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

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Abstract

We investigated the additive therapeutic effect that 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 Simvastatin with BMSCs starting at 24 hours 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 were 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.

Keywords

bone marrow stromal cell; simvastatin; arteriogenesis; angiogenesis; stroke

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

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Disclosures

None.

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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-Khalidi 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), 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; Chen et al., 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., 2008; Zacharek et al., 2007; Zacharek et al., 2009). However, there are no studies, which evaluate whether combination treatment of stroke with sub-therapeutic doses of Simvastatin and BMSCs amplify restorative therapy after stroke. In this study, we seek to test whether combination treatment of stroke with sub-therapeutic doses of Simvastatin and BMSCs induce additive functional improvement. We hypothesize, that Simvastatin promotes BMSC migration to the ischemic brain by up-regulating SDF1/CXCR4 and increases arteriogenesis and angiogenesis, and thereby improves functional outcome.

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'-dioleoyl-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 grams were used in all experiments. Transient (2 hour) right middle cerebral artery occlusion (MCAo) was induced (Chen et al., 2008). 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); d) combination Simvastatin and BMSCs (n=12/group). Our previous studies showed that the effective doses of Simvastatin (1 mg/kg) (Chen et al., 2008; 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; Chen et al., 2004; Chen et al., 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) was 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; Chen et al., 2004; Chen et al., 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 +1mm). A series of 6 μ m-thick sections was 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 [α SMA, a marker of smooth muscle cells (SMCs) and pericytes (Cui et al., 2008; Cui et al., 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; Cui et al., 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 $\times$ 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 α SMA-artery (mean diameter $\geq 20 \mu$ m), and the number of Ki67-VSMCs or Ki67-VECs in a total of 10 enlarged α SMA-vessels or vWF-vessels located in the IBZ were measured in each section using the MCID imaging analysis system, respectively (Chen et al., 2008; Cui et al., 2009). Data are presented as the number of α SMA-vessels or vWF-vessels/mm², 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 hemisphere 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 μ m) 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., 2008). The cells were treated with (n=6/group): 1) non-treatment for control, 2) Simvastatin 0.1 μ mol/L, and 3) Simvastatin 1 μ mol/L. The choice of Simvastatin dose is consistent with our previous study (Chen et al., 2008). Cells were treated for 3 hours or 24 hours 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., 2008; Zacharek et al., 2009). The membrane with protein samples was treated with blocking buffer for 1 hour at room temperature, followed by incubation with primary antibodies for anti- β -actin (1:2000; Sigma), anti-SDF1 (1 mg/L; Santa Cruz Biotechnology), and anti-CXCR4 (1 mg/L; Chemicon) for 16 hour at 4°C. The membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibody in blocking buffer. The bands were visualized with an enhanced chemiluminescence kit (SuperSignal).

RT-PCR

Total RNA isolation, PCR quantitation and RT-PCR were performed, as previously described (Chen et al., 2008; Zacharek et al., 2009). The following primers for real-time PCR were designed using Primer Express software (ABI). **SDF1**: FWD: CCC GGA TCC ATG AAC GCC AAG GTC GTG; REV: AGA GCT GGG CTC CTA CTG TGC GGC CGC GGG, **CXCR4**: FWD: GGC TGT AGA GCG ATG TTT GC; REV: GTA GAG GTT GAC AGT GTA, **GAPDH**: FWD: AGA ACA TCA TCC CTG CAT CC; REV: CAC ATT GGG GGT AGG AAC AC.

BMSC migration assay

To investigate whether Simvastatin enhances BMSC migration, transwell Polycarbonate Inserts with 8 μ m pore size (Corning Life Sciences) were coated with 50 mg/L Fibronectin (Chemicon) and 0.1% gelatin. DiI-BMSCs were placed in the upper chamber (5×10^4 /chamber) and treated with or without Simvastatin (0.1 μ mol or 1.0 μ mol) for 24 hour. To test whether

SDF1 attracts BMSC migration, SDF1 α (200 μ g/L, Sigma) was added in the lower chamber. The number of BMSC migrating to the lower-side of the insert was counted using MCID software (n=6 chamber/group) (Cui et al., 2007).

To further test whether SDF1/CXCR4 regulates Simvastatin-enhanced BMSC migration toward RBMECs or astrocytes, transwell co-culture of BMSCs with RBMECs or BMSCs with astrocytes were performed (Cui et al., 2007). DiI-BMSCs were pretreated with or without AMD3100 (a specific antagonist of CXCR4, 20 μ mol/L, AnorMed)(Cui et al., 2007) to block CXCR4 for 24 hours and then placed in the upper chamber (5×10^4 /well), whereas, RBMECs and astrocytes (5×10^5 /well) were placed in the lower chambers, the co-culture system was treated with or without Simvastatin (0.1 μ mol/L or 1.0 μ mol/L) for 24 hours. DiI-BMSC migration to the lower-side of the insert was then counted (n=6 chamber/group).

Statistical analysis

Comparisons within treatment groups of functional evaluation, arteriogenesis, angiogenesis, SDF1/CXCR4 mRNA and Western blot expression and BMSC migration data were analyzed using one-way ANOVA. Least Significant Difference analysis after Post Hoc Test was used for multiple comparison if an overall treatment group effect was detected at $p < 0.05$. Independent Samples T-Test was used for testing the number of BrdU-BMSCs and SDF1 expression in the ischemic brain between two groups. Pearson partial correlations after Bivariate correlation were used to analyze the correlation of neurological functional recovery (as the outcome) with the engrafted-BMSC number and the number/diameter of α SMA-arterioles (arteriogenesis) or the number/perimeter of vWF-vessels (angiogenesis) in the IBZ (as covariates) at 14 days after MCAo. Analysis began with testing for the individual covariate effect, followed by multivariable modeling. The final multivariable model included covariate(s) with $p < 0.05$. We calculated R^2 for the overall correlation by a set of covariates of arteriogenesis. The correlation is high if the correlation coefficient, $r \geq 0.75$. All data are presented as mean $\pm$ Standard Error (SE).

Results

Combination Treatment of Stroke Significantly Improves Neurological Outcome

Fig. 1A–C show that combination treatment had a significant improvement on mNSS (A), adhesive removal (B) and foot-fault (C) tests at 7 and 14 days after MCAo compared to MCAo control rats ($p < 0.05$, n=10/group). However, sub-therapeutic dose of Simvastatin alone or BMSCs alone did not improve functional outcome compared to MCAo control group. The multiple functional outcome tests showed an additive effect of combination treatment of stroke.

Combination treatment of stroke significantly increases arteriogenesis in the ischemic brain

The arteriogenesis response consists of the formation of new arterioles, which presumably occurs when preexisting capillaries acquire SMC coating, and these newly formed and/or pre-existing arterioles transform into channels with larger diameters (Buschmann and Schaper, 2000; van Royen et al., 2001). Fig. 2A–F show the sub-therapeutic dose of Simvastatin (B) or BMSC monotherapy (C) significantly increases arteriole density (E) and diameter (F) in the IBZ compared to MCAo control animals (A). Combination treatment (D) significantly enhances α SMA-arteriole density and α SMA-arteriole diameter compared to Simvastatin or BMSC monotherapy animals (E and F, $p < 0.05$, n=10/group). The diameter of α SMA-arterioles in the ischemic brain was significantly and highly correlated with improvement of the foot-fault test ($p < 0.05$, $r = -0.76$).

The stages of arteriogenesis consist of arteriolar thinning, followed by transformation of VSMCs from the contractile- into the proliferative- and synthetic phenotype (Scholz et al.,

2000). Fig. 2G and H show the percentages of Ki67-VSMCs in the arteries of the IBZ significantly increased in Simvastatin alone and combination treatment groups compared with MCAo control group. Moreover, combination treatment significantly increased the percentage of Ki67-VSMCs in the arteries in the IBZ compared with BMSC-treatment alone ($p < 0.05$, $n = 10/\text{group}$). These data indicate that combination treatment of stroke has an additive effect, and significantly enhances arteriogenesis.

Combination treatment of stroke significantly increases angiogenesis in the ischemic brain

Angiogenesis is defined as sprouting of new capillaries from pre-existing vessels resulting in new capillary networks (Heil et al., 2006). This process includes the proliferation of existing VECs in the walls. Sprouting of capillaries also leads to an increase of their density which is equivalent to a decrease of interspaces between neighboring vessels. Fig. 2I–L show that BMSC monotherapy (K and N) significantly increases the perimeter of vWF-vessels in the IBZ compared to MCAo control (I). Combination treatment (L) significantly enhances the density and perimeter of vWF-vessels compared to MCAo control ($p < 0.05$, $n = 10/\text{group}$). Moreover, combination treatment significantly enhances the density of vWF-vessels compared to Simvastatin monotherapy animals (J and M, $p < 0.05$, $n = 10/\text{group}$). Fig. 2O and P show the percentages of Ki67-VECs in the vessels of the IBZ significantly increase after BMSC monotherapy and combination treatment compared with the MCAo control group ($p < 0.05$, $n = 10/\text{group}$). These data indicate that combination treatment of stroke has an additive effect, and significantly enhances angiogenesis.

Combination treatment of stroke significantly increases the number of engrafted-BMSCs in the ischemic brain

No significant differences of ischemic lesion volumes in monotherapy alone (BMSCs: $25.9\% \pm 6.3\%$; Simvastatin: $29.1\% \pm 4.2\%$) and combination treatment ($21.4\% \pm 4.2\%$) groups were detected compared with MCAo control rats ($34.7\% \pm 5.5\%$), respectively. However, a significant increase in the number of BrdU-BMSCs was found in the ipsilateral and contralateral hemisphere in the combination treatment rats compared to BMSC monotherapy rats (Fig. 3A). In addition, double-fluorescence image showed that Dil-BMSCs (red) primarily aggregated around the vascular vessels (green) in the ischemic lesion area of the brain (Fig. 3B). The number of BrdU-BMSCs in the ischemic brain was correlated with improvement of foot-fault test ($p < 0.05$, $r = -0.72$) after adjusting for treatments. These data indicate that combination treatment of stroke significantly increases the number of engrafted-BMSCs in the ischemic brain and the numbers of BMSCs present in the ischemic may drive functional recovery.

Simvastatin upregulates the ischemic brain SDF1 and BMSC CXCR4 and promotes BMSC migration

To test whether Simvastatin enhancement of BMSC recruitment into the ischemic brain occurs via upregulation of the SDF1/CXCR4 pathway, SDF1 expression was measured using tissue immunostaining and Western blot, respectively. Fig. 3C–E show that SDF1-immunostaining significantly increased in the Simvastatin treatment group (D) compared with the MCAo control group (C) 14 days after stroke (E, $p < 0.05$, $n = 10/\text{group}$). Double-immunofluorescent staining shows SDF1 primarily co-localized with vWF-VEC and GFAP-astrocytes in the IBZ (Fig. 3F–K). Western blot assay shows SDF1 expression also significantly increased in the Simvastatin treatment group compared with MCAo control in the IBZ (Fig. 3L, $p < 0.05$, $n = 6/\text{group}$). SDF1 and CXCR4 gene and protein expression were measured in cultured RBMECs, astrocytes and BMSCs. Fig. 4A–F show that SDF1 mRNA and protein expression in RBMECs (A and B) and astrocytes (C and D), and CXCR4 mRNA and protein expression in BMSCs (E

and F) significantly increased in the Simvastatin treatment group compared with the non-treatment control group ($p < 0.05$, $n = 6/\text{group}$).

The transwell culture assay shows that Simvastatin and SDF1 α significantly increase BMSC migration (Fig. 4G, $p < 0.05$, $n = 6/\text{group}$). Moreover, stimulation of RBMECs or astrocytes with Simvastatin significantly increases BMSC migration compared with non-treatment BMSCs. However, inhibition of CXCR4 significantly attenuates Simvastatin-stimulated BMSC migration to RBMECs or astrocytes (Fig. 4H and I, $p < 0.05$, $n = 6/\text{group}$). These data indicate that Simvastatin treatment stimulates the SDF1/CXCR4 axis and enhances BMSC migration to RBMECs and astrocytes, coincident with in vivo data.

Discussion

Enhancement of growth of functional blood vessels is critical for the restoration of cerebral blood flow to ischemic brain, maintenance of neural function, and improvement functional outcome after stroke. The growth of blood vessels in adult organisms proceeds via two major processes, arteriogenesis and angiogenesis. Angiogenesis describes the growth of capillaries from pre-existing vessels. Arteriogenesis describes the remodeling of pre-existing arterio-arteriolar anastomoses to completely developed and functional arteries (Heil et al., 2006). In both the growth processes, the enlargement of vascular wall structures is caused by proliferation of existing wall cells and increases in luminal diameter in response to increased blood flow (Buschmann and Schaper, 2000; Heil et al., 2006; Heil and Schaper, 2004; van Royen et al., 2001). Arterial occlusion, stimulates the induction of vascular wall cell proliferation and migration, wall remodeling processes. For example, VECs and VSMCs proliferate, and VSMCs migrate to form a neo-intima (Scholz et al., 2000). In the present study, a sub-therapeutic dose of Simvastatin (0.5 mg/kg) evokes a significant increase of arteriogenesis but not angiogenesis after stroke. A sub-therapeutic dose of BMSCs (1×10^6) fails to induce a significant increase on SMC proliferation, and angiogenesis after stroke in rats. However, combination treatment of stroke with sub-therapeutic doses of Simvastatin and BMSCs shows an additive effect on arteriogenesis and angiogenesis, as demonstrated by increased vascular density and diameter/perimeter, and enhanced VEC and VSMC proliferation in the ischemic brain. Therefore, combination sub-therapeutic doses of Simvastatin and BMSCs treatment of stroke enhances angiogenesis and arteriogenesis which may, in concert, mediate functional improvements.

Growth and maturation of capillaries and arterioles proceed by the proliferation and migration of existing VECs and VSMCs (Heil et al., 2006). A similar role is currently discussed for circulating blood monocytes/macrophages and various populations of BMSCs and endothelial progenitor cells (EPCs) which incorporate into foci of neovascularization, consistent with postnatal vasculogenesis (Llevadot et al., 2001; Madeddu et al., 2008; Matsuo et al., 2008). The hypothesis that existing cells-after undergoing a (trans-)differentiation contribute to vascular remodeling has been challenged by a structural integration into the growing vessel wall (Heil et al., 2006). Many studies in various models of ischemia have demonstrated that BMSCs migrate into the injured brain tissue when transplanted systemically or locally, and BMSCs incorporate into new or remodeling vessels (Al-Khaldi et al., 2003; Chen et al., 2001; Chen et al., 2004; Chen et al., 2003a; Hossmann, 2006; Zacharek et al., 2007; Zhou et al., 2006). Paracrine effects may also underlie the therapeutic benefits of BMSC therapy. BMSCs augment angiogenesis and arteriogenesis through release of growth factors, proteases and chemokines, such as transforming growth factor-beta, basic fibroblast growth factor, VEGF, and stem cell homing factor (SDF1) (Borlongan et al., 2004; Caplan, 2009; Carmeliet, 2000; Crisan et al., 2008; da Silva Meirelles et al., 2008; Tang et al., 2005). These factors regulate EPCs and SMC progenitors proliferation and migration as well as structural remodeling of the extracellular compartment (Al-Khaldi et al., 2003; Borlongan et al., 2004;

Hossmann, 2006; Kinnaird et al., 2004a; Kinnaird et al., 2004b). Our previous study showed that Simvastatin enhances BMSC differentiation into endothelial cells and capillary tube-like formation in vitro (Xu et al., 2008). In the present study, we demonstrate that combination treatment significantly increases the number of engrafted-BMSCs and enhances arteriogenesis and angiogenesis in the ischemic brain, and the number of engrafted-BMSCs and arteriogenesis are significantly correlated with functional outcome after stroke. Thus, the increase in arteriogenesis and angiogenesis mediated by combination treatment may derive from incorporation of the administered cells into the vascular architecture and paracrine effects.

SDF-1 facilitates BMSC recruitment and entrapment, increases vascular density and promotes neovascularization in the ischemic brain and enhances functional local cerebral blood flow (Shyu et al., 2008; Zhou et al., 2006). BMSCs can be efficiently transduced to express CXCR4, and transduced BMSCs migrate rapidly toward SDF1 α (Bhakta et al., 2006; Cui et al., 2007). SDF-1 is also involved in the trafficking of hematopoietic stem cells from bone marrow to peripheral blood, and its expression is increased in the penumbra of the ischemic brain (Kucia et al., 2004). We have previously demonstrated that administration of AMD3100 antagonized the behavioral and histological responses in mice subjected to MCAo and treated with BMSCs in combination with DETA-NONOate, a nitric oxide donor (Cui et al., 2007). In this study, Simvastatin significantly increases ischemic brain SDF1 and BMSC CXCR4 expression, and promotes BMSC migration to RBMECs and astrocytes. Inhibition of CXCR4 in BMSCs significantly decreases Simvastatin-induced BMSC migration. Therefore, Simvastatin appears to primes the ischemic brain to generate a cell receptive microenvironment for BMSC therapy.

In summary, our data demonstrate that Simvastatin enhances the therapeutic potency of BMSCs, possibly via upregulation of SDF1/CXCR4 pathway and thereby increases BMSC migration into the ischemic brain, and promotes arteriogenesis and angiogenesis. This combination therapy mediated vascular remodeling may underlie the improved functional outcome after stroke.

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Fig. 1.

Combination Simvastatin and BMSCs treatment of stroke improves functional outcome in rats.

A: mNSS test. **B:** Adhesive-removal test. **C:** Foot-fault test.

Fig. 2.

Combination Simvastatin and BMSCs treatment of stroke enhances arteriogenesis and angiogenesis in the ischemic brain. **A–D:** α SMA-arteries in the IBZ in MCAo (A), Simvastatin (B), BMSCs (C) and combination treatment (D) groups. **E and F:** Quantitative data of arterial density (E) and diameter (F). **G:** Ki67-VSMCs of artery in the IBZ. **H:** Quantitative data of Ki67-VSMCs. **I–L:** vWF-vessels in the IBZ in MCAo (I), Simvastatin (J), BMSCs (K) and combination treatment (L) group. **M and N:** Quantitative data of the density (M) and perimeter (N) of vWF-vessels. **O:** Ki67-VECs in the IBZ. **P:** Quantitative data of Ki67-VECs. Bar in B and J=50 μ m, n=10/group.

Fig. 3.

Combination Simvastatin and BMSCs treatment of stroke up-regulates SDF1 and enhances BMSC migration to the ischemic brain. **A:** Quantitative data of BrdU-BMSCs in the ipsilateral and contralateral brain. **B:** DiI-BMSCs localize around vascular vessels in the ischemic area (green: FITC-vessels; red: DiI-BMSCs). **C** and **D:** SDF1-immunohistostaining in MCAo control (**C**) and Simvastatin treatment (**D**) animals. **E:** Quantitative data of SDF1-immunohistostaining. **F, G, I** and **J:** Double immunofluorescent staining of SDF1 (**F** and **I**) with vWF-VECs (**G**) and GFAP-astrocytes (**J**) in the IBZ. **H** and **K:** merged images from **F** and **G** (**H**) and **I** and **J** (**K**). **L:** Western blot assay of SDF1.

Fig. 4.

Simvastatin increases SDF1 expression in RBMECs and astrocytes and CXCR4 expression in BMSCs, and enhances BMSC migration to RBMECs and astrocytes. **A–D**: Quantitative data of SDF1 expression by RT-PCR (A and C) and Western blot (B and D) in RBMEC (A and B) and astrocytes (C and D). **E and F**: Quantitative data of CXCR4 expression by RT-PCR (E) and Western blot (F) in BMSCs. **G–I**: Quantitative data of BMSC migration (G) and BMSC migration to RBMEC (H) and astrocytes (I).