Neurorestorative Responses to Delayed Human Mesenchymal Stromal Cells Treatment of Stroke in Type 2 Diabetic Rats

Tao Yan, PhD*; Poornima Venkat, PhD*; Michael Chopp, PhD; Alex Zacharek, MS; Ruizhuo Ning, MD; Cynthia Roberts, BS; Yi Zhang, PhD; Mei Lu, PhD; Jieli Chen, MD

Background and Purpose—Comorbidity of diabetes mellitus and stroke results in worse functional outcome, poor long-term recovery, and extensive vascular damage. We investigated the neurorestorative effects and mechanisms of stroke treatment with human bone marrow–derived mesenchymal stromal cells (hMSCs) in type 2 diabetes mellitus (T2DM) rats.

Methods—Adult male Wistar rats were induced with T2DM, subjected to 2 hours of middle cerebral artery occlusion (MCAo) and treated via tail-vein injection with (1) PBS (n=8) and (2) hMSCs (n=10; 5×10⁶) at 3 days after MCAo.

Results—In T2DM rats, hMSCs administered at 3 days after MCAo significantly improves neurological function without affecting blood glucose, infarct volume, and incidence of brain hemorrhage in comparison to T2DM-MCAo PBS-treated rats. Delayed hMSC treatment of T2DM stroke significantly improves blood–brain barrier integrity, increases vascular and arterial density and cerebral vascular perfusion, and promotes neuroblast cell migration and white matter remodeling as indicated by increased doublecortin, axon, myelin, and neurofilament density, respectively. Delayed hMSC treatment significantly increases platelet-derived growth factor expression in the ischemic brain, decreases proinflammatory M1 macrophage and increases anti-inflammatory M2 macrophage compared to PBS-treated T2DM-MCAo rats. In vitro data show that hMSCs increase subventricular zone explant cell migration and primary cortical neuron neurite outgrowth, whereas inhibition of platelet-derived growth factor decreases hMSC-induced subventricular zone cell migration and axonal outgrowth.

Conclusions—In T2DM stroke treatment significantly improves neurological functional outcome and increases neurorestorative effects and M2 macrophage polarization. Increasing brain platelet-derived growth factor expression may contribute to hMSC-induced neurorestoration. (Stroke. 2016;47:2850-2858. DOI: 10.1161/STROKEAHA.116.014686.)

Key Words: diabetes mellitus, type 2 □ ischemia □ mesenchymal stromal cells □ platelet-derived growth factor □ stroke

Diabetes mellitus (DM) is a high-risk factor for patients with ischemic stroke and stroke, with DM battle higher mortality rates and poor long-term recovery than non-DM stroke patients.¹ In non-DM rodents, marrow stromal cell (MSC) therapy for stroke facilitates functional recovery by stimulating angiogenesis, vascular stabilization, and white matter (WM) remodeling in the injured brain.² However, MSCs therapy for stroke in non-DM rats does not successfully translate to DM rats.³ Diabetes mellitus type 2 (T2DM) rats subjected to stroke and treated with MSCs 24 hours later suffer from significantly increased brain hemorrhage and blood–brain barrier (BBB) leakage, and treated rats do not show functional improvement compared with T1DM stroke control rats.⁴ Because >90% of DM patients have type 2 DM (T2DM), in this study, we investigated the therapeutic effects of delayed (3 days) human MSC (hMSC) treatment in T2DM stroke rats.

MSCs secrete several angiogenic, trophic, and growth factors.⁵ Platelet-derived growth factor (PDGF) in particular, is a potent neuroprotective molecule and is secreted abundantly by MSCs.⁶ PDGF is highly expressed in WM and can induce axonal regeneration.⁷ PDGF-BB and its receptors are also expressed on endothelial cells and may play an important role in poststroke angiogenesis.⁸ Macrophages are major regulators of vascularization and axonal remodeling.⁹ Macropheres can be classified broadly into 2 types: the proinflammatory and angiogenic M1 phenotype and the anti-inflammatory and proangiogenic M2 phenotype.¹⁰ Whether hMSC treatment regulates PDGF expression and M2 macrophage polarization in T2DM stroke rats has not been investigated.
In this study, we investigate the therapeutic effects and underlying mechanisms of delayed hMSC treatment of stroke in T2DM rats. We hypothesize that delayed hMSC treatment of T2DM stroke significantly improves functional outcome and induces neurorestorative effects and PDGF, and M2 macrophage polarization may partially contribute to hMSC-induced neurorestoration.

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.

Diabetes Mellitus Induction

Adult male Wistar rats (175–200 g; Charles River) were induced with T2DM using a combination of high-fat diet and low dose of streptozotocin.6,10 Body weight, blood glucose, high-density lipoprotein, and total cholesterol were measured before and 10 days after streptozotocin injection. Rats with fasting (8 hours) plasma glucose ≥300 mg/dL were considered diabetic.

MCAo Model and Experiment Groups

T2DM rats were subject to transient (2 hours) middle cerebral artery occlusion (MCAo) by intraluminal suture model.5,10 Rats were randomized (n=10 per group) and treated 3 days after MCAo via tail-vein injection with (1) PBS and (2) 5×10^6 hMSCs (Cognate Bioservices, Inc.). T2DM stroke rats treated with PBS or hMSCs at 3 days after stroke were euthanized at 4 weeks after MCAo to evaluate long-term effects, and blood serum and brain tissues were collected for ELISA and immunostaining. Mortality rate was 20% in each group. The sample size, 10 per group, was predefined to detect an effect size of 1.33 with a power of 80%.

Functional Tests

To assess neurological functional outcome, a battery of tests including a modified neurological severity score test,11 adhesive removal test,3 and foot-fault test10 were performed before MCAo and after MCAo on days 1, 7, 14, 21, and 28 by an investigator who was blinded to the experimental groups.

Histological and Immunohistochemical Assessment

Rats were transcardially perfused with 0.9% saline; brains were immediately removed and fixed in 4% paraformaldehyde. A standard paraffin block was obtained from the center of the lesion (bregma –1 mm to +1 mm). Every 10th coronal section for a total of 5 sections (20–60 μm thick) was used for immunohistochemical staining. Antibody against α-smooth muscle actin, von Willebrand factor, and SMI) were digested under a ×20 or ×40 objective (Olympus BX40) using a 3-CCD color video camera (Sony DVC970MD) interfaced with an MCID image analysis system (Imaging Research).10 Data were analyzed in a blinded manner, and positive areas or positive cell numbers were counted and analyzed with regard to small and large vessels (≥10-μm diameter). The arterial density in the IBZ and the 10 largest arterial wall thicknesses and diameter were measured in the IBZ.

Arterial Density, Wall Thickness, and Diameter and Occluded Artery Measurement

The number of arteries stained with α-smooth muscle actin were counted and analyzed with regard to small and large vessels (≥10-μm diameter). The arterial density in the IBZ and the 10 largest arterial wall thicknesses and diameter were measured.

Cerebral Blood Perfusion Measurement

To test cerebral vascular perfusion, an additional set of animals (n=5 per group) were prepared and FITC-dextran (FD2000S, Sigma) 50 mg/rat in 2 mL. PBS was injected intravenously 5 minutes before euthanasia.12,13 Brain tissues were fixed by 4% paraformaldehyde for 48 hours and then were processed to acquire adjacent 100-μm-thick coronal sections using a vibratome. Five sections from the bregma (–1 mm to +1 mm) were imaged using a fluorescence microscope (Zeiss Axioptot 2), and FITC-dextran–labeled vessels were quantified using ImageJ.

Angiogenesis ELISA Array Assay

Mouse angiogenesis antibody array kit (R&D Systems), including 55 angiogenesis-associated proteins and cytokeneses, was used to test angiogenic protein level in ischemic brain tissue according to the manufacturer’s instructions.

Subventricular Zone Cell Migration Assay

Rats were subjected to 2 hours of MCAo, and subventricular zone (SVZ) explants were isolated from the ipsilateral side at 24 hours after stroke. To further test whether hMSCs affect neuroblast migration, we used a previously described in vitro SVZ explant culture model.14 The cultured SVZ explants were treated as per the following experimental groups: (1) control, (2) +50% hMSCs conditioned media, and (3) +50% hMSCs conditioned media+PDGF inhibitor (20 μg). The average linear distance of cell migration from the edge of the SVZ explant was measured using the MCID software.

Primary Cortical Neuron Axonal Outgrowth Assay

Axonal outgrowth was measured, as previously described.15 Briefly, primary cortical neurons were harvested from pregnant embryonic (day 18) Wistar rats. A microfluidic chamber (Standard Neuron Device, Xona Microfluidics) was used to separate axons from neuronal soma.16 The experimental groups included (1) control no treatment, (2) +50% hMSC conditioned media, (3) +50% hMSC conditioned medium+PDGF inhibitor (20 μg).

Statistical Analysis

All measurements and analyses were performed by normality of distribution, and the homogeneity of variances was tested including the functional tests, biochemistry, and immunostaining. The Global test using the generalized estimating equation was used to test effects on
Results

hMSC Treatment of Stroke in T2DM Rats Significantly Improves Functional Outcome but Does Not Affect Blood Glucose Level, Lesion Volume, and Brain Hemorrhage Transformation

Delayed hMSC treatment initiated at 3 days after MCAo significantly improves long-term neurological function compared with T2DM-MCAo control rats as indicated by modified neurological score test, foot-fault test, and adhesive removal test (P<0.05; n=8 per group; Figure 1A). Data were evaluated for normality, and ranked data were used for the analysis because the data were not normally distributed. The data show that the overall group effect was significant at 14, 21, and 28 days after stroke (P<0.05). However, hMSC treatment of stroke in T2DM rats does not significantly decrease lesion volume and brain hemorrhage and the blood glucose/lipid levels compared with nontreatment T2DM stroke controls (P>0.05; Table I in the online-only Data Supplement).

hMSC Treatment of Stroke in T2DM Rats Promotes Neurovascular Remodeling

To test the mechanisms of hMSC treatment-induced improvement of functional outcome after stroke in T2DM rats, vascular remodeling and cerebral perfusion were evaluated. We found that hMSC treatment of stroke initiated at 3 days after MCAo significantly decreases BBB leakage (Figure 1B) and increases cerebral vascular and cerebral arterial density (Figure 1C and 1D) in the IBZ compared with T2DM-MCAo rats after stroke (P<0.01). Figure 2A shows that the cerebral vascular perfusion is significantly increased in the T2DM-hMSCs rats compared with control group.

hMSC Treatment of Stroke in T2DM Rats May Have Potential Adverse Effects Such as Arteriosclerosis-Like Changes

hMSC treatment significantly increases cerebral artery wall thickness, artery intimae thickness, and internal carotid artery wall thickness and significantly decreases cerebral artery diameter compared with vehicle control in T2DM rats after stroke (Figure 2B and 2C). The data are consistent with our previous study that MSC treatment in T1DM MCAo rats increases atherosclerotic-like vascular changes.1

hMSC Treatment of Stroke in T2DM Rats Promotes Neural Progenitor Cell Migration and Axonal/WM Remodeling in IBZ

To test whether hMSC treatment affects axon/WM remodeling, bielschowsky silver (axon), luxol fast blue (myelin), SMI-31 (phosphorylated neurofilament), staining were performed. To test whether hMSC treatment regulates neural progenitor cell migration, DCX immunostaining was performed. Figure 3 shows that hMSC significantly increases SMI-31 (A), myelin (B), and axon (C) density in the striatum bundle of the IBZ and increases neural progenitor cell migration (D) after T2DM-MCAo.

hMSC Treatment of T2DM Stroke Rats Promotes M2 Macrophage Polarization in the Ischemic Brain

To investigate underlying mechanisms of hMSC treatment-induced neurorestorative effects in T2DM-MCAo rats, M2 macrophage polarization was evaluated. Figure 4 shows that hMSC treatment significantly decreases M1 macrophage ED1 (A) and significantly increases M2 macrophage CD163 (B) expression compared with PBS-treated T2DM-MCAo rats. The data suggest that hMSC treatment initiated at 3 days after MCAo in T2DM promotes M2 polarization and decreases proinflammatory effects post ischemia.

Inhibition of PDGF Attenuates hMSC-Induced Axonal/WM Remodeling and Decreases SVZ Explant Cell Migration

To test whether PDGF plays an important role in hMSC treatment-induced neurorestorative effects, in vitro studies were performed. We found that hMSCs treatment of T2DM-MCAo rats increases PDGF-AA and PDGF-BB levels compared with PBS-treated T2DM-MCAo rats (Figure 5A). Consistent with the ELISA array, immunostaining (Figure 5B and 5C) shows that PDGFRa and PDGFRaα expression are significantly increased in hMSC-treated T2DM-MCAo rats compared with PBS-treated T2DM-MCAo control.

Discussion

In this study, we present for the first time that delayed (3 days) treatment for T2DM stroke using hMSCs increases not only angiogenesis and axonal/WM remodeling but also neuroblast cell migration in the ischemic brain. We also specifically demonstrate the contribution of PDGF in hMSC-induced axonal/WM remodeling and in SVZ explant migration. Diabetes mellitus triggers a cascade of events leading to vascular endothelial cell dysfunction, increased vascular permeability, vigorous angiogenesis but dysfunctional neovascularization, and poor recovery after ischemic stroke.4,17 We have previously shown that MSCs secrete several growth and trophic factors, one of which is vascular endothelial growth factor (VEGF).5 It has been previously demonstrated that acute (1 hour after stroke) administration of VEGF enhances cerebral microvascular perfusion, increases BBB leakage, cerebral...
hemorrhage, infarction volume after stroke, whereas delayed (48 hours) administration of VEGF enhances angiogenesis in the ischemic penumbra and significantly improves neurological functional recovery. Because diabetic rats suffer from vascular damage, which is aggravated after an ischemic insult, the effects of VEGF may be exacerbated in diabetic stroke rats compared with nondiabetic stroke rats. Our data show that VEGF is significantly increased in the ischemic brain of T2DM rats compared with wild-type non-DM rats at 1 day after stroke (Figure II in the online-only Data Supplement). VEGF is significantly decreased by 3 days after stroke in the ischemic brain of T2DM stroke rats (Figure III in the online-only Data Supplement). In T1DM rats, when MSC therapy was initiated at 1 day after stroke, it induced brain hemorrhage and BBB leakage. In the acute phase after stroke, loss of BBB integrity and brain hemorrhage may over-ride the beneficial effects of MSC treatment such as vascular and axonal/WM remodeling. In the current study, we have demonstrated that treatment initiation at a delayed time point, ie, 3 days after stroke, in T2DM rats significantly improves neurological functional recovery.

Figure 1. In type 2 diabetes mellitus (T2DM)-middle cerebral artery occlusion (MCAo) rats, compared with PBS treatment, human bone marrow–derived mesenchymal stromal cell (hMSC) treatment 3 d post stroke significantly improves functional outcome indicated by (A) modified neurological severity score (mNSS) test, adhesive removal test, foot-fault test and significantly decreases blood–brain barrier (BBB) leakage as indicated by (B) FITC-albumin staining, and improves vascular remodeling as indicated by (C) von Willebrand factor (vWF) and (D) α-smooth muscle actin (αSMA) immunostaining and quantification data in the ischemic border zone.
functional outcome, does not increase BBB leakage and brain hemorrhage, and significantly promotes vascular remodeling, as demonstrated by increased vascular and arterial density and vascular perfusion. Hence, for cell therapy for DM subjects, treatment initiation time point is extremely critical and should be considered when treating diabetic stroke. Because the time point of hMSC treatment in the current T2DM stroke study (3 days after stroke) differs from that of the T1DM stroke treatment (1 day after stroke), we are unable to compare the apparent divergent outcomes between T1DM and T2DM MSC treatments. However, comparison of response to cell-based therapy performed under the identical treatment protocols in T1DM and T2DM rats with stroke warrants investigation.

Angiogenesis plays an important role in improving post-stroke neurological function. Angiogenic events increase blood supply to the ischemic brain tissue and are also tightly coupled to neurogenesis. In this study, we found that hMSC treatment of stroke increases vascular density and cerebral blood perfusion in T2DM rats. Post ischemia, there is an exuberant expansion of neural progenitor cells in the SVZ, differentiation into mature neurons, astrocytes, and oligodendrocytes, and migration to the IBZ. Neural progenitor cell migration is closely associated with blood vessels that serve as scaffolds and guide the migration of neural progenitor cells from the SVZ toward damaged brain regions. Our results show that hMSC therapy significantly increases SVZ explant cell migration and neural progenitor cell migration identified by increased DCX density in the IBZ. These data suggest that hMSC treatment of stroke in T2DM rats promotes neurovascular remodeling that may partially contribute to the functional outcome after stroke.

Stroke and diabetes mellitus cause axonal/WM damage, which induces long-term disability because of the brain’s limited capacity of axonal regeneration and inhibitory environment for axon regrowth, sprouting, and remyelination. Poststroke protection of neurons in the gray matter is not sufficient, as WM damage would still hinder neuronal connectivity and functioning. Therefore, targeting WM remodeling is crucial for improving long-term functional outcome after stroke. In this study, we found that hMSC treatment of stroke initiated at 3 days after MCAo in addition to promoting neurovascular remodeling also increases axonal/WM remodeling in the ischemic brain.

M1 macrophages are neurotoxic, whereas M2 macrophages promote a regenerative growth response in adult sensory axons. M2 phenotype polarization creates a conducive environment for axonal extension and functional recovery. Post ischemia, the local and infiltrating microglia and macrophages assume an anti-inflammatory and protective M2 phenotype. Extending this M2 phase of these macrophages and
microglia and delaying their transition into the proinflammatory M1 phenotype is a desirable effect. Macrophage invasion typically starts ≈24 hours post stroke and increases by 3 to 7 days after stroke; however, recent studies have indicated that the increased level of macrophage accumulation in the brain persists to at least 28 days after stroke.27 Our data show

Figure 3. Compared with PBS treatment, human bone marrow–derived mesenchymal stromal cell (hMSC) treatment 3 d post stroke in type 2 diabetes mellitus (T2DM)-middle cerebral artery occlusion (MCAo) rats significantly promotes white matter remodeling indicated by (A) SMI-31 (phosphorylated neurofilament marker), (B) luxol fast blue, and (C) bielschowsky silver immunostaining and quantification analysis. hMSC therapy also promotes neural progenitor cell migration indicated by (D) DCX (double-cortin) immunostaining.

Figure 4. Compared with PBS treatment, human bone marrow–derived mesenchymal stromal cell (hMSC) treatment 3 d post stroke in type 2 diabetes mellitus (T2DM)-middle cerebral artery occlusion (MCAo) rats significantly promotes macrophage polarization indicated by (A) decreasing M1 macrophage ED1 and (B) increasing M2 macrophage CD163.
that hMSC treatment of stroke in T2DM rats significantly induces M2 macrophage polarization and increases vascular integrity and axonal/WM remodeling in the ischemic brain. MSCs secrete several growth and trophic factors that may mediate several pathways and contribute toward neurorestoration after stroke in T2DM rats. Using the experimental design and methods used in this study, it is difficult to dissect whether M2 macrophage polarization has a direct or indirect (by creating a hospitable environment for brain repair) role in hMSC-induced neurorestorative effects after stroke in T2DM rats. This is an important question, and future studies are warranted.

Accumulating evidence suggests that the neurorestorative effects of cell therapy are primarily derived from the secretion of trophic and growth factors that stimulate endogenous brain repair and remodeling to induce neurological recovery. We acknowledge the similarities observed in treatment-derived benefits from our other cell-based treatment strategies such as human umbilical cord blood cells (HUCBCs). Accordingly, we have shown that for T2DM stroke, delayed (3 days) cell therapy with HUCBCs and hMSCs significantly promote long-term neurological functional outcome without affecting blood glucose and infarction volume. Similar to HUCBCs, hMSC treatment also promotes neurorestorative effects such as vascular and WM remodeling and induces anti-inflammatory effects such as M2 macrophage polarization (Figure II in the online-only Data Supplement). However, the mechanism of action is different for HUCBC and hMSCs. We have reported that HUCBC treatment in T2DM stroke promotes serum, brain, and brain endothelial cell micro-RNA-126 (miR-126) expression that stimulates vascular and WM remodeling in the ischemic brain. Compared with HUCBCs, hMSCs secrete significantly less miR-126 in vitro, and in T2DM stroke rats, treatment with hMSCs stimulates significantly less circulating miR-126 compared with treatment with HUCBCs (Figure III in the online-only Data Supplement). In the present study, we demonstrate that hMSC treatment of T2DM stroke significantly increases PDGF expression, which then plays a key role in promoting neurovascular and WM remodeling.

PDGF signaling mediates vascular SMC and pericyte function and regulates vessel integrity. PDGF treatment decreases axonal abnormalities and improves remyelination by promoting proliferation of oligodendrocytes and oligodendrocyte progenitor cells. Our data show that hMSC treatment of stroke in T2DM rats significantly increases PDGF expression in the ischemic brain and promotes neurovascular and WM remodeling.

Figure 5. A. Angiogenesis ELISA protein array shows that human bone marrow–derived mesenchymal stromal cell (hMSC) treatment of type 2 diabetes mellitus (T2DM) stroke increases platelet-derived growth factor (PDGF)-AA and PDGF-BB, and these results are confirmed by (B) PDGF receptor (PDGFR) a and (C) PDGFRaa immunostaining and quantification analysis.
remodeling. Inhibition of PDGF attenuates hMSC-induced SVZ cell migration and PCN axonal sprouting. However, PDGF signaling is also a key mediator of SMC proliferation and plays an important role in arteriosclerosis.\textsuperscript{30} PDGF triggers the switching of SMC from a quiescent, contractile phenotype to a proliferative, migratory, and synthetic phenotype.\textsuperscript{31} PDGF is a major stimulus for the abnormal migration and proliferation of SMCs and contributes critically to vascular disease. Our data show that hMSC therapy in T2DM stroke rats increases not only neurovascular and axonal/WM remodeling but also arteriosclerosis-like changes compared with PBS treatment. Therefore, increasing PDGF by hMSC treatment of stroke in T2DM rats not only promotes neurovascular and axonal/WM remodeling but also may induce vascular arteriosclerosis-like changes.

Conclusions
T2DM stroke treatment using hMSCs initiated 3 days after the ischemic insult significantly improves neurological functional recovery by promoting neurovascular and axonal/WM remodeling in the ischemic brain. Our data suggest that M2 macrophage polarization and increase of PDGF may contribute to the mechanisms underlying hMSC treatment-derived neurorestorative and atherosclerotic-like effects.

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Disclosures
None.

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## ONLINE SUPPLEMENT

Neurorestorative responses to delayed hMSC treatment of stroke in type 2 diabetic rats

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<td><strong>Hemorrhage</strong> (% positive area)</td>
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Supplemental Table I: Lesion volume, hemorrhage and biochemical estimates
Supplemental Figure I: VEGF is significantly increased in the ischemic brain of T2DM rats compared to wild type (WT) non-DM rats at 1 day after stroke. VEGF is significantly decreased by 3 days after stroke in the ischemic brain of T2DM stroke rats.

Supplemental Figure II: Comparison of end point parameters between HUCBCs and hMSC treatment for stroke in T2DM rats. HUCBCs and hMSCs significantly improve neurological functional outcome, promote white matter remodeling and increase M2 macrophage polarization after stroke in T2DM rats.
Supplemental Figure III: Comparison of mechanism between HUCBCs and hMSC treatment for stroke in T2DM rats. hMSCs secrete significantly less miR-126 in-vitro. In T2DM stroke rats, compared to HUCBC treatment, hMSC treatment induces significantly less circulating miR-126.