope (arrows). (B) Differentiated cells with calcium deposition stained red by alizarin red in osteogenic medium under a light microscope (arrow). Neuron-specific enolase (C), glial fibrillary acidic protein (D) and myelin basic protein (E) expressions (arrow) were obvious after neural induction under a fluorescent microscope.

Quantitative analysis of experimental animals

One week after tumor inoculation, 32 rat models were equally and randomly divided into four groups. Group 1 received contralateral ventricular injection of 5 μL PBS containing 5 × 10^5 umbilical cord mesenchymal stem cells labeled with CM-DiI; group 2 received equal number of umbilical cord mesenchymal stem cells at previous sites of tumor inoculation. As controls, group 3 and group 4 received 5 μL PBS via contralateral ventricular injection or intratumoral administration, respectively. Two weeks later, the rats were sacrificed and the brains were removed and sectioned for histological analysis and migration analysis. All animals were included in the final analysis.

Migratory capacity of umbilical cord mesenchymal stem cells *in vivo*

Histopathological analysis of the brains of glioma-bearing rats revealed that the gliomas mainly located in the right frontal lobe (Figure 4A, B) with infiltrating satellites distant from the tumor bed (Figure 4C). The right lateral ventricle was compressed and had lost its natural shape (Figure 4A, B).

In the contralateral ventricle injection group, the DiI-labeled umbilical cord mesenchymal stem cells migrated away from the initial injection site and targeted the glioma through the corpus callosum (Figure 4D).

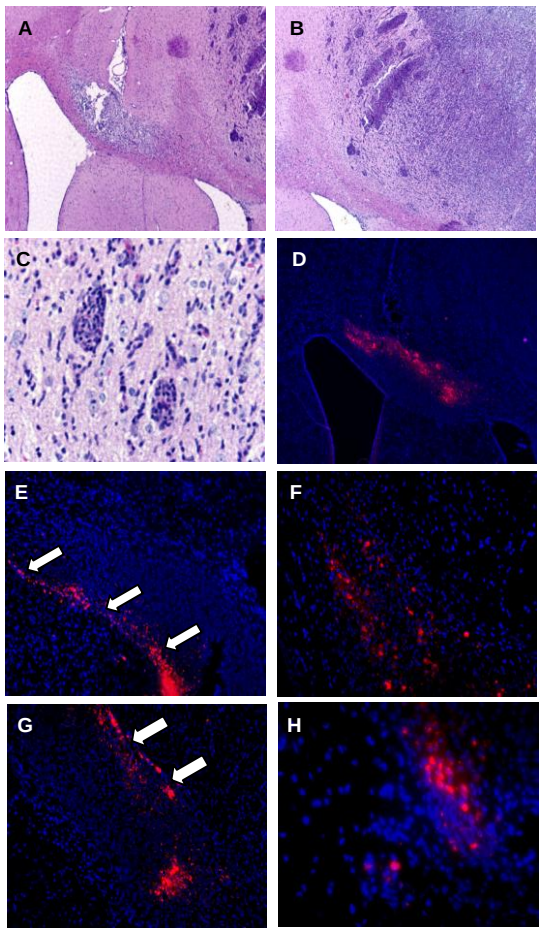

Figure 4 Glioma and glioma-tropic migration of human umbilical cord mesenchymal stem cells *in vivo*.

The rats were sacrificed 2 weeks after human umbilical cord mesenchymal stem cell transplantation. The brains were removed and sectioned for histological analysis using hematoxylin-eosin staining under light microscopy and for migration analysis by nuclei staining with Hoechst 33258 (blue) under fluorescent microscopy. DiI-labeled umbilical cord mesenchymal stem cells were red.

(A, B) Hematoxylin-eosin staining shows the right lateral ventricle was compressed and lost its natural shape because of glioma growth (× 40). (C) Infiltrating glioma satellites in normal parenchyma (× 200). (D) Migration of human umbilical cord mesenchymal stem cells from the initial injection site towards the glioma (× 40). (E) Distribution of human umbilical cord mesenchymal stem cells (arrows) along the interface of tumor and normal brain parenchyma (× 200), and (F) infiltration of human umbilical cord mesenchymal stem cells into the tumor mass after contralateral ventricular injection (× 200). (G) Migration of human umbilical cord mesenchymal stem cells into the interface of tumor and normal brain parenchyma (arrows; × 100) and (H) to the outgrowing tumor satellites away from tumor mass (× 100) after intratumoral injection.

They were largely distributed along the interface of the tumor and normal brain parenchyma (Figure 4E), and also infiltrated into the tumor bed (Figure 4F). After in-

oculation into the tumor, umbilical cord mesenchymal stem cells extensively distributed at the border zone between the tumor and tumor bed (Figure 4G) and migrated to the outgrowing glioma satellites distant from the tumor bed (Figure 4H). However, no DiI-labeled cells were observed in the control groups.

DISCUSSION

In the present study, we successfully isolated mesenchymal stem cells from human umbilical cord *via* explant culture, as demonstrated by the plastic-adherent characteristics, expression of specific mesenchymal stem cell surface phenotypes, and multi-directional differentiation capacities. Most importantly, umbilical cord mesenchymal stem cells showed excellent glioma-specific targeting capacity and extensive intratumoral distribution after intratumoral and contralateral intraventricular administration, indicating that they could serve as novel cellular vehicles for delivering therapeutic molecules in glioma treatment. To the best of our knowledge, this is the first report demonstrating the *in vivo* glioma-targeting capabilities of umbilical cord mesenchymal stem cells by intratumoral or contralateral ventricular administration.

Isolation and characterization of umbilical cord mesenchymal stem cells

In this study, we successfully isolated mesenchymal stem cells from human umbilical cord using a previously reported explant culture method[31-32]. Compared with a reported enzymatic digestion method, explant culture may be more clinically applicable as it involves minimal tissue handling, exerts negligible influence on cell viability, and is a less time-consuming procedure[31]. As demonstrated herein, the umbilical cord mesenchymal stem cells exhibited plastic-adherent characteristics, fibroblastic morphology, expression of various mesenchymal stem cell surface markers (CD13, CD29, CD44, and CD90), and were capable of multi-directional differentiation into adipocytes, osteocytes, and neural cells *in vitro*. Therefore, umbilical cord mesenchymal stem cells meet the criteria of mesenchymal stem cells defined by the International Society for Cellular Therapy[33]. Accordingly, human umbilical cord may become an alternative cell source of generally accepted mesenchymal stem cells.

Therapeutic potential of umbilical cord mesenchymal stem cells in neurological diseases

Recent studies have revealed the therapeutic potentials of umbilical cord mesenchymal stem cells in various neurological diseases. Umbilical cord mesenchymal stem cells have been demonstrated to increase axonal regeneration and reduce cavitations in spinal cord injury[30], increase endogenous neurogenesis and reduce infarct volume in experimental cerebral ischemia[34], inhibit inflammation and promote angiogenesis in intracerebral hemorrhage[35], rescue degenerating dopaminergic neurons and ameliorate apomorphine-induced rotations in Parkinson's disease[36], and enhance neuromotor function in brain injury[37]. The excellent migratory property of umbilical cord mesenchymal stem cells toward lesions of various neurological diseases prompted us to investigate whether umbilical cord mesenchymal stem cells possessed glioma-trophic capacity in a rat glioma model.

Advantages of umbilical cord mesenchymal stem cells

Although neural stem cells and various sources of mesenchymal stem cells[28-29, 38] have been investigated as cellular vehicles in experimental glioma, the demonstrated migration capacity of umbilical cord mesenchymal stem cells towards glioma in the present study is of particular interest due several advantages umbilical cord mesenchymal stem cells exhibit over their counterparts[39]. First, umbilical cord is routinely discarded at parturition, thus there are little legal limitations or ethical concerns associated with the harvesting of umbilical cord mesenchymal stem cells. Second, the extracorporeal nature of umbilical cord facilitates easier isolation by eliminating the invasiveness, discomfort and risks associated with adult mesenchymal stem cell isolation. Third, the comparatively abundant volume of umbilical cord and ease of physical manipulation increase the number of stem cells that can be extracted. Therefore, it is possible to get sufficient number of cells required for clinical transplantation without long-term culture and extensive expansion *ex vivo*. Fourth, due to the generation and sequestration of umbilical cord during early developmental period of ontogenesis, umbilical cord mesenchymal stem cells may be endowed with enhanced proliferation potency. In addition, umbilical cord mesenchymal stem cells have lower risk of bacterial and viral infections than mesenchymal stem cells isolated from adult tissue due to the presence of the placental barrier.

In contrast to the above mentioned advantages of umbilical cord mesenchymal stem cells, the extensively investigated adult bone marrow-derived mesenchymal stem cells may decrease expansion and differentiation potentials with advanced age[40], while the harvesting success rate of mesenchymal stem cells from human

umbilical cord blood may be as low as 6%, albeit at the cost of abundant hematopoietic stem cells[41]. Thus, umbilical cord mesenchymal stem cells may become an attractive novel cell source in cell-based therapy for currently refractory diseases, including glioma.

Glioma-tropic capability and possible underlying mechanism of mesenchymal stem cells

The excellent tumor-specific targeting ability and extensive intratumoral distribution of umbilical cord mesenchymal stem cells are prerequisites for the delivery of therapeutic molecules to tumor cells. It was postulated that this chemokinetic activity was mediated by an interaction between cytokines secreted by the glioma and corresponding receptors expressed on mesenchymal stem cells. Schichor *et al* [42] reported that the migratory behavior of mesenchymal stem cells was significantly increased by vascular endothelial growth factor-A, while glioma-cell-conditioned medium enhanced migration by twice as much as vascular endothelial growth factor-A alone, indicating that other mechanisms are involved in this process. A subsequent study demonstrated that interleukin-8, transforming growth factor-β1, and neurotrophin-3 were potent chemoattractants through interaction with their respective receptors CXCR 1, transforming growth factor-β1-RII, and TrkC, while other candidates, including epidermal growth factor, glial cell-derived neurotrophic factor, ciliary neurotrophic factor, brain-derived neurotrophic factor, and platelet-derived growth factor, were not involved in the mesenchymal stem cell recruitment by glioma[42-43]. In contrast, Hata *et al* [44] reported that platelet-derived growth factor-BB played a causal role in the localization and tropism of mesenchymal stem cells for glioma *via* interaction with platelet-derived growth factor receptor-β expressed on mesenchymal stem cells. Interestingly, the differential glioma-trophic migration of mesenchymal stem cells was due to different expression levels of matrix metalloproteinase-1[45]. Moreover, MCP-1/CCR2 and stromal-derived factor-1α/CXCR4 were also involved in the migration process[46]. Therefore, CXCR4 transfection may significantly enhance the chemotactic invasiveness of mesenchymal stem cells toward glioma by upregulation of CXCR4 on mesenchymal stem cells[47].

Migration analysis and comparison of various delivery routes

We speculated that the coculture system *in vitro* may be influenced by many influencing factors, such as fetal bovine serum, concentration of growth factors, cell viability and seeding density, which are unable to exactly simulate the tumor microenvironment *in vivo*. The subcutaneous flank xenograft model may be very different from the intracranial glioma model due to limitations in the penetration of therapeutic agents across the blood-brain barrier. Furthermore, mesenchymal stem cell survival in nude mice may be dissimilar to immunocompetent rats. Therefore, we directly investigated the glioma-targeting capacity of umbilical cord mesenchymal stem cells in an intracranial C6 glioma rat model. Our results revealed that the implanted umbilical cord mesenchymal stem cells demonstrated extensive distribution in the tumor bed and circumscribed along the tumor edge after intratumoral injection. More importantly, they showed excellent migratory capacity towards glioma tissue after contralateral administration. Interestingly, we also observed an association of umbilical cord mesenchymal stem cells with outgrowing glioma satellites, known as the “chasing down” phenomenon, which was first reported in neural stem cells[48]. Therefore, we can conclude that umbilical cord mesenchymal stem cells possess strong migratory capacity and glioma tropism, which implicates significant potential for *in vivo* targeted therapy of glioma.

As for the cell delivery routes, intravenous injection is undoubtedly less invasive and allows for the injection of larger amounts of cells in a single dose, however, the long-distance migration and the great proportion of cells trapped by systemic organs can cause a delayed engraftment into the brain parenchyma, inefficient infiltration into the lesion, and decrease in cell viability[49]. Although intra-arterial administration showed significantly increased migration and diffuse distribution pattern in the target brain compared with intravenous administration, the relatively high mortality associated with thrombosis and embolism poses a serious concern[50]. In addition, intracerebral cell implantation involves a relatively invasive procedure, with primary concerns of intracerebral hemorrhage and tumor cell dissemination along the needle tract, especially with repeated administration. Thus, we evaluated the effects of intraventricular transplantation because it is less invasive and a more clinically relevant procedure, and may facilitate easier repeated delivery of umbilical cord mesenchymal stem cells *via* Ommaya-reservoir. Furthermore, intraventricular administration has low risk of morbidity and does not infiltrate other organs, which is intriguing as mesenchymal stem cells expressing oncolytic viruses, chemotherapeutic precursors or cell trackers for diagnostic purpose can be administered. Additional studies are needed to demonstrate whether intrathecal administration of cells through cisterna magna or lumbar puncture

can achieve similar results, because these approaches would lead to a significant reduction in the invasiveness without reducing the benefits of the intraventricular approach.

The therapeutic potential and clinical application of glioma-targeting mesenchymal stem cells

Because of their glioma-tropic properties, umbilical cord mesenchymal stem cells may be particularly suitable for tracking gliomas that highly express related receptor ligands of umbilical cord mesenchymal stem cells to enhance the directional motility and recruitment. Employing this concept, genetically-modified mesenchymal stem cells expressing CXCR4[47] have been shown to strengthen the migration capacity of mesenchymal stem cells, prolong intratumoral retention, enhance antitumor effects, and improve the survival benefit of glioma-burdening animal models. The labeling of mesenchymal stem cells with superparamagnetic iron oxide or micrometer-sized iron oxide nanoparticles[51] may facilitate observation and monitoring of the migration and fate of transplanted mesenchymal stem cells in clinical settings using noninvasive MRI scanning.

Conclusion

Umbilical cord mesenchymal stem cells can be easily isolated from umbilical cord *via* explant culture. When considering the relative availability and ease of manipulation, excellent tumor-specific targeting ability, and extensive intratumoral distribution of umbilical cord mesenchymal stem cells, they may serve as cellular vehicles in delivering therapeutic molecules to exert anti-tumor effects on glioma. Future studies are needed to elucidate the mechanism underlying the glioma-tropic migration of umbilical cord mesenchymal stem cells to investigate whether they have direct cytotoxic effect on glioma, and to modify them using various therapeutic agents to exert greater anti-glioma effects.

MATERIALS AND METHODS

Design

In vitro cell culture, randomized, controlled animal study.

Time and setting

The experiments were conducted at the Peking University People's Hospital, China, from January 2011 to October 2012.

Materials

A total of 32 adult Sprague-Dawley rats, weighing 250–350 g, irrespective of age and gender, were provided by the Department of Laboratory Animal Science of Peking University Health Science Center in China (license No. SCXK (Jing) 2011-0012). Rats were housed in specific pathogen-free level barrier environment at 24 ± 3°C, relative humidity of 40–70%, on a 12-hour light/dark cycle, and allowed free access to food and water. Experimental procedures were performed in accordance to the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[52].

Methods

Cell isolation and primary culture

After informed consent, the umbilical cords (15–20 cm length) were freshly harvested from three healthy parturient women during cesarean section at Peking University People's Hospital in China, and placed in sterile containers. The arteries and veins were stripped and the remaining tissue was rinsed with PBS (Gibco, Camarillo, CA, USA). The tissues were cut into small fragments of 1–2 mm in diameter and plated in growth media consisting of Dulbecco's Modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mmol/L L-glutamine (Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco). Cultures were then maintained at 37°C in humidified atmosphere containing 5% carbon dioxide (v/v). Half of the culture media was changed 1 day after plating, and then the media was completely changed twice weekly. Daily progress was monitored under phase-contrast microscopy (Olympus, Tokyo, Japan) and photos were taken periodically.

Passaging and expansion

When the fibroblast-like cells reached 80–90% confluence, they were rinsed with PBS and detached with 0.20% trypsin (Gibco) at 37°C for 2 minutes. An equal volume of DMEM containing 10% fetal bovine serum was then added to neutralize trypsin activity and the cell suspension was centrifuged at 1 400 r/min with r = 13.5 cm for 5 minutes. The pellet was resuspended and plated at a dilution of 1:2.

Phenotypic analysis

To detect surface antigens, passage 3 cells were collected and resuspended in PBS at a concentration of 1 × 10^7 cells/mL. A total of 10 μL of the following antibodies was added to each tube containing 50 μL of cell suspension and were incubated at room temperature in the dark for 15–20 minutes. The antibodies included mouse monoclonal phycoerythrin (PE) or fluorescein isothiocyanate

(FITC)-labeled anti-CD13-PE, CD14-FITC, CD29- PE, CD31-FITC, CD34-PE, CD44-FITC, CD45-FITC, CD90-FITC (all from BD Biosciences, San Jose, CA, USA) and appropriate mouse FITC- or PE-conjugated IgG1 (BD Biosciences) were used as controls. The cells were washed with PBS, centrifuged at 1 000 r/min with r = 13.5 cm for 3 minutes and resuspended in 200 μL PBS before analyzing by flow cytometry (BD Biosciences) using 2.5 Win 95/NT 4.0 Cell Quest software (BD Biosciences).

Adipogenic, osteogenic, and neural differentiation analysis

The differentiation analysis was performed 24 hours after plating passage 3 umbilical cord mesenchymal stem cells into 6-well plates at a density of 3 000 cells/cm^2. For osteogenic induction, 0.1 μmol/L dexamethasone (Sigma), 0.05 mmol/L ascorbic acid-2-phosphate (Sigma) and 10 mmol/L β-glycerophosphate (Sigma) were added to the growth media. For adipogenic induction, 1 μmol/L dexamethasone, 5 μg/mL insulin, 0.5 mmol/L isobutylmethylxanthine (Sigma), and 60 μmol/L indomethacin (Sigma) were added to the growth media. For neural induction, 10 ng/mL basic fibroblast growth factor, 100 ng/mL brain-derived neurotrophic factor and 1% insulin-transferrin-selenium-A (Gibco) were added to the growth media. All the induction media were changed twice weekly and the cells were observed with phase contrast microscopy (Olympus).

After induction, the cells were fixed with 4% paraformaldehyde (Sigma) and stained with oil red O (Sigma) and alizarin red (Sigma) to view neutral lipid vacuoles in adipocytes and calcium deposition in osteocytes, respectively. The positive cells were observed and recorded with a light microscope (Olympus). The cells were subjected to immunofluorescence analysis after fixation with paraformaldehyde. The cells were washed twice with 100 mmol/L glycine in PBS and permeabilized in 0.1% Triton X-100 (Sigma) for 20 minutes at 4°C. Nonspecific binding was blocked with PBS containing 5% bovine serum albumin (Sigma) for 30 minutes. The cells were then incubated overnight at 4°C with mouse anti-human neuron-specific enolase, glial fibrillary acidic protein and myelin basic protein (1:100; all from Chemicon, Temecula, CA, USA). Then the cells were washed twice in PBS before incubation with the FITC-conjugated rabbit anti-mouse IgG (1:100; Chemicon) for 45 minutes at room temperature. The cell nuclei were stained with Hoechst 33258, followed by observation and recording of positive cells with a fluorescent microscope (Olympus).

Cell labeling and in vivo migration of umbilical cord mesenchymal stem cells in rat models of experimental glioma

The cell labeling procedures were performed according to the protocols of manufacturers. In brief, umbilical cord mesenchymal stem cells were incubated at the concentration of 1 μmol/L CM-DiI (Invitrogen, Eugene, OR, USA) in working solution for 5 minutes at 37°C, and an additional 15 minutes at 4°C. Then the cells were washed with PBS twice and resuspended in fresh PBS at a concentration of 1 × 10^5 /μL.

The cell implantation procedure was performed with a stereotactic technique[53]. Each animal was anesthetized with ketamine/xylazine and immobilized on a stereotactic apparatus. After disinfection and incision of the head skin, a blurred hole was made at the site 1 mm posterior to bregma and 3 mm right to midline, without damaging the dura. A 26-gauge needle was inserted 4 mm ventral from the dura, where 5 × 10^5 C6 tumor cells (Cell Resource Center, Shanghai Institute for Biological Sciences, part of the Chinese Academy of Sciences) in 5 μL of PBS were inoculated using a 10 μL Hamilton glass syringe (Hamilton Company, Reno, NV, USA).

One week after tumor inoculation, group 1 received contralateral ventricular injection of 5 μL PBS containing 5 × 10^5 umbilical cord mesenchymal stem cells labeled with CM-DiI at 1.3 mm posterior to bregma, 3 mm left to midline and 3.5 mm beneath the dura. Group 2 received equal number of umbilical cord mesenchymal stem cells at previous sites of tumor inoculation. As controls, group 3 and group 4 received 5 μL PBS without umbilical cord mesenchymal stem cells *via* contralateral ventricular injection or intratumoral administration, respectively. The rats were anesthetized and sacrificed 2 weeks after umbilical cord mesenchymal stem cell transplantation. The brains were removed and sectioned for histological analysis using hematoxylin-eosin staining under light microscopy, and migration analysis by staining nuclei with Hoechst 33258 (Sigma) under fluorescent microscopy.

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(Reviewed by Wallace M, Norman C, Wang D, Hu W)
(Edited by Mu WJ, Qiu Y, Li CH, Song LP, Liu WJ, Zhao M)