Human umbilical cord blood-derived mesenchymal stem cells inhibit C6 glioma via downregulation of cyclin D1

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Abstract

Aims and Background: Glioma is difficult to treat and despite advances, outcomes remain poor and new treatment modalities are required. We studied the inhibitive effects of human umbilical cord blood-derived mesenchymal stem cells (UCB1MSCs) on glioma growth. Material and Methods: UCB1MSCs were identified in mice by flow cytometric analysis, and neurogenic differentiation by immunohistochemistry. C6 cells were injected subcutaneously into the posterior right flank of each mouse. Dil1labeled UCB1MSCs were administrated by intravenous (IV) or intratumoral (IT) injection. Tumor blood vessel density was detected by counting the number of CD34-positive cells with endothelial morphology. Cyclin D1 protein expression was detected by immunohistochemistry and Western blot analysis. Results: A 26% reduction in overall tumor volume was observed after IV UCB1MSCs treatment, 36% in animals who received IT UCB1MSCs. UCB1MSC administration was associated with reduced neovascularization. We identified a 48% and 27% reduction in the number of cyclin D1-positive cells in mouse glioma tissues treated with UCB1MSCs IV and IT, respectively. Conclusion: We demonstrated that UCB1MSCs potently inhibit glioma growth, reduce neovascularization, and decrease cyclin D1 protein expression in vivo. IV or IT UCB1MSC administration significantly inhibits glioma growth, and may represent a promising new therapy.

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Introduction
Despite advances in the conventional treatment, malignant glioma continues to have a very poor prognosis. New trials combining chemotherapeutic agents in an orthotopic model and trialing new target agents have been attempted; the results of these trials have not been satisfactory. Therefore, it is necessary to develop innovative and effective treatments for glioma. Stem cells may perform crucial functions that might be used in designing novel treatments for malignant glioma.

Recent studies have demonstrated cytotoxicity of umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) against human malignant glioma cells in vitro. The most common method of administration is injection, both intravenously and intratumorally. It has been shown that administration of endometrial regenerative cells by intravenous (IV) or intratumoral (IT) injection leads to a significant inhibition of glioma growth. Aarif observed that in an in vivo model of Kaposi’s sarcoma (KS), IV injection of human mesenchymal stem cells (MSCs) hone to sites of tumorigenesis and potently inhibit tumor growth. UCB-MSCs have also demonstrated selective tropism for malignant cells, and have the ability to inhibit tumor growth unmanipulated.

Overexpression of cyclin D1 enhances cell cycle progression from the G1 to S phase and increases cell proliferation. Many studies have shown that cyclin D1 is frequently overexpressed in human cancers as a result of gene amplification, oncogene-induced signaling, or a mutation that disrupts degradation.

In this study, we aimed to elucidate the mechanism of neurogenic differentiation of UCB-MSC inhibition of C6 glioma in vivo. We compared the results of multiple IV and IT injections of UCB-MSCs to determine whether UCB-MSCs have the ability to hone to glioma cells. Finally, we assessed whether UCB-MSCs inhibit cyclin D1 expression in vivo.

### Material and Methods

Collection of human umbilical cord blood

Human UCB samples (n=6, gestational age, 36-40 weeks) were obtained from patients treated in the Department of Obstetrics and Gynecology of the first Affiliated Hospital of Zhenzhou University. UCB was collected from the umbilical veins after obtaining informed consent from patients. This study protocol was approved by our institutional ethics committee.

Culture of MSCs from UCB

To isolate mononuclear cells (MNCs), each UCB unit was diluted in a 1:1 ratio with phosphate buffered saline (PBS) and carefully loaded onto Ficoll-Hypaque solution (1.077 g/mL, Institute of Hematology, China Academy of Chinese Medical Sciences, China). After density gradient centrifugation at 800 g for 16 min at room temperature, MNCs were removed from the interphase, washed twice with PBS, resuspended in Dulbecco’s modified essential medium/nutrient mixture F-12 (DMEM/F12) at a 1:1 ratio (Invitrogen Corp., USA), and supplemented with 10% fetal bovine serum (FBS; Invitrogen Corp.). In addition, the medium also contained 5 ng/mL of fibroblast growth factor (bFGF; Beijing Double Heron Pharmaceutical, China). After counting, the cell suspension was seeded in 75 cm² tissue culture flasks (Corning, USA) at a concentration of 1 × 10⁶ cells/mL. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and the medium after 3 days.

Fluorescent dye CM-Dil labeling of UCB-MSCs

Chloromethyl-benzamidodialkylcarbocyanine (CM-Dil; Beyotime Institute of Biotechnology, Shanghai, China) was prepared in 1 mg/mL solution using dimethyl sulfoxide and used for labeling. UCB-MSCs from the third passage were digested with 0.25% trypsin, suspended in PBS, quantified, labeled with CM-Dil (2 mL/10⁶ cells), incubated at 37°C for 10 min, washed twice with PBS, and resuspended for future use.
Identification of UCB-MSCs by flow cytometric analysis

UCB-MSCs were harvested and washed in ice-cold PBS. To characterize cell surface expression of the typical marker proteins, the cells were labeled with the following human antibodies: CD29-PE, CD44-FITC, HLA-ABC-FITC, HLA-DR-FITC, CD34-PE, CD45-PerCP, FITC-IgG1, PerCP-IgG1, and PE-IgG2a (BD Bioscience, USA). Approximately 10,000 cells were measured using a FACScan flow cytometer (Becton Dickinson), and the results were analyzed with CellQuest software (Becton Dickinson). Three independent experiments were performed to confirm the results.

Culture of C6 cells

C6 cells (a gift from Professor Fusheng Liu, Beijing Neurosurgical Institute, China) were cultivated in DMEM (Gibco BRL, Gaithersburg, MD, USA) containing 10% FBS (Gibco BRL) in a 5% CO₂ cultivator at 37°C. C6 cells were a glioblastoma cloned from a rat glial tumor induced by N-nitrosomethylurea after a series of alternate culture and animal passages. S-100 production increases 10-fold as cells grow from low density to confluency (Organ: brain; Disease: glioma; Cell type: glial cell).

Animal model

Mice (BALB/C, nu/nu) were obtained from Shanghai SLACCAS Laboratory Animal Company, Ltd. Animals from 6 to 10 weeks of age were used, with more than a 2-week separation in each experiment. The mice were housed in microisolator cages under sterile conditions and observed for at least 1 week to ensure good health before initiating experiments. Lighting, temperature, and humidity were centrally controlled and recorded on a daily basis. The mice were implanted subcutaneously with 2 × 10⁶ C6 cells (suspended in 100 µL PBS) on day 0, in their right posterior flanks. The animals were divided into 3 groups: Group A (n=6, control group) received 20 µL sterile PBS without UCB-MSCs in area of C6 implantation on day 2. Group B (n=6) received 4 × 10⁶ Dil-labeled UCB-MSCs in 1 mL sterile PBS via the caudal vein (IV) on days 0, 5, and 10. Group C (n=6) received 2 × 10⁶ Dil-labeled UCB-MSCs in 20 µL sterile PBS in the area of C6 implantation (IT, subcutaneously), on day 2. The injections were uneventful with no adverse reactions. It takes 6 days for a tumor to manifest after injecting C6 cell line in mice. Tumor growth and progression were monitored by biweekly sliding caliper measurements. All the experiments were performed according to protocols approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute (NHLBI) and by the Institutional Animal Care and Use Committee of Zhenzhou University. All the mice were sacrificed on day 30.

Tumor volume measurement

Using Vernier caliper measurements, tumor volume was calculated according to the following formula: \( V = \frac{1}{2} \times C \times D^2 \). \( V, C, \) and \( D \) represent tumor volume, length, and width, respectively.

Fluorescence microscopic observation

After sacrifice, tumor tissue was frozen, embedded, cut into 10-20 µm sections, and observed under fluorescent and optical microscopy.

Immunohistochemistry staining

To investigate whether UCB-MSCs can differentiate into neuron-like cells, immunocytochemistry was performed for nestin, GFAP, and NSE protein expression. Frozen sections were collected, thawed, and air dried for 30 min, fixed in 100% acetone for 5 min at room temperature, rinsed in PBS (pH 7.4) 3 times for 5 min, incubated in blocking solution (4% nonfat milk and 2% normal horse serum) for 60 min, and incubated with nestin, GFAP, NSE (mouse monoclonal anti-human antibody; Beijing Zhong Shan Golden Bridge Biotechnology Co. Ltd., China), CD34 (C-18) (1:200, goat poly-IgG; Santa Cruz), cyclin D1 (1:200, diluted in 2% milk, mouse monoclonal IgG, Santa Cruz, Delaware, USA) overnight at 4°C. The next day, sections were rinsed in PBS, biotinylated horse anti-goat antibodies (Vector Laboratories, Burlingame, USA.) were applied, goat anti-mouse antibodies (sc-2031, Santa Cruz) were applied at 1:200 for 2 h and 1% H 2 O 2 /PBS for 10 min, and the
avidin-biotin complex kit (ABC, Vector Laboratories) was used and visualized with diaminobenzidine (DAB). Sections were counterstained with hematoxylin, viewed with the microscope, and the pictures were stored in the computer for analysis. Paraffin slides were deparaffinized and placed in a pressure cooker with Antigen Unmasking Solution (Vector H-3300, Vector Laboratories) according to the manufacturer’s instructions, before blocking. The assessment of microvessel density was performed in the following manner: CD34-stained sections were scanned at a low magnification (40×) to determine the areas with the highest number of microvessels, or "hot spots." Microvessels were counted at 200× magnification in 2 hot spots on each section as well as average microvessel density (MVD).

Western blot analysis

C6 glioma tissues of 3 groups of nude mice were harvested and homogenized in volumes of homogenization buffer. Samples (40-50 mg of total protein) was directly isolated by lysing the cells into Laemmli buffer containing 2% SDS, 10% glycerol, 2% 2-mercaptoethanol, and 0.002% bromphenol blue in 75 mM Tris-HCl. The samples were heated to 95°C for 10 min before separating on 10% Tris/Glycine/SDS acrylamide gels (Bio-Rad, Hercules, USA). The proteins were subsequently transblotted to polyvinylidene difluoride (PVDF; Millipore) membranes and blocked for 2 h at room temperature in 5% dry milk. Immunoblots were incubated for 2 h at 37°C with rabbit anti-cyclin D1 (Santa Cruz). After 3 washes with TBS/0.05% Tween-20, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz) for 1 h at 37°C. Protein signals were visualized using the SuperSignal West Pico Chemiluminescent Substrate (PIERCE). Images of blots were captured with an Apple scanner, and densitometric analysis of bands was performed using Scion software for Macintosh. Background values were subtracted, and multiple blots were combined for statistical analysis. β1Actin protein was visualized and detected as above.

Statistical analysis

All the data were expressed as the mean ± standard deviation (SD) and were analyzed by using SPSS 13.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) and a post least significant difference (LSD) method was performed. A P value of less than 0.05 was considered to be statistically significant.

Results

Immunophenotype of UCB1MSCs by flow cytometric analysis

UCB-MSCs were positive for CD29, CD44, and HLA-ABC and negative for CD34, CD45, and HLA-DR. Representative histograms and their respective isotype controls are shown with dotted lines in [Figure 1].

Nestin, GFAP, and NSE protein expression in UCB1MSCs

Immunocytochemistry demonstrated nestin, GFAP, and NSE positivity in UCB-MSCs [Figure 2], indicating that UCB-MSCs can differentiate into neural-like cells.

UCB-MSCs administration inhibits C6 tumor growth

A 26% and 36% reduction in tumor volume was observed after IV and IT injection of UCB-MSCs (P < 0.05), respectively [Figure 3].

Fluorescent microscopy

Red fluorescence-labeled UCB-MSCs were not seen in the control group. A small number of Dil-labeled UCB-MSCs were seen in gliomas after IV treatment, and a large number after the IT treatment [Figure 4].
UCB-MSC administration associated with reduced neovascularization

Tumor blood vessel density was detected by counting CD34-positive cells with endothelial morphology. As seen in [Figure 5], fewer blood vessels were seen in tumors from animals treated with IV and IT UCB-MSCs as compared with the controls. Specifically, we found a 29% vs 57% reduction in blood vessel density as compared with the control, IV, and IT groups (Group A, B, and C: 78 ± 18, 55 ±14, 33 ± 8; P < 0.05). (Figure 5)

Reduction of cyclin D1-positive C6 cells in UCB-MSC-treated animals

Cyclin D1 protein was expressed in the endochylema. Under a 400× visual field, the number of cyclin D1-positive cells (10 fields/section, 20 sections/group) was calculated and compared among groups. There was a 48% and 27% reduction in cyclin D1-positive cells in mouse glioma tissues treated with IV and IT UCB-MSCs (12.7 ± 2.7 and 18.0 ± 2.0, respectively, P < 0.05) as compared with the controls (24.6 ± 5.6; [Figure 6]). (Figure 6)

Western blot analysis of cyclin D1 protein

There was a significant reduction (P < 0.05) in cyclin D1 expression after both IV and IT treatment, as compared with the controls (F = 31.68, 30 days after injection of UCB-MSCs). [Figure 7]a and b illustrates the effects of UCB-MSCs on cyclin D1 expression in each group, including 30 days after injection and statistical analysis. (Figure 7)

Discussion

Our fibroblast-like cells showed characteristic features of UCB-MSCs: plastic-adherent capabilities. Their immunophenotype, similar to that of MSCs reported by Kern et al, [15] was negative for CD34, CD45, and HLA-DR and positive for CD29, CD44, and HLA-ABC [Figure 1]. In addition, these MSC-like cells have the capability to differentiate into neural-like cells [Figure 2] that can inhibit the growth of C6 glioma. Therefore, we believe that these cells most probably represent UCB-MSCs.

Kang et al[9] found that UCB-MSCs, with or without activation, demonstrate significant cytotoxicity against human malignant glioma cells in vivo. In addition, when cytokine-activated, they have significantly higher cytotoxicity than unactivated UCB-MSCs. Differentiated immune effectors from cytokine-activated UCB-MSCs did not increase. Likewise, activated UCB-MSCs secreted more immune response-related proteins (interleukin-4, interferon-γ) than unactivated UCB-derived MSCs. We believe that neurogenic differentiation of UCB-MSCs enables secretion of more immune response-related proteins (interleukin-4, interferon-γ) that inhibit the growth of glioma. When direct cell-cell contact occurs, in mediating the effects of UCB-MSCs on C6 cells, inhibition is more obvious.

After the injection of Dil-labeled UCB-MSCs into the caudal vein, visible UCB-MSCs were seen in the tumor tissue [Figure 2], suggesting a tumor-tropic action of UCB-MSCs. Ho et al[16] showed that stem cell migration toward brain gliomas was related to MMP-1 expression, because MMP-1 or its protease-activated receptor inhibition significantly weakened the ability of stem cells to migrate toward the tumors. Kim et al[17] demonstrated that UCB-MSCs exhibit a stronger ability to migrate into the brain glioma cell lines (U-87 MG, LN 18, U138, U251) than into the bone marrow mesenchymal stem cells. RT-PCR and immunohistochemistry results demonstrated that UCB-MSCs expressed interleukin 8, CXCR1 receptor, and CXCR2 receptor, [18] which provides theoretical evidence for venous treatment of gliomas.

Conclusion

Human umbilical cord blood-derived mesenchymal stem cells inhibit C6...
The treatment of C6 gliomas with IV or IT UCB-MSCs profoundly inhibits tumor growth. Suppression of tumor growth was not associated with necrosis, but was characterized by fewer new blood vessels identified morphologically and by anti-CD34 staining. Given that conditioned media of UCB-MSC cultures stimulates human umbilical vein endothelial cell proliferation in vitro, [19] we hypothesized that UCB-MSCs may inhibit tumor growth; as a result, less angiogenesis was present.

Inappropriate cyclin D1 overexpression has been shown in human gliomas. [20],[21],[22] Cyclin D1 expression significantly correlates with the degree of malignancy, invasion, and prognosis of patients in a variety of human carcinomas, including glioma. [23],[24],[25] We demonstrated that IV- or IT-induced UCB-MSCs inhibit cyclin D1 expression in mouse glioma. This finding suggests that UCB-MSCs may provide a novel treatment for malignant glioma. The underlying mechanism remains poorly understood and requires further investigation.

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References


