Simvastatin combined with bone marrow stromal cells treatment activates astrocytes to ameliorate neurological function after ischemic stroke in rats

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1. Introduction
Research indicates that astrocytes play an important role in secreting antioxidants, regulating extracellular levels of ions and neurotransmitters, reabsorbing glutamate as energy substrate in support of neuronal metabolism, being involved in the control of cerebral blood flow, participating in regulation of blood–brain barrier (BBB) permeability, and forming glial scars after cerebral ischemia (Sofroniew and Vinters, 2010; Barreto et al., 2011; Kowiański et al., 2013). Previously, Li et al. (2008) found that infarct size was larger in glial fibrillary acidic protein (GFAP) knockout mice than in wild-type mice, and this was attributed to the reduction of glutamate uptake and decrease of plasminogen activator inhibitor-1 and endothelin B receptor expression. Under pathological conditions, astrocytes could release neurovascular trophic factors, e.g., glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), stromal cell-derived factor-1 (SDF-1), and vascular endothelial growth factor (VEGF)-A (Miyazaki et al., 2001; Argaw et al., 2012; Mele and Jurič, 2014; Shin et al., 2014). As has been well demonstrated, SDF-1, BDNF, and GDNF were capable of activating the phosphatidylinositol 3-kinase (PI3K)/AKT and/or extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signaling pathways in glia, while the reactive PI3K/AKT signal pathway could further improve astrocytes to express VEGF and angiopoietin-1 (Ang1) (Lu et al., 2009; Lui et al., 2012; Jang et al., 2014). Due to their pivotal role in the nervous system, astrocytes are now recognized as key participants in brain development, function, and disease.

Studies indicated that bone marrow stromal cell (BMSC) transplantation was a promising approach for the treatment of stroke. Intravenous BMSC administration could significantly enhance neurological outcome, which is related to BMSCs migrating to injured tissue and releasing neurovascular trophic factors (Chen et al., 2001; Li et al., 2002). Moreover, BMSCs could induce astrocytes and mouse brain endothelial cells (ECs) to express VEGF and Ang1, resulting in angiogenesis and vascular stabilization.
As a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, simvastatin exhibits neuroprotective action in acute stroke. Some studies demonstrated that simvastatin could decrease infarct volume and improve neurological function through reducing neuronal excitotoxicity and cytotoxic edema, decreasing oxidative stress response, protecting the BBB, promoting angiogenesis and neurogenesis, and ameliorating stroke-induced peripheral immunodepression after cerebral ischemia (Chen et al., 2009; Zacharek et al., 2009; Jin et al., 2013; Campos-Martorell et al., 2014; Zhu et al., 2014). Recently, some reports found that combination treatment with simvastatin and BMSCs more significantly improved neurological function scores and arteriogenesis and angiogenesis and reduced infarct volume and brain edema superior to any single treatment method; furthermore, simvastatin could advance BMSCs’ migration and differentiation (Cui et al., 2009; Xu et al., 2009; Pirzad Jahromi et al., 2012). However, the exact effects and mechanism of simvastatin combined with BMSC treatment on astrocytes after ischemic stroke are still indistinct and need to be elucidated. In this research, we investigated whether combination treatment with simvastatin and BMSCs could enhance reactive astrocytes to express SDF-1α, VEGF, BDNF, and GDNF in the IBZ, and mediate astrocytes’ Akt/ mTOR and ERK 1/2 pathways in the therapeutic procedures.

2. Materials and methods

2.1. Materials
Simvastatin was obtained from Sigma Chemical Co. (USA). Astrocytes were supplied by the American Type Culture Collection (ATCC); GIBCO Dulbecco’s modified Eagle medium/F12 (DMEM/F12) was the product of Life Technologies (USA). Primary antibodies of AKT, p-AKT, mTOR, p-mTOR, ERK 1/2, and p-ERK 1/2 were products of Cell Signaling Technology, Inc. (USA). The other materials and reagents were from commercial sources.

2.2. BMSC culture and identification
BMSCs were isolated from fresh complete bone marrow of 3-week-old male Sprague Dawley (SD) rats’ tibias and femurs (n = 4), and the culture protocol was done according to our previous report (Zhao et al., 2013). In general, the cells were cultured with DMEM/F12 cell medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin for 48 h. Subsequently, cell medium of BMSCs was replaced every 48 h and passage was done during the cell logarithm growth period with cell fusion of 80%. At passage 3, digestive BMSCs were incubated with fluorescence-conjugated antibodies including CD44-PE, CD90-PE, CD45-FITC, CD34-PerCP, and phosphate-buffered saline (PBS; negative control) in a black chamber at 4 °C for 30 min. Then the cells were washed by PBS and fixed with 4% paraformaldehyde, and the flow cytometry with the Cellquest system (Becton, Dickinson and Company, USA) was used to analyze purity.

2.3. Middle cerebral artery occlusion model establishment and group division
The present investigation conforms to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised in 1996. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Macau University of Science and Technology. Male SD rats (n = 45) weighing 240–260 g and supplied by the Laboratory Animal Center of Sun Yat-Sen University (Guangzhou, PR China) were anesthetized with 10% (w/v) chloral hydrate (3.0 mL/kg, i.p.) and a permanent middle cerebral artery occlusion (MCAo) model was established according to the method described by Longa et al. (1989). Briefly, the middle cerebral artery of SD rats was obstructed with a 4–0 surgical nylon suture (length of 20–22 mm, determined by body weight) coated with polysilane, which was inserted into the internal carotid artery from the external carotid artery, and then a 5-point scale neurological deficit score as reported by Yilmaz et al. (2006) was used to evaluate the MCAo model. Only animals with a score of 2 (circling to the right) were selected for group division. A total of 45 MCAo rats were included in this study, and the rats were randomly divided into: 1) MCAo group (n = 15), 2) BMSC group (n = 15), and 3) simvastatin (Sim) + BMSC group (n = 15). One milliliter of PBS or BMSC suspension solution (2 × 10^6 cells/mL) was respectively injected into rats in the MCAo or BMSC and Sim + BMSC groups via caudal vein at 3 h after the model establishment; simvastatin (1 mg/kg) (Cui et al., 2009) was gavaged daily for 7 days in the Sim + BMSC group. A sham-operated group (n = 15) was also established to exclude the influence of the operation process on the therapeutic effect.

2.4. Neurological functional evaluation
We evaluated neurological function in each group at 3 h and at 1, 3, 5, and 7 days after operation following the principle of Garcia et al.’s (1995) neurological score. The principle include six sections: 1) evaluating animals’ spontaneous activity; 2) symmetry of four limbs’ movements when rat was held suspended by the tail and symmetric forepaws
were used as the control.

2.5. Immunofluorescence staining

On day 7 after the operation, the rats in the MCAo, BMSC, and Sim + BMSC groups were sacrificed, and brains were harvested and fixed in fresh 4% paraformaldehyde for histological studies. Fresh frozen coronal sections of 40 µm in thickness were cut on a cryostat microtome (Thermo Fisher Scientific Shandon Cryotome FSE). Every 5th slice for a total of 6 sections was used for immunohistochemical staining. To identify whether astrocytes were activated and expressed chemokine and neurovascular trophic factors after stroke, antibody against GFAP (1:1000) was respectively couple-stained with SDF-1α (1:100), VEGF (1:200), BDNF (1:1000), and GDNF (1:20) according to the manufacturer’s protocol. In brief, the slices were first incubated with primary antibodies at 4 °C overnight, followed by secondary antibodies including Alexa Fluor 488 goat antirabbit IgG (H+L) (1:200) or goat antirabbit IgG H&L (Cy3) (1:100) preadsorbed secondary antibodies for 1 h at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). A LSM710 laser-scanning confocal microscope (Carl Zeiss, Germany) was used to detect fluorescent labeling of every slice. Five nonoverlapping fields of one slice at the penumbra cortex were observed for each rat under a magnification of 10 × 10 and 10 × 40 in confocal images. Image-Pro Plus software (Media Cybernetics) was applied to analyze the proteins’ expression. In control experiments, primary antibodies were replaced with PBS.

2.6. Astrocytes induced by oxygen–glucose deprivation and group division

Culture medium of astrocytes was exchanged with glucose-free feeding medium, and cells were incubated under hypoxic atmosphere (95% N₂ and 5% CO₂) in an anaerobic chamber (HP015, HITECH, P.R. China) for 1 h at 37 °C. After oxygen–glucose deprivation (OGD), astrocytes were immediately rinsed with PBS and the medium was replaced with fresh DMEM/F12 feeding medium. Subsequently, astrocytes (5 × 10⁵ cells/mL) induced by OGD were incubated with: 1) DMEM/F12 culture medium alone, 2) DMEM/F12 culture medium from BMSCs that had been cultured for 12 h, and 3) conditional medium from BMSCs that had been treated with simvastatin (1 µmol/L) (Cui et al., 2009) for 12 h. OGD-induced astrocytes were respectively incubated with different culture mediums for 5 min and 60 min, and western blot assay was performed to determine p-AKT/AKT, p-mTOR/mTOR, and p-ERK1/2/ERK1/2. Normal cultured astrocytes with DMEM/F12 were used as the control.

2.7. Western blot assay

Protein samples were extracted from cultured astrocytes in four groups (normal group, OGD group, BMSC group, and Sim + BMSC group) with TRIzol (Invitrogen, USA), and protein concentration was determined by enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, P.R. China) according to the method suggested by the manufacturer. Subsequently, protein samples were electrophoresed on gradient sodium dodecyl sulfate-polyacrylamide gel and electrotransferred to PVDF membranes in Tris–glycine transfer buffer. Membranes were blocked in 5% (w/v) nonfat dry milk for 1 h at room temperature, followed by incubation with primary antibodies including anti-AKT (1:1000), anti-p-AKT (1:2000), anti-mTOR (1:1000), anti-p-mTOR (1:1000), anti-ERK 1/2 (1:1000), and anti-p-ERK 1/2 (1:2000) at 4 °C overnight. Then the membranes were gently washed with TBST (Tris-HCl pH 8.0, 125 mm NaCl, 0.1% Tween 20) three times and incubated with goat antirabbit IgG (H+L) secondary antibodies (1:5000) at room temperature for 1 h. After the final washes, the membranes were scanned and the signals of reactive bands were quantified using the Odyssey Infrared Imager (LI-COR Corporate, USA). Phosphorylation levels of the targeted proteins were analyzed by total levels of corresponding proteins. Western blots were duplicated three independent times.

2.8. Statistical analysis

All results were expressed as means ± standard deviation. The significance for the difference among groups was analyzed with SPSS 19.0 (USA) by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. Differences were considered to be statistically significant at P < 0.05.

3. Results

3.1. BMSC morphology and characterization

As illustrated in Figure 1, 72 h after the initial seeding, the morphology of adherent cells seemed to be fibroblastic-like mononuclear cells and it took a logarithmic growth appearance; from passage 2, the cells became uniform and grew in whirlpool, radial, or parallel patterns. We thus choose passage 3 of cells to identify characterization of BMSCs by flow cytometry analysis. The results indicated that the rate of CD34-positive cells (as a hematopoietic stem cell marker) was 2.32%, of CD45-positive cells (as a lymphocyte antigen) was 7.24%, and of CD44 and CD90 (both as characteristic BMSC surface markers) was as high as 97.56% and 99.07%, respectively (Figure 2). The flow cytometry results demonstrated that our primary BMSCs were highly purified.
Figure 1. BMSC morphology under microscope. A: BMSCs were primary-cultured for 72 h; B: BMSCs at passage 2 (scale bar: 200 µm).

Figure 2. BMSC phenotype identification by flow cytometry. The rate of CD34-positive cells was 2.32%, of CD45-positive cells was 7.24%, and of CD44 and CD90 was respectively 97.56% and 99.07%.
3.2. Simvastatin combined with BMSCs promoted poststroke outcome of neurological function

To evaluate whether combination treatment with simvastatin and BMSCs could attenuate neurological injury after stroke, a neurological functional test was performed. As observed in Figure 3, neurological scores in the combination treatment group began to markedly enhance from day 1 compared with those in the MCAo and BMSC groups, and the increasing tendency continued to the 7th day (P < 0.01 vs. MCAo group; P < 0.05 vs. BMSC group). In addition, neurological scores in the BMSC group were notably higher than those in the MCAo group at 3 and 7 days (P < 0.05). These data indicated that combination treatment promoted neurological functional recovery superior to BMSC alone treatment.

3.3. Simvastatin combined with BMSCs activated astrocytes and improved astrocyte-derived SDF-1α, VEGF, BDNF, and GDNF expressions

As one of a family of proteins of intermediate filament, GFAP's expression has been regarded as the hallmark of reactive gliosis. To test whether combination treatment could activate astrocytes in the IBZ after stroke, GFAP expression was measured by immunofluorescence staining. As shown in Figure 4, GFAP-positive signals (green) in the combination treatment group were higher compared with those in the BMSC and MCAo groups (P < 0.01), and this result demonstrated that simvastatin plus BMSCs could notably activate astrocytes.

Our triple fluorescent immunostaining images displayed that SDF-1α and VEGF (green) primarily aggregated in the GFAP-positive (red) area in the IBZ (Figure 5A). The quantitative analysis showed that the expressions of SDF-1α and VEGF overlapping the GFAP-positive expression area in the Sim + BMSC group were higher compared with those in the MCAo and BMSC groups (P < 0.01). This suggested that combination treatment of simvastatin and BMSCs could increase astrocyte-derived SDF-1α and VEGF expressions. Some research reports stated that reactive astrocytes could express BDNF and GDNF; furthermore, transplanted BMSCs possessed the ability to facilitate the expressions of BDNF and GDNF in the IBZ (Chen et al., 2005; Béjot et al., 2011; Wei et al., 2012). We observed images of GFAP double-staining with BDNF and GDNF on the 7th day after stroke and found that the overlaps of GFAP-positive (red) and BDNF/GDNF-positive (green) signals were primarily distributed around the ischemic core, as shown in the BMSC and Sim + BMSC groups. It illustrated that BMSC could promote astrocyte-derived BDNF/GDNF expressions and combination treatment might possess an additive effect (Figure 5B). Quantitative analysis displayed that simvastatin plus BMSCs could significantly enhance BDNF and GDNF expressions in reactive astrocytes (P < 0.01 compared with MCAo and BMSC groups).

3.4. Simvastatin plus BMSCs activated OGD-induced astrocytes' AKT/mTOR signal cascade

To further study the intracellular mechanism of the effect of simvastatin plus BMSCs on astrocytes, we investigated AKT/mTOR and ERK 1/2 pathway proteins' activation status in astrocytes by western blot assay. As shown in

Figure 3. Neurological functional outcome from 3 hours to 7 days post-surgery. Garcia neurological score evaluation was performed at 3 h and 1, 3, 5, and 7 days after the operation in each group. Data as presented as mean ± standard deviation. *P < 0.05, **P < 0.01 vs. MCAo at corresponding time; #P < 0.05 vs. BMSC group at corresponding time.
Figure 4. Combination treatment with simvastatin and BMSCs promoted astrocytes to express GFAP in the IBZ on the 7th day after ischemic stroke. Immunofluorescence staining of GFAP (green) in the IBZ (magnification 100×) and quantitative analysis. Data are expressed as mean ± standard deviation (n = 15). *P < 0.05, **P < 0.01 compared with MCAo; ##P < 0.01 compared with BMSC group. The experiment was repeated 3 times, and representative pictures are shown. Scale bar: 100 µm.

Figure 5. Combination treatment with simvastatin and BMSCs enhanced reactive astrocytes to express SDF-1α, VEGF, BDNF, and GDNF in the IBZ on the 7th day after stroke. A: Immunofluorescence staining of SDF-1α (green) and VEGF (green) overlapping GFAP (red) in astrocytes is presented (magnification 400×); quantitative analysis of SDF-1α and VEGF expressions in GFAP-positive area. B: Positive cells of GFAP (red) double-immunofluorescence stained with BDNF (green) and GDNF (green) (magnification 400×); relative expressions of BDNF and GDNF in GFAP-positive area were analyzed. Data are expressed as mean ± standard deviation (n = 15). **P < 0.01 compared with MCAo; #P < 0.05, ## P < 0.01 compared with BMSC group. The experiment was repeated 3 times, and representative pictures are shown. Scale bar: 20 µm.
Figure 6A, phosphorylated expressions of AKT and mTOR in the Sim + BMSC group increased significantly after 5 min of treatment (P < 0.01 vs. BMSC and OGD groups), and phosphorylated expression of mTOR in the Sim + BMSC group was still higher than that in the OGD group at 60 min (P < 0.05). We concluded that simvastatin combined with BMSC activated the AKT/mTOR pathway in OGD-induced astrocytes. We also found that combination treatment did not enhance phosphorylated expression of ERK 1/2 after 5 min of incubation, and moreover, the Sim + BMSC group obviously decreased ERK 1/2 expression in astrocytes at 60 min (Figure 6B).

4. Discussion

Studies had demonstrated that simvastatin plus BMSCs could improve angiogenesis and reduce infarct volume and brain edema, which would obviously contribute to neurological deficits amelioration after stroke (Cui et al., 2009; Xu et al., 2009; Pirzad Jahromi et al., 2012). In our present study, we further verified that combination treatment with simvastatin and BMSC could promote astrocyte-derived SDF-1α, VEGF, GDNF, and BDNF expressions in IBZ, as well as activate the astrocytic AKT/mTOR signal pathway, which helped to decrease neurological functional injury.

As the most abundant nonneuronal cells in the brain, astrocytes play important roles in cerebral ischemia. Pekny and Pekna (2004) reported when the GFAP gene was knocked out in transgenic mice, reactive gliosis began to attenuate and the signs of central nervous system degeneration became more prominent. By investigating GFAP expression, our present study firstly confirmed that combination treatment with simvastatin and BMSC could significantly activate astrocytes in the ischemic zone. Reactive astrocytes expressed a variety of chemokine and neurovascular trophic factors including SDF-1α, VEGF, GDNF, and BDNF, which are involved in the tolerance of the body to severe ischemic injury, angiogenesis, neurogenesis, and neurite outgrowth after stroke (Swanson et al., 2004; Gao et al., 2005; Shen et al., 2010; Shin et al., 2014).

The chemokines SDF-1α and VEGF have important effects on the migration and angiogenesis of stem cells (e.g., neural progenitor cells/stem cells, endothelial progenitor cells, and mesenchymal stem/stromal cells). Xu et al. (2007) reported that SDF-1α and VEGF from hypoxia-induced astrocytes could promote neural progenitor cells migration in vitro. Moreover, SDF-1α cooperated with VEGF-modulated circulating MSCs’ recruitment and entrapment as a vital step of neovascularization, and SDF-1α increased VEGF expression to induce MSCs’ differentiation into ECs from multiple approaches (Zhou et al., 2007; Wang and Zou, 2014). Among families of neurotrophic factors, BDNF and GDNF are found to be the major factors that improve stroke recovery and brain plasticity after ischemia (Béjot et al., 2011; Kuric et al., 2013). In our present study, we demonstrated that combination treatment was obviously capable of enhancing SDF-1α, VEGF, BDNF, and GDNF expressions in astrocytes compared with BMSC alone treatment. In this sense, we think that the combination treatment indeed amplified the therapeutic effect.

To further understand the mechanism of combination treatment effect on reactive astrocytes, we examined the AKT/mTOR and ERK 1/2 pathways in OGD-induced...
Figure 6. Combination treatment of simvastatin and BMSCs activated AKT/mTOR signaling pathway in OGD-induced astrocytes. A: Representative western blot results for p-Akt, Akt, p-mTOR, and mTOR at 5 and 60 min and quantitative analysis. B: Western blot results and analysis of ERK1/2 proteins at 5 and 60 min in OGD-induced astrocytes. Data are expressed as means ± standard deviation. *P < 0.05, **P < 0.01 vs. OGD; ##P < 0.01 vs. BMSC; &&P < 0.01 vs. normal.
astrocytes in vitro. Previous reports showed that the reactive AKT/mTOR signal pathway in astrocytes was beneficial to regulate glutamate transporter 1 and VEGF gene transcription (Wu et al., 2010; Sun et al., 2013). As a major phosphorylating target of mTOR, p70S6K is associated with apoptosis of astrocytes (Pastor et al., 2009). Therefore, rapamycin (mTOR inhibitor) or deletion of p70S6K1 and p70S6K2 notably promoted OGD-induced cell apoptosis in microglia or astrocytes (Pastor et al., 2009; Shang et al., 2011). This suggested that mTOR activation might protect astrocytes against ischemia-induced apoptosis. In addition, a study found that microglia stimulated with SDF-1α could increase Akt and ERK1/2 phosphorylation to regulate interleukin-6 (Lu et al., 2009). In our present study, we found that combination treatment with simvastatin and BMSCs dramatically activated the Akt/mTOR signaling pathway in OGD-induced astrocytes; it should benefit to decrease glutamate toxicity and apoptosis of astrocytes, improve therapeutic angiogenesis, and modulate inflammation after stroke. The efficacy of ERK1/2 activation on neuroprotection is in dispute at present. Szydlowska et al. (2010) observed that the inhibition of ERK1/2 contributed to attenuating glutamate-induced death of astrocytes. Our experimental results showed that combination treatment inhibited the ERK1/2 pathway; it might be related to regulation of glutamate.

In summary, our present study demonstrated that combination treatment with simvastatin and BMSCs could obviously improve reactive astrocytes to express chemokine and neurovascular trophic factors, as well as activate the astrocytic Akt/mTOR signaling pathway. All of the above processes benefit neurological functional recovery. Due to synergic functions of this combination treatment method, our approach might be a more effective therapy for ischemic stroke.

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References


