Effective adenovirus transduction of human umbilical cord-derived mesenchymal stem cells (hUCMSCs) via tetrameric cell-permeable peptides and implantation of glial cell line-derived neurotrophic factor-overexpressing hUCMSCs in newborn mice with hypoxic-ischemic brain injury

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Directed by Professor Kook In Park

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ABSTRACT

Effective adenovirus transduction of human umbilical cord-derived mesenchymal stem cells (hUCMSCs) via tetrameric cell-permeable peptides and implantation of glial cell line-derived neurotrophic factor-overexpressing hUCMSCs in newborn mice with hypoxic-ischemic brain injury

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Mesenchymal stem cells (MSCs) transplantation has been shown to be beneficial in treating neonatal hypoxic-ischemic (HI) brain injury. However, as naïve MSCs transplantation showed only modest therapeutic benefits, some genetic modification of MSCs has been considered to enhance their therapeutic outcomes. This study examined the therapeutic potential of MSCs overexpressing glial cell-line derived neurotrophic factor (GDNF), which is known as a potent neuroprotective agent in ischemic brain tissue. Human umbilical cord-derived mesenchymal stem cells (hUCMSCs) were isolated from Wharton's jelly and cultured in dishes. The cultured hUCMSCs were transfected with adenoviral vectors encoding GDNF or GFP (GDNF-MSCs or GFP-MSCs) in the presence of tetrameric cell permeable peptides (CPPs) to improve the efficacy of viral transduction. The effects of GDNF-MSCs on neuronal survival and neurite outgrowth were assessed *in vitro*. GDNF-MSCs or GFP-MSCs were transplanted into neonatal mouse brain with HI injury. Following transplantation, animal's behavioral performance was evaluated using neurological and cylinder tests, and the levels of the infarction volume,

apoptosis, neurogenesis, and gliogenesis were assessed via immunohistochemistry. Using tetrameric CPPs, the efficient gene transduction of hUCMSCs via adenovirus was shown to act at 15- fold lower concentration of virus. GDNF-MSC-transplanted mice demonstrated significantly decreased cerebral infarction volume and improved neurological function as compared with vehicle- or GFP-MSC-injected groups. GDNF-MSC transplantation after HI brain injury reduced cellular apoptosis but increased neurogenesis and gliogenesis. The treatment of SH-SY5Y cells with GDNF-MSC-derived conditioned medium (CM) significantly enhanced neurite outgrowth and promoted neuronal survival compared with GFP-MSC-derived CM. These results suggest that GDNF-overexpressing hUCMSCs transplantation could be more advantageous for treating neonatal HI brain injury.

Key words: hypoxic-ischemic brain injury, mesenchymal stem cells, glial cell line-derived neurotrophic factor, adenovirus, tetrameric cell-permeable peptides, neuroprotection, transplantation

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I. INTRODUCTION

Neonatal hypoxic ischemic encephalopathy (HIE) is one of the major cause of severe brain injury in both preterm and term infants.[1] HIE occurs in 1-3 per 1000 term births, and the frequency has not decreased despite medical advances in fetomaternal care.[2] Although therapeutic hypothermia has become the standard therapy for HIE, there are still many infants who die or develop severe neurologic impairments, such as cerebral palsy, mental retardation, and seizures.[3, 4] Current therapeutic intervention strategies are very limited and restricted to supportive intensive care. Therefore, a treatment strategy using a new therapeutic agent is needed.

Neonatal HIE induces cell death that is exacerbated by abnormal expression and activation of the ATP-dependent Na+-K+ pump, oxidative stress, and aberrant excitatory neurotransmission due to insufficient oxygen and blood

flow.[1,4] Furthermore, loss of highly vulnerable axons, oligodendrocyte progenitors, and neurons disrupts neural network maturation which has a destructive impact on brain structure and connectivity.[5]

Recent advances in regenerative medicine suggest that stem cells transplantation may repair brain damage. In animal models, neural stem cells or mesenchymal stem cells (MSCs) have been applied to many neurologic disorders, including adult cerebral ischemia, Alzheimer's disease, Parkinson disease, and also neonatal hypoxic-ischemic (HI) brain injury.[6-10] Furthermore, umbilical cord blood cells transplantation into patients with neonatal HIE shows safety and feasibility in phase 1 clinical trial.[11]

MSCs transplantation into both neonatal and adult ischemic brain injury models has been reported to promote endogenous repair processes, reduce lesion size, and improve functional outcomes.[6, 12-17] Although MSCs can differentiate into neuronal or glial cells, their beneficial effects of cell therapy are less likely to result from replacement of lost cells by MSCs. Transplanted MSCs repair damaged brain tissue via the release of trophic factors and stimulate endogenous repair processes such as neurogenesis, angiogenesis, and synaptogenesis.[12, 18]

Among the various source of MSCs, the human umbilical cord (especially Wharton's jelly) is a promising source for cell-based regenerative medicine. The human umbilical cord-derived MSCs (hUCMSCs) have a relatively high harvest rate compared with MSCs derived from cord blood or bone marrow, making it possible to obtain a substantial number of cells after a few passages without long culture periods. Furthermore, harvesting MSCs from Wharton's

jelly does not need invasive procedure and is performed using the tissues that are generally discarded. Thus, hUCMSCs are easily harvested and manipulated without harm to the baby or mother.[19] Overall, the umbilical cord might be an ideal source of stem cells for cell-based regenerative medicine, especially for neonatal disease.

Transplantation of naïve MSCs into the ischemic brain has only modest effects, although it is safe and feasible. In addition, in cases of severe HIE resulting in death or permanent neurologic deficits, the effect of cell therapy is insufficient. Recent studies show that the application of genetically-modified MSCs to ischemic brain injury has more therapeutic potential than naïve MSCs.[20]

Glial cell-derived neurotrophic factor (GDNF) is a 33-45kDa glycosylated disulfide-bonded homodimer, that belongs to the transforming growth factor-β superfamily. The molecule was identified in conditioned media from a glial cell line based on its ability to promote survival and increase cell size and neurite length in mesencephalic dopaminergic neurons.[21, 22] Subsequent studies revealed that GDNF functions as a trophic factor for other neurons in the central and peripheral nervous systems. GDNF regulates cellular activity by interacting with GDNF family receptor-α1 receptors, which might signal through transmembrane Ret tyrosine receptors or neural cell adhesion molecules, to promote cell survival, neurite outgrowth, and synaptogenesis.[23] However, GDNF protein is difficult to administer in the clinical setting because it cannot cross the blood-brain barrier and has a short half-life.[24] To maximize the neuroprotective effect, GDNF should be released over a longer

duration in a stable condition. Thus, it would be more efficient to deliver GDNF-secreting cells instead of delivering only GDNF. Stem cells are good means of delivering GDNF, because they have self-renewal capabilities and effectively release paracrine factors.

To date, some *ex vivo* gene therapies using different viral vectors and stem cells have been attempted in rodent animal models of stroke. In a rat middle cerebral artery occlusion model, administrating GDNF-expressing cells reduced infarction size, enhanced striatal neurogenesis, reduced ischemic brain edema, and reduced the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) positive neurons.[17, 25-27] Additionally, GDNF improves outcomes in adult intracranial hemorrhage animal models.[28] However, the therapeutic potential of GDNF in neonates with CNS diseases has not been studied.

Adenoviruses are widely used for gene delivery. These viruses have broad cellular tropism, can produce high levels of transgene expression, are associated with a low risk of insertional mutation, are easy to produce with high titers, and can infect both dividing and non-dividing cells.[29] However, adenovirus have serious shortcomings, such as immunogenicity, promiscuous tropism, and the inability to efficiently infect certain cell types, including MSCs. Although replication-deficient recombinant adenovirus (rAds) lacking the E1 gene are used for *in vivo* therapeutic gene delivery, rAd particles directly cause an immediate inflammatory response and acute tissue injury.[30] Because MSCs are inefficiently transduced with rAds, the use of very high virus concentration for transfection is unavoidable. Therefore, the use of

adenovirus alone for gene transfer into MSCs is not ideal for clinical translation.

Cell-permeable peptides (CPPs) are small, polybasic peptides that cross the plasma membrane of several mammalian cell types. These peptides have been used to deliver various bioactive substrates, including proteins, antisense oligonucleotides, liposomes, plasmid DNAs and small interfering RNAs into cells. CPPs (Tat, HP4, or Hph) also used in viral or nonviral gene delivery. In recent studies, oligomerized tetrameric CPPs (4Tat or 4Hph) significantly enhanced the rAd transduction efficiency into MSCs. Higher transduction efficiencies were achieved using 3000-5000-fold less tetrameric CPPs than that of monomeric CPPs.[31, 32]

Here, we aimed to examine the therapeutic potentials of hUCMSCs overexpressing GDNF implanted into neonatal mice with HI brain injury. In the process of gene delivery to hUCMSCs, we established efficient and safe viral gene transduction in hUCMSCs using rAd vectors in the presence of tetrameric CPPs.

II. MATERIALS AND METHODS

1. Preparation and culture of hUCMSCs

Human umbilical cord tissue was obtained from healthy full-term babies of healthy mothers. Written informed consent was obtained before elective

Cesarean section and prior to tissue collection, in accordance with the approval of the Research Ethics Committee of Yonsei University College of Medicine, Seoul, Korea (4-2011-0010). The detailed MSC harvest and culture method was as follows: Part of umbilical cord (5-10 cm), which was supposed to be discarded, was retrieved from a newborn infant immediately after birth in the delivery room. Umbilical cord tissue was collected in cold 1x H-H buffer solution for transport to the laboratory. After dissecting the umbilical cord, blood vessels and amnion were removed. Stromal tissue was collected, and the tissue was dissociated with papain. The tissue pieces (umbilical cord subamnion and umbilical cord stroma) were transferred to 10 cm^2 plate containing CMRL 1660 medium (Sigma, St. Louis, MO, USA) supplemented with L-glutamine and 10% fetal bovine serum, and were incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2. Cell surface antigens were analyzed by flow cytometry using culture cells, and mesodermal lineage differentiation assays were performed to confirm that the cultured cells were multipotent MSCs: Von Kossa stain for osteogenesis, Oil Red O stain for adipogenesis, and Safranain O stain for chondrogenesis.

2. Adenoviral vector construction

Adenoviral vector was constructed under the control of the CAG promoter with internal ribosome entry site-based bicistronic green fluorescent protein (GFP) expression. AdEasy Adenoviral Vector System (Stratagene, La Jolla, CA, USA) was used to generate adenoviral particles. The full-length human

GDNF coding sequence was inserted into the vector to produce an adenovirus encoding GDNF and GFP. (Ad-GDNF/GFP) To obtain the control cell group, an adenovirus encoding only GFP was prepared. (Ad-GFP) Ad-GDNF/GFP and Ad-GFP were transfected into human embryonic kidney 293 cells, expanded, and purified by cesium chloride ultracentrifugation. hUCMSCs were transduced with either Ad-GDNF/GFP or Ad-GFP.

3. Transduction of adenovirus into hUCMSCs in the presence of tetrameric CPPs

hUCMSCs were plated at a density of 1×10^5 cells/mL in 6 well plate. 4Hph-1 tetrameric CPP (Peptron Co., Daejeon, Korea) was used to enhance the efficiency of viral transduction. Ad-GDNF/GFP + CPP, and Ad-GFP + CPP were combined at a 1:1 ratio in serum-free media and incubated for 30 min at room temperature. 3.5 hours after plating hUCMSCs, the cells were treated with the Ad-GDNF/GFP + CPP or Ad-GFP + CPP mixture at multiplicity of infection (MOI) of 10, 15, 20, 25, 30, 35, 40 for 2 hours. After 48 h incubation, cell morphology and GFP expression were analyzed by fluorescence microscopy. GFP expression was also analyzed using flow cytometry. The optimal virus/CPP concentration for MSC transfection was determined based on flow cytometry.

4. Quantification of the GDNF protein by Enzyme-Linked Immunosorbent Assay (ELISA)

At 48 h incubation after the transfection, the conditioned media (CM) derived from Ad-GDNF/GFP-transduced MSCs (GDNF-MSCs) or Ad-GFP-transduced MSCs (GFP-MSCs) were collected and cleared via centrifugation at 3000 rpm for 3 min. Human GDNF protein levels in the CM were measured using the GDNF DuoSet ELISA Development kit (R & D systems, Minneapolis, MN, USA).

5. Immunocytochemistry

For immunocytochemistry, GDNF-MSCs or GFP-MSCs were plated into each well of a 8-well chamber slide system (Nunc, Roskilde, Denmark) coated with 0.1 mg/mL Poly-D-Lysine (PDL, Sigma). 48 h after plating, the cultures were fixed with 4% paraformaldehyde for 15 min at room temperature , washed with phosphate-buffered saline (PBS), and incubated with blocking solution [3% bovine serum albumin (BSA), 10% normal donkey serum (NDS) and 0.2% Triton X-100 in PBS] for 1 h at room temperature in a humidified chamber. Next, the cells were incubated with rabbit anti-GDNF antibody (1: 50, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-GFP antibody (1: 100, Invitrogen, Tokyo, Japan) overnight at 4 ℃. After washing three times with PBS for 10 min, the cells were incubated with anti-mouse antibody conjugated with fluorescein (1:200, Vector Labs, Burlingame, CA, USA) and anti-rabbit antibody conjugated with TexasRed (TR, 1:200, Vector Labs) at 37 ℃ for 70 min. After rinsing 3 times with PBS, specimens were mounted using VECTASHIELD mounting medium with

4,6-diamino-2-phenylindole (DAPI, Vector Labs) and were analyzed by immunofluorescence microscopy (BX51, Olympus, CenterValley, PA, USA)

6. *In vitro* neuronal survival and neurite outgrowth assay

SH-SY5Y human neuroblastoma cells were plated at a density of 3×10^4 cells/well in 48-well plates in growth medium (10% fetal bovine serum and 1% P/S in DMEM/F12). Differentiation into neuron was induced with 10 μM retinoic acid (Sigma) for 5 days. The cells were washed and treated with CM derived from GDNF-MSCs (GDNF-CM) or GFP-MSCs (GFP-CM). After 1 h, cells were challenged with 5 mM glutamate and incubated for another 24 h. Cell viability was determined using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan). For neurite outgrowth assays, SH-SY5Y cells were cultured overnight at 1×10^6 cells/2 mL in poly L-lysine-coated 6-well plates in growth media. Next, the cells were incubated with GDNF-CM or GFP-CM for 24 h. For immunodepletion studies, GDNF-CM were preincubated with 5 μg/mL anti-GDNF antibody (Santa Cruz Biotechnology) for 1 h. As a control, GDNF-CM were preincubated with 5 μg/mL of isotype-matched IgG antibodies (ChromPure rabbit IgG, Jackson ImmunoResearch, West Grove, PA, USA). To quantify neurite length, cells are observed under Olympus IX71 microscope and measured using NeuronJ software.

7. Animal model

At postnatal day 7, CD-1 (ICR) mice pups underwent unilateral hypoxic ischemic brain injury by permanent right common carotid artery ligation under isoflurane anesthesia, followed by exposure to 8% oxygen in nitrogen for 90 min at 37 ℃ on a hot plate in a closed plastic chamber. After the injury, the pups were returned to their mothers and were given routine care. All animals were maintained under a 12 h light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yonsei University College of Medicine and in accordance with the *Guideline for the Care and Use of Laboratory Animals* issued by the National Institutes of Health.

8. Transplantation of MSCs into HI-injured brain in neonatal mouse

Three days after HI brain injury, HI tissue damage in the right cortical hemisphere was visually confirmed. Mice showing moderate infarct size (30-60 % of total hemisphere volume) were selected for experiments. Mice were randomly assigned to three groups, which were injected with 8 μL of GDNF-MSCs (4 x 10^4 cells/μL), GFP-MSCs (4 x 10^4 cells/μL), or H-H buffer (vehicle), respectively, into the center of the infarcted region of a unilaterally (right-sided) asphyxiated mouse brain using a glass micropipette (diameter, 0.3 mm). Pups from three to four different litters were randomly distributed across all experimental groups to reduce potential bias due to litter-specific variation.

Males and females were equally divided among the experimental groups. Cyclosporine (10 mg/kg) was intraperitoneally administered to all mice once per day beginning 1 d before injection and until euthanization.

9. Neurological function test

Neurological function was assessed 1, 2, and 3 weeks after MSC transplantation. Neurological severity scoring (NSS) and cylinder tests were used. NSS was assessed via the following reflexes: (i) when suspended by the tail, the mouse remained upright and extended its forelimbs; (ii) when a mouse was placed on its side, it immediately turned over to rest in the normal position with all four feet on the ground; (iii) when the dorsum of the paw contacted the edge of the table, the mouse immediately placed its paw on the surface; and (iv) when a mouse was suddenly placed or bounced on a board, it spread its toes. Each exam was scored as ‘‘0’’ if the response was normal and ‘‘1’’ if not. For the cylinder test, animals were individually placed in a transparent acrylic cylinder (10 cm diameter × 20 cm long) and observed for 3 min in the housing room. The initial forepaw (left/right/both) preference for weight-bearing contact during full rearing was recorded. The relative proportion of left (ipsilateral) forepaw contacts was calculated as follows: (left-right)/(left+right+both) ×100.

10. Assessment of lesion volume

Three weeks after cell transplantation (1 month after birth), mice were anesthetized, transcardinally perfused with cold PBS and fixed with 4% paraformaldehyde. Brains were removed, post-fixed, cryoprotected with 30% sucrose in PBS, and frozen in O.C.T compound (Sakura Finetek, Torrance, CA, USA). Brains were sectioned coronally at 16-μm thickness using a cryostat. Brain sections were stained with hematoxylin-eosin to reveal the infarct area. The size of each lesion was calculated as the ([area of the left contralateral hemisphere – area of the remaining right ipsilateral hemisphere]/area of the left contralateral hemisphere) ×100. Areas were measured using ImageJ software.

11. Immunohistochemistry

The brain sections were washed in PBS and blocked with 3% BSA (Sigma), 10% NDS, and 0.3% Triton X-100 (Sigma) in PBS for 20 min at room temperature. Sections were incubated with anti-microtubule-associated protein 2 (anti-MAP2, 1:1000, Sigma) or anti-myeline basic protein (anti-MBP, 1:200, Abcam, Cambridge, MA, USA) antibodies overnight at 4 ℃. After washing three times with PBS, the sections were incubated with anti-mouse antibody (for MAP2, 1:400, Jackson) or anti-rabbit antibody (for MBP, 1:200, Jackson) and 70 min at 37 ℃. After rinsing three times with PBS, specimens were mounted using VECTASHIELD mounting medium with DAPI (Vector labs).

Fluorescent staining images were obtained at the level of penumbra cortex and striatum for MAP-2, and at the level of subcortex and corpus callosum for MBP, using immunofluorescence microscopy (BX51, Olympus). Using ImageJ softeware, the immunodensity of MAP-2 or MBP was assessed by measuring the mean grey value, subtracted by the mean value of the background staining. Level of the animals or GFP- or GDNF-injected group were normalized levels in the vehicle-injected group.

12. Apoptosis

The brain sections were employed for TUNEL assay, using an i*n Situ* cell death detection kit (Roche Life Science, Basel, Switzerland). The samples were washed with PBS and incubated with blocking solution for 10 min at room temperature. After rinsing, the tissues were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate buffer for 15 min at 4 ℃. After three washes, 50 μL TUNEL reaction mixture was added to each sample and incubated for 60 min at 37 ℃. Peri-infarct areas in the ipsilateral cortex were analyzed for TUNEL+ and DAPI+ cells using immunofluorescence microscopy (BX51, Olympus).

13. Statistical Analysis

Statistical analyses were performed using SPSS version 23 (IBM Corp., Armonk, NY, USA). Continuous data were expressed as mean ± SEM, and

numeric data were expressed as number (%). Data were analyzed by one-way ANOVA with Bonferroni *post hoc* test for normally distributed data, or Kruskal-Wallis test for nonparametric data.

III. RESULTS

1. *In vitro* characterization of hUCMSCs

The phenotype of hUCMSCs appeared to be spindle-fibroblast-shaped. The MSCs were positive for CD29, CD44, CD90, CD105, and CD73, and negative for CD14, CD31, CD34, CD45, and CD106 (Figure 1). The ability to differentiate into mesodermal cells was also confirmed. hUCMSCS were successfully differentiated into osteocytes adipocytes, and chondrocytes when they were exposed to differentiation conditions. (Figure 2).

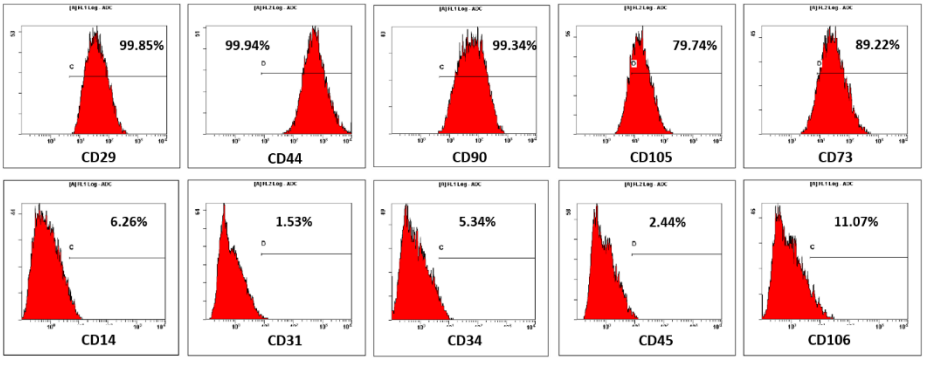


Figure 1. Florescence-activated cells sorting (FACS) analysis of cell surface markers typical of mesenchymal stem cells.

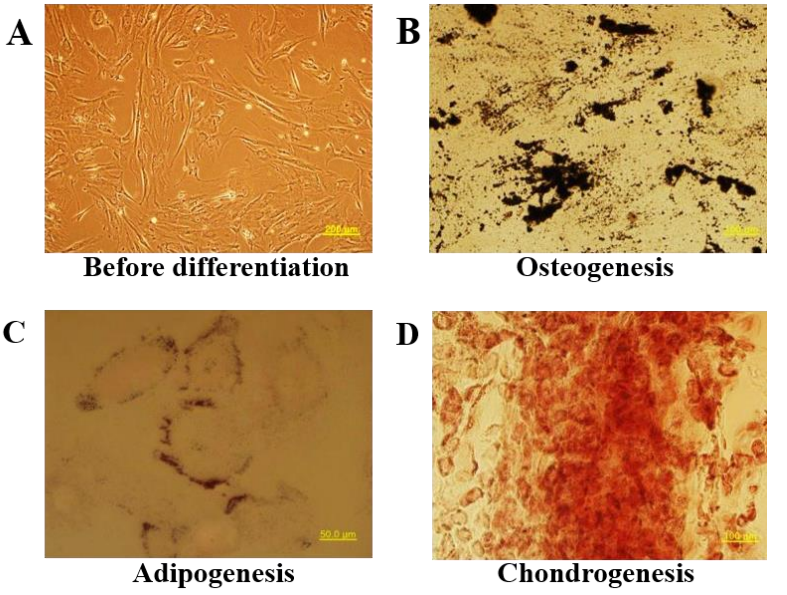
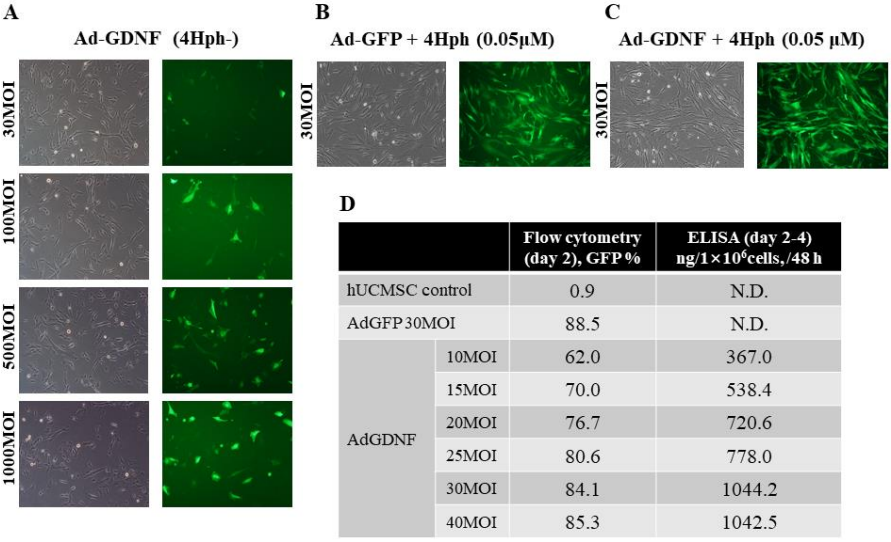


Figure 2. Differentiation potential of UCMSCs. A. Spindle-fibroblast-like shape of naïve hUCMSCs. B. Osteogenesis after Von Kossa staining. C. Adipogenesis after Oil Red O staining. D. Chondrogenesis after Safanain O staining. Scale bars, 200 μm (A.B) 50 μm (C) 100 μm (D)

2. Enhanced adenoviral gene transduction via tetrameric CPPs

We compared GFP positivity of Ad-GDNF-transduced hUCMSCs under the 30, 100, 500, and 1000 MOI without CPPs to 10, 15, 25, 30, 40, and 50 MOI using 0.05 uM 4Hph. The images of GFP-positive cells were compared using a fluorescence microscope 48 h after transduction. When hUCMSCs were transduced with Ad-GDNF in the absence of CPPs, GFP positivity slightly increased as the MOI increased from 100 to 1000, but GFP fluorescence was

much dimmer compared to all 4Hph methods (Figure 3). In addition, many of cells showed apoptotic morphology due to high dose viral infection. Flow cytometry performed 48 h after transduction showed that GFP positivity of GDNF-MSCs was peaked at 84% from 30 MOI adenovirus and 0.05μM of 4Hphs. ELISA showed that the GDNF protein concentration also peaked at 1044.2 ng/1×10^6 cells using GDNF-CM from 30 MOI adenovirus 48 h after transduction. Successful GDNF expression was confirmed via immunocytochemistry (Figure 4).



D

		Flow cytometry (day 2), GFP %	ELISA (day 2-4) ng/1×10^6cells,/48 h
hUCMSC control		0.9	N.D.
AdGFP 30MOI		88.5	N.D.
AdGDNF	10MOI	62.0	367.0
	15MOI	70.0	538.4
	20MOI	76.7	720.6
	25MOI	80.6	778.0
	30MOI	84.1	1044.2
	40MOI	85.3	1042.5

Figure 3. Construction of glial cell-line derived neurotrophic factor/human umbilical cord-derived mesenchymal stem cells (GDNF-MSCs). Fluorescent microscopy images show GFP fluorescence (A, B, C). (A) GDNF transduction without 4Hph at 30, 100, 300, 500, and 1000 MOI adenovirus. (B) GFP transduction with 0.05 μM of 4Hph and 30 MOI adenovirus. (C) GDNF transduction with 0.05 μM of 4Hph and 30 MOI adenovirus. (D) The efficiency of gene transduction using adenovirus with 4Hph: GFP positive

cells in GFP-MSCs and GDNF-MSCs via flow cytometry and GDNF protein concentration in conditioned media from GDNF-MSCs of different MOI adenovirus.

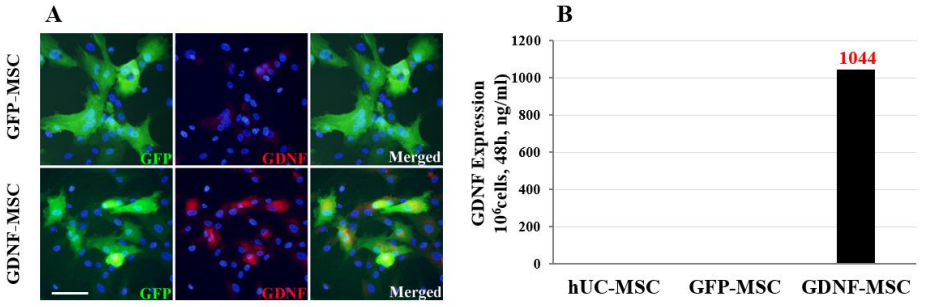


Figure 4. *In vitro* characterization of GDNF-MSCs. (A) Compared to GFP-MSCs (GFP, green; DAPI+, blue), GDNF-MSCs express GDNF (red). Scale bar: 100 μm. (B) An ELISA of GDNF in hUCMSC-CM, GFP-CM, and GDNF-CM.

3. Improved neuronal survival and neurite outgrowth by GDNF-MSCs

We investigated whether GDNF-MSC-secreted factors stimulate neurite outgrowth of SH-SY5Y cells and can protect the cells against glutamate induced excitotoxicity. Neurite outgrowth of SH-SY5Y cells was also promoted by both GDNF-CM and GFP-CM (Control, 54.5 ± 2.5; MSC-CM, 61.2 ± 2.3; GDNF-CM, 77.0 ± 3.8, P <0.05 and P <0.001, respectively, Figure 5A, 5B), and GDNF-CM showed more significant promotion in neuronal outgrowth compared to MSC-CM (P<0.05, Figure 5A, 5B). When anti-GDNF

antibody was added to GDNF-CM, the effect on neurite outgrowth was blocked, and adding isotype-matched IgG to GDNF-CM showed similar effect as GDNF-CM, which confirmed that neutralization of GDNF resulted loss of promoting effect on neurite outgrowth. (GDNF-CM+anti-GDNF antibody, 44.6 ± 2.5; GDNF-CM + IgG, 74.9 ± 4.2, P <0.05, Figure 5A, 5B). GDNF-MSC- and GFP-MSC-derived CM both significantly improved SH-SY5Y cell survival compared to glutamate-only treatment. (Control, 1 ± 0.01%; Control + Glu, 0.71 ± 0.01%; GFP-CM + Glu, 0.93 ± 0.01%; GDNF-CM + Glu, 1.07 ± 0.01%, P< 0.001 in each group, Figure 5C) and GDNF-CM was shown to be more protective than GFP-CM (P<0.05; Figure 5C).

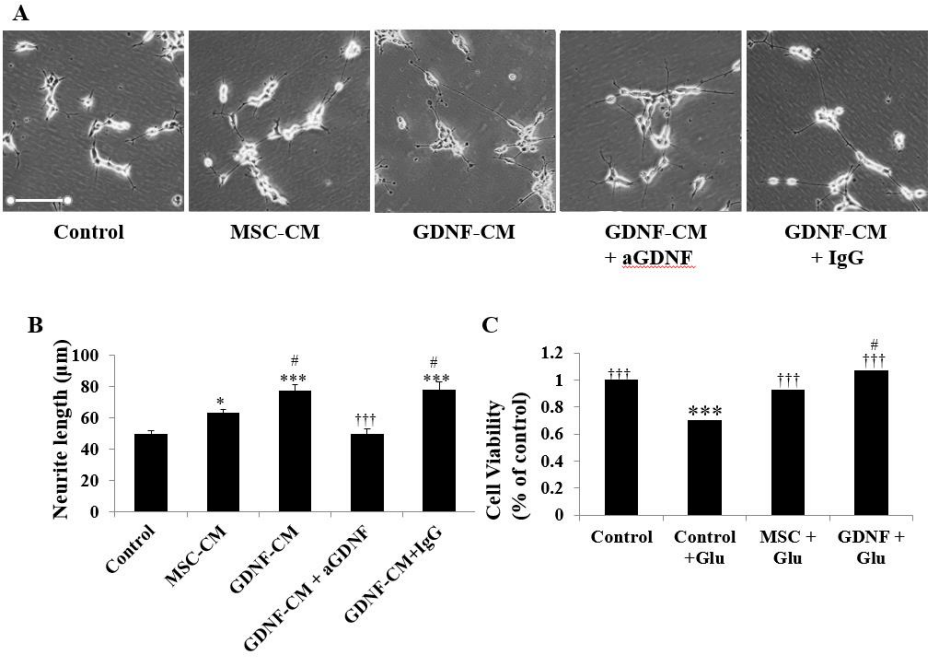


Figure 5. Secreting factors from GDNF-MSCs promote SH-SY5Y neurite

outgrowth and cell survival. (A) Representative images of CM-treated SH-SY5Y cells. Scale bar, 100 μm. (B) Quantification of average neurite length under different experimental condition. (Control, n = 109; MSC-CM, n =109; GDNF-CM, n = 71; GDNF-CM + anti-GDNF antibody, n = 119; GDNF-CM + IgG, n = 97) (C) Neuronally differentiated SH-SY5Y cells were exposed to glutamate in the presence of MSC-CM, GDNF-CM (n = 5 per group). After 24 h of glutamate treatment, the effect of CMs on the neuronal survival was assessed as the percentage of cell viability (glutamate-untreated group was considered 100% viable). Data represent the mean ± SEM. * *P* <0.05 *** *P* <0.001 vs control, # *P* <0.05 vs MSC-CM, ††† <0.001 vs GDNF-CM in data of (B); *** *P* <0.001 vs control, # *P* <0.05 vs MSC + Glu, ††† *P* <0.001 vs Control + Glu in data of (C).

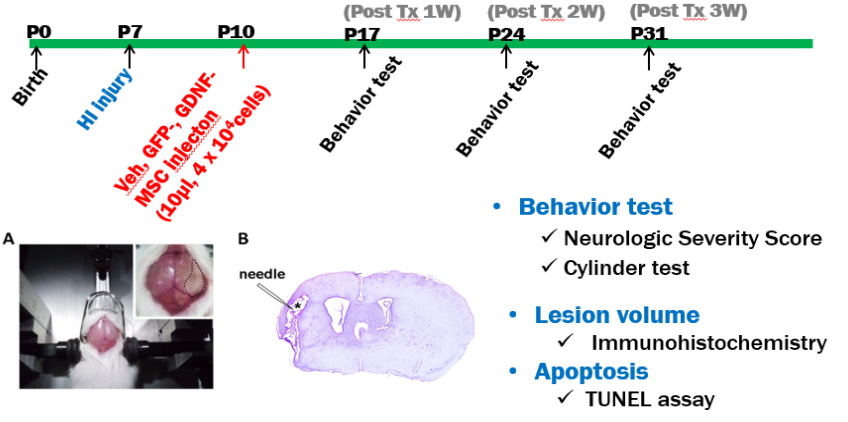


Figure 6. Schematic view of *in vivo* experiment protocol

4. Transplantation of GDNF-MSCs into neonatal HI brain injury

Vehicle, GFP-MSCs, and GDNF-MSCs were transplanted into the HI-injured brains of neonatal ICR mice 3 days after HI injury. Serial neurobehavioral assessment and immunohistochemical analysis were performed 3 weeks after transplantation. (Figure 6). One week after transplantation, both GFP-MSCs and GDNF-MSCs were well grafted into the peri-infarct (Figure 7A), which almost disappear at 3-week post-transplantation.

5. Transplanted GDNF-MSCs showed robust engraftment, reduced infarction volume, and recovery from neuronal & glial loss

Three weeks after the transplantation, the infarction volume was significantly smaller in the GDNF-MSC group than in the vehicle or GFP-MSC group. (Control, n =24, 42.2 ± 1.0%; GFP-MSC, n = 28, 39.4 ± 1.1%; GDNF-MSC, n = 22, 31.7 ± 1.9%, $P < 0.001$, Figure 7B and C). A significant increase in MAP2 positivity was observed in GDNF-MSC group compared with vehicle and GFP-MSC group. (Vehicle, n =24, 100 ± 2.3%; GFP-MSC, n = 28, 122.1 ± 2.5%; GDNF-MSC, n = 22, 150.4 ± 2.9%, $P < 0.001$, Figure 7D and E). Loss of MBP was also notably attenuated in GDNF-MSC group (Vehicle, n =24, 100 ± 2.1%; GFP-MSC, n = 28, 125 ± 2.1%; GDNF-MSC, n = 22, 146.0 ± 2.2%, $P < 0.001$, Figure 7D and F).

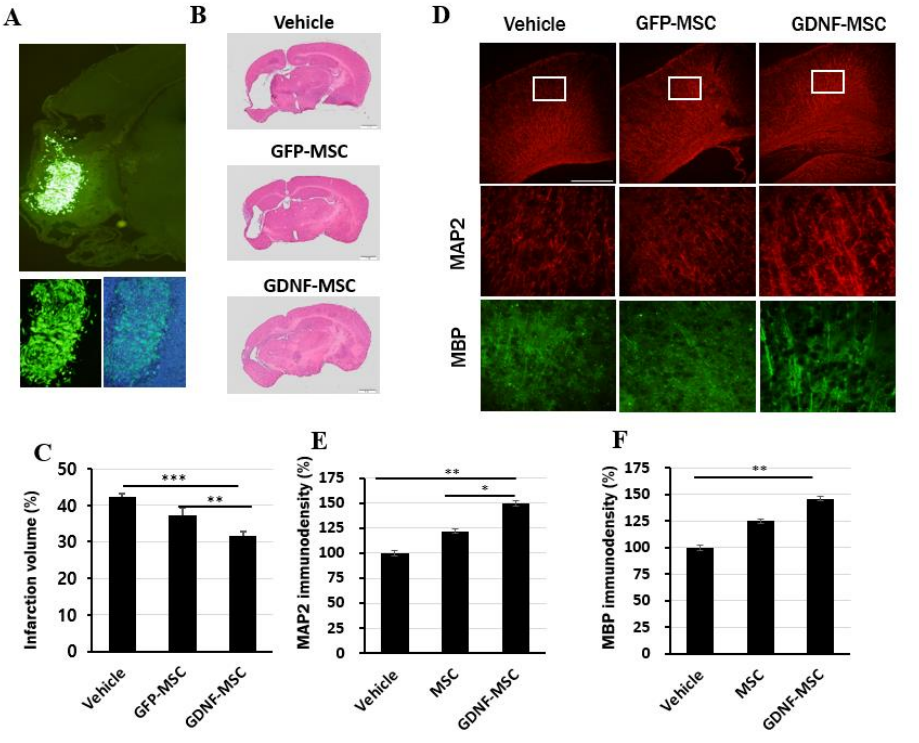


Figure 7. Transplantation of GDNF-MSC into HI brain injury in neonatal mice and immunohistochemical analysis. (A) Representative images of engrafted GDNF-MSCs within the peri-infarct area of the HI-injured mouse brain at 1 week post-transplantation. (B) Representative images of infarct volume in veihicle, GFP-MSC, and GDNF-MSC –transplanted mice, H-E stain at three weeks after MSC treatment. (C) Infarction volume was significantly reduced in GDNF-MSC group compared with vehicle and GFP-MSC groups. (D) Representative images of MAP2 and MBP staining from vehicle, GFP-MSC, and GDNF-MSC-transplanted mice. (E) Ipsilateral MAP2 immunodensity was significantly increased in GDNF-MSC group compared with vehicle and GFP-MSC groups. (F) Ipsilateral MBP immunodensity was significantly attenuated in GDNF-MSC group compared with vehicle and

GFP-MSC groups. *P < 0.05, **P < 0.01, *** P < 0.001. Data are presented as the mean ± SEM.

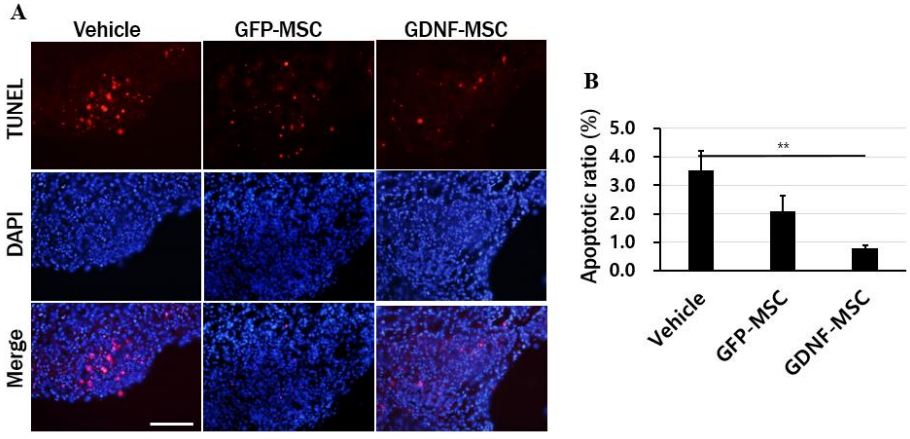


Figure 8. Effects of GDNF-MSC transplantation on apoptosis. (A) Representative images of TUNEL$^+$ cells within the ipsilateral cortex in the vehicle-, GFP-MSC-, and GDNF-MSC-transplanted mice. Scale bars, 50 μm. (B) Quantitative analysis of TUNEL$^+$ apoptotic cells. **P < 0.01 vs. vehicle. Data are presented as the mean ± SEM.

6. GDNF-MSC transplantation reduced cellular apoptosis

TUNEL assays showed that the apoptotic ratio, defined as the number of TUNEL+ cells among all DAPI+ cells, were significantly lower in GDNF-MSC-transplanted mice compared to the vehicle-injected group (0.8 ± 0.1 %vs. 3.5 ± 0.7 %, P = 0.002), though not statistically significantly different from the GFP-MSC group (2.1 ± 0.6, P = 0.176) (Figure 8A and B).

7. GDNF-MSC transplantation facilitates functional recovery

We assessed whether GDNF-MSC transplantation promoted recovery from neurological damage. The cylinder test results showed that only GDNF-MSC transplantation group showed a significantly higher performance level than the vehicle or GFP-MSC group at 1 week and 3 weeks of assessment. (0.5 % vs 13.6 % vs. 20.8 %, and 6.3 % vs 19.1 % vs. 30.4 %, $P < 0.05$, respectively, Figure 9A). The neurological severity score was similar at 1 weeks in the three groups, and improved only in the GDNF group, which was statistically different from the vehicle group (1.6 ± 0.7 % vs 2.6 ± 0.5 %, $P < 0.05$, Figure 9B).

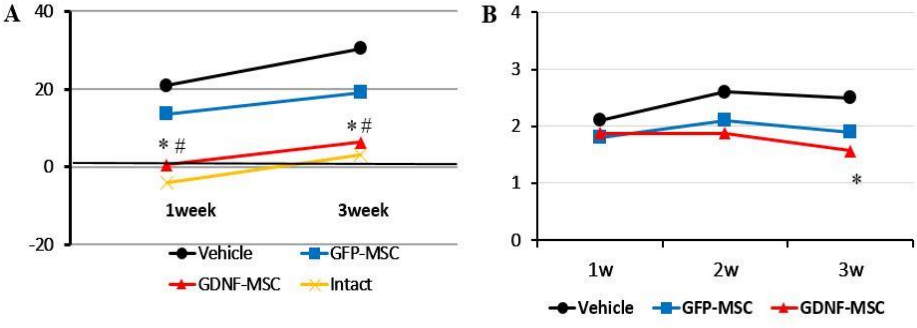


Figure 9. Behavioral performance of GDNF-MSC-, GFP-MSC-, and vehicle-injected mice with HI brain injury. (A) Cylinder tests performed at 1 week and 3 weeks after transplantation. (B) Neurologic severity score was assessed at 1 week, 2 weeks, and 3 weeks after transplantation. Data represent the mean ± SEM. GDNF-MSC, n = 20; GFP-MSC; n = 24; vehicle, n = 22; intact, n = 10. * $P < 0.05$ vs. vehicle; # $P < 0.05$ vs. GFP-MSCs.

IV. DISCUSSION

The present study showed enhanced efficiency of adenoviral gene delivery into hUCMSCs in the presence of CPPs (4Hph-1) and evaluated therapeutic potential of GDNF-overexpressing hUCMSCs in neonatal HI brain injury model. Tetrameric CPPs significantly enhanced transduction efficiency approximately at 30-fold lower concentrations of adenovirus, compared to transduction with adenovirus alone. GDNF overexpressing hUCMSCs improved neuron survival and reduced cytotoxicity. Transplantation of GDNF-MSCs into areas of neonatal HI brain injury reduced brain lesion volume, attenuated apoptosis and facilitate neurobehavioral recovery. To our best knowledge, this is the first report of GDNF overexpressing hUCMSCs as a therapeutic agent for neonatal HI brain injury, as well as a cell and gene therapy using oligomeric CPPs applied in neonatal ischemic brain model.

The concept of stem cell therapy in neonatal HI brain injury is minimizing cell death and assisting the brain in regenerative process after injury by immunomodulation and formation of microenvironment. The therapeutic potential of MSCs for brain injury has been reported in various experimental models rodent neonatal ischemic brain, demonstrating immune modulatory properties and neuroprotective potential. MSCs secrete a plethora of biological action compounds, such as chemokine, anti and proinflammatory cytokines, angiogenic factors, growth factors, and growth factor-binding proteins.[33] Currently, MSC is considered as the most promising cell therapy material for near-future use in humans in terms it’s paracrine effects and

favorable immunological profile.[34] Among the origin of MSCs, hUCMSCs are relatively easy to isolate without invasive procedures, and have advantages for the possibility of autologous transplantation in neonatal brain injury. hUCMSCs also have been shown to be safe for allogenic transplantation, given their favorable immunological profile in previous experimental studies and few clinical studies for various diseases.[35, 36] However, there are still concern about lacking neuroprotective and regenerative effect in clinical setting of naïve MSCs. Given the severity of neonatal HIE, the effectiveness of MSC treatment is still minimal to modest. To maximize therapeutic potentials of MSCs, genetically modified MSCs have been tried in animal models and resulted in diverse outcome.[37]

We previously reported the neuroprotective effect of GDNF expressing human fetal brain-derived primary neural stem/progenitor cells by transplanting into the adult spinal cord injury rat models, which resulted promoted functional recovery and histological improvement showing neurite outgrowth and axonal extension, increasing stem cell migration and myelination, modulation of astroglial scars.[38] We explored the possibility of therapeutic potentials of cell and gene therapy through adenoviral gene delivery of GDNF into stem/progenitor cells. GDNF was originally identified by its capacity to promote the survival of cultured midbrain dopaminergic neurons.[21] Upregulation of GDNF mRNA in the rodent brain has been shown to occur after excitotoxicity induced by glutamate, kainite, or ischemia.[39-41] GDNF has been shown to protect neurons against oxidative stress in cultured mesencephalic neurons and glial cells, against ischemia- or hypoxia-induced

brain injury in neonatal rats, after brain injury following permanent or transient focal cerebral ischemia in rats or mice.[25, 42] GDNF promotes cell survival in multiple ways; downregulation of NMDA-induced neuronal death from study with cultured cerebrospinal neurons[43] and upregulation of Bcl-2 and Bcl-X_1 in apoptosis-induced rat neurons resulting in a reduction in caspase activation in MCAO model were suggested.[41] GDNF was reported to improved neuronal survival in dorsal root ganglions in neonatal peripheral nerve injury rat models.[44, 45]

Exogenous GDNF administration into neonatal HI brain injury in animal model was previously attempted via lipid coated microbubbles[46] or GDNF secreting cells as transfer tools.[47] Like other neurotrophic factors, the GDNF molecule is a protein that does not readily pass the BBB. GDNF expressing bone marrow-derived MSC transplantation into central nervous system showed neuroprotective effects on intracerebral hemorrhage in adult rat model and promoted functional recovery in animal model of degenerative disease as Parkinson's disease.[48-50]

Replication-deficient recombinant adenovirus lacking the *E1* gene have long been used for therapeutic gene delivery in diverse preclinical researches including stem cell therapy. However, adenovirus particles directly cause an immediate inflammatory response and acute tissue injury, for which limits the efficiency and safety of gene delivery.[30] Park et al showed the synthesis of branched tetrameric CPPs by conjugating the C termini of the peptides to lysine linkers and dramatically increased CPP-mediated rAd transduction efficiency into MSCs due to increased CPP/rAd complex formation and

efficient simultaneous binding of tetrameric CPPs to both the cell membrane and rAd compared to monomeric CPPs, which was independent of CPP type.[31] In our study, we tested 2 type of CPPs (4Tat and 4Hph-1) at various concentration during adenoviral gene transduction. Both tetrameric CPPs resulted similar transduction efficiency and similar toxicity at optimal concentration. As Tat is protein of HIV-1 virus and Hph-1's origin is human transcription factor, 4Hph-1 was finally chosen for subsequent experiment considering the clinical application of this cell-based gene therapy. We could dramatically reduce adenoviral toxicity during gene transduction. The transduction efficiency reached almost maximum at 30 MOI, albeit poor gene transduction through classic method even at 1000 MOI. The viability and stability of MSCs were also much improved with transduction in the presence of CPPs, probably due to less toxicity of viral infection. Despite the specific pathways involved in facilitating transduction remain elusive, non-cell specific CPPs can rapidly enter the cells, by electrostatic interaction of CPPs with anionic elements, such as glycosaminoglycans on the cell surface followed by direct translocation, micropinocytosis, a more energy-dependent and slower process.[51]

In this study, GDNF-MSCs successfully secreted GDNF to environment around the cells, which was shown to have neuroprotective effect through increased cell survival and promoted neuronal outgrowth of SH-SY5Y cells treated with GDNF- CMs. In other words, the neurotrophic factors secreted from MSCS after the overexpression of GDNF, might be much enriched and have protective effects. We didn't examine whole profiles of secretory

molecules of GDNF-MSCs, so there is a limitation to interpret that whether the advantage of GDNF-MSCs is only from increased GDNF secretion or from further modification of intracellular signaling that can alter multiple therapeutic potential of MSCs.

When transplanted into neonatal HI brain injury model, GDNF-MSCs showed stable engraftment, but survived for no longer than 3 weeks, compared to transplantation with neuronal progenitor cells (NPCs). in our previous study, the survival of cells was shorter. However, unlike NPCs, the cells did not show robust migration or further differentiation. This means that there is very little risk of prolonged cell survival or *in vivo* differentiation into mesodermal cells, although careful iterative experiments are essential to assess these types of cell and gene therapies in the clinical.

The most remarkable outcome of our study was behavioral improvement, which is the most critical scale for the clinical implication. We observed beneficial effects exceeding the previous modest effect of stem cell therapy in neonatal HI brain injury. Our study demonstrated significant recovery in sensory-motor function in the GDNF-MSC-transplanted group compared to the GFP-MSC-transplanted group. This result was supported by immunohistochemical analysis, which showed reduced apoptosis and lesion volume, and suggested the possibility of decreased cortical and subcortical tissue loss after injury.

V. CONCLUSION

Tetrameric CPPs enabled efficient and safe gene transduction via adenovirus to MSCs which could be leveraged for cell and gene therapy using hUCMSCs. GDNF-overexpressing hUCMSCs provided better functional recovery and neuroprotection in neonatal HI brain injury than naïve hUCMSCs

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ABSTRACT(IN KOREAN)

4량체 세포투과성 펩타이드 및 아데노바이러스 벡터 매개 유전자 전달 연구와 신생아 저산소성 허혈성 뇌손상 모델에서 GDNF-과발현 탯줄유래 중간엽 줄기세포를 이용한 세포-유전자 치료

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신 정 은

신생아 저산소성 허혈성 뇌손상 (neonatal hypoxic ischemic brain injury) 은 만삭아 1000명 중 1-3명에서 발생하며, 중증 신경학적 장애의 주요 원인이지만 현재까지 제한적 저체온 요법 외 신경 보호제가 없다. 그동안 신생아 저산소성 허혈성 뇌손상 동물 모델에서 중간엽 줄기 세포 (mesenchymal stem cells) 이식의 치료 효과가 보고되어 왔으나, 임상 적용을 위해서는 유효성의 획기적 개선이 필요하다. 본 연구는 탯줄 유래 중간엽 줄기세포에 신경 보호 물질인 glial cell line-derived neurotrophic factor (GDNF) 를 과 발현 시킨 후 신생아 저산소성 허혈성 동물 모델에 이식하여 새로운 세포-유전자 치료 가능성을 연구하고자 하였다. 또한 중간엽 줄기세포로의 GDNF 유전자 전달 과정에서, 재조합 아데노바이러스 벡터와 사량체 세포 투과성 펩타이드를 이용하여 유전자 전달 효율을 극대화하고자 하였다.

건강한 만삭아의 탯줄 와튼 젤리(Wharton’s jelly) 에서 탯줄 유래 중간엽 줄기세포를 분리, 배양한 후 아데노바이러스 벡터를 이용하여 GDNF 유전자를 전달하였으며 (GDNF-MSCs), 대조군으로 GFP 유전자를 전달하였다 (GFP-MSCs). 사량체 세포 투과성 펩타이드 4Hph-1 를 아데노바이러스의 세포 감염 시

함께 사용하여 GDNF 유전자 전달을 위한 조건을 수립하였다. 그 결과, 4Hph-1 0.05 μM 과 아데노바이러스 30 MOI 에서 최대 감염 효율 (84%) 과 최대 GDNF 단백 발현이 확인되었으며, 세포 투과성 펩타이드가 없는 경우에 비하여 아데노바이러스 감염 농도를 1/15 로 낮출 수 있었다. GDNF-MSCs 배양 배지를 SH-SY-5Y 신경세포주에 처리한 결과 GFP-MSCs 배지 조건과 비교하여 신경 독성이 호전되고 neurite outgrowth 가 의미 있게 증대되어 GDNF에 의한 신경 보호 효과가 있음을 확인하였다.

저산소성 허혈성 뇌손상 발생 마우스 모델에 손상 3일 후 GDNF-MSCs 와 GFP-MSCs를 각각 이식하여 이식 1주 후 부터 3주 신경학적 기능의 변화를 관찰하였으며, 전체 뇌 경색 범위 및 피질과 피질 하 조직의 손상 정도, 손상 부위의 세포 사멸에 대해 면역화학 염색을 통해 분석하였다. 그 결과, GDNF-MSCs의 이식은 vehicle 및 GFP-MSCs 대조군에 비교하여 의미 있는 신경학적 기능 호전을 보였으며, GDNF-MSC 이식 군에서 뇌 경색 부위, 특히 피질과 피질 하 조직의 손상 범위가 GFP-MSCs 이식 군 대비 호전되고 세포 사멸이 억제 됨을 확인할 수 있었다.

이상의 연구 결과로 신생아 저산소 허혈성 뇌손상에서 GDNF 과발현 탯줄 유래 중간엽 줄기세포 이식을 통해 신경 보호 효과 및 신경학적 기능 호전을 확인하여, 세포-유전자 치료를 통한 치료 유효성의 증대를 확인하였으며, 사량체 세포 투과성 펩타이드가 중간엽 줄기세포에 아데노바이러스를 이용한 유전자 전달의 효율을 극대화 시킬 수 있음을 제시하였다.

핵심되는 말: 저산소성 허혈성 뇌손상, 중간엽줄기세포, glial cell line-derived neurotrophic factor, 신경 보호, 세포 치료, 유전자 치료

PUBLICATION LIST

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