Neurorestorative Therapy of Stroke in Type 2 Diabetes Mellitus Rats Treated With Human Umbilical Cord Blood Cells

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Background and Purpose—Diabetes mellitus is a high-risk factor for ischemic stroke. Diabetic stroke patients suffer worse outcomes, poor long-term recovery, risk of recurrent strokes, and extensive vascular damage. We investigated the neurorestorative effects and the underlying mechanisms of stroke treatment with human umbilical cord blood cells (HUCBCs) in type 2 diabetes mellitus (T2DM) rats.

Methods—Adult male T2DM rats were subjected to 2 hours of middle cerebral artery occlusion (MCAo). Three days after MCAo, rats were treated via tail-vein injection with (1) PBS and (2) HUCBCs (5×10^6), n=10 per group.

Results—HUCBC stroke treatment initiated 3 days after MCAo in T2DM rats did not significantly decrease blood–brain–barrier leakage (P=0.1) and lesion volume (P=0.078), but significantly improved long-term functional outcome and decreased brain hemorrhage (P<0.05) when compared with the PBS-treated T2DM MCAo control group. HUCBC treatment significantly promoted white matter remodeling as indicated by increased expression of Bielschowsky silver (axons marker), Luxol fast blue (myelin marker), SMI-31 (neurofilament), and Synaptophysin in the ischemic border zone. HUCBC promoted vascular remodeling and significantly increased arterial and vascular density. HUCBC treatment of stroke in T2DM rats significantly increased M2 macrophage polarization (increased M2 macrophage, CD163 and CD206; decreased M1 macrophage, ED1 and inducible nitric oxide synthase expression) in the ischemic brain compared with PBS-treated T2DM MCAo controls (P<0.05). HUCBC also significantly decreased proinflammatory factors, that is, matrix metalloproteinase 9, receptor for advanced glycation end products and toll-like receptor 4 expression in the ischemic brain.

Conclusion—HUCBC treatment initiated 3 days after stroke significantly increased white matter and vascular remodeling in the ischemic brain as well as decreased neuroinflammatory factor expression in the ischemic brain in T2DM rats and promoted M2 macrophage polarization, HUCBC reduction of neuroinflammation and increased vascular and white matter axonal remodeling may contribute to the HUCBC-induced beneficial effects in T2DM stroke rats. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.115.009870.)

Key Words: diabetes mellitus, type 2 ■ human umbilical cord blood ■ infarction, middle cerebral artery ■ matrix metalloproteinase 9 ■ neurorestorative therapy ■ stroke ■ vascular remodeling

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Mortality rates in diabetic stroke mice were significantly greater than in wild-type mice. With T2DM being the most common form of diabetes mellitus and the success of HUCBC treatment in nondiabetic and T1DM stroke animals, our aim in this study is to evaluate the therapeutic efficiency and underlying mechanism of delayed HUCBC treatment in T2DM rats when treatment is initiated 3 days after stroke.

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 Induction

T2DM was induced using a combination of 2-week high-fat diet followed by low dose of streptozotocin (35 mg/kg, Sigma Chemical Co, St. Louis, MO) intraperitoneal injection in adult male Wistar rats and continued a high-fat diet for another 2 weeks. Two weeks after streptozotocin injection, the fasting blood glucose level was tested using a glucose analyzer (Accu-Chek Compact System; Roche Diagnostics, Indianapolis, IN) and animals with fasting blood glucose >300 mg/dL underwent 2 hours of transient middle cerebral artery occlusion (MCAo).

MCAo Model and Experiment Groups

Twenty-four T2DM rats were subjected to transient (2 hours) right MCAo via intraluminal vascular occlusion, as previously described. The exclusion criteria were rats with modified neurological severity score (mNSS) >6 (possibly small to no lesion) or >13 (poor survival) at 24 hours after MCAo (before treatment). Accordingly, 4 rats were excluded from this study. The rats were randomly assigned to different groups and treated with (1) PBS as vehicle control (n=10); (2) HUCBCs (5×10^6, n=10); Saneron CCEL Therapeutics) via tail vein injection starting at 3 days post MCAo. The treatment time point of 72 hours after stroke in T2DM rats was used to investigate HUCBC-induced neurorestorative effects as well as to accommodate a wide treatment window. Rats were euthanized 28 days after MCAo for immunostaining quantification analysis.

Neurological Functional Tests

An investigator was blinded to the experimental groups to perform a battery of functional tests, including foot-fault, adhesive removal, and evaluation of mNSS before MCAo and after MCAo on days 1, 7, 14, 21, and 28.

Histological and Immunohistochemical Assessment

Brains were fixed using transcardial perfusion with saline, followed by immersion and 4% parafomaldehyde. Then, the brains were embedded in paraffin and a standard block was obtained from the center of the lesion (bregma –2 mm to +2 mm). A series of 6-μm thick sections were cut from the block. Hematoxylin and eosin stained 7 coronal sections of tissue were used for lesion volume calculation and presented as a percentage of lesion compared with the contralateral hemisphere.

Brain coronal tissue sections were prepared and antibody against α-smooth muscle actin (mouse monoclonal IgG, 1:800; Dako); Von Willebrand factor (1:400; Dako); SMI-31 (Neurofilaments, phosphorylated monoclonal, 1:1000; Covance); Synaptophysin (monoclonal, 1:500; Millipore); RAGE (1:400; Dako); toll-like receptor 4 (TLR4; goat polyclonal IgG, dilution 1:100; Santa Cruz Biotechnology); MMP9 (1:500; Santa Cruz Biotechnology); CD163 (1:500; Abcam Cambridge, MA); ED1 (a mouse mAb against rat microglia/macrophages, monoclonal, 1:30; AbD Serotec); CD 206 (1:3000; Abcam); and inducible nitric oxide synthase (1:200; Millipore) were used. Antibody against albumin (albumin-fluorescein isothiocyanate polyclonal, 1:500; Abcam) was used to demonstrate blood–brain–barrier (BBB) leakage and Prussian blue staining used to evaluate hemorrhage. Bielschowsky silver immunostaining was used to demonstrate axons, and luxol fast blue (LFB) staining was used to demonstrate myelin. Control experiments consisted of staining brain coronal tissue sections as outlined above, but nonimmune serum was substituted for the primary antibody.

Quantification Analysis

All the immunostaining quantification analysis was performed by an investigator who was blinded to the experimental groups. Five slides from each brain, with each slide containing 8 fields from striatum of the IBZ were digitized under a ×20 objective (Olympus BX40) using a 3-CCD color video camera (Sony DYC-970MD) interfaced with an MCID image analysis system (Imaging Research, St. Catharines, Canada). For Bielschowsky silver and LFB measurements, positive areas of immunoreactive cells were measured in the WM bundles of the striatum in the IBZ. For other immunostaining (albumin-fluorescein isothiocyanate Prussian blue, Synaptophysin, SMI-31, RAGE, MMP9, and TLR4), positive areas of immunoreactive cells were measured in the IBZ, and for CD163, inducible nitric oxide synthase, ED1, and CD206 positive cell number was measured in the IBZ.

Vascular Density Measurement

To measure the vascular density in the IBZ, 8 fields of view of Von Willebrand factor immunostaining from the IBZ were digitized using a ×20 objective via the MCID software. The α-smooth muscle actin–stained arteries were analyzed with regard to small and large vessels (≥10 μm diameter). The arterial density in the IBZ was measured.

Statistical Analysis

One-way ANOVA was used for the evaluation of functional outcome and histology, respectively. Contract/estimate statement was used to test the group difference. If an overall treatment group effect was detected at P<0.05, pair-wise comparisons were made. All data are presented as mean±SE.

Results

HUCBC Treatment Significantly Improved Long-Term Functional Outcome but Did Not Significantly Decrease BBB Leakage and Lesion Volume

Long-term functional benefit derived from HUCBC treatment initiated at 3 days after MCAo in T2DM rats was assessed using a battery of behavioral tests. n=10 per group was used and with a mortality rate of 20% (between days 7–14 after MCAo), 8 rats per group survived at the end of experiments in both treatment and control groups. Figure 1A presents mNSS, adhesive removal, and foot-fault data for 28 days after stroke that indicate significantly improved functional outcome in T2DM MCAo rats treated with HUCBCs compared with PBS-treated control group (P<0.05).

HUCBC treatment significantly decreased hemorrhage in the brain identified by Prussian blue staining...
(P<0.05; Figure 1D) compared with PBS-treated T2DM stroke rats. HUCBC treatment in T2DM rats did not alter body weight and blood glucose level and did not significantly decrease lesion volume (P=0.078; Figure 1C) and BBB leakage as indicated by fluorescein isothiocyanate albumin immunostaining (P=0.1; Figure 1B) compared with PBS-treated T2DM MCAo rats.

**HUCBC Stroke Treatment Significantly Promoted Vascular Remodeling**

To test the beneficial effects of HUCBC treatment, vascular remodeling was evaluated using α-smooth muscle actin and Von Willebrand factor immunostaining. Figure 2 indicates that HUCBC significantly enhanced cerebral artery density (α-smooth muscle actin; Figure 2A) and vascular density (Von Willebrand factor; Figure 2B). The figures show that HUCBC treatment significantly increased the density of cerebral arteries and vessels compared to PBS-treated T2DM MCAo rats.

**Figure 1.** Human umbilical cord blood cell (HUCBC) treatment 3 days post stroke in type 2 diabetes mellitus (T2DM) middle cerebral artery occlusion (MCAo) rats significantly improved functional outcome (A) modified neurological severity score (mNSS), adhesive removal test, and foot-fault; (B) did not significantly decrease blood–brain–barrier (BBB) leakage, (C) body weight, blood glucose, or lesion volume but significantly decreased (D) brain hemorrhage compared with PBS-treated T2DM MCAo rats.
Willebrand factor; Figure 2B) compared with PBS-treated control T2DM stroke rats (P<0.05).

**HUCBC Stroke Treatment Promoted White Matter Remodeling and Increased Axonal and Synaptic Plasticity**

Bielschowsky silver and LFB staining were used to evaluate the beneficial effects of HUCBC treatment on WM remodeling. HUCBC treatment significantly increased Bielschowsky silver and LFB expression (Figure 3A and 3B) in the WM bundles compared with PBS-treated T2DM MCAo rats (P<0.05). HUCBC treatment also regulated axonal and synaptic plasticity, indicated by significantly increased SMI-31 (Figure 3C) and Synaptophysin (Figure 3D) expression levels in the IBZ compared with PBS-treated T2DM MCAo rats (P<0.05).

**HUCBC Stroke Treatment Significantly Decreased Neuroinflammatory Factor Expression and Increased M2 Macrophage Polarization in the IBZ**

To understand the mechanisms of HUCBC therapy–derived benefits, neuroinflammation was evaluated in the ischemic brain. Expression levels of inflammatory factors TLR4, MMP9, and RAGE were measured in the IBZ. As indicated in Figure 4, HUCBC treatment significantly decreased TLR4, MMP9, and RAGE (P<0.05) expression compared with PBS-treated T2DM stroke rats (P<0.05).

To test the effect of HUCBC treatment on macrophage polarization, expression levels of M2 macrophage markers, CD163 and CD206, and M1 macrophage markers, ED1 and inducible nitric oxide synthase, were measured. Figure 5 shows that HUCBC treatment in T2DM MCAo rats significantly increased M2 and decreased M1 macrophage expression in the ischemic brain in comparison with PBS-treated T2DM stroke rats (P<0.05).

**Discussion**

HUCBCs have received a great deal of attention as a treatment option for hematologic disorders and malignancies as they are a rich source of hematopoietic stem cells. The driving advantages in using HUCBCs include easy availability as it is discarded post birth, lack of ethical conflicts, and none to low severity effects like graft versus host disease from donor-recipient human leukocyte antigen mismatch. This tolerance of HUCBC therapy to donor-recipient human leukocyte antigen mismatch aided by cord blood’s naïve and immature immune function plays a key role in translating this therapy to the clinic. We found that HUCBC therapy in T2DM rats initiated intravenously at 3 days post stroke, did not significantly decrease BBB leakage and lesion volume, but significantly decreased brain hemorrhagic transformation and significantly increased vascular and WM remodeling and improved functional recovery. The underlying mechanisms of HUCBC induced benefits maybe decreasing neuroinflammatory effects and promotion of M2 macrophage polarization.

Several stroke treatments in the diabetic rat population have failed, including bone marrow stromal cells and tissue-type plasminogen activator. These failures were associated with increased BBB leakage, brain hemorrhagic transformation, and inflammation in T1DM and T2DM rats. In this study, significant functional benefit was derived on HUCBC therapy, although we found that HUCBC treatment initiated 3 days after stroke in T2DM did not significantly decrease BBB leakage and lesion volume compared with T2DM PBS-treated stroke rats. These data suggest that HUCBC-induced therapeutic and functional benefits in T2DM stroke rats may primarily be derived from the regulation of vascular and WM remodeling. In nondiabetic rats, hemorrhagic transformation and BBB leakage occur early after stroke, but in diabetic rats, these events have an extended time window and are present at 14 days after stroke. Although the 28-day time point is not ideal for studying BBB permeability and hemorrhagic transformation which occur acutely after stroke, because we observed HUCBC treatment–induced functional benefits starting at 14 days after stroke, we elected not to perform these measurements at an early time point.

**Figure 2.** Human umbilical cord blood cell (HUCBC) treatment 3 days post stroke in type 2 diabetes mellitus (T2DM) middle cerebral artery occlusion (MCAo) rats significantly improved vascular remodeling as indicated by (A) α-smooth muscle actin (α-SMA) and (B) Von Willebrand factor (vWF) immunostaining and quantification data in the ischemic border zone compared with PBS-treated T2DM MCAo rats.
A multifaceted mechanism of action has been suggested by several studies using cell-based therapies to promote poststroke long-term beneficial effects; minor benefit derived from a small portion of infused cells migrating to the brain and differentiating into neuronal cells, and major benefit derived by promoting various aspects of neurorestoration like WM remodeling, vascular remodeling, synaptogenesis, and neurogenesis by (1) enhancing endogenous brain repair mechanisms and (2) secreting trophic and growth factors. Stroke decreases cerebral blood flow and triggers vascular remodeling to improve blood supply via angiogenesis and arteriogenesis. Increasing angiogenesis and arteriogenesis are correlated with neurological functional outcome after stroke. In T2DM mice, poor functional outcome was correlated with exacerbated WM and vascular damage compared with nondiabetic stroke mice. HUCBC treatment increased cerebral arterial and vascular density in the IBZ. These results indicate that HUCBC stroke treatment induces an increase in cerebral vascular remodeling in T2DM rats.

WM of brain is highly susceptible to ischemic stress primarily because of its relatively limited blood supply. Hence, after a stroke, WM recovery is critical for sustained long-term functional recovery. HUCBC treatment enhanced the expression of Bielschowsky silver, SMI-31, and LFB in the IBZ indicative of enhanced axonal plasticity and myelin regeneration. Several studies point toward axonal remodeling to improve brain repair, attenuate stroke-induced neurological deficits, and to contribute long-term benefits in functional improvement. At the same time, worse outcomes in DM stroke subjects have been associated with defective or restricted axonal regeneration. The importance of enhanced axonal myelination centers on faster communication and sensory/motor reflexes which helps restore lost neurological function and decreases stroke-induced paralytic symptoms. Stroke not only leads to loss of myelin in the peri-infarct area at 48 hours after onset but also stimulates the formation of new myelin around the damaged and sprouting axons. This endogenous
The brain repair process is significantly enhanced by HUCBC treatment to promote the expression of LFB, a myelin marker, in the ischemic brain regions. HUCBC treatment also enhanced synaptic plasticity, indicated by increased Synaptophysin expression in the IBZ. Intercellular communication between neurons and other neurons/cells is facilitated via a synapse in the central nervous system, and improved synaptic plasticity indicated by enhanced Synaptophysin expression has been reported to mediate stroke treatment benefits. Enhanced axonal and myelin remodeling and axonal and synaptic plasticity may contribute to the observed HUCBC treatment induced post stroke recovery in T2DM rats.

Regulation of the inflammatory responses induced by stroke early after onset lasting up to weeks later is crucial for effectiveness of stroke treatments. While mild inflammation can be favorable for brain repair in a chronic stage, in the acute phase, on uncontrolled inflammation the activated microglia, astrocytes, and macrophages can exacerbate damage or death to the injured brain by releasing proinflammatory factors and by creating an inhospitable environment for neurovascular plasticity. MMP9 has been implicated in enhancing T2DM-induced WM and axonal damage. TLR4 and RAGE are both inflammatory factors typically increased in diabetic stroke animals and have been implicated in exacerbating brain damage. HMGB1 (high-mobility group box 1) is an inflammatory mediator secreted on injury by immune cells or injured cells. HMGB1 release can trigger an inflammatory cascade and binds to its receptors, TLR4 and RAGE. It has been reported that in cerebral ischemia HMGB1 triggers MMP9 increase in neurons and astrocytes mainly through TLR4. Hence, treatments that can regulate the HMGB1/TLR4 signaling pathway can potentially decrease tissue damage by controlling postischemic inflammatory responses. HUCBC treatment significantly decreased the expression levels of these detrimental inflammatory factors (TLR4, RAGE, and MMP9) in the IBZ and induced restorative effects in T2DM stroke rats.

M2 macrophage polarization has been associated with decreased neuroinflammation and enhanced axon growth in injured mouse spinal cord. Our data show that M2 macrophage polarization was significantly increased by HUCBC treatment in the IBZ of T2DM MCAo rats. M2 macrophage polarization, marked by increased M2 macrophage CD163 and decreased M1 macrophage ED1 expression, was evident with HUCBC treatment. The M2 macrophage polarization mechanism can improve functional outcome post stroke. Microglia and macrophages on ischemic insult can assume an anti-inflammatory M2 activation and protect neurons; which is a potential target for neurorestorative therapies. Soon after focal cerebral ischemia, the local and infiltrating macrophages assume the M2 phenotype and decrease the expression of inflammatory factors thereby extending a protective effect to the neurons and improving their survival in the ischemic environment. Extending the M2 phase of these macrophages and microglia and delaying their transit into M1 phenotype, which is detrimental to the ischemic brain because of increased proinflammatory factor production, is a desirable effect. HUCBC treatment promotes M2 macrophage polarization which may contribute to improved neurological outcome. Although it is common knowledge that macrophage invasion starts around...
24 hours after stroke and increases by 3 to 7 days after stroke, recent studies have revealed that the increased level of macrophage accumulation in the brain persists to at least 28 days after stroke, lasting ≤1 year after stroke. The links between M2 polarization and HUCBC-induced regulation of neuroinflammation and WM and vascular remodeling leading to beneficial effects are not clear, and further studies are warranted.

Conclusions
Treatment of stroke initiated 3 days post the ischemic insult via intravenous administration of HUCBCs in T2DM rats significantly improves functional recovery by enhancing WM axonal and vascular remodeling in the ischemic brain. Our data suggest that decreasing neuroinflammatory factors and increasing M2 macrophage polarization may be contributing mechanisms underlying HUCBC treatment–derived beneficial effects.

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Disclosures
Dr Chen is a consultant to Saneron CCEL Therapeutics, Inc. Also, Dr Sanberg and N. Kuzmin-Nichols are inventors on cord blood patents/applications. Dr Sanberg is Sr. VP of R&D, and N. Kuzmin-Nichols is the President and COO at Saneron CCEL Therapeutics, Inc. The other authors report no conflicts.

References


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