

Therapeutic Benefit of Human Umbilical Cord Derived Mesenchymal Stromal Cells in Intracerebral Hemorrhage Rat: Implications of Anti-inflammation and Angiogenesis

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Key Words

Human umbilical cord • Mesenchymal stromal cells • Intracerebral hemorrhage • Anti-inflammation • Angiogenesis

Abstract

Cell-based therapy represents a promising strategy in the treatment of neurological disorders. Human umbilical cord tissue has recently been recognized as an ideal source of mesenchymal stromal cells due to accessibility, vast abundance and safety. Here, an intracerebral hemorrhage (ICH) rat model was established by injection of bacterial collagenase VII and CM-Dil labeled human umbilical cord tissue derived mesenchymal stromal cells (UC-MSC) were intracerebrally transplanted into rat brain 24h after ICH. The results demonstrated that UC-MSC treatment significantly improved neurological function deficits and decreased injury volume of ICH rats. Leukocytes infiltration, microglial activation, ROS level and matrix metalloproteinases (MMPs) production were substantially reduced in peri-ICH area in cell-treated group as compared with PBS control at day 3 post-transplantation. In addition, UC-MSC treatment significantly increased vascular density in peri-ICH area and transplanted UC-MSC were found to be able

to incorporate into cerebral vasculature in ipsilateral hemisphere at 14 days after transplantation. In summary, intracerebral administration of UC-MSC could accelerate neurological function recovery of ICH rat, the underlying mechanism may ascribe to their ability to inhibit inflammation and promote angiogenesis. Thus UC-MSC may provide a potential cell candidate for cell-based therapy in neurological disorders.

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Introduction

Intracerebral hemorrhage (ICH), which results from the spontaneous rupture of an intracranial vessel, is a subtype of stroke with high morbidity and mortality accounting for about 15% of all deaths from stroke and occurs frequently as a major complication of thrombolytic therapy for acute ischemic stroke [1-3]. Increasing evidence suggests that inflammatory mechanisms are involved in ICH-induced brain injury [4, 5]. Infiltrated leucocytes and activated microglia and accumulated inflammatory factors such as reactive oxygen species (ROS) and Matrix metalloproteinases (MMPs) after ICH are detrimental to neural cells [6-8]. In this regard, anti-

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inflammatory strategies have been tested in preclinical trials and proven to be effective in the treatment of ICH [7, 9, 10].

Mesenchymal stromal cells (MSC), also known as mesenchymal stem cells [11], have distinct immunosuppressive properties in addition to their multi-differentiation capacity [12, 13]. MSC could interact with both innate and adaptive immune cells and have a profound inhibitory effect on their functions [14, 15]. Moreover, evidence of the immunosuppressive and anti-inflammation effects of MSC were presented in neuroimmunological and neurodegenerative diseases such as experimental autoimmune encephalomyelitis (EAE), amyotrophic Lateral Sclerosis (ALS) and Parkinson's disease [16-18]. Therefore, MSC transplantation may inhibit inflammation after ICH and reduce subsequent neurological damage.

Angiogenesis is considered to be a natural protective mechanism in response to cerebral ischemia [19, 20]. Interestingly, a recent study reported that cerebral angiogenesis also occurred after collagenase-induced ICH in rats [21]. Thus, therapeutic angiogenesis provides a promising strategy in the treatment of stroke. MSC possess angiogenic potentials which is indicated not only by the ability to integrate into blood vessels but also by their effect to induce host angiogenesis after injury by secretion of angiogenic factors [22, 23]. Recently, we also demonstrated that MSC could promote angiogenesis in rat cerebral ischemia [24]. Thus, we speculate that MSC treatment may also be able to enhance angiogenesis after ICH induced injury.

We have recently established a method to readily isolate MSC from human umbilical cord tissue (UC-MSC) [25], a better MSC source to bone marrow in regard to the accessibility, cell number and safety. Here, we aim to address whether UC-MSC transplantation could suppress inflammation and promote angiogenesis in rats after ICH, and leads to improved neurological function.

Materials and Methods

Isolation and identification of UC-MSC

Human umbilical cords from both sexes were collected from full-term deliveries with informed consent of the mother after caesarian section and aseptically stored at 4°C in sterile PBS supplemented with 200 U/ml penicillin-streptomycin (Sigma). Tissue collection for research was approved by the institutional review board of the Chinese Academy of Medical Science and Peking Union Medical College. MSC were isolated using the method as described previously [25]. Briefly, the cord

was cut with scissor into pieces and digested with 0.075% collagenase II (Sigma, St Louis, MO, USA) for 30 minutes and with 0.125% trypsin (Gibco, Grand Island, NY, USA) for another 30 minutes with gentle agitation at 37°C. The supernatant was centrifuged at the speed of 250g for 15 minutes and the cells were suspended in fresh media and plated onto non-coated plastic flasks after rinsed with PBS for three times. Nonadherent cells were removed through changing the medium after 3 days. DMEM/F12 (1:1) (Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin-streptomycin (Sigma), 1% glutamine (Sigma), and 10ng/ml epidermal growth factor (Sigma) was used as the maintenance and expansion system. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and passaged at 60–80% confluency. Flow cytometric analysis of cell surface markers and osteogenic, adipogenic and chondrogenic induction were used to identify these cells as described previously [24, 25].

ICH model construction

Male Sprague-Dawley rats (Experimental Animal Center of Academy of Military Medical Science, Beijing, China) weighing 230 to 260 g were used in this study. All experimental procedures were approved by the Care of Experimental Animals Committee of the University Hospital and by the institutional review board for the use of human cells. ICH was induced by stereotaxic, intrastriatal administration of bacterial collagenase by previously described methods with modification [21]. In brief, after anesthetized by intraperitoneal injection of 10% chloral hydrate (30 mg/kg), the rats were placed in a stereotaxic frame. A burr hole was made, and a 10-μl needle was inserted through the burr hole into the striatum (coordinates: 0.5 mm posterior, 6.0 mm ventral, and 3.0 mm lateral to the bregma). ICH was induced by the administration of collagenase type VII (0.5U in 2 μl sterilized PBS, Sigma) over a period of 5 minutes. After placement for another 5 minutes, the needle was gently removed. The burr hole was sealed with bone wax, and the wound was sutured. For the sham-operated group, 2 μl PBS without the collagenase was injected to the same site. During the procedure, body temperature was maintained at 37±0.5°C by use of a thermistor controlled heat blanket.

UC-MSC labeling and transplantation procedure

After trypsinized and washed with PBS, UC-MSC were labeled with 3 μg/ml CM-DiI (Molecular Probes, Eugene, OR, USA) at 37°C for 10 minutes and then at 4°C for another 15 minutes. After washed with PBS for three times, UC-MSC were diluted at the concentration of 2×10⁴ cells/μl and transplanted into rat peri-ICH area stereotaxically (coordinates: 0.5 mm posterior, 3.5 mm ventral, and 3.0 mm lateral to the bregma) at 24 hours after ICH. In cell-treated group (n=20), each rat received 10 μl cell-PBS mixture with a total number of 2×10⁵ UC-MSC at the speed of 1 μl/minute and placed for another 5 minutes thereafter. Equal volume of PBS was administrated in control group (n=16) and rats in sham-group (n=16) received no treatment. In order to evaluate the immunogenicity of human UC-MSC in rat brain, equal number of cells were also injected

into normal animals (n=10) and the immune response was detected by CD11b (for macrophage and activated microglia; Chemicon, Temecula, CA, USA) and Myeloperoxidase (MPO, for leukocytes; abcam, Cambridge, UK) immunostaining 3 days after injection. Rats did not receive any immunosuppressive medications.

Neurological Evaluation

Neurological evaluation, including modified neurological severity score (mNSS) and Morris water maze test [26, 27], was performed before and at 1, 7, 14, 21, 28 days after ICH by 2 individuals blinded to the experimental groups.

The mNSS [26] is a composite of motor, sensory, reflex, and balance tests. Neurological function was graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). In the severity scores of injury, 1 score point is awarded for the inability to perform the test or for the lack of a tested reflex; thus, the higher the score, the more severe is the injury.

Morris Water Maze test [27] was used to measure learning and memory. Briefly, the rat was placed in a 4-ft-diameter water tank that is visually separated into four quadrants. In the center of one quadrant, the goal quadrant, a platform is hidden 1 inch below the waterline. The rat was placed in the tank at one of four start locations (north, south, east, west) and swam for up to 60 seconds until it found the platform. Once the animal located the platform, it remained on it for 20 seconds. In the event that an animal did not locate the platform, the observer positioned the animal on the platform at the end of the 60-second swim period. During a 2- to 4-minute intertrial interval, the animals were kept warm by infrared heating lamps. The temperature of the water maze was $21 \pm 0.5^\circ\text{C}$. The latency and path length of the rat were measured by a video tracking system interfaced to a computer (Olympus, Tokyo, Japan).

Histology

Luxol fast blue/cresyl violet (Sigma) staining and the Fluoro-Jade B (FJB, Sigma) staining were performed according to published protocols [28, 29]. Cells permeable to FJB were marked for cell death. FJB can sensitively and selectively detect degenerating neurons. At 3 days after treatment, rats were over anaesthetized and decapitated and coronal frozen sections of brain were prepared for FJB staining, degenerating neurons were counted by sampling an area $0.5 \times 0.5 \text{ mm}^2$ immediately adjacent to the haematoma in at least three fields using a magnification of 200 in at least three sections per animal and expressed as cells/ mm^2 , areas with large blood vessels were avoided. Quantitative analysis of injury volume was conducted at 28 days after treatment. Rat brains were dissected into 2-mm coronal sections and the total injury volume of each brain was calculated with Image-Pro Plus software (Media Cybernetics, Carlsbad, CA) by summation of the injured area of all slices. Cell counting and volume quantitation were conducted by two observers blinded to the experimental treatment.

In situ detection of ROS

Production of reactive oxygen species (ROS) after ICH was investigated by *in situ* detection of oxidized hydroethidine [30]. Hydroethidine (a redox-sensitive probe) is oxidized by

superoxide to ethidium, which intercalates within the DNA and the nucleus fluoresces bright red. Hydroethidine (in DMSO; Molecular Probes) was diluted to 20mM/ml in PBS and sonicated. At 1 or 3 days after ICH, rats were injected intraperitoneally with 2.5ml of hydroethidine. Brains were harvested 2 h later and sectioned at 20 μm . The brain frozen sections were incubated with 300nM DAPI (Molecular Probes) in PBS to identify nuclear for 2 min in a dark chamber. Ethidium was visualized on a fluorescent microscope (excitation, 510 nm; emission, 580 nm) and photographed using a digital camera system and double exposure to produce images of ethidium and DAPI. Ethidium, indicative of the presence of ROS, was quantified using the same methods as FJB staining analysis.

In situ zymography

In situ zymography was used to detect MMPs activity as previous study described using a commercial kit (EnzChek Gelatinase Assay kit; Molecular Probes) [10]. Briefly, fresh frozen sections (10 μm thick) obtained at 3 days post-transplantation were incubated with DQ gelatin conjugate, a fluorogenic substrate, at 37°C for 1 h and washed and fixed in 4% paraformaldehyde in PBS. Cleavage of DQ gelatin by MMPs results in a green fluorescent product (excitation, 495 nm; emission, 515 nm).

In vitro induction of UC-MSC differentiation to endothelial cells

Confluent UC-MSC were cultured in DMEM/F12 medium supplemented with 2% FCS, 100 U/ml penicillin-streptomycin, 50 ng/ml vascular endothelial growth factor (VEGF; Sigma) and 10 ng/ml basic fibroblast growth factor (bFGF; Sigma) and under hypoxic (3% O_2) condition oriented to endothelial cell differentiation, Medium was changed every 3 days. After induced for 7 days, Immunostaining was performed to detect the expression of endothelial specific marker vWF.

Uptake of DiI-Ac-LDL (Molecular Probes), which is a well known function of mature endothelial cells [31], was also conducted to verify cellular differentiation towards endothelial phenotype by incubating the post-induced UC-MSC with 10 $\mu\text{g}/\text{ml}$ DiI-Ac-LDL in serum-free medium at 37°C for 4 hours.

Immunohistochemical Assessment

Animals were reanesthetized with 10% chloral hydrate (30 mg/kg) and rat brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde, and dehydrated with 30% sucrose in 0.1 M PBS for overnight, and frozen in powdered dry ice. A series of adjacent 10 μm coronal cryostat sections were cut from the brain tissue corresponding to the coronal coordinates bregma 0.5mm to -1.5mm. To visualize the CM-DiI labeled UC-MSC expression of neuron/glia specific markers, sections were processed for immunofluorescence using primary antibodies against neuronal specific enolase (NSE; Chemicon), microtubule associated protein 2 (MAP2; Chemicon), glial fibrillary acidic protein (GFAP; Dako, Carpinteria, CA, USA), α -smooth muscle actin (ASMA; Chemicon), von Willebrand factor (vWF; Dako), myeloperoxidase (MPO, Abcam, Cambridge, UK) and CD11b

(Chemicon). Fluorescein isothiocyanate conjugated antibody (FITC; Chemicon) was employed for immunofluorescence identification. Negative control sections from each animal received identical preparations for immunohistochemical staining, except that primary antibodies were omitted.

Vascular density was analyzed at 14 days post-transplantation with ASMA immunostaining to visualize vascular regions. Five equidistant slices (AP=-1.5 to AP=0.5 from the bregma) from five animals per group were processed for analysis. Per slice, five random areas of peri-ICH region were photographed at a magnification of 200 and used for measurement. Vascular areas were calculated using Image-Pro Plus software package and vascular density was expressed by the percentage of vascular areas in randomly selected regions.

Statistical analysis

Data are presented as mean±SD. Comparisons of continuous variables between more than two groups were performed by a one-way ANOVA. If the *F* distribution was significant, a *t*-test was used to specify differences between groups. *p*<0.05 was considered statistically significant. The SPSS software package (SPSS, Chicago, Illinois, USA) was used for the statistical tests.

Results

Characterization of UC-MSC

UC-MSC are plastic-adherent and appear bipolar spindle-like morphology in standard culture conditions. Flow cytometric analysis demonstrated that UC-MSC could express CD105(SH2), CD73(SH3), CD90, CD29, CD44 and HLA-ABC, and lack expression of CD34, CD45, CD14 and HLA-DR (Fig. 1A). After induction with osteogenic, adipogenic or chondrogenic conditional medium, UC-MSC could differentiate into osteocytes, adipocytes and chondrocytes, as detected by positive staining of von Kossa, Oil Red O and Alcian blue, respectively (Fig. 1B).

Neurological benefits after UC-MSC treatment

ICH is usually accompanied by characteristic behavioral deficits. ICH rats received UC-MSC treatment showed significant neurological improvement on mNSS from 7 days post-transplantation and the improvement lasted for up to 4 weeks post-transplantation as compared with PBS control (Fig. 2A). The neurological benefit was also observed in Morris water maze test (Fig. 2B), a test for learning and memory ability. The primary consequence of ICH is haematoma, which can lead to secondary brain injury resulting in severe neurological deficits and sometimes delayed fatality [32]. At 14 days after treatment, we found that the injury area was significantly

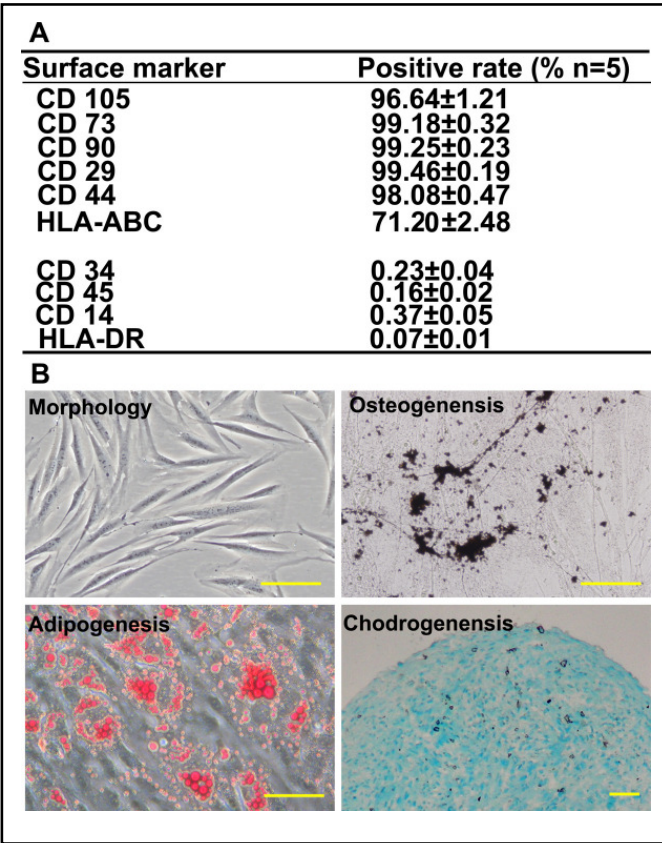


Fig. 1. Charaterization of UC-MSC. A, Cell surface phenotype profile of UC-MSC. B, Morphology of UC-MSC and osteogenic, adipogenic and chondrogenic differentiation of UC-MSC *in vitro*. Scale bars=100 μm.

reduced in UC-MSC-treated group compared to the PBS control as shown by Luxol fast blue/cresyl violet staining (Fig. 2C). Quantitative analysis revealed that the injury volume in PBS-treated group was 44.12±5.78 mm³, while it reduced to 31.87±7.26 mm³ in UC-MSC treated group (Fig. 2D, *P*<0.05).

FJB can sensitively and selectively detect degenerating neurons [29]. Compared with control, the number of degenerating neurons in peri-ICH area in UC-MSC received rats was decreased dramatically at 3 days after treatment. The number of FJB-positive cells were 302±46 and 398±30 per mm² in cell-treated group and control, respectively (*P*<0.01) (Fig. 3A,B). These data suggest UC-MSC are neuroprotective for ICH-induced brain damage.

In vivo differentiation of transplanted UC-MSC

In order to evaluate immunogenicity of UC-MSC in case of xenotransplantation, UC-MSC were injected into normal rat brain and at 3 days after injection, host immune reaction were detected. We found no obvious immune

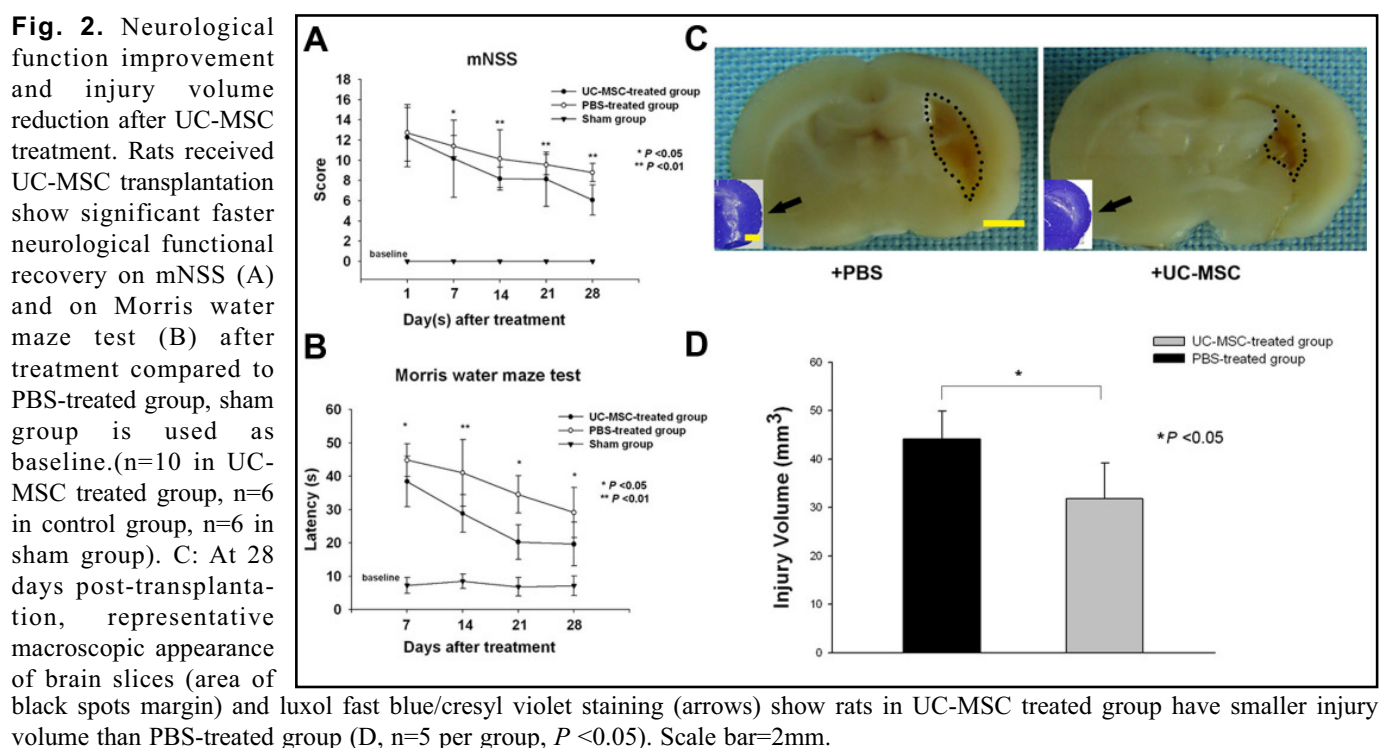
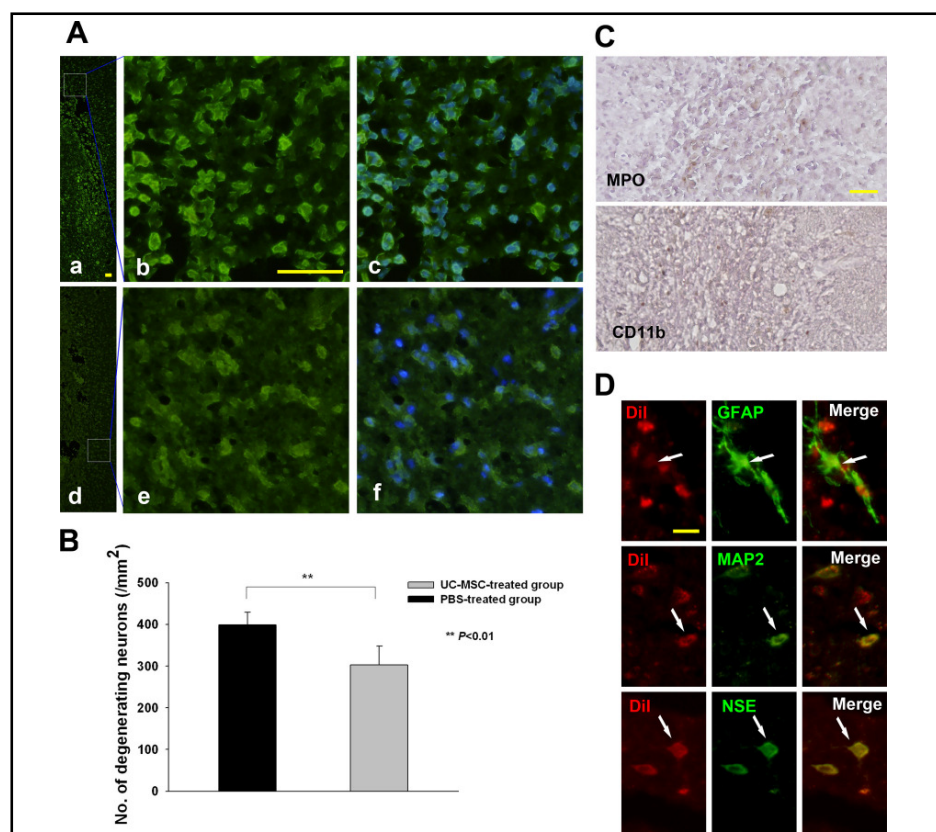


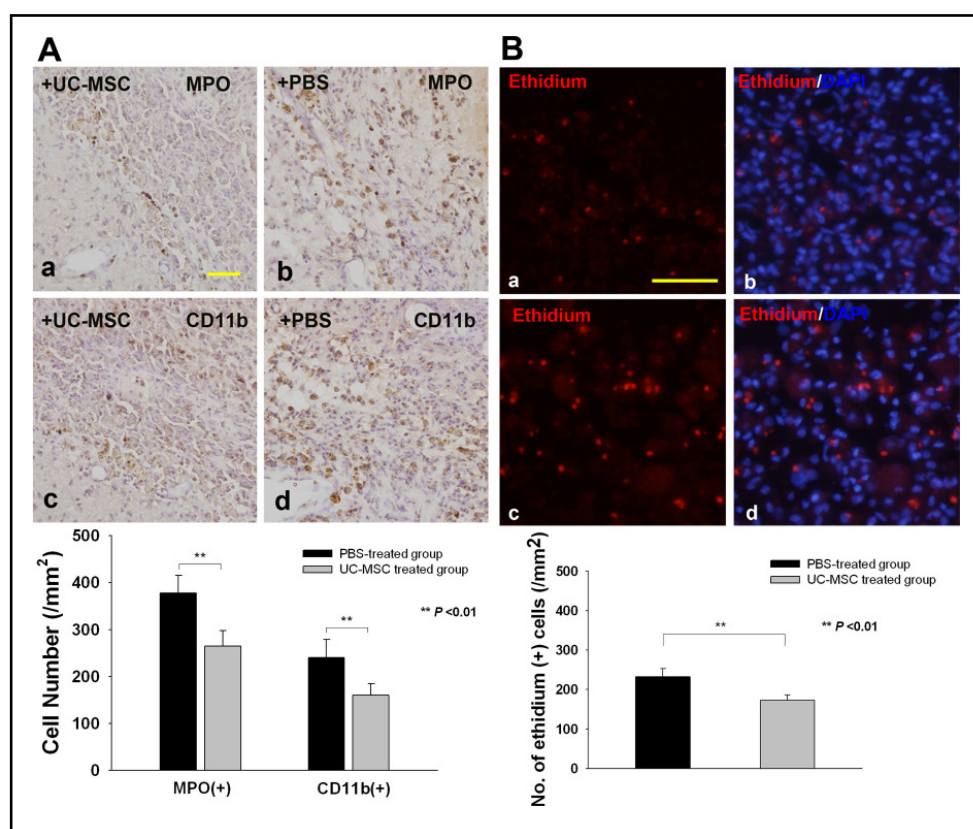
Fig. 3. Neuroprotection of UC-MS-C treatment and UC-MS-C *in vivo* differentiation. A: Neurons degenerating in peri-ICH were substantially reduced in UC-MS-C received rats (d) compared with PBS control (a) as shown by FJB staining (b, e are magnification of boxed areas in a and d, respectively. c and f were merged image of b and e with DAPI), this difference is significant (B, n=5 per group). C shows that UC-MS-C do not elicit immune response in rat brain as detected by MPO and CD11b immunostaining. D: CM-DiI (red) labeled UC-MS-C colocalize with neural/glial cell markers including GFAP, MAP2 and NSE (green) in rat brain. Scale bar: A, C=50 μ m, D=20 μ m.



response against human MSC in rat brain as shown by MPO and CD11b immunostaining (Fig. 3C). With the use of fluorescent CM-DiI for UC-MS-C labeling, we found these cells could survive at least 4 weeks in injured rat

brain, apart from a part of cells clustered in injection site, the majority of cells spread to peri-ICH areas in injured hemisphere (data not shown). A small amount of donor cells showed positive staining with GFAP ($\approx 2.5\%$), MAP2

Fig. 4. Anti-inflammatory effects of UC-MSC in rats after ICH. A: MPO and CD11b immunostaining show that UC-MSC treatment (a, c) obviously attenuates leucocytes infiltration and microglial activation around ICH area compared to PBS control (b, d) at 3 days after treatment. (n=5 per group) B: ROS production in peri-ICH region of UC-MSC -treated rats(a) and control (c) as shown by ethidium (red), b and d are merged images with DAPI of a and c, respectively (n=5 per group). Scale bars=50 μ m.



($\approx 4\%$) or NSE ($\approx 3\%$) antibody (Fig. 3 D), which demonstrated UC-MSC had potential to differentiate to neuron/glia cell types under *in vivo* condition, though with limited frequency.

Anti-inflammation effects of UC-MSC in ICH

Leukocytes infiltration and microglial activation are two important features of the ensuing pathological change after ICH, the degree of which correlates with the outcome of the ICH-induced injury [33, 34]. It has been reported that leukocytes infiltration and microglial activation reached a maximum at 3 to 7 days after ICH [10, 33]. In cell-treated rats, leukocytes infiltration and microglial activation as shown by MPO and CD11b immunostaining [10, 35] were substantially attenuated at 3 days post-transplantation compared with PBS control (Fig. 4A). The number of MPO and CD11b positive cells around ICH area in cell treated group and control were 264 ± 33 and 378 ± 37 per mm² ($P < 0.01$), 159 ± 24 and 239 ± 39 per mm² ($P < 0.01$), respectively (Fig. 4A).

ROS, a major factor in the pathogenesis of neuronal damage, is generated by various cells after ICH [34, 36]. On day 1 after collagenase injection, the peri-ICH region exhibited significantly increased level of oxidized hydroethidine compared with the contralateral uninjected side of the brain (data not shown). ROS level around

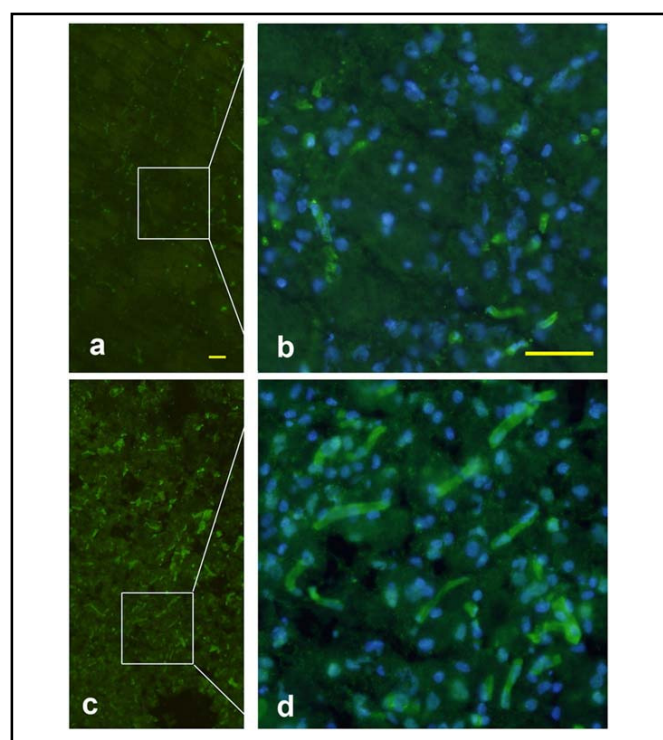


Fig. 5. UC-MSC treatment downregulates MMP production in ICH rats. At 3 days post-transplantation, *in situ* zymography shows that MMP activity (green) is dramatically reduced in UC-MSC received rats (a, b) as compared with PBS control (c, d), b and d are magnification of boxed area of a and c and merged with DAPI. Scale bars=50 μ m.

lesion remained high in PBS-treated rats at 3 days after injection (Fig. 4B-c), while the number and intensity of ethidium positive cells was drastically decreased in UC-MSC treated rats (Fig. 4B-a). The number of ethidium positive nuclear in cell treated group and control were 173 ± 13 and 232 ± 31 per mm^2 , respectively ($P < 0.01$).

In addition, MMPs activity as assessed by *in situ* zymography was also dramatically reduced after UC-MSC treatment in peri-ICH region as compared with control (Fig. 5).

Vascular density increase after UC-MSC treatment and endothelial cell differentiation of UC-MSC

Vascular structures were recognized by vWF immuostaining. We found that UC-MSC treatment significant increased the vascular density in ICH boundary zone at 14 days after treatment compared to PBS treated group ($11.01 \pm 2.29\%$ versus $7.47 \pm 1.53\%$, $P < 0.05$, Fig. 6A). In addition, the majority of blood vessels around ICH region contained transplanted UC-MSC which either incorporated into cerebral vasculature or located in vessel wall and a part of these incorporated donor cells expressed endothelial cell specific protein vWF (Fig. 6B), which is consistent with previous reports [37, 38].

In vitro induction also revealed angiogenic potential of UC-MSC. Under hypoxic condition and in presence of VEGF and bFGF, UC-MSC could differentiate into endothelial cells as shown by immuno-positive of vWF (Fig. 6 C-a) and by uptake of LDL-ac-DiI (Fig. 6 C-c), which is considered to be one of the typical functions for mature endothelial cells [31].

Discussion

Recent advancement and clinical experience suggest that MSC are promising cell candidate in cell based therapy in a variety of diseases. This promise is based not only on the evidence that MSC transplantation could be beneficial in the repair of tissue injury, but also on that MSC are poorly recognized by HLA-incompatible hosts due to their limited immunogenicity [39], which allows them to be used in allogeneous or xenogeneous conditions. In addition, MSC could be isolated from almost all organs and tissues [40] and the immunoprivileged property of MSC seem to be independent of their origin [41, 42], making it prospective and necessary to search for an ideal cell source of MSC for the future therapeutic use. In this setting, we and others suggest that UC-MSC may provide

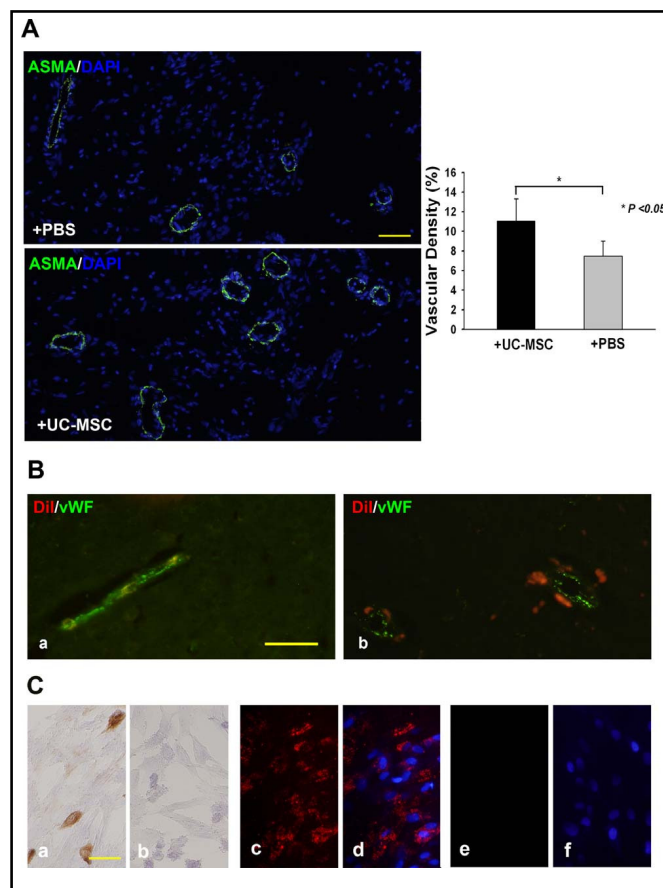


Fig. 6. Increased vascular density in peri-ICH region after UC-MSC treatment. Representative images indicate that vascular density around ICH areas in UC-MSC treated group is obviously higher than control group at 14 days after treatment (A, $n=5$ per group). B: CM-DiI labeled UC-MSC could incorporate in endothelium as shown by colocalization with vWF (a) and some of them also stay in the blood vessel wall (b). C: UC-MSC are immuno-positive for vWF (a) and capable of LDL-ac-DiI uptake (c) under specific condition *in vitro*. b and e are controls of a and c, respectively; d, f were merged image of c, e with DAPI. Scale bars=50 μm .

more utility than MSC from other tissues such as bone marrow and cord blood on the basis of accessibility, successful rate of isolation, cell abundance, proliferation rate and safety [25, 43, 44].

We demonstrated that UC-MSC treatment reduced injured lesion and accelerated functional recovery in rats after ICH, while it seems hard to attribute these effects to cell replacement since transdifferentiation was not a common event in injured brain. Though MSC possesses potential to differentiate into neural cells *in vitro* [25, 45], observations from our and other groups revealed that donor cells did not frequently transform to neural cell identity after transplantation [24, 26, 46, 47]. Also, whether

these engrafted cells establish functional synaptic connections with host tissue remains indeterminate.

Since cell replacement was not responsible for the functional improvement of ICH animals, there should be other pathways that mediated the benefit of UC-MSC transplantation. Recently, several studies reported that MSC treatment could lead to therapeutic effects by downregulating inflammation in a variety of diseases such as graft-versus-host disease (GVHD) [48], lung injury [49], myocardial infarction [50] and neural diseases [18]. In ICH-induced brain injury, inflammation is also regarded as an important role in the pathologic process [4, 5]. Thus anti-inflammation effect of MSC may be of value in the treatment of ICH. We previously observed that UC-MSC could inhibit immune cells proliferation and inflammatory factors production *in vitro* (unpublished data). Consistently, in present study we found significant decrease of leukocytes infiltration and microglial activation in injured brain after UC-MSC treatment. The *in vivo* results are also supported by MSC transplantation in EAE and Parkinson's disease mice [16, 18]. Patients of ALS, a progressive fatal neurodegenerative disease in which reactive microglia activation play a main role in the pathogenesis, also gained significant neurological improvement by intraspinal injection of MSC [17]. In line with the reduction of infiltrated leukocytes and activated microglia, the neurotoxic factors ROS and MMPs which are mainly derived from these inflammatory cells were also decreased after UC-MSC treatment. Therefore, anti-inflammation effects of UC-MSC may contribute to some extent to the improved outcome in the treatment of ICH.

However, it is unknown how donor MSC interact with these inflammatory cells and regulate their functions in ICH. Evidence from *in vitro* studies by coculture of MSC with inflammatory cells implies that the anti-inflammatory effect of MSC may involve cell-cell contact and/or paracrine mechanisms [14, 42, 51]. In a model of bleomycin induced lung injury, it has been documented that MSC could inhibit inflammation and protect lung tissue by production of interleukin 1 receptor antagonist [49]. Another group reported that MSC could recruit lymphocytes and inhibit their functions through a concert of chemokines and nitric oxide in a GVHD model [52]. These data reveal that both cell-cell contact and soluble factors may be involved as to the *in vivo* anti-inflammation effect of MSC. Although the inflammatory process is different among various diseases, it is reasonable to speculate that the transplanted MSC faced a foreign, inflammatory environment in ICH brain

and induced immunosuppressive milieu through a combination of cell-cell contact with paracrine mechanism, which not only enhanced their survival, but also rescued neural cells from dying.

Another mechanism that mediated the benefit of UC-MSC for ICH may attribute to the effect to promote angiogenesis. Angiogenesis, the process of new blood vessel formation from pre-existing ones, is fundamental to brain development and repair [19]. It has been demonstrated that cerebral angiogenesis was upregulated after ICH [21] thus providing implications in treatment with pro-angiogenic strategies. MSC are capable of differentiating into vascular cells [23] and producing various angiogenic cytokines [42, 53], and have been proven to be able to promote angiogenesis in various diseases including neural injury [22, 38, 54]. Accordingly, we have also reported that UC-MSC transplantation in myocardial infarction [37], critical limb ischemia [55] and cerebral ischemia animals [24] could lead to enhancement of angiogenesis. Not surprisingly, we observed similar results by transplantation of UC-MSC in ICH rats. Therefore, pro-angiogenic effect of UC-MSC may represent one of the mechanisms underlying their benefit to ICH. In addition, we observed many donor cells located in vessel wall or differentiated into endothelial cells in peri-ICH region, suggesting increased angiogenesis by MSC may be associated with direct incorporation into cerebral vasculature [24, 55].

Recently, perivascular region is regarded as the *in vivo* niche for MSC in terms of their extensive distribution and the similarity between MSC and pericytes [56, 57]. And it is proposed that MSC are responsible for stabilizing blood vessels, maintaining tissue and immune system homeostasis under physiological conditions, and they become more active in the repair of focal tissue injury [56]. Consistent with this hypothesis, our data demonstrated that UC-MSC could repress the immune response and inhibit inflammatory cells-mediated destruction of the injury site in ICH. In another aspect, UC-MSC were able to incorporate into cerebral vasculature to stabilize blood vessels and promote angiogenesis. It is notable that endogenous MSC were probably activated after ICH and participated into the neural recovery, while the extent to which may be insufficient to elicit remarkable recovery. By additional exogenous MSC supply, this recovery process may be amplified, which subsequently accelerated the neurological improvement.

In conclusion, we demonstrated that intracerebral

administration of MSC from human umbilical cord tissue was beneficial to rats after ICH, and the underlying mechanisms may correlate with anti-inflammatory and pro-angiogenic effects of these cells. Further studies were warranted to find out the soluble factors and signaling pathways which involved in these processes, as such UC-MSC based therapy in neurological diseases would be facilitated.

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