Dose-dependent efficacy of ALS-human mesenchymal stem cells transplantation into cisterna magna in SOD1-G93A ALS mice

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Abstract
Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by motor neuron loss. Although the underlying cause of the disease remains unclear, a variety of pathogenic mechanisms have been proposed. Despite promising preclinical studies showing the modification of the disease progression, most trials have failed to demonstrate any significant improvement in outcome. Stem cell therapy therefore has been proposed as an alternative therapy for ALS. In this study, we evaluated the dose-dependent effects of human bone marrow mesenchymal stem cells (hMSCs) obtained from an ALS patient into SOD1 mice via intrathecal injection and showed its practicality for hMSCs. We transplanted different doses (1 × 10^2, 2 × 10^3, and 1 × 10^4) of ALS-hMSCs into the cisterna magna and performed clinical observations including symptom onset, survival time, and locomotor performance using the rotarod test. Nissl staining was performed to evaluate motor neurons in lumbar spinal cord sections at 109 days, and transplanted cells were evaluated by immuno-fluorescence staining at the end stage. A cell dose of 1 × 10^2 cells significantly prolonged life span and delayed the decline of motor performance. At this dose, the average number of motor neurons was significantly higher than those of the untreated and 1 × 10^4 cell treated groups. Most injected hMSCs distributed in the ventricular system and subarachnoid space, while some migrated into the brain and spinal cord. These data suggest that intrathecal injection with an optimized cell number could be a potential route for stem cell therapy in ALS patients.

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the cisterna magna. Using the ALS mouse model, we evaluated the dose-dependent effect of hMSCs obtained from an ALS patient (ALS-hMSCs) and showed the practical possibility of intrathecal injection for treatment of ALS.

We used ALS-hMSCs remained after a human trial. The patient provided written informed consent and understood all issues involving the use of MSCs and CSF for basic research. This study was approved by the Institutional Review Board for Human Investigation of Hanyang University Hospital.

To obtain ALS-hMSCs, mononuclear cells were isolated from bone marrow aspiration (the iliac crest) with a density gradient (Histopaque, density 1.077 g/ml; Sigma–Aldrich, St. Louis, USA) and washed twice with Dulbecco’s Modified Eagle’s Medium containing low glucose (DMEM-LG). The cells at a density of 2 × 10^5 cells/cm^2 were cultured in DMEM-LG with 10% fetal bovine serum at 37 °C, seeded at a density of 4 × 10^3 cells/cm^2 and expanded up to 80–90% confluence. The immunophenotype of the cells was confirmed by flow cytometry (negative for CD34, CD45 and HLA-DR and positive for CD29, CD44, CD73, CD105 and CD49). In this study ALS-hMSCs at third-passage were used.

The patient's CSF was collected through a lumbar spinal puncture. After using the CSF for the clinical applications, the remaining CSF was stored in a deep freezer at −70 °C and used after thawing.

All mice (B6SJ-Tg(SOD1-G93A)1Gur/J) over-expressing human SOD1 containing the Gly93 → Ala mutation) were obtained from the Korea Research Institute of Bioscience and Biotechnology (Chunbuk, Korea). They were identified by PCR according to Jackson Laboratory's genotyping protocol and evaluated transgene copy numbers using real time quantitative PCR (ΔACT = 6.948 ± 0.053) [1,13]. We used 26 wild-type mice and 139 SOD1 mice in this study. The SOD1 mice were randomly assigned to five groups: a control group (n = 27), a CSF group (treated with 10 μL of CSF, n = 27), and three groups that received different doses of cells [1 × 10^6 (n = 28), 2 × 10^5 (n = 29), and 1 × 10^6 (n = 28), respectively]. For the latter three groups, the cells were suspended in CSF at cellular density of 1 × 10^5, 2 × 10^5, 1 × 10^6 cells/μL, respectively, and the volume of cell suspension administered to each group was 10 μL. 1 × 10^5 cells was the maximum cellular density in 1 μL of CSF, and 10 μL of CSF + 1 × 10^5 cells was the volume to be transplanted into the cisterna magna of mice as determined by our preliminary study (data not shown).

At 60 days, the mice were anesthetized with tiletamine/zolazepam (60–80 mg/kg, IP) and xylazine (5–10 mg/kg, IP) and positioned in a stereotaxic apparatus (ASI instruments, Heidelberg, Germany) such that the cisterna magna was the highest point. Under an operating microscope, the atlanto-occipital membrane was exposed and 10 μL of CSF from the ALS patient or CSF + ALS-hMSCs was injected using a Hamilton syringe (25 μL, 31 G) and a pump (1 μL/min). A drop of saline was placed on the top of the injection point to avoid cell leakage [13]. The needle was withdrawn after 10 min period and the incision was closed. All animals that underwent this operation were injected with cyclosporine (10 mg/kg IP per day) from the day prior to transplantation to the end point of the experiment.

All animal experiments were performed according to the Hanyang University guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Hanyang University.

All mice were observed daily for disease progression. Symptom onset was defined as the presence of a hind limb tremor when the mouse was suspended by the tail. The end point was defined as the time when the mouse was unable to right itself within 30 s when placed on its side. Its age was considered the survival time. We evaluated body weight and motor function twice a week. Motor function was tested using an acceleration rotarod device (4–40 r.p.m. 180 s Rota-Rod 7650; Ugo Basile, Comerio, Italy). Each animal was given three consecutive trials, and the longest latency for each animal to fall was recorded [7].

Animals were euthanized by an overdose of isoflurane inhalation. They were then perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brain and spinal cord were carefully dissected, post-fixed for 24 h in the same solution, and cryoprotected overnight in 30% sucrose solution in 0.1 M PB. The tissue was frozen in cryostat medium and cut in coronal 20-μm-thick serial sections.

For motor neuron counting, six mice (three females and three males) were euthanized at 109 days (49 days after transplant) in each group and Nissl staining was performed in the lumbar spinal cord region sections. We examined every tenth section at 40× magnification and counted large motor neurons bi-laterally in 10 sections. Transplanted cells were detected by immuno-fluorescence staining using a human-specific marker. Every tenth section was blocked with 10% goat serum in 0.01 M PBS for 40 min at room temperature and incubated overnight with the mouse anti-human mitochondria monoclonal antibody (1:100 dilution; Chemicon, CA, USA). After washing, the sections were incubated with a goat anti-mouse IgG conjugated with tetramethyl rhodamine isothiocyanate as the secondary antibody (1:100 dilution; Invitrogen, CA) for 3 h. For negative control staining, the sections of the control and CSF groups were incubated without the primary antibody.

Data are expressed as mean ± SEM, and p-values < 0.05 was considered significant. The Kaplan–Meier method (log rank test) was used to evaluate survival time. The other data were analyzed by repeated measures ANOVA, followed by a Tukey’s multiple comparisons test. All data were analyzed using SPSS Statistics 17.0 for Windows (SPSS Inc., IL, USA).

Of the 165 used in this study, we evaluated 20 wild-type and 109 SOD1 mice. The SOD1 mice were divided into five groups: control mice [female to male ratio (F:M) = 10:11], CSF only (11:10), and three groups receiving different concentrations of cells [1 × 10^4 (11:11), 2 × 10^5 (11:12), and 1 × 10^6 (12:10) cells, respectively]. The symptom onset, defined as a hind limb tremor, was observed at 88.9 ± 1.3, 89.2 ± 0.9, 88.8 ± 0.9, 90.5 ± 0.8, and 89.5 ± 0.4 days, respectively. There was not a statically significant difference among the groups. The survival time of the group that received 2 × 10^5 cells (131.7 ± 1.2 days) and 1 × 10^6 cells (134.0 ± 2.0 days) was significantly prolonged compared to the control (125.8 ± 1.1 days, p = 0.001) and CSF (127.6 ± 1.1 days, p < 0.016) groups. The survival time of the group that received 1 × 10^6 cells was also significantly extended compared to the group that had received 1 × 10^5 cells (128.0 ± 1.8 days) (χ^2 = 4.527, p = 0.033, Fig. 1A).

In the rotarod test, there were no significant differences between the control group, the CSF group, or the groups that received 1 × 10^4 and 2 × 10^5 cells (Fig. 1B). Administration of 1 × 10^6 cells, however, significantly slowed the decline in motor performance compared to the control group, the CSF group, and the group that had received 1 × 10^4 cells (p < 0.05). Of the group that had received 1 × 10^6 cells, 18% were able to run in the rotarod test until approximately day 133. In addition, no gender differences were observed in this group in terms of life span or motor performance, therapeutic effectiveness was found in both male and female group.

In all five of the tested groups, no significant differences were observed in body weight from day 59 to day 116.

We counted large motor neurons at 109 days (49 days after transplant) in the ventral horns of the lumbar cord using Nissl staining. All SOD1 mice presented with a significant and
Fig. 1. Effect of ALS-hMSC transplantation on disease progression. Survival analysis (A) and rotarod test (B) of SOD1 mice. Survival time in the group that received $1 \times 10^6$ cells was significantly prolonged for 7–11 days compared to untreated mice (A), and motor performance had a significant decrease in decline compared to the control and CSF group and the group treated with $1 \times 10^4$ cells.

Fig. 2. Motor neurons in the ventral horn of the spinal cord. Nissl stained sections from wild-type mice (A), CSF mice (B), and mice that had received $1 \times 10^6$ cells (C) at 109 days. Motor neurons of SOD1 mice significantly decreased compared to wild-type and mice that received $1 \times 10^6$ cells showed a significant decreased in motor neuron loss compared to the other SOD1 mice groups (D). *p < 0.05 (when compared to wild-type mice); §p < 0.05 (when compared to the control group); ¶p < 0.05 (when compared to the control and CSF groups and the group that received $1 \times 10^6$ cells). Scale bar: 100 μm.
progressive reduction in motor neurons compared to the wildtype group (Fig. 2). The group treated with $1 \times 10^6$ cells had a significantly decreased motor neuron loss compared to the control, the CSF, and the group treated with $1 \times 10^4$ cells ($6.1 \pm 0.1$ cells vs. $3.4 \pm 0.1$ cells, $4.5 \pm 0.4$ cells, and $3.7 \pm 0.2$ cells, respectively). The group that had received $2 \times 10^5$ cells also showed a decreased motor neuron loss when compared to the control group ($4.8 \pm 0.3$).

We next performed immuno-fluorescence staining using a human-specific marker and observed the distribution of the ALS-hMSCs that were transplanted into the cisterna magna (Fig. 3). The most transplanted cells were found in the ventricular system (Fig. 3A and B) and subarachnoid space (Fig. 3C). Some cells migrated into the brain (Fig. 3D) and spinal cord (Fig. 3E and F). No signal was detected in the control or CSF groups.

Mesenchymal stem cells are “non-hematopoietic multi-potent stem-like cells” that are able to differentiate into various cell types, including mesenchymal and non-mesenchymal phenotypes [11]. They are also able to avoid allorecognition and modulate other immune responsive cells, such as B cells and dendritic cells [4]. Moreover, MSCs have been studied for stem cell therapy because they can be easily collected, expanded, and engineered to deliver trophic and growth factors [11]. In ALS stem cell research, neuronal protection strategies to modulate a non-neuronal environment have been recently suggested instead of cellular replacement strategies [3,21].

We transplanted $1 \times 10^4$, $2 \times 10^5$, and $1 \times 10^6$ hMSCs obtained from an ALS patient into SOD1 mice using intrathecal injection. Although there was no significant difference in the symptom onset time, the group that had received $1 \times 10^6$ cells had prolonged survival time and a slower decline motor performance and motor neuron loss ($p<0.05$). Garbuzova-Davis et al. [10] transplanted different doses of hUCBs ($1 \times 10^7$, $2.5 \times 10^7$, $5 \times 10^7$ cells) into SOD1 mice using intravenous injection. They found that a cell dose of $2.5 \times 10^7$ cells ameliorated disease progression and decreased pro-inflammatory cytokines, a cell dose of $1 \times 10^7$ cells was not sufficient to modulated disease progression, and a cell dose of $5 \times 10^7$ had no beneficial effect compared to a cell dose of $2.5 \times 10^7$. This might suggest that an optimized cell dose would be important to determine the efficacy of cell therapy for ALS patients. Here, we found that a cell dose of $1 \times 10^6$ cells effectively ameliorated disease progression when transplanted into the cisterna magna.

Amyotrophic lateral sclerosis is a multifocal disease affecting the cerebral cortex, brain stem, and spinal cord. Using stem cell therapy via intrathecal injection should be considered as an alternative to intraspinal cord injection, which is an invasive, localized injection [23]. Although intrathecal injection is less invasive and able to deliver cells extensively by CSF [23], it has been unclear whether intrathecally injected cells migrate to the damaged spinal cord parenchyma [13,14,21]. We transplanted hMSCs obtained from an ALS patient using intrathecal injection and found most had migrated into the ventricular system, the subarachnoid space, and some in the spinal cord. In contrast to our findings, transplantation of human bone marrow-derived mesodermal stromal cells or hUCBs into the cisterna magna in SOD1 mice did not improve clinical disease outcomes, and most of the cells were found within the subarachnoid space [13]. Multiple injections of hMSCs into the cisterna magna, however, extended survival time and improved motor performance despite the few hMSCs in the lumbar spinal cord parenchyma [23]. It is possible that the intrathecally injected cells distributed in the CSF protected motor neurons by releasing neurotrophic factors instead of replacing damaged cells [3,21,23]. Intrathecal injection has been suggested to potentially be a practical route for stem cell therapy for ALS patients if an optimized...
number of cells is used. Further study is needed to investigate the neuroprotective mechanisms of ALS-hMSCs when they are transplanted into the cisterna magna.

Our data showed dose-dependent effect. It is therefore important to determine the maximum number of hMSCs that could be used for therapy. In our preliminary study performed to determine the maximum cell number, we transplanted 2 × 10^6 ALS-hMSCs/20 μl into the cisterna magna in five mice. All mice, however, died in the middle of the injection. Since there is a limit to a single dose, further study is needed to investigate the effect of repeated intrathecal injections.

We transplanted hMSCs that were obtained from a patient with ALS. For the best outcome of stem cell therapy for ALS, a selection of optimal stem cell sources as well as an optimal dose of ALS. For the best outcome of stem cell therapy for ALS, a selection of optimal stem cell sources as well as an optimal dose of stem cells is important [5,6]. Yet for unknown reasons, autologous hMSC transplants have had mixed results in human trials. This suggests that autologous stem cells that originate from an ALS patient could be compromised compared to healthy donor cells. In this study, we only demonstrated the importance of dose-dependent effects of stem cell therapy using ALS-hMSCs. Further studies focused on comparing the therapeutic efficacy between healthy hMSCs and ALS-hMSCs should be performed using the ALS mouse model.

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References