Introduction

Mesenchymal stem cells (MSCs) exist in adult bone marrow and occupy less than 0.01% of all nucleated bone marrow cells [1]. Even though the main MSC differentiation is of mesodermal lineage, several experimental studies have revealed that these cells can differentiate into neuronal and glial ectodermal cells [2-4]. Animal and human trials have suggested that MSC administration exerts beneficial effects in ischemic heart disease, stroke, and even progressive neurodegenerative diseases, regardless of their autologous or allogeneic origin [5-7].

Neurogenins (Ngn) are a family of basic helix-loop-helix (bHLH) transcription factors involved in neuronal or glial differentiation, and their subtypes are classified into Ngn 1, 2, and 3 [8]. Among Ngn subtypes, Ngn1 facilitates neuronal differentiation as an activator for downstream transcription factors such as NeuroD [9]. A previous study demonstrated that intracerebral (IC) administration of Ngn1-expressing MSCs (MSCs/Ngn1) produced restorative augmentation of behavioral functions and induced the expression of neuron-specific proteins and voltage-gated Ca\(^{2+}\) and Na\(^{+}\) channels, suggesting survival of the grafted MSCs [10].

Animal experiments have shown that intracerebral (IC) transplantation is a reliable route for stem cell transplantation [11-13]. IC cell grafting is avoided in the clinical field due to its hazardous complications, such as seizure, chronic subdural hematoma, cortical vein occlusion, and postprocedural clinical deterioration [14-16]. On the other hand, with intravenous (IV) administration the majority of MSCs are entrapped within the lung and liver before they are targeted to the brain, which potentially reduces their therapeutic potential [5, 17].

Theoretically, intra-arterial (IA) cell grafting is more feasible in clinical applications because of more predictable engrafting (vs. IV approach) and less invasiveness (vs. IC approach). An animal study reported that IA administration of bone marrow stromal cells can facilitate axonal sprouting and remyelination in the cortical ischemic boundary zone and improve neurological function [18]. Recent animal studies showed that IA administration is a safe and more effective method than IV administration for treating cerebral ischemia [19, 20]. Nonetheless, there are no comparative studies on the engrafting effectiveness of IA versus IC stem cell administration. Therefore, the purpose of this study was to investigate behavioral changes, infarct volume, cell distribution, and stem cell identification in IA and IC MSC/Ngn1 groups in a rat model with ischemic stroke.
Materials and methods

MSCs/Ngn1 preparation
MSCs were isolated from human bone marrow with approval of the Institutional Review Board of Ajou University Medical Center (Suwon, Republic of Korea). Human MSCs were transduced to prepare the MSCs/Ngn1 as previously described [10]. Briefly, MSCs were transduced with Ngn1-expressing retrovirus for 8 hours in the presence of 4 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA) and 10 ng/mL basic fibroblast growth factor (bFGF, Dong-A Pharmaceutical Co., Youngin, Republic of Korea). The transduced cells were enriched for 2 weeks in the presence of 2 μg/mL puromycin (Sigma-Aldrich, St. Louis, MO, USA) and maintained by subculturing every 5 to 7 days in the growth medium, which was composed of Dulbecco’s modified Eagle’s medium (Welgene, Daegu, Republic of Korea) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin (Gibco, Grand Island, NY, USA).

Middle cerebral artery occlusion (MCAO)
All animal protocols were approved by the Institutional Animal Care and Use Committee of Ajou University Medical School (Suwon, Republic of Korea). A transient focal ischemia model of 120-minute MCAO with an intraluminal filament was performed in male Sprague Dawley rats (approximately 250 g) according to a modified procedure originally described by Longa et al. [21]. Briefly, anesthesia was induced with 5% isoflurane in 70% N₂O and 30% O₂ and maintained at 3% isoflurane. A 4-0 monofilament nylon suture with a rounded tip was introduced into the external carotid artery (ECA) lumen and gently advanced into the internal carotid artery (ICA) until it blocked the bifurcating origin of the MCA. The suture was removed two hours after MCAO. A double screen-out method was employed to ensure consistent ischemic injuries between rats. First, test subjects with negligible or moderate ischemic symptoms on any type of behavior tests on day 1 were excluded. Second, rats with a small infarct only involving the striatum or cortex were excluded after magnetic resonance imaging (MRI) analysis on day 2 (Supplementary Fig. 1). Twenty two animals showing a similar degree of neurologic deficit on behavior tests and similar infarct pattern and volume on MRI were selected and randomly assigned to the following groups: (I) to receive normal saline (NS) via the IA route (n = 5), (II) to receive NS via the IC route (n = 5), (III) to receive MSCs/Ngn1 via the IA route (n = 5), and (IV) to receive MSCs/Ngn1 via the IC route (n = 5) (Table 1).

Transplantation
Three days after MCAO the animals were anesthetized with 5% isoflurane in 70% N₂O and 30% O₂ using an induction chamber and maintained at 3% isoflurane using a face mask. After the skull was opened, MSCs/Ngn1 (1.0 x 10⁶ cells) or NS in a total fluid volume of 10 μL were IC transplanted into the striatum (anteroposterior [AP], 0.5; mediolateral [ML],...
Behavioral testing

Tests were performed to measure motor and sensory behaviors. The battery consisted of the Rotarod test and adhesive removal test. All animals were trained for 7 days before MCAO induction. Only the animals capable of remaining on the Rotarod cylinder for more than 300 s and removing adhesive dots within 10 seconds were used for the experiment. In the Rotarod test, the Rotarod cylinder (Ugobasile, Comerio, Italy) was accelerated from 4 to 40 rpm within 5 minutes, and the amount of time each animal remained on the Rotarod was measured with a cut-off time of 300 seconds. The data are presented as the percentage of the mean duration from three trials before MCAO. For adhesive removal tests, square dots of adhesive-backed paper (100 mm²) were used as bilateral tactile stimuli occupying the distal-radial region on the wrist of each forelimb. The time taken for each animal to remove each dot was recorded, and the animals were given 3 trials with a cut-off time of 300 seconds. The data are presented as the mean time to remove the dots. We performed behavior tests at 1, 7, 14, and 28 days after MCAO.

Infarct volume measurement

MRI scanning was performed using a 3.0 T whole-body MRI scanner (Achieva 3.0T X-Series Qasar Dual, Philips Healthcare, Amsterdam, Netherlands) equipped with a gradient system capable of 35 milliteslas/m at 3, 7, 14, and 28 days after MCAO. A fast-spin echo imaging sequence was used to acquire T2-weighted anatomical images of the rats’ brains in vivo, using the following parameters: repetition time, 4,000 milliseconds; effective echo time, 96 milliseconds; field of view, 55 x 55 mm²; image matrix, 256 x 256; slice thickness, 1.5 mm; flip angle, 90°; number of excitations, 2; and pixel size, 0.21 x 0.21 mm². A 300-mm diameter quadrature 16-ring birdcage coil arrangement was used for RF excitation, and a 40-mm diameter saddle coil was used for signal detection. A total of 15 slices were scanned to cover the entire brain. For each slice, the ischemic area from each T2-weighted image was manually marked and calculated using the software program Osiris (University of Geneva, Geneva, Switzerland).

Relative infarct volume (RIV) was normalized as described by Neumann-Haefelin et al. [23] using the equation $RIV = \left( \frac{LT}{RT} - 1 \right)$, in which LT and RT represented the areas of the left and right hemispheres, respectively, in square millimeters; RI was the infarcted area in square millimeters; and d was the slice thickness (1.5 mm). RIV was expressed as a percentage of the right hemispheric volume.

Statistical analysis

Comparisons between the two groups were achieved using Student’s unpaired t-test. Multiple comparisons for each treatment group were analyzed using one-way analysis of variance (ANOVA) with the independent variables being treatment groups and days of testing. These were followed by Tukey’s post hoc tests. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA), and the results were used for multiple comparisons.

Results

Therapeutic effects of MSCs/Ngn1 according to administration modality

The effectiveness of MSCs/Ngn1 administration in a rat stroke model was evaluated, followed by a comparison of the efficacy between IA and IC administration. Control animals that received IA or IC normal saline spontaneously recovered to a limited degree in the first seven days and then no further improvement was observed during the 28 day study.
The IC MSCs/Ngn1-transplanted animals showed significantly better performance in Rotarod testing than the IC control group on day 28 (Tukey’s post hoc test, p < 0.0001 on day 28 only). The IA MSCs/Ngn1-transplanted animals showed significantly better Rotarod performance than the IA control group from days seven to 28 (Tukey’s post hoc test, all p < 0.05). The adhesive removal test revealed greater improvement for the IA MSCs/Ngn1 group compared to the IA control group (Tukey’s post hoc test, p < 0.05 for days 21 and 28). Interestingly, the IC MSCs/Ngn1 group showed a decrease in functional score on the Rotarod test on day seven compared to other groups (including control groups; this was the periprocedural period after IC stereotactic administration). Functional recovery was higher in the IA MSCs/Ngn1 group than in the IC MSCs/Ngn1 group for both the Rotarod and adhesive removal tests. On day 28, the IA MSCs/Ngn1 administration group achieved significantly higher functional scores in the Rotarod test (unpaired t-test, p = 0.003) and adhesive removal test (unpaired t-test, p = 0.009), compared to the IC MSCs/Ngn1 transplantation group (Fig. 2A, 2B).

Serial changes in infarct volume according to administration modality

The RIV of the rat brains were monitored using MRI analysis over the 28-day experimental period (Fig. 2C) and histologic assessments of ischemic rat brains were performed on day 28 (Fig. 2D). No differences were seen in the initial infarct volume ratios between the four groups (p = 0.966). Although the IC-MSCs/Ngn1 administration group showed a tendency toward a reduced infarct volume ratio compared to the IA control (p = 0.061) and IC control (p = 0.223), the IA MSCs/Ngn1 administration group showed a significant

Figure 2. Comparison of functional behavior and serial infarct volume in MRI during 28 days. Behavioral improvements of the animals transplanted with normal saline or MSCs/Ngn1 stem cells via intra-cerebral or intra-arterial route were evaluated by the A. Rotarod test or B. adhesive removal test. MSC/Ngn1 stem cells showed a therapeutic effect compared with the control and the intra-arterial route had better functional recovery compared with the intra-cerebral route. C. Quantitative analysis of infarct volume was evaluated using brain magnetic resonance imaging. Statistical significant differences between the groups were determined by analysis of variance (*p < 0.05 compared with IA-control group; #p < 0.05 compared with IC-MSCs/Ngn1 group). Arrows indicate transplantation time of cells. D. Representative MR imaging from 2 to 28 days after ischemia. E. Cresyl violet staining on day 28 after ischemia. The MSCs/Ngn1 transplanted groups showed more reduced infarct volume on MRI images and histology slides.
reduction in infarct volume compared with IA controls \((p = 0.044)\) and a trend toward that reduction compared with IC controls \((p = 0.174)\) (Table 2) at 28 days.

**MSCs/Ngn1 distribution according to administration modality**

SPIO labeling was evaluated in MSCs/Ngn1 stained with Prussian blue after ferridex and protamine sulfate incubation for 12 hours (Fig. 3A). The distribution patterns of transplanted MSCs/Ngn1 were discretely different. IA-transplanted SPIO-labeled MSCs/Ngn1 were widely distributed in the ischemic area, whereas IC-transplanted SPIO-labeled MSCs/Ngn1 on MRI were localized to the stereotactically injected site after four hours. Serial MRI imaging studies on days 1, 4, and 7 showed that each distribution pattern was not dramatically changed from the initial pattern on MRI (Fig. 3B). To identify the SPIO signal on MRI, animals were sacrificed on day 7. The IA MSCs/Ngn1-transplanted ischemic rat brain specimens were stained with Prussian blue to identify SPIO-labeled cells. Identification of MSCs/Ngn1 and phagocytic immune cells was performed with immunohistochemistry using an hMT antibody and ED1 antibody, respectively (Fig. 3C). Some SPIO remained within the injected MSCs/Ngn1, and other

![Figure 3. MSCs/Ngn1 tracing by using SPIO labeling. A. MSCs/Ngn1 labeled with ferridex and protamine sulfate. Prussian blue staining for the identification of incorporation of ferridex into the MSCs/Ngn1. B. Distribution of MSCs/Ngn1 was monitored by SPIO labeling on MRI. Intra-arterial transplanted SPIO labeled MSCs/Ngn1 was widely distributed in the ischemic area. Each distribution pattern persisted during the serial MRI imaging studies on days 1, 4, and 7. C. Prussian blue staining 7 days after the ischemic brain transplantation with intra-arterial MSCs/Ngn1 cells. Immunostaining to identify transplanted SPIO labeled MSCs/Ngn1 cells and phagocytic activity (microglia/macrophages), by using anti hMT (human Mitochondria) and ED1 antibodies respectively, was performed in the ischemic boundary (I) and core (II) regions. SPIO correlated with hMT (red arrowheads) and ED1 (black arrowheads). Scale bars = 20 mm.](image)

**Table 2. Behavioral tests results and MRI analysis.**

| Group | IC-NS (n = 5) | IA-NS (n = 5) | IC-MSCs/Ngn1 (n = 5) | IA-MSCs/Ngn1 (n = 5) | P value |
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All data were presented as mean ± SD. *p < 0.05, ANOVA; #p < 0.05, Student’s unpaired t-test. Abbreviations: ANOVA, analysis of variance; IA, intra-arterial; IC, intra-cerebral; MRI, magnetic resonance imaging; MSCs/Ngn1, Ngn1-expressing mesenchymal stem cell; NS, normal saline; SD = standard deviation.
SPIO stained the immune cells, suggesting that immune cells had phagocyted some MSCs/Ngn1.

MSCs/Ngn1 transdifferentiation in a rat stroke model

To assess transdifferentiation of the IA-transplanted MSCs/Ngn1, we identified hMT immunoreactive cells in the ischemic core and border zone on day 28. Most hMT positive cells expressed MAP2 (a neuronal marker) but did not express GFAP (an astrocyte marker).

Discussion

The experiment demonstrated that MSCs/Ngn1 have therapeutic properties in an acute ischemic stroke model and provide more benefits when they are administered intra-arterially. SPIO-labeled MSCs/Ngn1 delivered via the IA route were more evenly distributed within the infarcted area on MRI, whereas they remained at the injection site in the IC group. Moreover, neuronal differentiation of the IA-transplanted MSCs/Ngn1 in the ischemic area was observed.

Stem cell therapy has emerged as a promising strategy for treating neurological diseases [25, 26]. MSCs are of particular clinical interest because they are easily isolated from bone marrow, adipose tissue, and umbilical cord blood and can be expanded through as many as 50 population doublings in about 10 weeks [27]. In previous studies, MSCs were shown to ameliorate neurologic dysfunction in patients with acute ischemic stroke [5, 17, 28]. One study reported that IC administration of Ngn1-expressing MSCs was more beneficial than parenterally delivered MSCs in an acute animal stroke model [10].

Figure 4. Transdifferentiation of MSCs/Ngn1 4 weeks after intra-arterial grafting. Confocal images of the transplanted cells 4 weeks after ischemia. A. Neuronal transdifferentiated MSCs/Ngn1 cells were identified by the anti-MAP2 (microtubule-associated protein) antibody (neuronal marker) and hMT (human mitochondrial) antibody. Arrowheads denote the donor-derived cells co-localized with cell type specific antigens in enlarged orthographic images. B. None of the MSCs/Ngn1 that identified with anti-hMT antibody (arrows) co-stained with antibody recognizing glial fibrillary acidic protein (GFAP). Scale bars = 20 mm.

Figure 5. Cerebral infarct in rat model. Infarction of rat brain resulted from the occlusion of middle cerebral artery (MCAo) is documented by T2 and diffusion weight image (DWI) of brain magnetic resonance image (MRI).
neuronal proteins such as vesicular glutamate transporter 2 (VGLUT2), NF200, and Tau [10]. However, whether the injected stem cells via IA can be moved into the ischemic lesion and can be differentiated into neuronal cells or not is questionable. This neuronal differentiation of mesenchymal stem cells showed the capability of neuronal induction by genetic engineering through transfection of the Ngn1 gene. In addition to the restorative paracrine effect, neuronal replacement by differentiation into neuronal cell with mature function may be another mechanism to recover the injured neuronal function by stem cell therapy with MSCs/Ngn1.

Although the IA-MSCs/Ngn1 group showed a significant reduction in infarct volume on MRI compared with IA controls, IA administration of MSCs/Ngn1 did not significantly reduce RIV on MRI compared to the IC-MSCs/Ngn1 group in this study. Since we performed behavior tests on day 1 and the brain MRI study on day two serially to verify and include MCAO rat models with similar neurologic impairment and similar infarct volume into our study, we could not avoid the delay of stem cell administration and MSCs/Ngn1 that were administrated on day three from stroke onset in this study. This late treatment with MSCs/Ngn1 may have affected the results, for example leading to low differences in the size of ischemic lesion volume between IA and IC groups.

It is known that IA delivery of MSCs enables efficient cell administration to an infarcted area and it results in significant functional recovery after ischemic stroke in a rat model [29, 30]. However, the major issue of IA administration is microembolization due to aggregation of the cells in the blood vessels [31]. The major determinants of the safety of IA stem cell administration are cell size, velocity of injection, and cell dose [22, 32]. Janowski et al. [22] showed that administration of MSCs with an infusion velocity over 1 mL/minute often resulted in stroke whereas a lower velocity (0.2 mL/minute) was safe. They also showed that stroke lesions occurred frequently when injecting 2 x10^6 MSCs, but not after lowering the dose to 1 x 10^6 cells. In this study, we administered a low dose of MSCs/Ngn1 (1x10^6 cells/1.2 mL) with a lower infusion velocity (0.24 mL/min) to avoid microembolism. We did not find any newly developed microembolic infarcts on MRI scans after MSCs/Ngn1 administration via the IA route, but further assessment of the optimum cell administration procedure is required to evaluate the adverse effects. To minimize the risk of distant embolism in human patients undergoing an endovascular approach, a skilled interventionist and a well-equipped clinical setting are essential for ensuring the safety of IA administration of MSCs or intensified MSCs. Safe IC administration in human patients is technique sensitive and requires general anesthesia. Moreover, the direct deposit of grafting cells via the IC route may inevitably result in damage to the brain parenchyma. For these reasons, previous human trials with the IC route reported both serious and non-serious adverse effects [14-16]. Although IV injection is a clinically relevant and minimally invasive technique, only a small amount of injected cells reach the targeted brain area due to the first pass effect in the lungs and liver [19]. Since the IA route increases the chances of MSC migration and distribution, it facilitates efficient engrafting of stem cells to the target brain areas [33].

The present study compared the therapeutic effect after IC and IA administrations of stem cells. We also showed the hemispheric distributions of injected stem cells on serial MRIs and observed neuronal differentiation on immunohistochemical analyses. The results require cautious interpretation because of the lack of comparisons with surrogate biomarkers for paracrine effects of injected MSCs.

Although we did not find newly formed microembolic strokes on MRI scans after IA delivery of stem cells, we cannot exclude the possibility of microembolism. It is possible that the microembolisms caused by MSC infusion might be masked by the large ischemic lesions on the MRI. Therefore, continuous stirring should be implemented to prevent distant embolism due to the tendency of MSCs to aggregate. Further human stem cell trials are required to investigate the potential clinical benefits of the IA approach in acute ischemic stroke patients.
Conclusions

This study in an acute ischemic stroke model suggests that MSCs/Ngn1 stem cells have promising therapeutic effectiveness. IA administration would be a feasible grafting modality compared to IC administration, based on the behavior test results and MRI analysis.

Acknowledgments

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