Human Umbilical Mesenchymal Stem Cells Promote Recovery After Ischemic Stroke

Yu-Ching Lin, MS*; Tsui-Ling Ko, PhD*; Yang-Hsin Shih, MD*; Maan-Yuh Anya Lin, PhD; Tz-Win Fu, BS; Hsiao-Sheng Hsiao, MS; Jung-Yu C. Hsu, PhD†; Yu-Show Fu, PhD†

Background and Purpose—Stroke is a cerebrovascular defect that leads to many adverse neurological complications. Current pharmacological treatments for stroke remain unclear in their effectiveness, whereas stem cell transplantation shows considerable promise. Previously, we have shown that human umbilical mesenchymal stem cells (HUMSCs) can differentiate into neurons in neuronal-conditioned medium. Here we evaluate the therapeutic potential of HUMSC transplantation for ischemic stroke in rats.

Methods—Focal cerebral ischemia was produced by middle cerebral artery occlusion and reperfusion. The HUMSCs treated with neuronal-conditioned medium or not treated were transplanted into the ischemic cortex 24 hours after surgery.

Results—Histology and MRI revealed that rats implanted with HUMSCs treated with neuronal-conditioned medium or not treated exhibited a trend toward less infarct volume and significantly less atrophy compared with the control group, which received no HUMSCs. Moreover, rats receiving HUMSCs showed significant improvements in motor function, greater metabolic activity of cortical neurons, and better revascularization in the infarct cortex. Implanted HUMSCs, treated or not treated, survived in the infarct cortex for at least 36 days and released neuroprotective and growth-associated cytokines, including brain-derived neurotrophic factor, platelet-derived growth factor-AA, basic fibroblast growth factor, angiopoietin-2, CXCL-16, neutrophil-activating protein-2, and vascular endothelial growth factor receptor-3.

Conclusions—Our results demonstrate the therapeutic benefits of HUMSC transplantation for ischemic stroke, likely due to the ability of the cells to produce growth-promoting factors. Thus, HUMSC transplantation may be an effective therapy in the future. (Stroke. 2011;42:2045-2053.)

Key Words: magnetic resonance imaging | MCAO | positron emission tomography | transplantation

To date most pharmacological treatments for stroke have confined therapeutic windows. Thrombolytic therapy, for example, using tissue plasminogen activator is effective only when given within 3 hours of a stroke. One alternative strategy to treat stroke is the transplantation of pluripotent stem cells. However, the use of human embryonic stem cells is controversial, partially due to moral and ethical concerns. In this regard, adult human stem cells appear to be a favorable option for cell replacement therapies. Nevertheless, lack of sufficient adult human tissue and appropriately differentiated cells has limited their therapeutic applications for stroke.

Human umbilical mesenchymal stem cells (HUMSCs) in the Wharton’s jelly of the umbilical cord possess stem cell properties and can be cultured in abundance in vitro. We previously have demonstrated that HUMSCs can differentiate into neuron-like cells when cultured with neuronal-conditioned medium (NCM) for 6 days. Moreover, transplantation of dopaminergic neurons derived from HUMSCs into the striatum of parkinsonian rats alleviates lesion-induced, amphetamine-evoked rotation behavior. HUMSCs are a suitable stem cell source for transplantation but their therapeutic effectiveness in ischemic stroke has not yet been evaluated.

In this study, we take the beneficial neuronal properties of differentiated HUMSCs to test the hypothesis that transplantation of HUMSCs facilitates the healing of the infarct cortex and functional recovery after ischemic stroke in rats.
treated HUMSCs or HUMSCs treated with NCM for 6 days, which induced their differentiation into neuron-like cells, were implanted into the ischemic cortex after middle cerebral artery occlusion (MCAO) and reperfusion. Our investigation may lead to novel therapeutic approaches to ischemic stroke.

Methods
The use of human tissue and laboratory animals in this study was approved by the Research Ethics Committee at Taipei Veterans General Hospital and the Animal Research Committee of the College of Medicine at National Yang-Ming University. The timeframe for various experiments is illustrated in Figure 1A. More detailed information about the materials and methods used in this study is available in Supplemental Methods in the Supplemental Material (http://stroke.ahajournals.org).

Preparation of HUMSCs and NCM
With written consent from the parents of the infants, human umbilical cords were collected in cold Hanks balanced salt solution. The mesenchymal cells in Wharton’s jelly were dissociated aseptically with collagenase and trypsin and were cultured by 10% fetal bovine serum in Dulbecco Modified Eagle Medium. Sprague-Dawley rats, obtained from the Laboratory Animal Center at National Yang-Ming University, at the age of 7 days were deeply anesthetized and their brains were removed and triturated in Ca²⁺/Mg²⁺-free buffer. The dissociated cells were resuspended in 10% fetal bovine serum in Dulbecco Modified Eagle Medium and incubated at 37°C. The medium was collected 5 days later as NCM for the culture of HUMSCs.

Animal Surgery
Adult Sprague-Dawley rats (weighing 280 to 360 g) were anesthetized with chloral hydrate (400 mg/kg body weight, intraperitoneally). We performed MCAO surgery and reperfusion as previously described.9 Briefly, a hole was drilled by the right orbit to expose the right middle cerebral artery. The artery was ligated at the same time as both common carotid arteries were clamped simultaneously for 90 minutes, after which the ligature and clamping clips were removed to

Figure 1. Changes of the ischemic cortex after MCAO in the control rats. The diagram shows the time course for various experiments in this study (A). Changes in cerebral blood flow is measured by a laser Doppler flowmeter (B). I, Before ligations; (II) onset of the ligations; (III) blood flow obstruction (90 minutes); (IV), removal of the ligations; (V) restoration of blood flow. Representative 2,3,5-triphenyltetrazolium chloride (TTC)-stained images of rat brains are shown after MCAO (C; arrowheads: damaged areas). The infarct volume, measured from TTC-stained sections is the largest 1 day after MCAO due to extensive edema but is reduced thereafter (D). The time points examined for TTC staining are indicated on the abscissa. *P<0.05 compared with 1 day after MCAO. Scale bar=1 cm. MCAO indicates middle cerebral artery occlusion.
restore blood flow. Cortical blood flow at the right frontoparietal region was measured during the surgery by a laser Doppler flowmeter (OxyLab LDF; Oxford Optronix).

Transplantation of HUMSCs
Cultured HUMSCs used in this study were collected after 8 to 10 passages. A total of $5 \times 10^5$ HUMSCs, treated with NCM for 6 days or without NCM treatment, were grafted into the infarct cortex of each rat ($n=25$/group) 24 hours after MCAO by 2 injections (bregma +1.2 mm/+2.8 mm, medial-lateral +5.2 mm/+6.2 mm, dorsal-ventral −4.0 mm/−5.0 mm). The control group ($n=25$) received only phosphate-buffered saline injections. Another group of rats were grafted with bis-benzimide-labeled HUMSCs to trace the distribution of these cells.

Infarct Cortex Identification
Rats ($n=4$/group) were deeply anesthetized and decapitated. Coronal sections of the brains were sliced at 2 mm, immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) (T8877; Sigma), and then fixed with 10% formalin. The size of the infarct area, which was devoid of red staining, was determined on the digital images using ImagePro software.

Magnetic Resonance Imaging
T2-weighted images of the whole brain ($n=5$/group) were acquired by a high-resolution 3-T MRI system (Biospec; Bruker Companies) 1, 8, 15, 22, 29, and 36 days after HUMSC transplantation. On the MRIs, white areas in the infarct cortex were indicative of edema. Cortical atrophy was determined by subtracting the volume of the residual cortex of the right hemisphere from that of the left (unaffected) hemisphere. The infarct volume of the cortex was defined as the total volume of edema and atrophy.

Behavioral Tests
Two behavioral tests were performed ($n=25$/group). A cylinder test evaluated the frequency at which a rat used each of its forearms to explore the wall of a transparent cylinder to determine the asymmetrical uses of the contralateral (affected) forearm ([contralateral uses/(contralateral+ipsilateral uses)]×100). A rotarod test evaluated the coordinated movements of the limbs by measuring the latency at which the rats remained on a slowly accelerated spinning rod ([latency after MCAO/latency before MCAO]×100).

Positron Emission Tomography
Rats ($n=4$/group) were anesthetized and received intravenous injection of 10 mCi $2^{-18F}$-fluoro-2-deoxy-D-glucose 45 minutes before the image of the head was taken by a GE/Scanditronix positron emission tomography camera (PC4096-15WB; General Electric Company, Uppe-sala, Sweden). The uptake of $2^{-18F}$-fluoro-2-deoxy-D-glucose in the image of the head was taken by a GE/Scanditronix positron emission tomography camera (PC4096-15WB; General Electric Company, Uppe-sala, Sweden). The uptake of $2^{-18F}$-fluoro-2-deoxy-D-glucose in the infarct cortex was compared with that in the contralateral intact cortex and was expressed in percentages.

Histology and Immunohistochemistry
Rats ($n=5$/group) were anesthetized and perfused transcardially with 4% paraformaldehyde. Frozen sections were cut at 30 µm in a cryostat and stained with cresyl violet for light microscopy. Engrafted HUMSCs were identified by immunohistochemistry using mouse antihuman specific nucleus antigen antibody (1:100, MAB1281; Chemicon, Temecula, CA) followed by the biotinylated secondary antibody. The immunolabeling was visualized by biotin–avidin peroxidase reaction using 3,3’-diaminobenzidine as the chromogen.

Analysis of Blood Vessels
Rats ($n=4$/group) were anesthetized and received intracardial injections of 50 µg/mL fluorescent isothiocyanate–dextran amine (FD2000S; Sigma) to label the blood vessels 36 days after transplantation. The rat brains were removed, rapidly fixed in 4% paraformaldehyde, and cut at 30 µm in a cryostat. Vessel density, defined as the proportion of the area occupied by the labeled blood vessels to that of the entire infarct cortex, was measured using ImagePro.

Human Cytokine Array
A human protein cytokine kit (RayBiotech Inc, Norcross, GA) was used to screen the expression of 174 human cytokines. The homogenates of cortices ($n=3$/group) were incubated with the membranes containing an array of human cytokine antibodies. The levels of cytokine expression were determined by the intensity of immunoreactivity, relative to that of the standard controls, following the manufacturer’s instructions.
Statistical Analysis
One-way or 2-way analysis of variance was used to compare all means followed by least significant difference posteriori tests. All data are presented as means±SEM. A statistically significant difference was defined at P<0.05. Detailed statistical results are available in Supplemental Tables.

Results
MCAO Surgery Alters the Blood Flow and Causes Cortical Infarction
Blood flow of the cortex was decreased approximately from 350 down to 40 blood perfusion units during the 90-minute ligation and returned to approximately 200 blood perfusion units after removing the ligation (Figure 1B), indicating that an ischemia–reperfusion stroke model was successfully established in our experiments.

As shown by TTC staining, the infarct cortex of the control rat was swollen and edematous considerably 1 day after MCAO (Figure 1C). The total infarct volume, however, was reduced significantly at 8 days after MCAO and remained stable thereafter because the gradual decrease in cortical edema was compensated by increased development of cortical atrophy (Figure 1D). Our results demonstrate a temporal change in cortical morphology from edematous to atrophic after MCAO in control rats.

HUMSC Transplantation Reduces the Damaged Area of the Infarct Cortex
We likewise used TTC and cresyl-violet staining to determine the therapeutic effect of HUMSCs on the infarct cortex. The damaged areas of the infarct cortices were minor in the untreated-HUMSC and NCM-treated HUMSC groups relative to those of the control group, which received phosphate-buffered saline injection, at 8 days and 36 days after transplantation (Figure 2).

We further analyzed the infarct volume of the cortex both qualitatively and quantitatively using MRI (Figure 3). Cortical edema became obvious in the infarct cortex 1 day after transplantation in all 3 groups. Starting from 8 days after transplantation, however, the 2 groups grafted with HUMSCs showed significantly minor cortical atrophy than the control group (Figure 3D). These results demonstrate that HUMSCs, treated with NCM or not treated, have a protective role in preventing the infarct cortex from atrophy after MCAO.
HUMSC Transplantation Enhances Neuronal Metabolic Activity and Improves Motor Function

With the use of positron emission tomography scan, we found that the 2-[18F]-fluoro-2-deoxy-D-glucose radioactivity of the infarct cortex was significantly greater in the groups receiving untreated HUMSCs or NCM-treated HUMSCs than in the control group at both 1 and 29 days after transplantation (Figure 4A–B), suggesting that engrafted HUMSCs enhance neuronal metabolic activities in the infarct cortex after MCAO.

In the cylinder test (Figure 4C; Supplemental Videos 1 to 4), rats in the 2 groups grafted with HUMSCs used their contralateral (affected) forelimbs significantly more frequently than the control group at all time points tested after MCAO, suggesting that engrafted HUMSCs enhance motor function after MCAO. In the rotarod test, rats grafted with untreated HUMSCs or NCM-treated HUMSCs spent significantly longer time on the rotarod than the control group from 8 days after transplantation onward (Figure 4D; Supplemental Videos 5 to 8). Our results indicate that HUMSCs, treated with or without NCM, may improve motor function after MCAO.

Engrafted HUMSCs Survive and Migrate in the Infarct Cortex

Bis-benzimide-labeled HUMSCs migrated along the rostrocaudal axis from the 2 implantation sites 36 days after transplantation (Figure 5A). Moreover, immunostaining showed that the engrafted HUMSCs, not treated or treated with NCM, were distributed near the injection sites 15 days after transplantation but scattered in the infarct cortex 36 days after transplantation (Figure 5B–C). These results demonstrate the survival and migration of the engrafted HUMSCs in the infarct cortex, regardless of having been treated with NCM or not treated.

Figure 4. Enhanced cortical metabolic activity and functional improvements after HUMSC transplantation. Neuronal metabolic activities, determined by glucose uptake using positron emission tomography (A, upper rows: horizontal sections, lower rows: coronal sections), are higher in the 2 groups receiving HUMSCs at 1 day and 29 days after transplantation (B, *P < 0.05 compared with the control on the same day). The infarct cortex is demarcated by blue dotted lines, whereas the contralateral unaffected cortex is marked by black dotted lines. The 2 groups grafted with HUMSCs also show better functional recovery in the cylinder test (C) and rotarod test (D) than the control group (*P < 0.05 compared with the control group at the same day; #P < 0.05 compared with the normal value obtained before MCAO of the same group). d indicates days. HUMSC indicates human umbilical mesenchymal stem cell; MCAO, middle cerebral artery occlusion.
**HUMSC Transplantation Promotes Angiogenesis**

The 2 groups receiving HUMSCs not only manifested more widespread newly formed blood vessels on the surface of the cortex, but also showed significantly greater vascular density in the infarct areas than the control group 36 days after transplantation (Figure 6A–B), suggesting that engrafted HUMSCs, treated with NCM or not treated, may promote angiogenesis in the ischemic cortex, leading to the beneficial outcome after transplantation.

**Engrafted HUMSCs Express Growth-Related Cytokines in the Ischemic Cortex**

We next performed human cytokine array to identify factors underlying the growth-promoting features of the engrafted HUMSCs (Figure 6C). Our analyses showed that the levels of human brain-derived neurotrophic factor, neutrophil-activating protein-2, angiopoietin-2, CXCL-16, and platelet-derived growth factor-AA were significantly increased in the infarct cortices of the 2 groups grafted with HUMSCs (Figure 6D). The group receiving NCM-treated HUMSCs showed significantly higher expression of basic fibroblast growth factor than the untreated HUMSC group. On the other hand, the untreated-HUMSC group demonstrated significantly greater levels at the expression of platelet-derived growth factor-AA and human vascular endothelial growth factor receptor-3 than the NCM-treated HUMSC group. These findings suggest that engrafted HUMSCs can release growth-associated human cytokines to preserve the infarct cortex, regardless if these HUMSCs are treated with NCM.

**Discussion**

Until now, several kinds of stem cells, including bone marrow mesenchymal stem cells, human umbilical cord blood mesenchymal stem cells, and human embryonic or fetal neural stem/progenitor cells, have been examined for effectiveness in treating stroke in rodents within the first week after stroke onset.\(^\text{10}\) These stem cells, when delivered intracerebrally or intravenously to animals with stroke, can reduce neurological deficits and improve functional recovery.\(^\text{11–14}\) The effectiveness of engrafted bone marrow mesenchymal stem cells, for instance, is derived from increased expression of trophic factors, enhanced neurogenesis, reduced cell death, and upregulated antiinflammatory cytokines and downregulated proinflammatory cytokines in the infarct cortex.\(^\text{15–18}\) Likewise, transplantation of umbilical mesenchymal stem cells increases the regional expression of various growth-associated trophic factors, activates angiogenic remodeling, and reduces immunoreaction and inflammation in the lesion.\(^\text{19–22}\) Similar therapeutic mechanisms are found after transplantation of human umbilical cord blood cells, which are also rich in mesenchymal stem cells.\(^\text{23,24}\) The effectiveness of HUMSC transplantation has been proven to be successful in treating diabetes, liver fibrosis, Parkinson disease, and spinal cord injury in rats.\(^\text{8,25–28}\) Our studies here further demonstrate that transplantation of HUMSCs, treated with NCM or not treated, substantially facilitates the repair of the infarct cortex and improves functional outcome after ischemic stroke in rats.

The HUMSCs are collected from the umbilical cord, which is generally considered to be a “leftover” after childbirth and can be obtained with little effort. The amount of HUMSCs collected can be increased greatly in cultures. Moreover, transplanted HUMSCs are immunologically compatible in recipients. With the least ethical dilemmas, if any, and with moderate technical requirements, HUMSCs appear to be a better source of cells for transplantation than other stem cells mentioned, although the therapeutic outcomes of these different types of stem cells are similar.

In this study, one of the most intriguing observations was that implanted HUMSCs, treated with NCM or not treated, showed similar therapeutic outcomes up to 36 days after stroke onset in rats. Previously, we found that HUMSCs treated with NCM start to express neurofilaments after 3 days...
and exhibit more neuronal properties, including the elaboration of neuronal processes and the expression of kainate receptor mRNA and glutamate decarboxylase, after 6 days of treatment.

It is likely that NCM-treated HUMSCs, supposed to differentiate into neurons, were not directly integrated into the neuronal network of the host, but rather released certain nourishing factors to enhance indirectly the endogenous mechanisms of tissue repair as did the untreated HUMSCs. This speculation is supported by our findings that a wide variety of growth-associated human cytokines were produced at high levels by both groups of engrafted HUMSCs, treated with NCM or not treated. We have previously shown that HUMSCs express different cytokines when engrafted into fibrotic liver versus the injured spinal cord, depending on different pathological microenvironments. It is likely that NCM-treated HUMSCs, supposed to differentiate into neurons, were not directly integrated into the neuronal network of the host, but rather released certain nourishing factors to enhance indirectly the endogenous mechanisms of tissue repair as did the untreated HUMSCs. This speculation is supported by our findings that a wide variety of growth-associated human cytokines were produced at high levels by both groups of engrafted HUMSCs, treated with NCM or not treated. We have previously shown that HUMSCs express different cytokines when engrafted into fibrotic liver versus the injured spinal cord, depending on different pathological microenvironments.

As revealed by our human cytokine assay, HUMSCs grafted in the infarct cortex released brain-derived neurotrophic factor, basic fibroblast growth factor, and platelet-derived growth factor-AA, which are neuroprotective and facilitate neurogenesis, whereas substantial amounts of angiopoietin-2, vascular endothelial growth factor receptor-R3, and CXCL-16 may promote angiogenesis.

Although neuronal death and degeneration occur after ischemic brain injury, neurogenesis starts spontaneously by endogenous precursors residing in the subventricular zone and hippocampus. These neuronal precursors can prolif-
egrate and migrate toward the damaged area of a stroke.34 However, only a small fraction of the dead neurons (approximately 0.2%) is replaced by new neurons after a stroke.33 Therefore, it is conceivable that the significantly high level of neuronal activity and better improvements in rats engrafted with HUMSCs are associated with the HUMSC-released neurotrophic factors that promote neurogenesis and neuroprotection.

After HUMSC transplantation, significantly increased density of newly formed blood vessels in the infarct cortex is in part due to the differentiation of the engrafted cells into endothelial cells.20 We also believe that the high levels of angiopoietin-2 is upregulated in the infarct area after ischemic stroke55 and is expressed at sites of vascular remodeling to initiate neovascularization synergistically with other angiogenic factors.36 Likewise, CXCL-16 and vascular endothelial growth factor receptor-R3 may stimulate the proliferation, chemotaxis, and tube formation of endothelial cells and are essential for the development of blood vessels.37,38 All these factors, therefore, are likely to facilitate the formation of new blood vessels in the infarct cortex after HUMSC transplantation.

Conclusions

Our study demonstrates the therapeutic benefits of HUMSC transplantation for the treatment of ischemic stroke in rats. Additional research is needed to better define the therapeutic potential of HUMSCs when transplanted at chronic stages of ischemic stroke. With abundant availability and easy accessibility, HUMSCs are a promising and reliable source for future therapeutic interventions.

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Disclosures

None.

References

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Preparation of HUMSCs

With written consent from the parents of the infants, human umbilical cords were collected in Hanks’ balanced salt solution (HBSS) (14185-052, Gibco, Grand Island, New York) at 4°C. After disinfection with 75% ethanol for 30 seconds, the umbilical cord vessels were cleared off in HBSS. The mesenchymal tissue in Wharton’s jelly was then diced into cubes 0.5 cm on each side and centrifuged at 250 g for 5 minutes. After removal of the supernatant fraction, the precipitate that contained mesenchymal tissue was washed with serum-free Dulbecco’s Modified Eagle’s Medium (DMEM) (12100-046, Gibco) and centrifuged at 250 g for 5 minutes. After aspiration of the supernatant fraction, mesenchymal tissue in the precipitate was treated with collagenase at 37°C for 18 hours, washed, and further digested with 2.5% trypsin (15090-046, Gibco) at 37°C for 30 minutes. Fetal bovine serum (FBS) (SH30071.03, Hyclone, Logan, Utah) was added to neutralize the excess trypsin. The dissociated mesenchymal cells were further dispersed by treatment with 10% FBS-DMEM and counted under a microscope by a hemocytometer. These HUMSCs then were used for further cultures or stored in liquid nitrogen for later use.

Preparation of Neuronal-Conditioned Medium

Sprague-Dawley rats at the age of 7 days were anesthetized by intraperitoneal injection of 10% chloral hydrate. The brain was removed, placed in Ca\(^{2+}\)/Mg\(^{2+}\)-free buffer (14185-052, Gibco), and centrifuged at 900 rpm for 5 minutes. After removal of the supernatant fraction, 10% FBS-DMEM was added to the precipitate, which was triturated 15 times to dissociate single cells.
The cells were resuspended in 10% FBS-DMEM and incubated at 37°C in 5% CO₂ and 95% O₂. To inhibit the growth of glial cells, 2 μM cytosine arabinofuranoside (c-6645, Sigma-Aldrich, St. Louis, Missouri) was added on the next day. On the fifth day of culture, the medium was collected as NCM. The HUMSCs were cultured solely in NCM, which was replaced every other day.

Animal surgery

Adult Sprague-Dawley rats (weighing 280 to 360 g) were used in this study. The rats were anesthetized with chloral hydrate (400 mg/kg body weight, i.p.) and fixed on a stereotaxic frame. We performed MCAO surgery and reperfusion to induce cerebral infarction by ligating the right middle cerebral artery and bilateral common carotid arteries, as previously described ⁹. Briefly, a curved, 1-cm skin incision was made vertically by the right orbit, and the temporalis muscle was removed to uncover the junction of the zygomatic arch and the squamous bone, where a 3-mm² burr hole was drilled to expose the middle cerebral artery. The artery was ligated with a 10-0 suture while both common carotid arteries were clamped simultaneously by nontraumatic aneurysm clips for 90 minutes, after which the ligature and clips were removed to restore blood flow. Cortical blood flow was measured during the surgery by a laser Doppler flowmeter (OxyLab LDF™, Oxford Optronix) through a 1-mm burr hole at the right frontoparietal region to ensure occlusion of the arteries and reperfusion.

Transplantation of HUMSCs

Cultured HUMSCs were collected after 8-10 passages. The HUMSCs treated with NCM for 6 days were trypsinized at 37°C for 5 minutes with 0.25% trypsin, and the dissociated cells
were resuspended in PBS. A total of $5 \times 10^5$ cells were grafted into the infarct cortex of each rat ($n = 25$) by two injections based on the coordinates relative to Bregma and the skull surface (first injection: anteroposterior +1.2mm, lateral +5.2mm, ventral −4.0mm; second injection: anteroposterior −2.8mm, lateral +6.2mm, ventral −5.0mm). The needle was withdrawn after a 10-minute wait to prevent injected cells from leaking. Another group of stroke rats ($n = 25$) were grafted with HUMSCs without NCM treatment, whereas the control group ($n = 25$) received only phosphate buffered saline (PBS) injections in the infarct cortex. A separate group of rats were grafted with HUMSCs incubated with 1 $\mu$g/mL bis-benzimide (B2883, Sigma-Aldrich) for 24 hours to trace the distribution of these cells in the infarct cortex. No immunosuppressant was given to any group of rats after transplantation.

**Identification of the infarct cortex**

Rats ($n = 4$/group) were deeply anesthetized with chloral hydrate (400 mg/kg body weight, i.p.) and decapitated 1, 8, 15, and 29 days after MCAO surgery. The brains were removed carefully and dissected into 2-mm-thick coronal sections using a brain slicer. These fresh brain slices were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) (T8877, Sigma) in normal saline for 30 minutes and then fixed with 10% formalin at 4°C. The size of the infarct area, which was devoid of red staining, was determined by analyzing the digital images of the brain slices using ImagePro software.

**Magnetic resonance imaging examinations**

T2-weighted images of the whole brain were acquired by a high resolution 3-Tesla magnetic resonance imaging (MRI) system (Biospec, Bruker Companies, Ettlingen, Germany) at
1, 8, 15, 22, 29, and 36 days after transplantation (n = 5/group). With pulse sequences generated by spin echo (repetition time 3500 ms; echo time 62 ms), 20 coronal and transverse images of the brain were obtained at 1-mm intervals and analyzed using ImagePro. On the MRI images, white areas in the infarct cortex were indicative of edema. Atrophy of the infarct cortex was determined by subtracting the volume of the residual cortex of the right hemisphere from that of the left (unaffected) hemisphere. The infarct volume of the cortex was defined as the total volume of edema and atrophy.

**Behavioral tests**

Two behavioral tests were performed to evaluate the motor function before MCAO and 1, 4, 8, 15, 22, 29, and 36 days after HUMSC transplantation (n = 25/group). A cylinder test evaluated asymmetric use of the forelimbs. The rats were placed in a transparent cylinder 20-cm in diameter and 30-cm high for 3 minutes and the frequency at which a rat used each of its forearms to explore the wall in an upright posture was recorded. The uses of the contralateral (affected) forearm were calculated and expressed in percentages \(((\text{contralateral uses}/(\text{contralateral + ipsilateral uses})) \times 100)^2\). A rotarod test evaluated the coordinated movements of the limbs and body through balance on a spinning rod that accelerated slowly from 4 to 40 rpm within 5 minutes.

There were three habituation trials before the surgery. The time the rats remained on the rotarod was recorded and averaged from three daily trials. Percentages for the time on the rotarod after HUMSC transplantation, relative to that before the MCAO surgery, were calculated.

**Positron Emission Tomography**
Rats (n=4/group) were anesthetized and received intravenous injection of 2-[\textsuperscript{18}F]-fluoro-2-deoxy-D-glucose (\textsuperscript{18}F-FDG). An image of the head was taken 45 minutes after an intravenous bolus injection of 10 mCi of sterile \textsuperscript{18}F-FDG. A GE/Scanditronix positron emission tomography (PET) camera (PC4096-15WB, General Electric Company, Uppsala, Sweden) was used to make positron images. We used a VAX computer (Digital Equipment Corporation, Maynard, Massachusetts) as the network server at our center and visualized PET images with a VAX workstation, and we used DECnet with Pathwork (Digital Equipment Corporation) to connect PCs to the VAX so that PET images could be visualized with software running on Windows 3.1 (Microsoft Corporation, Redmond, Washington). PET images were obtained preoperatively and visually analyzed to identify areas of localized \textsuperscript{18}F-FDG uptake compared with uptake in the contralateral intact cortex. All PET images were interpreted by observers blinded to the identity and treatment of the animals.

**Histology and immunohistochemistry**

Rats (n=5/group) were anesthetized with chloral hydrate and fixed by transcardial perfusion with 4\% paraformaldehyde in PBS. The brains were removed and dissected and immersed in the same fixative for 24 hours at 4°C. After cryoprotection with 30\% sucrose in PBS, frozen sections were cut at a thickness of 30 µm in a cryostat and stained with cresyl violet for light microscopy. Sections containing the graft of bis-benzimide (B2883, Sigma-Aldrich) -labeled HUMSCs were examined using an epifluorescence microscope.

Engrafted HUMSCs were identified by immunohistochemistry. Frozen sections were rinsed in 0.1M PBS and treated with blocking and permeabilizing solution containing 5\% normal goat serum, 3\% bovine serum albumin, and 0.05\% Triton X-100 for 30 minutes to prevent
nonspecific immunobinding. Mouse anti-human specific nucleus antigen (1:100, MAB1281, Chemicon, Temecula, California) was used as the primary antibody. After 18 hours of incubation at 4°C, sections were rinsed in PBS and reacted with biotinylated secondary antibody at room temperature for 1 hour. The immunolabeling was visualized by biotin-avidin peroxidase reaction using an ABC kit (PK-4000; Vector Laboratories, Burlingame, California) in combination with 0.05% 3,3′-diaminobenzidine as the chromogen and 0.01% H₂O₂ in 50 mM Tris buffer. Sections were dehydrated and coverslipped with Permount.

Analysis of blood vessels

Rats (n=4/group) were anesthetized and received intracardial injections of (50 mg/mL) fluorescein isothiocyanate (FITC)-dextran (FD-2000S, Sigma) to label the blood vessels 36 days after transplantation. The rats were decapitated after 2 minutes and the brains were removed, rapidly fixed in 4% paraformaldehyde at 4°C overnight, and immersed in 30% sucrose in PBS for cryoprotection. Frozen sections were cut at 30 µm in a cryostat. Vessel density, defined as the proportion of the area occupied by the labeled blood vessels to that of the entire infarct cortex, was measured using ImagePro.

Human Cytokine Array

To elucidate which human cytokines were involved in the repair of the infarct cortex, a human protein cytokine kit (AAH-CYT-2000, RayBio® Human Cytokine Antibody Array C Series 2000, RayBiotech, Inc. Norcross, Georgia) was used to screen the expression of 174 human cytokines. Rats (n = 3/group) were deeply anesthetized and decapitated 15 days after HUMSC transplantation. The cortex tissue was homogenized in lysis buffer and centrifuged at
1,500 g to separate cell debris. The supernatant was harvested and then incubated with the membranes containing an array of human cytokine antibodies for 2 hours at room temperature. The levels of cytokine expression were determined by the intensity of immunoreactivity, relative to that of the standard controls, using enhanced chemiluminescence following the manufacturer's instructions.

**Statistical Analysis**

One-way or two-way analysis of variance was used to compare all means, followed by least significant difference (LSD) posteriori tests. All data are presented as means ± SEM. A statistically significant difference was defined at $p < 0.05$. 

## SUPPLEMENTAL TABLES

### Supplemental table 1. The functional improvements after HUMSC transplantation.

<table>
<thead>
<tr>
<th>Days</th>
<th>Group</th>
<th>Control (n=25)</th>
<th>Untreated-HUMSCs (n=25)</th>
<th>NCM-HUMSCs (n=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tests</td>
<td>Cylinder test</td>
<td>Rotarod test</td>
<td>Cylinder test</td>
</tr>
<tr>
<td>-2D (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.38±2.65 #</td>
<td>49.25±0.49</td>
<td>18.23±4.17 #</td>
<td>15.13±3.71 #</td>
<td>24.86±3.58 #</td>
</tr>
<tr>
<td># p = 3.26x10^-10</td>
<td>100</td>
<td># p = 3.54x10^-7</td>
<td># p = 4.04x10^-27</td>
<td># p = 2.31x10^-5</td>
</tr>
<tr>
<td>-1D (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.44±3.55 #</td>
<td>20.90±3.63 #</td>
<td>43.30±8.18 #</td>
<td>32.34±4.03*,#</td>
<td>53.11±4.98 #</td>
</tr>
<tr>
<td>p = 7.99x10^-7</td>
<td># p = 6.53x10^-7</td>
<td># p = 9.03x10^-14</td>
<td># p = 0.036 #</td>
<td># p = 1.05x10^-13</td>
</tr>
<tr>
<td>1D (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.80±2.74 #</td>
<td>41.27±5.40 #</td>
<td>33.10±2.79*,#</td>
<td>58.46±4.61*,#</td>
<td>36.24±3.45*,#</td>
</tr>
<tr>
<td># p = 1.40x10^-7</td>
<td># p = 1.65x10^-14</td>
<td># p = 0.042 #</td>
<td># p = 0.016 #</td>
<td># p = 0.003 #</td>
</tr>
<tr>
<td>4D (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.17±2.33 #</td>
<td>32.94±4.25 #</td>
<td>35.60±3.50*,#</td>
<td>55.03±3.89*,#</td>
<td>37.91±4.24*,#</td>
</tr>
<tr>
<td># p = 3.40x10^-8</td>
<td># p = 1.31x10^-17</td>
<td># p = 0.034 #</td>
<td># p = 0.004 #</td>
<td># p = 0.002 #</td>
</tr>
<tr>
<td>8D (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22.12±1.95 #</td>
<td>33.47±3.91 #</td>
<td>35.76±3.07*,#</td>
<td>59.73±3.06*,#</td>
<td>37.10±2.70*,#</td>
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<tr>
<td># p = 2.05x10^-9</td>
<td># p = 4.80x10^-17</td>
<td># p = 0.038 #</td>
<td># p = 6.34x10^-5</td>
<td># p = 0.017 #</td>
</tr>
<tr>
<td>15D (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.14±2.66 #</td>
<td>35.35±5.68 #</td>
<td>36.27±3.59*,#</td>
<td>58.09±4.27*,#</td>
<td>37.26±4.07*,#</td>
</tr>
<tr>
<td># p = 1.20x10^-9</td>
<td># p = 5.61x10^-16</td>
<td># p = 0.0001</td>
<td># p = 0.001</td>
<td># p = 0.001</td>
</tr>
<tr>
<td>22D (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23.28±2.47 #</td>
<td>36.51±2.89 #</td>
<td>38.35±3.25*,#</td>
<td>62.24±3.88*,#</td>
<td>39.73±4.03*,#</td>
</tr>
<tr>
<td># p = 4.50x10^-8</td>
<td># p = 1.40x10^-15</td>
<td># p = 0.002 #</td>
<td># p = 2.74x10^-6</td>
<td># p = 0.001</td>
</tr>
<tr>
<td>29D (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26.59±2.81 #</td>
<td>37.86±3.47 #</td>
<td>40.24±3.78*,#</td>
<td>63.24±4.05*,#</td>
<td>40.91±4.12*,#</td>
</tr>
<tr>
<td># p = 5.57x10^-9</td>
<td># p = 1.40x10^-15</td>
<td># p = 0.002 #</td>
<td># p = 2.74x10^-6</td>
<td># p = 0.001</td>
</tr>
<tr>
<td>36D (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05, compared with the control group at the same day. 
# p < 0.05, compared with the normal value obtained before MCAO of the same group.
Supplemental table 2. Infarct volume (edema+atrophy) examined by magnetic resonance imaging (MRI).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Days (mm³)</th>
<th>1D</th>
<th>8D</th>
<th>15D</th>
<th>22D</th>
<th>29D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrophy (mm³)</td>
<td>ND</td>
<td>30.00±7.63</td>
<td>67.35±8.67</td>
<td></td>
<td>109.20±6.67</td>
<td>111.47±5.43</td>
</tr>
<tr>
<td>Infarct (mm³)</td>
<td></td>
<td>287.33±6.34</td>
<td>164.37±10.71</td>
<td>172.30±18.26</td>
<td>178.71±7.77</td>
<td>171.62±5.57</td>
</tr>
<tr>
<td>Atrophy (mm³)</td>
<td></td>
<td>9.58±2.08</td>
<td>37.07±7.60</td>
<td>44.84±12.32</td>
<td>46.36±9.70</td>
<td></td>
</tr>
<tr>
<td>Untreated HUMSCs (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrophy (mm³)</td>
<td></td>
<td>11.39±5.29</td>
<td>34.15±7.01</td>
<td>39.58±3.92</td>
<td>39.91±4.64</td>
<td></td>
</tr>
<tr>
<td>Infarct (mm³)</td>
<td></td>
<td>286.61±36.79</td>
<td>155.84±32.29</td>
<td>142.42±18.05</td>
<td>136.45±22.53</td>
<td>130.78±18.65</td>
</tr>
<tr>
<td>NCM-HUMSCs (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrophy (mm³)</td>
<td></td>
<td>165.03±18.24</td>
<td>180.87±1.84</td>
<td>153.81±16.72</td>
<td>150.20±13.74</td>
<td></td>
</tr>
<tr>
<td>Infarct (mm³)</td>
<td></td>
<td>332.86±30.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* $p < 0.05$ in atrophy volume, compared with the control group at the same day.

# $p < 0.05$ in infarct volume, compared with that of the same group 1 day after transplantation.

$\Delta p < 0.05$ in atrophy volume, compared with that of the same group 8 days after transplantation.

$\bullet p < 0.05$ in atrophy volume, compared with that of the control group 15 days after transplantation.
Supplemental table 3. Enhanced cortical metabolic activity measured by positron emission tomography after HUMSC transplantation.

<table>
<thead>
<tr>
<th>Days</th>
<th>Groups</th>
<th>Control (n=4)</th>
<th>Untreated HUMSCs (n=4)</th>
<th>NCM-HUMSCs (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>69.38±5.12</td>
<td>86.24±3.39*</td>
<td>88.60±5.89*</td>
</tr>
<tr>
<td>1 D (%)</td>
<td>Untreated HUMSCs (n=4)</td>
<td>5.12</td>
<td>p = 0.016</td>
<td>p = 0.007</td>
</tr>
<tr>
<td></td>
<td>NCM-HUMSCs (n=4)</td>
<td>86.01±3.90*</td>
<td>92.66±1.85*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.39</td>
<td>p = 0.045</td>
<td>p = 0.012</td>
</tr>
<tr>
<td>29 D (%)</td>
<td>Control (n=4)</td>
<td>74.70±4.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05, compared with the control at the same day

Supplemental table 4. Increased newly formed blood vessels in the infarct cortex after HUMSC transplantation.

<table>
<thead>
<tr>
<th>Side</th>
<th>Groups</th>
<th>Control (n=4)</th>
<th>Untreated HUMSCs (n=4)</th>
<th>NCM-HUMSCs (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14.19±0.61%*</td>
<td>13.30±0.64%*</td>
<td>14.31±0.96%*</td>
</tr>
<tr>
<td>Contralateral cortex</td>
<td></td>
<td>* p = 0.002</td>
<td>* p = 0.002</td>
<td>* p = 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.15±0.20%</td>
<td>17.10±2.23%*</td>
<td>17.02±1.65%*</td>
</tr>
<tr>
<td>Ipsilateral cortex</td>
<td></td>
<td>* p = 0.002</td>
<td>* p = 5.52 x10⁻⁵</td>
<td>* p = 0.0001</td>
</tr>
</tbody>
</table>

* p <0.05, compared with the ipsilateral cortex (infarct) of the control group.
Supplemental table 5. Increased growth-promoting cytokines in the infarct cortex after HUMSC transplantation.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Groups</th>
<th>Normal (n=3)</th>
<th>Control (n=3)</th>
<th>Untreated HUMSCs (n=3)</th>
<th>NCM-HUMSCs (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td></td>
<td>74.63±7.53</td>
<td>67.37±14.23</td>
<td>163.24±12.56* p = 0.0001</td>
<td>176.8±1.84* p = 6.61 x10⁻⁵</td>
</tr>
<tr>
<td>NAP-2</td>
<td></td>
<td>300.75±10.38</td>
<td>352.96±0.37</td>
<td>431.58±2.45* p = 0.001</td>
<td>420.45±19.47* p = 0.003</td>
</tr>
<tr>
<td>Angiopoietin-2</td>
<td></td>
<td>135.12±7.79</td>
<td>134.47±2.49</td>
<td>182.40±11.71* p = 0.008</td>
<td>206.42±13.92* p = 0.001</td>
</tr>
<tr>
<td>bFGF</td>
<td></td>
<td>148.87±1.19</td>
<td>151.75±9.23</td>
<td>155.09±0.51</td>
<td>204.25±3.73* p = 7.66 x10⁻⁵</td>
</tr>
<tr>
<td>CXCL-16</td>
<td></td>
<td>122.62±0.90</td>
<td>135.69±3.23</td>
<td>172.50±8.34* p = 0.001</td>
<td>170.22±4.63* p = 0.001</td>
</tr>
<tr>
<td>PDGF-AA</td>
<td></td>
<td>51.83±1.99</td>
<td>59.18±8.89</td>
<td>175.41±1.48* p = 7.11 x10⁻⁵</td>
<td>88.58±19.97* p = 0.046</td>
</tr>
<tr>
<td>VEGF-R3</td>
<td></td>
<td>115.35±4.08</td>
<td>102.45±8.22</td>
<td>208.88±34.58* p = 0.003</td>
<td>142.57±1.50</td>
</tr>
</tbody>
</table>

* p < 0.05, compared with the control group.

# p < 0.05, compared between groups receiving untreated HUMSCs or NCM-treated HUMSCs.
SUPPLEMENTAL REFERENCES


LEGENDS FOR THE VIDEO FILES

Supplementary video 1: Normal – the cylinder test of normal rats before MCAO.

Supplementary video 2: Control group – the cylinder test of control group at 36 days after PBS injection.

Supplementary video 3: Untreated-HUMSCs group – the cylinder test of the untreated-HUMSCs group at 36 days after transplantation.

Supplementary video 4: NCM-HUMSCs group – the cylinder test of NCM-treated HUMSCs group at 36 days after transplantation.

Supplementary video 5: Normal – the rotarod test of the normal rat before MCAO.

Supplementary video 6: Post-stroke – the rotarod test of the control rat after MCAO.

Supplementary video 7: Untreated-HUMSCs group – the rotarod test of the rat receiving untreated-HUMSCs at 36 days after transplantation.

Supplementary video 8: NCM-HUMSCs group – the rotarod test of the rat receiving NCM-treated HUMSCs at 36 days after transplantation.
Human Umbilical Mesenchymal Stem Cells Promote Recovery After Ischemic Stroke

Yu-Ching Lin, MS1; Tsui-Ling Ko, PhD4; Yang-Hsin Shih, MD5,7; Maan-Yuh Anya Lin, PhD3,8; Tz-Win Fu, BS8; Hsiao-Sheng Hsiao, MS2; Jung-Yu C. Hsu, PhD9; Yu-Show Fu, PhD2,10

1 Institute of Anatomy and Cell Biology, 2 Department of Anatomy and Cell Biology, and 3 Department of Pharmacology, School of Medicine, National Yang-Ming University, Taipei, Taiwan; 4 Department of Anatomy and 5 School of Medicine, Taipei Medical University, Taipei, Taiwan; 6 Department of Neurosurgery and 7 Neurological Institute, 8 Taipei Veterans General Hospital, Taipei, Taiwan; 9 Department of Cell Biology and Anatomy, National Cheng-Kung University College of Medicine, Tainan, Taiwan; and 10 Department of Education and Research, Taipei City Hospital, Taipei, Taiwan

Abstract

ヒト臍帯間葉系幹細胞は虚血性脳卒中後の回復を促進する

Human Umbilical Mesenchymal Stem Cells Promote Recovery After Ischemic Stroke

Yu-Ching Lin, MS1; Tsui-Ling Ko, PhD4; Yang-Hsin Shih, MD5,7; Maan-Yuh Anya Lin, PhD3,8; Tz-Win Fu, BS8; Hsiao-Sheng Hsiao, MS2; Jung-Yu C. Hsu, PhD9; Yu-Show Fu, PhD2,10

1 Institute of Anatomy and Cell Biology, 2 Department of Anatomy and Cell Biology, and 3 Department of Pharmacology, School of Medicine, National Yang-Ming University, Taipei, Taiwan; 4 Department of Anatomy and 5 School of Medicine, Taipei Medical University, Taipei, Taiwan; 6 Department of Neurosurgery and 7 Neurological Institute, 8 Taipei Veterans General Hospital, Taipei, Taiwan; 9 Department of Cell Biology and Anatomy, National Cheng-Kung University College of Medicine, Tainan, Taiwan; and 10 Department of Education and Research, Taipei City Hospital, Taipei, Taiwan

Human umbilical mesenchymal stem cells (HUMSC) have been shown to promote recovery after ischemic stroke. In this study, we investigated the potential of HUMSC for the treatment of ischemic stroke. HUMSC were isolated from human umbilical cords and were characterized by their immunophenotypic and functional properties. The cells were then transplanted into a rat model of ischemic stroke, and the effects on neuronal survival, angiogenesis, and functional recovery were assessed. Our results indicate that HUMSC transplantation may be a promising therapeutic approach for the treatment of ischemic stroke. Further studies are needed to clarify the mechanism of action and to evaluate the feasibility of HUMSC transplantation in clinical settings.