Evaluation of the Safety and Therapeutic Effect of Hypoxia-specific, VEGF-expressing Neural Stem Cells in a Model of Spinal Cord Injury

Bo Young Cho

Department of Medicine

The Graduate School, Yonsei University

Evaluation of the Safety and Therapeutic Effect of Hypoxia-specific, VEGF-expressing Neural Stem Cells in a Model of Spinal Cord Injury

Directed by Professor Do Heum Yoon

Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Bo Young Cho

June 2011

This certifies that the Master's Thesis (Doctoral Dissertation) of Bo Young Cho is approved.

Thesis Supervisor : Do Heum Yoon

[Kook In Park: Thesis Committee Member#1)

[Jong Eun Lee: Thesis Committee Member#2)

[Keung Nyun Kim: Thesis Committee Member#3)

[Seung Hwan Yoon: Thesis Committee Member#4)

The Graduate School
Yonsei University

June 2011

ACKNOWLEDGEMENTS

It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here. Above all, this thesis would not have been possible without the help, support and patience of my principal supervisor, Prof.Do Heum Yoon. I also thank Prof. Kook In Park and Prof. Jong Eun Lee for their support and assistance from the start of my work.

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

Evaluation of the Safety and Therapeutic Effect of Hypoxia-specific, VEGF-expressing Neural Stem Cells in a Model of Spinal Cord Injury

Bo Young Cho

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Do Heum Yoon)

We established three stable neural stem cell (NSC) lines to explore the possibility of using EpoSV-VEGF NSCs to treat spinal cord injury (SCI). The application of EpoSV-VEGF NSCs into the injured spinal cord after clip compression injury not only showed therapeutic effects such as extended survival, angiogenesis, and functional recovery, but also displayed its safety profile as it did not cause unwanted cell proliferation or angiogenesis in normal spinal cord tissue, as EpoSV-VEGF NSCs consistently showed hypoxia-specific VEGF expression patterns. This suggests that our EpoSV-VEGF NSCs are both safe and therapeutically efficacious for the treatment of SCI. Furthermore, this hypoxia inducible gene expression system may represent a safe tool suitable for gene therapy delivery.

Key words: hypoxia-specific gene expression, VEGF, safety, efficacy, neural stem cells

Evaluation of the Safety and Therapeutic Effect of Hypoxia-specific, VEGF-expressing Neural Stem Cells in a Model of Spinal Cord Injury

Bo Young Cho

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Do Heum Yoon)

I. INTRODUCTION

Spinal cord injury (SCI) leads to cell death, loss of blood vessels, demyelination, axonal degeneration, and scar formation[1]. Among these phenomena, the destruction of blood vessels gives rise to a hypoxic environment, thereby decreasing the survival of transplanted stem cells[2]. Moreover, it has been known that new blood vessel formation is a critical component of the wound healing process[3]. To improve the survival of transplanted stem cells and enhance angiogenesis in the injured spinal cord, many gene therapy strategies have been investigated [2, 4, 5]. It has been known that vascular endothelial growth factor (VEGF) plays a role in angiogenesis and has neuroprotective effects [6, 7], and the transplantation with VEGF-expressing stem cells was shown to significantly improve functional recovery, angiogenesis, and cell survival in the injured spinal cord[8-10]. However, uncontrolled VEGF expression as the environment of the injured spinal cord transitions from hypoxic to normoxic may contribute to

tumor formation by uncontrolled angiogenesis [11]. With the goal of making gene therapy safer, the hypoxia-inducible gene expression system was established to control the expression of the chosen therapeutic gene in normal conditions [12]. Even though this gene expression system has been introduced in previous studies [5, 13-15], the present study is the first to report the safety of VEGF side effects in uncontrolled expression. In this study, the hypoxia-inducible VEGF expression system was introduced into mouse neural stem cells (NSCs) to establish a controlled, VEGF-expressing stable neural stem cell line (EpoSV-VEGF NSCs). VEGF expression was then compared to uncontrolled, VEGF-expressing stable neural stem cell line (SV-VEGF NSCs) under normoxic (1% O_2) and hypoxic (1% O_2) conditions. *In vitro*, we investigated whether EpoSV-VEGF NSCs show controlled expression in normoxic conditions when compared to SV-VEGF NSCs, as well as confirmed the neuroprotective effect of both EpoSV-VEGF NSCs and SV-VEGF NSCs in hypoxia mimicking injury model. Additionally, in an animal model of spinal cord injury, we investigated whether transplantation with both VEGF expressing NSCs improves angiogenesis and functional recovery compared to normal NSCs. Moreover, we evaluated the safety of hypoxia-inducible gene expression system and its ability to control VEGF expression in normal tissue.

II. MATERIALS AND METHODS

Cell culture & Establishment of stable neural stem cell line

Mouse neural stem cells (NSCs) were purchased from the American Type Culture Collection (ATCC, CRL-2925). NSCs were cultured in Dulbecco's-modified Eagle's medium (DMEM/F12, Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS, Hyclone, Logan, UT) and 1% penicillin/streptomycin (100 unit/mL, Invitrogen, Carlsbad, CA) maintained at 37°C in a humidified atmosphere containing 5% CO_2. Stable cell lines expressing VEGF were established using a specifically constructed plasmid (Fig.1). The protocol followed to establish a stable NSC line is as follows: Step 1: plasmid DNA (pBudCE4.1-DsRed, 1 μg/μL) was mixed with 100 μL Opti-MEM (Invitrogen, Carlsbad, CA). Step 2: 2.5 μL Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA) was mixed with 100 μL Opti-MEM and incubated for 5 minutes at room temperature. Step 3: The solutions from Step 1 and Step 2 were combined and incubated for 20 minutes at room temperature. Step 4: The mixed solution of Step 3 was added to the cultured cells, and cells were then maintained at 37°C in a humidified atmosphere containing 5% CO_2 for 4 hours. Afterward, the media was changed to growth culture medium. After twenty-four hours incubation, Zeocin (200ug/mL, Invitrogen, Carlsbad, CA) was added to the growth culture medium. Single Zeocin-resistant cells were isolated by cloning rings and expanded.

RT-PCR

Total RNA was isolated from each sample using Trizol (Invitrogen, Carlsbad, CA) and cDNA was synthesized using Accupower RT/PreMix (Bioneer, Seoul, Korea). RT-PCR was performed using following primer sets, forward (5′-CCCAAGCTTGAAACCATGAACTTGCT-3′) and a reverse (5′-GCTCTAGATCATTCATTCATCACCGCCT-3′). DNA was amplified by the following steps: 94℃ for 5 minutes, 30 cycles at 94℃ for 30 seconds, 51℃ for 30 seconds, 72℃ for 30 seconds, and 72℃ for 10 minutes.

MTT Assay

After cell cultivation under each condition for each time point, media was changed with DMEM/F12 containing 10% MTT solution (5 mg/ml, Sigma, St. Louis, MO), then incubated for an additional 5 hours. After removing the media containing MTT solution, formazan crystals were dissolved by adding dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) to each well. After transferring the dissolved products to 96 well plates (Nunc, Roskilde, Denmark), the absorbance was measured at 550 nm.

Spinal Cord Injury and Cell Transplantation

The SCI animal model involved adult male Sprague-Dawley rats (250-300 g; OrientBio, Gyeongi-do, Korea). All protocols were approved by the Animal

Care and Use Committee of Yonsei University College of Medicine. All experiments were performed according to international guidelines on the ethical use of animals, and the number of animals used was minimized. Animals were anesthetized by sodium penthobarbital (20 mg/kg; Choongwae Pharma, Seoul, Korea). After anesthesia, laminectomy was performed at the T9 level. The spinal cord was injured by clip compression at the T9 level for 10 minutes.

For the behavioral test, NSCs were transplanted into the injured spinal cord immediately. The groups are as follows: Group 1: PBS injection; Group 2: DsRed-NSCs ($3x10^5$ cells/5μL), Group 3: SV-VEGF NSCs ($3x10^5$ cells/5μL), Group 4: EpoSV-VEGF NSCs ($3x10^5$ cells/5μL). Cells were injected into the epicenter of the injured spinal cord using a 26 gauge Hamilton syringe. All animals were double-blind tested using the Basso, Beattie, and Bresnahan (BBB) hind limb locomotor scale every week for 6 weeks. Apart from these groups, in order to confirm controlled VEGF expression, both EpoSV-VEGF NSCs ($3x10^5$ cells/5ul) and SV-VEGF NSCs ($3x10^5$ cells/5ul) were transplanted into the normal and injured spinal cord.

Tissue Extraction and Immunofluorescence Staining

To obtain spinal cord tissue, all rats were sacrificed at 1 day, 2 weeks, or 6 weeks by heart perfusion with saline (pH 7.4) and 4% paraformaldehyde (pH 7.4, PFA, Merck, Germany). Spinal cord tissue was then removed, including the injury epicenter. After fixation in 4% paraformaldehyde for 24 hours, all tissue

was maintained in 30% sucrose until sinking to the bottom. After freezing with OCT compound, tissues were cut into 20 μm-thick sections.

Tissue samples were fixed in methanol for 10 minutes at -20℃. After washing with ice-cold PBS, cells were incubated with 0.5% Triton X-100 (Invitrogen, Carlsbad, CA) in PBS for 15 minutes at room temperature. Samples were blocked with normal donkey serum (Jackson Immuno Research, West Grove, PA) in PBS for 1 hour at room temperature. Samples were incubated with primary antibody, rabbit anti-vWF (1:250, abcam), mouse anti APC-CC1 (1:250, Millipore), mouse anti GFAP (1:1000, Millipore), mouse anti-Neun (1:200, Millipore), for 1 hour. After incubation, samples were washed with PBS and incubated with FITC-conjugated anti-rabbit immunoglobulin G (1:250, Jackson Immuno Research, West Grove, PA) for 1 hour at room temperature. The samples were washed three times with ice cold PBS and then covered with a cover slip after drops of Vectashield mounting medium containing 4',6'-diamino-2-phenylindole (DAPI, Vector, Burlingame, CA) were added. Samples were analyzed using a BX51 Olympus fluorescence microscope (Olympus, Japan) and laser confocal (LSM 700, ZEISS).

Optical Density Analysis

To confirm the survival of transplanted NSCs, tissue samples from each group (Group 1: DsRed-NSCs, $3x10^5$cells/5μL; Group 2: SV-VEGF NSCs, $3x10^5$ cells/5μL; and Group 3: EpoSV-VEGF NSCs, $3x10^5$ cells/5μL) were obtained

by saline perfusion at two weeks after transplantation. A total of 100 longitudinal cross sections with a thickness of 10 μm were made from all spinal cord tissue. Because all stable NSCs expressed the DsRed protein, immunostaining for tracking of NSCs not performed. To distinguish between the injected NSC-DsRed and host cells, DAPI staining (Vector Laboratories, Inc., Burlingame) was performed as a counter-staining method. After taking a picture using the BX51 Olympus fluorescence microscope (Olympus, Japan), NSC-DsRed cells were counted from 20 serial sections out of a total of 100 sections.

Statistical Analysis

Student's t-test was used to compare data between the two experimental groups. One-way ANOVA test was used to compare data among the three experimental groups. Data were expressed as the mean ± standard deviation (SD). A p value less than 0.05 was considered statistically significant.

III. RESULTS

Cell Characterization & VEGF Expression

In this study, three stable neural stem cell lines (DsRed-NSCs, SV-VEGF NSCs and EpoSV-VEGF NSCs) were established (Fig.1A). To confirm whether EpoSV-VEGF NSCs showed hypoxia-specific gene expression, these three NSC cell lines were incubated in normoxia (21% O_2) and hypoxia (1% O_2) for 24hourrs. Specifically, we investigated whether VEGF expression of EpoSV-VEGF NSCs showed controlled expression of VEGF under normoxia (21% O_2). VEGF expression in SV-VEGF NSCs was high in both normoxia and hypoxia, but the expression of VEGF in EpoSV-VEGF NSCs showed specific regulation under normoxia. The relative expression of VEGF in normoxia was significantly different in EpoSV-VEGF NSCs compared to SV-VEGF NSCs (Fig.1B). To investigate whether EpoSV-VEGF NSCs also showed controlled VEGF expression when the oxygen concentration changes from hypoxia to normoxia, EpoSV-VEGF NSCs were incubated in the hypoxic chamber for 24 hours, and then transferred to the normoxic chamber, where they were then incubated for 24 hours. High VEGF expression in the EpoSV-VEGF NSC group under hypoxia was decreased by re-oxygenation in normoxia (Fig.1C), indicating that EpoSV-VEGF NSCs not only show hypoxia-specific VEGF expression, but also may reduce the side effect of uncontrolled VEGF expression during recover from hypoxia to normoxia.

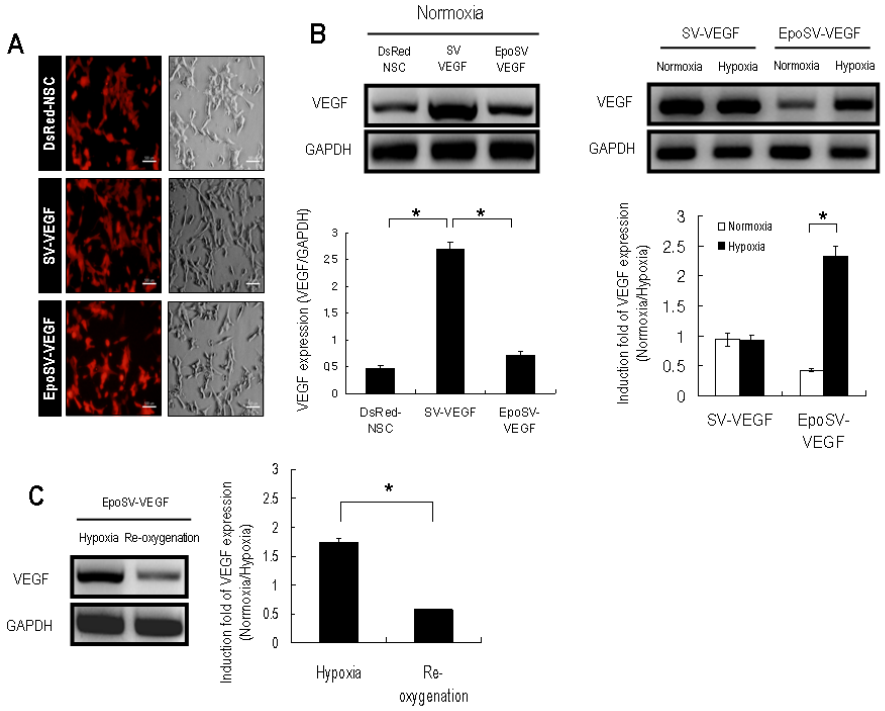

Fig.1 Hypoxia-specific VEGF expression and stable neural stem cell lines

(A) The morphology of three stable neural stem cell lines (DsRed-NSCs, SV-VEGF NSCs, and EpoSV-VEGF NSCs) is represented. (B) VEGF expression of SV-VEGF NSCs showed non-specific VEGF expression in both normoxia and hypoxia. In contrast, EpoSV-VEGF NSCs showed hypoxia-specific VEGF expression pattern. (C) VEGF expression of EpoSV-VEGF NSCs was down regulated during the transition from hypoxia to normoxia. Scale bar =500um, *: significant difference between each group ($p < 0.05$). Data are shown as mean ± SD.

Cell Proliferation and Viability

To investigate whether VEGF expression affects cell proliferation and viability, three NSC cell lines were incubated under normoxia and hypoxia for 24 hours, then underwent MTT assay and RT-PCR (with apoptosis related genes, such as bax, screened). The proliferation of SV-VEGF NSCs in normoxia significantly improved compared to DsRed NSCs and EpoSV-VEGF NSCs. The proliferation of EpoSV-VEGF NSCs in normoxia was similar to that of DsRed-NSCs, and was lower compared to SV-VEGF NSC viability. Moreover, the proliferation of both SV-VEGF NSCs and EpoSV-VEGF NSCs in hypoxia was significantly higher compared that of DsRed-NSCs (Fig.2A), ki67 expression showed the similar pattern to MTT assay (Fig.2B). It indicates that EpoSV-VEGF NSCs showed hypoxia-specific VEGF expression, with the cell proliferation of EpoSV-VEGF NSCs regulated in normoxia.

To investigate whether VEGF expression affected cell viability, three NSCs lines were cultured in serum free DMEM/F12 media containing 500uM hydrogen peroxide (H_2O_2) in hypoxia for 24 hours, followed by RT-PCR. The viability of SV-VEGF NSCs and EpoSV-VEGF NSCs was significantly in the *in vitro* injury model than those cultures in hypoxia without H_2O_2. In accordance with the MTT assay (Fig.2A), VEGF expression of both SV-VEGF NSCs and EpoSV-VEGF NSCs was significantly higher in the *in vitro* injury model. However, the expression of Bax, an apoptosis-related gene, was decreased to counter the VEGF expression pattern (Fig.2C, D). These results

indicate EpoSV-VEGF NSCs and SV-VEGF NSCs show an equally potent neuroprotective effect under hypoxia with H_2O_2 injury.

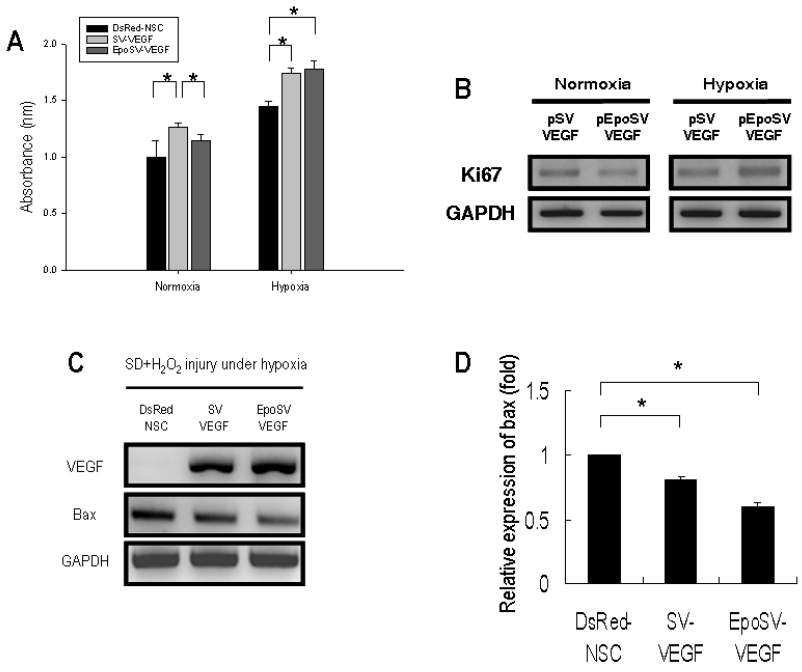

Fig.2 Cell proliferation and viability

Cell proliferation was investigated using the MTT assay and RT-PCR. (A) Proliferation of SV-VEGF NSCs was consistently high in both normoxia and hypoxia. However, the proliferation of EpoSV-VEGF NSCs was similar to that of DsRed-NSCs under normoxia and was significantly increased in hypoxia. (B) Ki67 expression pattern showed similar to MTT assay result. (C and D) In serum deprivation (SD) and hydrogen peroxide (500uM, H_2O_2) injury under hypoxia, both SV-VEGF and EpoSV-VEGF NSCs highly expressed VEGF, while the expression of *bax* in VEGF-expressing NSCs was low compared to that of DsRed-NSCs. *: significant difference between each group ($p < 0.05$). Data are shown as mean ± SD.

VEGF Expression of Transplanted NSCs

To confirm whether the transplanted EpoSV-VEGF NSCs also show a similar controlled expression pattern in normal tissue as was seen in the *in vitro* study, three NSCs lines (DsRed-NSCs, SV-VEGF NSCs and EpoSV-VEGF NSCs) were transplanted into the injured or normal spinal cord, followed by RT-PCR after 24 hours. Transplanted SV-VEGF NSCs expressed higher than normal amount of VEGF in both the normal and injured spinal cord. In contrast, EpoSV-VEGF NSCs transplanted into the uninjured spinal cord showed consistent, controlled expression of VEGF; in the injured spinal cord, VEGF expression was almost equal toVEGF expression by SV-VEGF NSCs in the injured spinal cord (Fig.3). These results indicate EpoSV-VEGF NSCs sufficiently express VEGF to a similar degree as SV-VEGF NSCs in injured tissue. At the same time, by regulating VEGF expression when the hypoxic environment returns to normoxia, EpoSV-VEGF NSCs also provide a greater measure of safety, minimizing any side effects of VEGF expression during normoxia.

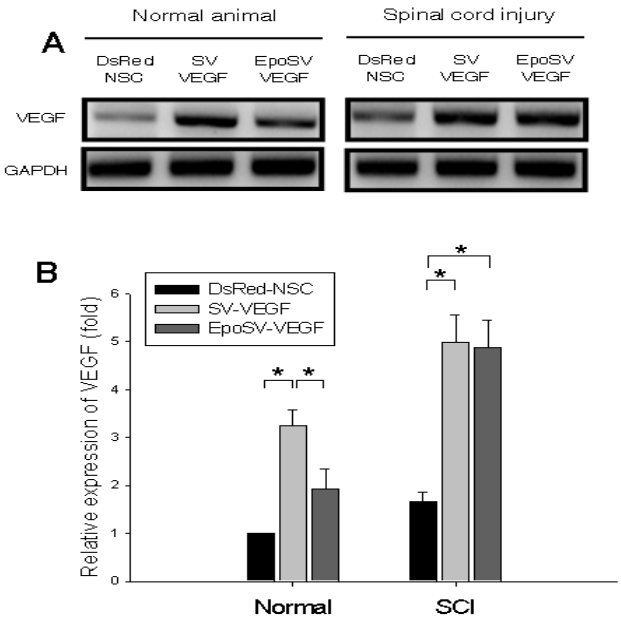

Fig.3 Hypoxia-specific VEGF expression of EpoSV-VEGF NSCs *in vivo*

VEGF expression of transplanted SV-VEGF NSCs was high in both normal and injured spinal cord. In contrast, EpoSV-VEGF NSCs showed consistent hypoxia-specific VEGF expression. *: significant difference between each group ($p < 0.05$). Data are shown as mean ± SD.

Safety Evaluation of Controlled VEGF-expressing NSCs

To confirm the safety of transplantation with controlled, VEGF-expressing NSCs, both SV-VEGF and EpoSV-VEGF NSCs were transplanted into the normal spinal cord, followed by cell survival and angiogenesis analysis 2 weeks after transplantation. The optical density of EpoSV-VEGF NSCs transplanted into the normal spinal cord was lower compared to that of SV-VEGF NSCs (Fig.4A). EpoSV-VEGF NSCs show controlled VEGF expression in the normal spinal cord, with high bax expression (Fig.4B-D). Two weeks after transplantation, tissues were stained with vWF antibody, and then the optical density of vWF-positive structures in the transplanted area was measured. In the normal spinal cord, vWF-positive vessels were frequently observed in the boundary of the NSC transplanted area. The density of vWF-positive vessels in the boundary of the SV-VEGF NSC transplanted area was significantly higher than in that of EpoSV-VEGF NSCs (Fig.4E and F). While tumor formation was not observed in the SV-VEGF NSC transplanted group, our results suggest that controlled VEGF expression (as exemplified in the EpoSV-VEGF NSC group) may provide the safer treatment by preventing unwanted neovascularization.

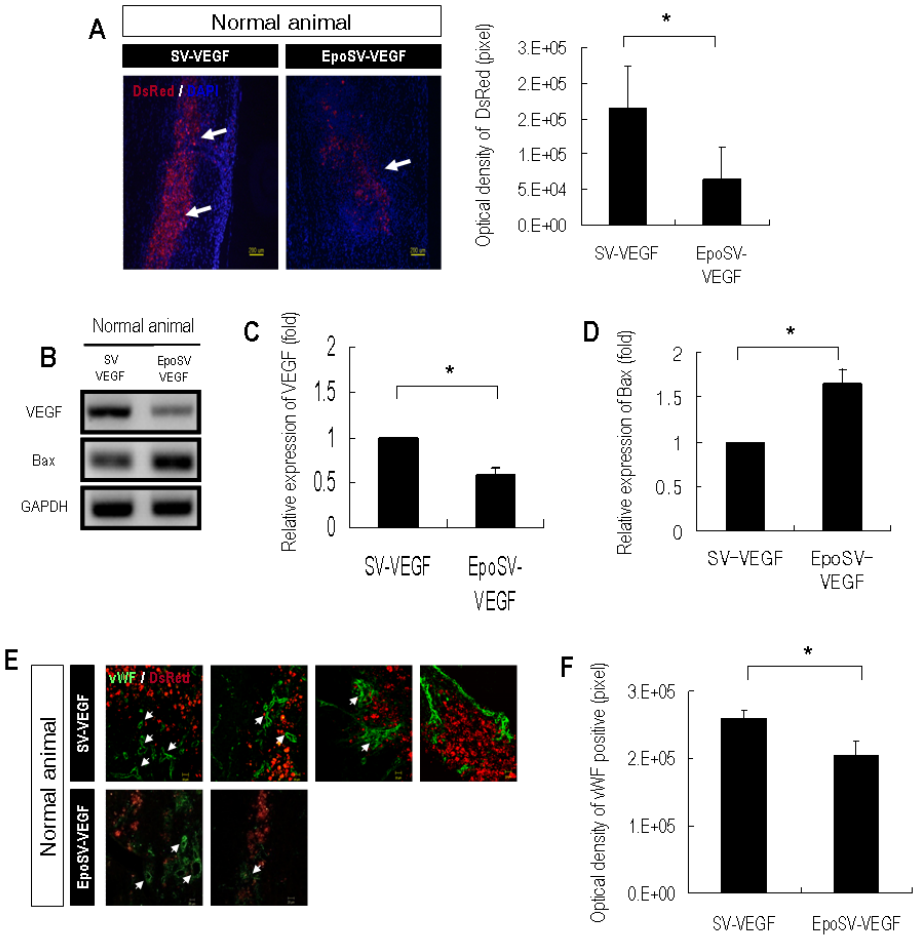

Fig.4 Safety evaluation of controlled VEGF-expressing NSCs

(A) Two weeks after transplantation into the normal spinal cord, the optical density of DsRed in SV-VEGF NSCs group was greater than that of the EpoSV-VEGF NSCs group. (B-D) The VEGF expression of EpoSV-VEGF NSCs was significantly lower than that of the SV-VEGF NSCs. In contrast, the *bax* expression of EpoSV-VEGF NSCs was significantly higher compare to that of the SV-VEGF NSCs. (E and F) Immunofluorescence staining with vWF two

weeks after transplantation into the normal spinal cord. The vWF-positive vessels in the area of the transplanted SV-VEGF NSCs were significantly greater than in that of the EpoSV-VEGF NSCs. Scale bar =200um (Fig.4A), 20um (Fig.4E), *: significant difference between each group ($p < 0.05$). Data are shown as mean ± SD.

Therapeutic Effect of Hypoxia-specific VEGF-expressing NSCs

To confirm the therapeutic effect of transplantation with controlled VEGF expression, three NSC cell lines (DsRed, SV-VEGF, and EpoSV-VEGF) were transplanted into the injured spinal cord, followed by cell survival and angiogenesis analysis 2 weeks after transplantation. The number of DsRed-positive NSCs in both the SV-VEGF and EpoSV-VEGF groups was higher than in the DsRed control group (Fig.5A). Bax expression was existent in all three groups. However, SV-VEGF and EpoSV-VEGF NSC-treated groups showed low *bax* expression compared to the control NSC treated group (Fig.5B-D). Two weeks after transplantation, tissues were stained with vWF antibody, followed by the measurement of vWF optical density in the NSC transplanted area. The density of vWF-positive vessels in the boundary of the transplanted area was significantly higher in the SV-VEGF and EpoSV-VEGF NSC treated groups than in the DsRed-NSC treated group (Fig.5E and F). These results indicate, in a model of spinal cord injury, VEGF affects both cell survival and angiogenesis, with our hypoxia-inducible, VEGF-expressing NSCs showing an equal therapeutic effect when compared to SV-VEGF NSCs.

The cell survival and angiogenesis analysis was performed at 6 weeks after transplantation. The number of DsRed-positive NSCs in three groups was not significantly difference (Fig.5G). The density of vWF-positive vessels was significantly higher in EpoSV-VEGF NSC treated groups than in the DsRed-NSC treated group (Fig.5H).

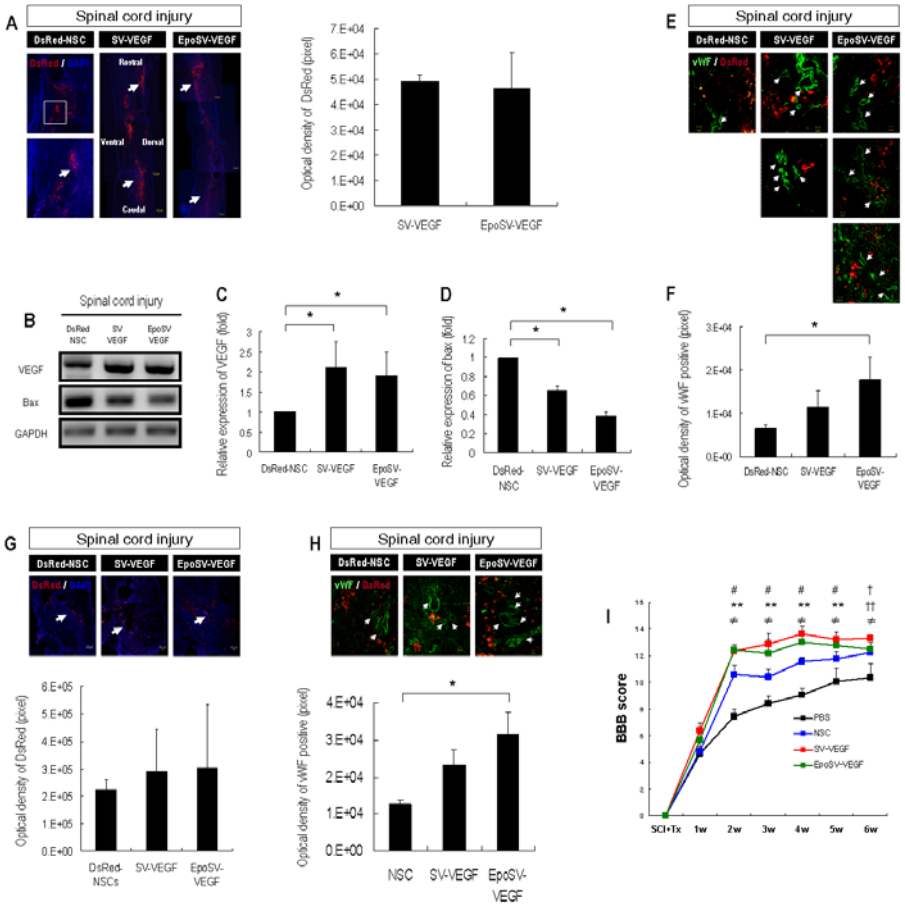

Fig.5 Therapeutic effect of hypoxia-specific VEGF expressing NSCs

(A) Two weeks after transplantation into the injured spinal cord, the optical density of DsRed in both the SV-VEGF and EpoSV-VEGF NSC groups was greater than that of the DsRed-NSC group. (B-D) The VEGF expression of both SV-VEGF and EpoSV-VEGF NSCs was significantly greater compared to that of the DsRed-NSCs. *Bax* expression of both SV-VEGF and EpoSV-VEGF NSCs was significantly higher than that of DsRed-NSCs. (E and F) Immunofluorescence staining with vWF two weeks after transplantation into the

injured spinal cord. The vWF-positive vessels in the area of the transplanted SV-VEGF and EpoSV-VEGF NSCs were significantly greater than in the DsRed-NSC transplanted area. (G) Six weeks after transplantation into the injured spinal cord, the optical density of DsRed was not significantly difference among three groups. (H) Immunofluorescence staining with vWF six weeks after transplantation into the injured spinal cord. The vWF-positive vessels in the area of the transplanted EpoSV-VEGF NSCs were significantly greater than in the DsRed-NSC transplanted area. (I) Functional recovery in the cell injected groups rapidly improved compared to the PBS injected group. Specifically, the transplantation of SV-VEGF and EpoSV-VEGF NSCs significantly improved functional recovery when compared to the DsRed-NSCs injected group. Scale bar =200um (Fig.5A), 20um (Fig.5E). #: SV-VEGF significaltly difference compare with NSC and PBS; **: EpoSV-VEGF significaltly difference compare with NSC and PBS; ≠: NSC significantly difference compare with PBS; †: SV-VEGF significantly difference compare with EpoSV-VEGF, NSC and PBS; ††: EpoSV-VEGF significantly difference compare with PBS ($p < 0.05$). Data are shown as mean ± SD (C, D and F) and SEM (G)

Functional Recovery

To confirm whether the treatment with VEGF-expressing NSCs affected hind limb functional recovery after spinal cord injury, behavioral testing was performed for 6 weeks after transplantation (Fig.5I). PBS-injected animals showed plantar stepping with occasional weight bearing after spinal cord injury, yet they did not show forelimb-hindlimb coordination. NSC transplantation showed significant improvement in hind limb function compared to the PBS-injected group. NSC-transplanted animals showed frequent weight supported stepping from 2 weeks after transplantation and showed occasional forelimb-hindlimb coordination. Specifically, within 2 weeks after transplantation, SV-VEGF and EpoSV-VEGF NSC transplanted groups showed significant functional recovery compared to DsRed-NSC and PBS injected groups and showed frequent forelimb-hindlimb coordination.

Differentiation potency of transplanted cells

Two weeks after post transplantation, the double immunostaining was performed with anti-GFAP, APC-CC1 and Neun. Transplanted NSCs were differentiated into the GFAP, APC-CC1 and Neun-positive neurons respectively (Fig.6).

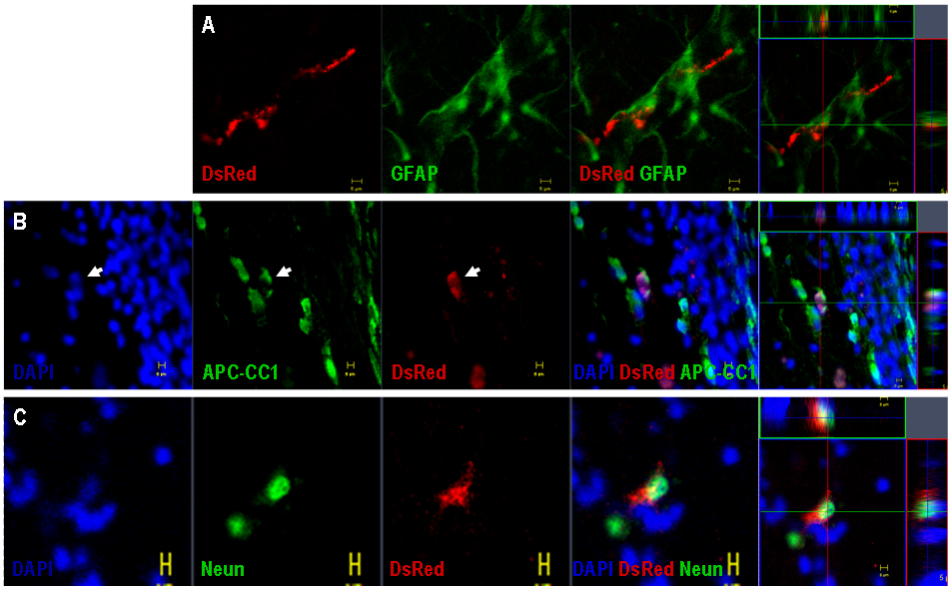

Fig.6 Differentiation potency

Two weeks after transplantation into spinal cord, the double mmunofluorescence staining was performed respectively using anti-GFAP, APC-CC1 and Neun antibody. A few NSCs were differentiated into the GFAP-positive astrocyte (A) and APC-CC1-positive oligodendrocyte (B) and Neun –positive neuron (C). Scale bar =5um

IV. DISCUSSION

We verified the safety and therapeutic efficiency of combined therapy with a hypoxia-specific VEGF expression system and neural stem cells in a model of spinal cord injury. Even though we previously confirmed many times the therapeutic effect of VEGF using a hypoxia-specific gene expression, investigated gene transfection efficiency by injection of the VEGF plasmid into a neuroblastoma cell or the injured spinal cord [13, 14], we had not evaluated the therapeutic effect using our hypoxia-specific VEGF expression system with neural stem cells in spinal cord injury. Moreover, the evaluation of the safety of our hypoxia-specific VEGF expression system had not been performed until now. Therefore, we established a stable neural stem cell line to maximize the synergistic effect by combining neural stem cell and VEGF therapy.

RTP801 promoter also had been introduced as hypoxia-inducible gene expression system, this promoter showed the high gene expression pattern in hypoxia [15]. We also tried to establish the hypoxia-specific VEGF expressing neural stem cell using RTP801 promoter. Although, RTP801-VEGF NSCs showed the high gene expression more than EpoSV-VEGF NSCs in hypoxia, it's not showed the controlled gene expression in normoxia. Therefore, we established the EpoSV-VEGF NSCs.

In a previous study, a stable neural stem cell line with a hypoxia-specific luciferase expression system had been established, and we confirmed the general characterizations of stable, hypoxia-specific, luciferase expressing

neural stem cells both *in vitro* and *in vivo*. Luciferase expression of hypoxia inducible luciferase expressing neural stem cells transplanted into the injured spinal cord was increased compare with transplanted into the normal spinal cord[16]. It's indicate that spinal cord injury lead to hypoxic condition, our hypoxia inducible gene expressing neural stem cells show the hypoxia specific expression. Therefore, in this study, we established a stable neural stem cell line that equally expresses VEGF as well as SV-VEGF NSC in hypoxia. In addition, we also showed the controlled expression of VEGF in normoxia.

Here we report EpoSV-VEGF NSCs consistently showed hypoxia-specific VEGF expression *in vitro*. Consistent with this result, the EpoSV-VEGF NSCs transplanted into the uninjured spinal cord also showed controlled VEGF expression compared to SV-VEGF (which expressed high amounts of VEGF in both injured and uninjured spinal cords). The VEGF expression of EpoSV-VEGF NSCs was similar to that of SV-VEGF NSCs *in vitro* under hypoxic conditions as well as in our *in vivo* spinal cord injury model. This indicates that EpoSV-VEGF NSCs equally express VEGF as much as SV-VEGF NSCs in injured tissue. At the same time, EpoSV-VEGF NSCs also provide a better safety profile given that they minimize the side effect of uncontrolled VEGF expression while the spinal cord transitions from hypoxic to normoxia.

It has been reported that VEGF treatment has therapeutically beneficial effects, such as angiogenesis and cell survival, in a variety of disease models [10, 17-19]. Our results demonstrate the anti-apoptotic effect of VEGF by the viability of

both SV-VEGF and EpoSV-VEGF NSCs being greater than that of control NSCs *in vitro*. Moreover, the number of SV-VEGF and EpoSV-VEGF NSCs transplanted into the injured spinal cord was greater than the number of DsRed-NSCs two weeks after transplantation. Additionally, the density of vWF-positive vessels surrounding transplanted cells was greater in both SV-VEGF and EpoSV-VEGF transplanted groups than in the DsRed-NSCs transplanted group. This indicates that transplantation with EpoSV-VEGF NSCs showed a similar SV-VEGF NSC anti-apoptotic effect.

In the behavioral test, only animals that received cell transplants showed significant improvement of hindlimb recovery within two weeks after transplantation; this hindlimb score was maintained until 6 weeks after injury. The difference in cell survival between VEGF-expressing NSCs and control NSCs in the injured spinal cord was gone two weeks after transplantation. In a previous study, hypoxic-specific luciferase expressing cells not only consistently down regulated the luciferase gene in the normal spinal cord, but also highly expressed luciferase in the injured spinal cord. Luciferase expression was maintained for two weeks after injection, although the expression level slowly decreased over time [16]. In fact, it is widely known that the survival of injected cells decreases over time [20, 21]. It may be that survival of transplanted cells correlates with functional recovery, given that the animals that received cell therapy had higher BBB scores compared to PBS injected animals at the same time point (two weeks after transplantation). From Week 2 to Week

6, the functional outcome of the cell injected groups did not improve, as the difference in functional outcome was reduced during this timeframe. The cell number was not significantly different among DsRed, SV-VEGF and EpoSV-VEGF NSCs at 6 weeks post-transplantation. Therefore, the data suggests that the survival of transplanted cells correlates with the functional recovery. As such, maintaining the number of transplanted cells through combined therapy may be necessary to improve recovery.

In this study, we also focused on the safety of EpoSV-VEGF NSCs. Although stem cell therapy and VEGF gene therapy show promise, the risk of tumor formation must be considered. It has been reported that unregulated VEGF expression in normal conditions may lead to tumor formation[11]. In the present study, it seems that proliferation of NSCs correlated with VEGF expression. The proliferation of EpoSV-VEGF NSCs was regulated compared to that of SV-VEGF, which showed greater proliferation in both normoxia and hypoxia. The proliferation of SV-VEGF NSCs transplanted into normal tissue improved more than that of EpoSV-VEGF NSCs. Moreover, vWF-positive vessels around SV-VEGF NSCs injected into the normal spinal cord were higher than those of EpoSV-VEGF NSCs.

In fact, the proliferation of cells transplanted into ischemic tissue may be necessary to increase the efficacy of stem cell transplantation therapy. However, angiogenesis and endless cell proliferation due to abnormally high expression of VEGF in normal conditions may lead to tumors. Although tumor formation was

observed neither in SV-VEGF nor EpoSV-VEGF NSC transplanted groups, our data seems to suggest that VEGF expression should be regulated to prevent unwanted side effects in normal tissue.

V. CONCLUSION

In this study, we investigated the safety and therapeutic effect of hypoxia-specific, VEGF-expressing NSCs in spinal cord injury. EpoSV-VEGF NSCs consistently showed hypoxia-specific gene expression pattern *in vitro* and *in vivo*. In our spinal cord injury model, transplantation with EpoSV-VEGF NSCs not only equally improved NSC survival, angiogenesis and functional recovery to the same degree as SV-VEGF NSC, but also regulated cell proliferation and angiogenesis in normal spinal cord tissue. This combined system with a hypoxia-specific gene expression system and stem cells may represent a safe tool as well as a suitable form of gene therapy.

REFERENCES

1. Hagg T, Oudega M. Degenerative and spontaneous regenerative processes after spinal cord injury. Journal of neurotrauma. 2006;23(3-4):263-80.

2. Song H, Kwon K, Lim S, Kang S, Ko Y, Xu Z, Chung J, Kim B, Lee H, Joung B. Transfection of mesenchymal stem cells with the FGF-2 gene improves their survival under hypoxic conditions. Mol Cells. 2005;19(3):402-07.

3. Casella G, Marcillo A, Bunge M, Wood P. New vascular tissue rapidly replaces neural parenchyma and vessels destroyed by a contusion injury to the rat spinal cord. Experimental neurology. 2002;173(1):63-76.

4. Bhang S, Cho S, Lim J, Kang J, Lee T, Yang H, Song Y, Park M, Kim H, Yoo K. Locally Delivered Growth Factor Enhances the Angiogenic Efficacy of Adipose Derived Stromal Cells Transplanted to Ischemic Limbs. Stem Cells. 2009;27(8):1976-86.

5. Kim SH, Moon HH, Kim HA, Hwang KC, Lee M, Choi D. Hypoxia-inducible Vascular Endothelial Growth Factor-engineered Mesenchymal Stem Cells Prevent Myocardial Ischemic Injury. Molecular Therapy.2011;19(4):741-50

6. Jin KL, Mao XO, Greenberg DA. Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. Proceedings of the National Academy of Sciences of the United States of America. 2000;97(18):10242-7.

7. Sadat S, Gehmert S, Song YH, Yen Y, Bai X, Gaiser S, Klein H, Alt E. The cardioprotective effect of mesenchymal stem cells is mediated by IGF-I and VEGF. Biochemical and biophysical research communications. 2007;363(3):674-9.

8. Kim H, Hwang D, Lee J, Kim S, Kim B. Ex vivo VEGF delivery by neural stem cells enhances proliferation of glial progenitors, angiogenesis, and tissue sparing after spinal cord injury. PLoS One. 2009;4(3):4987.

9. Lee H, Kim K, Park I, Kim S. Human neural stem cells over-expressing VEGF provide neuroprotection, angiogenesis and functional recovery in mouse stroke model. PLoS One. 2007;2(1):156.

10. Wang Y, Guo X, Qiu M, Feng X, Sun F. VEGF overexpression enhances striatal neurogenesis in brain of adult rat after a transient middle cerebral artery occlusion. Journal of neuroscience research. 2007;85(1):73-82.

11. Lee R, Springer M, Blanco-Bose W, Shaw R, Ursell P, Blau H. VEGF gene delivery to myocardium: deleterious effects of unregulated expression. Circulation. 2000;102(8):898-901.

12. Lee M, Lee E, Kim Y, Choi B, Park S, Park H, Park H, Kim S, Ha Y. Ischemic injury-specific gene expression in the rat spinal cord injury model using hypoxia-inducible system. Spine. 2005;30(24):2729-34.

13. Choi B, Ha Y, Huang X, Park S, Chung J, Hyun D, Park H, Park H, Kim S, Lee M. Hypoxia-inducible expression of vascular endothelial growth factor for the treatment of spinal cord injury in a rat model. Journal of Neurosurgery: Pediatrics. 2007;7(1):54-60.

14. An S, Jin H, Kim K, Kim H, Kim D, Cho J, Liu M, Oh J, Yoon D, Lee M. Neuroprotective effect of combined hypoxia-induced VEGF and bone marrow-derived mesenchymal stem cell treatment. Child's Nervous System.*2010;*26(3):323-31.

15. Lee BW, Lee M, Chae HY, Lee S, Kang JG, Kim CS, Lee SJ, Yoo HJ, Ihm SH. Effect of hypoxia inducible VEGF gene expression on revascularization and graft function in mouse islet transplantation. Transplant International.2011;24(3):307-14

16. Liu M, Oh J, An S, Pennant W, Kim H, Gwak S, Yoon D, Kim K, Lee M, Ha Y. Controlled nonviral gene delivery and expression using stable neural stem cell line transfected with a hypoxia inducible gene expression system. The Journal of Gene Medicine.12(12):990-1001.

17. Zhang ZG, Jiang Q, Zhang R, Davies K, Powers C, van Bruggen N, Chopp M. VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. Journal of Clinical Investigation. 2000;106(7):829-38.

18. Yang J, Zhou W, Zheng W, Ma Y, Lin L, Tang T, Liu J, Yu J, Zhou X, Hu J. Effects of myocardial transplantation of marrow mesenchymal stem cells transfected with vascular endothelial growth factor for the improvement of heart function and angiogenesis after myocardial infarction. Cardiology. 2006;107(1):17-29.

19. Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A, Greenberg DA. VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. Journal of Clinical Investigation. 2003;111(12):1843-52.

20. Okada S, Ishii K, Yamane J, Iwanami A, Ikegami T, Katoh H, Iwamoto Y, Nakamura M, Miyoshi H, Okano H. In vivo imaging of engrafted neural stem cells: its application in evaluating the optimal timing of transplantation for spinal cord injury. The FASEB journal. 2005;19(13):1839-41.

21. Parr A, Kulbatski I, Tator C. Transplantation of adult rat spinal cord stem/progenitor cells for spinal cord injury. Journal of neurotrauma. 2007;24(5):835-45.

ABSTRACT(IN KOREAN)

척수 손상 동물모델에서, 저산소 특이적으로 VEGF 유전자를 발현하는 신경줄기세포주의 안전성 및 치료효과 평가

<지도교수 윤도흠>

연세대학교 대학원 의학과

조 보 영

본 실험은 저산소 특이적으로 VEGF 유전자를 발현하는 신경줄기세포주를 이용하여 척수손상 동물모델에서 그의 치료 효율성과 안전성을 평가하였다. 저산소 특이적으로 VEGF 유전자를 발현하는 신경 줄기세포주는 정상산소 조건에서는 VEGF 유전자의 발현을 충분히 감소하는 특징을 보여주었고, 오직 저산소 특이적으로 VEGF 유전자를 발현하는 양상을 보였다. 척수손상 동물모델에서는, 저산소 특이적으로 VEGF 유전자 발현하는 신경줄기세포주의 특징으로 인해 세포의 생존, 혈관생성 및 행동학적 기능회복이 향상되는 것을 확인하였다. 또한 정상조직에서는 감소된 VEGF 유전자로 인해 혈관생성이 감소되는 것을 확인하였다. 따라서 저산소 특이적 VEGF 유전자 발현 신경 줄기세포주가 치료 효율성과 안전성 모두를 겸비한 세포임을 확인하였다.

핵심되는 말: 저산소 특이적 유전자 발현, 혈관세포 성장 인자, 안전성, 효율성, 신경줄기세포