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Chemokine, vascular and therapeutic effects of combination Simvastatin and BMSC treatment of stroke

Xu Cui [a], Michael Chopp [a,d], Alex Zacharek [a], Cynthia Roberts [a], Mei Lu [b], Smita Savant-Bhonsale [c], Jieli Chen [a,*]

[a] Department of Neurology, E&R Bldg., Room 3091, Henry Ford Hospital, 2799 W Grand Blvd, Detroit, MI 48202, USA
[b] Department of Biostatistics and Research Epidemiology, Henry Ford Hospital, Detroit, MI 48202, USA
[c] Department of Neurobiology, Theradigm, Inc. Baltimore, MD 21227, USA
[d] Department of Physics, Oakland University, Rochester, MI 48309, USA

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ABSTRACT

We investigated the additive therapeutic effect of the combination treatment of stroke with sub-therapeutic doses of Simvastatin, a HMG-CoA reductase inhibitor, and bone marrow stromal cells (BMSCs). Rats were administered Simvastatin (0.5 mg/kg), BMSCs (1×10^6) or combination of Simvastatin and BMSCs starting at 24 h after stroke. Combination treatment significantly improved neurological outcome, enhanced angiogenesis and arteriogenesis, and increased the number of engrafted-BMSCs in the ischemic brain. The number of engrafted-BMSCs and arteriogenesis was significantly correlated with functional outcome. Simvastatin significantly increased stromal cell-derived factor-1 (SDF1) expression in the ischemic brain and chemokine (CXC motif) receptor-4 (CXCR4) in BMSCs, and increased BMSC migration to RBMECs and astrocytes. Combination treatment of stroke upregulates the SDF1/CXCR4 axis and enhances BMSC migration into the ischemic brain, amplifies arteriogenesis and angiogenesis, and improves functional outcome after stroke.
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Introduction

Stroke patients with higher density of cerebral blood vessels fare better and survive longer than those with lower vascular density (Wei et al., 2001). Arteriogenesis and angiogenesis serve as efficient mechanisms to restore perfusion with oxygen and nutrition in the ischemic brain and promote long-term functional recovery in patients treated with or without thrombolysis for stroke (Christoforidis et al., 2005; Wei et al., 2001). Therefore, stimulating arteriogenesis and angiogenesis may provide a treatment strategy for patients with stroke.

Adult bone marrow stromal cells (BMSCs) selectively target the injury site, participate in arteriogenesis and angiogenesis, and induce a neovascular response resulting in a significant increase in blood flow to the ischemic area which aids in repair of injured brain (Al-Khaldi et al., 2003; Chen et al., 2003a; Cui et al., 2008; Zacharek et al., 2007). However, the effect of BMSC transplantation on stroke is dose-dependent (Borlongan et al., 2004; Chen et al., 2001), and the success of a vascular route for BMSC treatment may be limited by the low migration efficiency of the transplanted-BMSCs into the lesioned area (Borlongan et al., 2004; Muller-Ehmsen et al., 2006). Thus, strategies which promote BMSC migration into the ischemic brain may augment the BMSC regenerative treatment of stroke. Chemokines are important factors controlling cellular migration. Stromal cell-Derived Factor-1 (SDF1) and its unique receptor chemokine CXC motif receptor 4 (CXCR4) play an important role controlling cellular migration (Cui et al., 2007).

Combining BMSCs and pharmacological therapy is an attractive approach for neurorestorative treatment of stroke (Chen and Chopp, 2006). HMG-CoA reductase inhibitors (statins) are a class of drugs originally used to lower cholesterol. Statins also possess cholesterol-independent benefits including increasing vascular endothelial growth factor (VEGF), brain-derived neurogrowth factor (BDNF) expression and endothelial nitric oxide synthase (eNOS), and tissue plasminogen activator (tPA) activity, which augment cerebral blood flow, promote angiogenesis and improve functional outcome after stroke (Asahi et al., 2005; Chade et al., 2006; Chen et al., 2005, 2003b). Our previous studies have shown that treatment of stroke with a therapeutic dose of Simvastatin (1 mg/kg) amplifies angiogenesis and vascular stabilization, and promotes arteriogenesis in rats (Chen et al., in press; Zacharek et al., 2007, 2009). However, there are no studies, which evaluate whether combination treatment of stroke with sub-therapeutic doses of Simvastatin and BMSCs amplifies restorative therapy after stroke. In this study, we seek to test whether combination treatment of stroke with sub-therapeutic doses of Simvastatin and BMSCs induces additive functional improvement. We hypothesize, that Simvastatin promotes BMSC migration to the ischemic brain by upregulating SDF1/CXCR4 and increases arteriogenesis and angiogenesis, and thereby improves functional outcome.

* Corresponding author. Fax: +313 916 1318.
E-mail address: jieli@neuro.hfh.edu (J. Chen).
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Materials and methods

All experiments were conducted in accordance with the standards and procedures of the American Council on Animal Care and Institutional Animal Care and Use Committee of Henry Ford Health System.

BMSC culture and labeled with 5-bromo-2′-deoxyuridine (BrdU) or DiI

Rat BMSCs (R-048, P4. Cognate BioServices) were incubated and labeled with BrdU (30 μg/ml, Sigma-Aldrich) for 3 days, as previously described (Cui et al., 2007) or labeled with 1,1″-diolecyl-3,3,3″,3″-tetramethylindodicarbo-cyanine methanesulfonate (Δ^9-DiI, AnaSpec) for 30 min, respectively. Passage 4–5 BMSCs were used.

Animal middle cerebral artery occlusion model and experimental groups

Adult male Wistar rats (Jackson Laboratory) weighing 270 to 300 g were used in all experiments. Transient (2 h) right middle cerebral artery occlusion (MCAo) was induced (Chen et al., in press). Twenty-four hours after MCAo, these rats were treated with either: a) a single dose of phosphate buffered solution (PBS), intravenously injected via a tail vein one time ($n=15$/group); b) sub-therapeutic dose of Simvastatin (0.5 mg/kg, Sigma), gavaged daily for 7 days ($n=13$/group); c) a single sub-therapeutic dose of BMSCs (1×10^6), intravenously injected via a tail vein one time ($n=12$/group); or d) a combination of Simvastatin and BMSCs ($n=12$/group). Our previous studies showed that the effective doses of Simvastatin (1 mg/kg) (Chen et al., in press; Zacharek et al., 2009) and BMSCs (3×10^6) (Chen et al., 2001; Shen et al., 2007; Zacharek et al., 2007) enhance angiogenesis, vascular stabilization and improve functional outcome after stroke in rats, however, a sub-therapeutic dose of BMSC (1×10^6) has marginal or no functional benefits (Chen et al., 2001, 2004, 2003a). Here, sub-therapeutic doses of Simvastatin (0.5 mg/kg) and BMSCs (1×10^6) were used. One set of rats ($n=10$/group) were euthanized 14 days after MCAo for immunostaining; the second set of rats from MCAo control and Simvastatin group ($n=6$/group) were euthanized 3 days after MCAo for Western blot assay. Two rats from the MCAo control group were euthanized 7 days after MCAo for rat brain microvascular endothelial cell (RBMEC) culture. Four rats from BMSCs alone and combination group ($n=2$/group) were euthanized 7 days after MCAo for fluorescent image capture.

Neurological functional tests

A series of functional tests including a modified neurological severity score (mNSS), adhesive-removal test and foot-fault evaluation were performed before MCAo and at 1, 7 and 14 days after MCAo by an investigator who was blinded to the experimental groups, as previously described (Chen et al., 2001, 2004, 2003b).

Histological and immunohistochemical assessment

Rats were sacrificed 14 days after MCAo ($n=10$/group). The brains were fixed in 4% paraformaldehyde and embedded in paraffin. Seven coronal sections of tissue were processed and stained with hematoxylin and eosin for calculation of the lesion volume.

For immunostaining, a standard paraffin block was obtained from the center of the lesion (bregma −1 to +1 mm). A series of 6 μm-thick sections were cut from the block. Every 10th coronal section for a total of 5 sections was used for immunohistochemical staining. Antibody against Alpha smooth muscle actin [αSMA, a marker of smooth muscle cells (SMCs) and pericytes (Cui et al., 2008, 2009), 1:800, DAKO], von Willebrand factor (vWF, a marker of endothelial cells, 1:200, Santa Cruz Biotechnology), Ki-67 (a marker of proliferating cells (Cui et al., 2008, 2009), 1:300, LabVision/NeoMarkers), BrdU (1:100; Boehringer Mannheim), and SDF1 (1:250; Santa Cruz Biotechnology) immunostaining were performed. Control experiments consisted of staining brain coronal tissue sections as outlined earlier, but omitted the primary antibodies.

Quantitation

Lesion volume evaluation was performed, as previously described (Cui et al., 2009).

For quantification of vascular density, perimeter, diameter and vascular SMC (VSMC) or vascular endothelial cell (VEC) proliferation, five slides from the standard reference coronal section of each brain, with each slide containing 8 fields from the ischemic border zone (IBZ) (Cui et al., 2008) were digitized under a 40× objective (BX40; Olympus Optical) using a 3-CCD color video camera (DXC-970MD, Sony) interfaced with a Micro Computer Imaging Device (MCID) software (Imaging Research). The total number of vessels was divided by the total tissue-area to determine vascular density. The perimeter of a total of 20 enlarged vWF-vessels or the diameter of αSMA-artery (mean diameter ≥ 20 μm), and the number of Ki67-VSMCs or Ki67-VECs in a total of 10 enlarged αSMA-vessels or vWF-vessels located in the IBZ were measured in each section using the MCID imaging analysis system, respectively (Chen et al., in press; Cui et al., 2009). Data are presented as the number of αSMA-vessels or vWF-vessels/mm^2, the percentage of the Ki67-VSMCs or Ki67-VECs to total VSMCs or total VECs. For quantitative measurements the number of BMSCs engrafted in the ischemic brain, the total numbers of BrdU-BMSCs both in the ipsilateral and contralateral hemispheres were counted. For SDF1 quantification, the percentage of the SDF1-positive area was measured in the ischemic border area.

Double immunofluorescence and fluorescent vessels staining

To identify whether SDF1-reactive cells co-localized with brain VECs or astrocytes, double immunofluorescence labeling for SDF1 (anti-SDF1-FITC, 1:250; Santa Cruz) with vWF (anti-vWF-Cy3, 1:400, DAKO), and SDF1 with glial fibrillary acidic protein (GFAP, a marker of astrocytes, anti-GFAP-Cy3, 1:1000, DAKO) were performed, as previously described (Cui et al., 2007).

Four rats from BMSC treatment alone and combination group were injected FITC-dextran (1 mL, 50 g/L, 2×10^6 molecular weight; Sigma) via the tail vein 5 min before sacrifice at 7 days after MCAo. Vibratome sections (40 μm) were prepared. Double immunofluorescent images were acquired using fluorescent microscopy (Axiophot2, HB0100 W/2, Carl Zeiss MicroImaging Inc.) with a digital camera (C4742-95, Hamamatsu).

Rat brain microvascular endothelial cell (RBMEC), astrocyte and BMSC culture

To test whether Simvastatin regulates RBMEC or astrocyte SDF1, or BMSC CXCR4 gene and protein expression, RBMEC, astrocyte (CRL-2006, ATCC) and BMSC (R-048, Cognate BioServices) cultures were employed, respectively. RBMECs obtained and cultured, as previously described (Chen et al., in press). The cells were treated with ($n=6$/group): 1) non-treatment for control, 2) Simvastatin 0.1 μmol/L, and 3) Simvastatin 1 μmol/L. The choice of Simvastatin dose is consistent with our previous study (Chen et al., in press). Cells were treated for 3 h or 24 h before harvesting for real-time PCR (RT-PCR) and Western blot assay, respectively.

Western blot

The method of Western blot assay is as previously described (Chen et al., in press; Zacharek et al., 2009). The membrane with protein samples was treated with blocking buffer for 1 h at room temperature,

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