Transplantation of hypoxia induced GM-CSF expressing mouse neural stem cell in spinal cord injury model

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Directed by professor Do Heum Yoon

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Lastly, I wish Professor Jung Yong Ahn a speedy recovery…

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ABSTRACT

Transplantation of hypoxia induced GM-CSF expressing mouse neural stem cell in spinal cord injury model

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Granulocyte-macrophage colony stimulating factor (GM-CSF) is a hematopoietic cytokine and identified to stimulate the differentiation and function of hematopoietic cells. GM-CSF is recently being suggested to play important roles in the nervous system. Present study intended to understand hypoxia induced GM-CSF effects on neural stem cells (NSCs) in a model of spinal cord injury (SCI). GM-CSF over-expressing NSCs were engineered utilizing a hypoxia-inducible gene expression plasmid, which included the Epo enhancer ahead of the SV promoter (EpoSV-GM-

CSF). The resulting cells were then subjected either to hypoxia (pO_2, 1%) or a hypoxia-mimicking reagent ($CoCl_2$) *in vitro*. The expression of GM-CSF was tested in time dependent manner in hypoxia by Real-Time PCR and ELISA assay. Using hydrogen peroxide(H_2O_2), the viability of EpoSV-GM-CSF transfected NSCs were compared with SV-GM-CSF transfected NSCs. After retinoic acid(RA) treatment for 7 days, β-Ⅲ tubulin expression increased in EpoSV-GM-CSF transfected NSCs. EpoSV-GM-CSF transfected NSCs or SV-GM-CSF transfected NSCs were transplanted into rat spinal cord injury model to assess its effect on NSC survival and restoration of function. Over-expression of GM-CSF in both undifferentiated and differentiated NSCs created resistance to H_2O_2-induced apoptosis in hypoxia. Moreover, after transplantation, a significantly higher amount of surviving NSCs, neuronal differentiation, as well as a significant improvement in locomotor function was observed in the EpoSV-GM-CSF treated group. These results show GM-CSF over-expression by the Epo enhancer in hypoxia was beneficial for transplanted NSC survival, neuronal differentiation, and behavioral improvement, pointing toward a possible role for GM-CSF in spinal cord injury treatment.

Key words: Neural Stem Cell, Spinal Cord Injury, GM-CSF

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Ⅰ. INTRODUCTION

Neural stem cells (NSCs) have been shown to migrate to and differentiate within and near injury sites in both the brain and spinal cord [1,2]. As such, NSCs have increasingly received attention as a cell replacement strategy to treat various central nervous system (CNS) disorders, including spinal cord injury (SCI).

While NSCs show promise as donor cells for cell replacement after neurological damage, the environment of the injured spinal cord is not a supportive surrounding for transplanted cells. The pathological primary and secondary cellular mechanisms triggered by CNS trauma includes minimally transient hypoxia, increased

extracellular calcium and potassium concentrations, increased glutamate release, and overproduction of reactive oxygen species[3-5]. Although many methods have been developed to overcome the environmental challenges faced by NSC transplants and improve their engraftment with host tissue [6-8], hypoxia remains a challenging condition of the injured spinal cord, but this spinal cord hypoxia might also be useful for activating specific gene expression patterns within cell transplants.

Previous reports compared several elements to regulate gene expression in post-translational gene control using hypoxic environment[9-12]. Hypoxia-inducible factor (HIF)-1 is considered as a endogenous hypoxia maker. It consists of HIF-1a and HIF-1b. HIF-1 activity is depend on HIF-1a stability by oxygen-dependent proline hydroxylases (PHD). Hypoxic environment blocks PHD activity and allow HIF-1a to form heterodimerization with HIF-1b. Dimerized HIF-1 translocates to nuclear and binds to hypoxia-responsive elements (HRE) in promoter and enhancer region of target genes. Somewhat ironically, methods have been developed to use hypoxia to induce gene expression. A plasmid with the erythropoietin (EPO) enhancer and SV 40 promoter lead to tightly controlled luciferase expression in a model of ischemic injury[13]. Additionally, vascular endothelial growth factor (VEGF) gene expression was shown to increase in murine N2a cells and SCI using hypoxia-induced expression

system[14]. Gene therapy with hematopoietic growth factors is an interesting strategy for the treatment of CNS traumatic disorders, which result in various degrees of hypoxia and ischemic neural tissue damage.

The central nervous system (CNS) was believed as a place of immune privilege by protection from the blood-brain and the blood-cerebrospinal fluid barriers[15]. In CNS injury, it considered the impact by inflammation mounts up the damage. However, according to 'the brain-repair system', neural cells express the passive major histocompatibility comples (MHC) molecules and innate-self maintenance program is activated in degenerative disorders. More recently, inflammatory signals enclosed as a regulator for neurogenesis and gliogenesis. T cells specific to myelin basic protein can enhance autoimmune encephalomyelitis and protection of injured CNS neurons at once. Macrophages and microglia eliminate myelin lamellae from axons as a maker in autoimmune demyelination and promote remyelination by phagocytosing myelin debris simutaneously. As these hidden side, pro-inflammatory cytokines such as interferon r and tumor necrosis factor (TNF)-α revealed neuronal protective effect not just for inflammation activators. EPO and Granulocyte-colony stimulating factor (G-CSF), hematopoietic growth factors, also have actively researched in neurogenesis, EPO enhanced oligodendrogenesis and recovery of

neurological function after neonatal hypoxic/ischemic brain injury and described as a neuroprotective agent. G-CSF decreased brain amyloid burden in alzheimer's mice and ameliorated hippocampal neurogenesis after irradiation-induced suppression in adult mice. Like other hematopoietic growth factors [16,17], granulocyte-macrophage colony stimulating factor (GM-CSF) has been suggested to have roles not only in hematopoietic cell generation, but also in the survival of CNS dopaminergic neurons[18], inhibition of CNS glial scar formation, and as a neuroprotective treatment after SCI [19].

GM-CSF can function as a neurotrophic factor and induce neural progenitor cells (NPCs) proliferation *in vitro*. The biological effects are activated through receptors of target cells. Several cells (*e.g.* granulocytes, erythrocytes, megakaryocytes, macrophage progenitor cells, mature neutrophils, monocytes, macrophages, dendritic cells, plasma cells, certain T lymphocytes, vascular endothelial cells, uterine cells, and myeloid leukemia cells) express GM-CSF receptor on the surfaces. High affinity binding of GM-CSF to their receptors induces a number of key events at the cell surface and within the cytoplasm necessary for receptor activation. These include receptor oligomerization, activation of tyrosine kinase, phosphorylation of receptor, and the recruitment of proteins with src-homology domains[20]. GM-CSF stimulates bone marrow hematopoietic stem cell proliferation and reduces leukocyte apoptosis,

thus increasing white blood cell numbers in the peripheral blood. Because of these hematopoietic stimulating effects, GM-CSF has been used as a therapeutic cytokine in patients suffering from diseases related to bone marrow suppression. In the normal quiescent CNS, GM-CSF appears to be mainly released from astrocytes and regulates the functions of microglia. Following a peripheral nerve lesion, fibroblasts, induced by tumor necrosis factor-a(TNF-a) and IL-1a, produce GM-CSF which in turn activates macrophsges and schwann cells with concomitant increase in the cell surface expression of MAC-2, an activation marker[21]. GM-CSF can enhance the proliferation of neural precursor cells *in vitro* and been suggested to facilitate recovery of locomoter function after SCI in an adult mouse[22]. Therefore, we hypothesized hypoxia-specific GM-CSF over-expression may give NSCs a greater window to survive in the inhospitable environment of the injured spinal cord after transplantation. In short, GM-CSF over-expression by NSCs would be activated via the EPO enhancer and SV40 promoter as a result of the hypoxic conditions within the injured spinal cord.

In this paper, we have also used an erythropoietin (EPO) enhancer as a hypoxia-inducible gene expression tool to overcome the potentially inhospitable milieu of the injured spinal cord and improve the engraftment of NSCs with host neural tissue.

Ⅱ. MATERIALS AND METHODS

1. Plasmid Construction

pBudSV-luciferase and pBudEpoSV-luciferase plasmids were used as mother vectors[23] for SV-GM-CSF and EpoSV-GM-CSF construct, respectively. The luciferase sequence from pBudSV-luc and pBudEpoSV-luc each was digested by Hind3 and Hpa1, and human GM-CSF CDS from pcDNA3-GM-CSF was obtained by Hind3 and EcoRV (the pcDNA3-GM-CSF plasmid was a generous gift from Prof. Choi in the department of Biomedical and Bioengineering Sciences, Inha University). Each plasmid was ligated to GM-CSF (Fig. 1A) for 1hour at room temperature, and E.coli, DH5a strain, was used for competent cell transformation.

2. Cell Culture and Transfection

Mouse neural stem cells established from a 9-day old mouse embryo (ATCC CRL-2925) were cultured in DMEM/F12 with 10% fetal bovine serum (FBS). Transfection was performed using Lipofectamine 2000 (Invitrogen, CA, USA) as previously described. Briefly, in a 6-well plate, one day after seeding (300,000 cells/well), the transfection mixture of plasmid/Lipofectamine 2000 (1:2.5, 4 μg DNA/well) was

dropped in culture media. Media was changed after 4 hours of incubation in normoxia (pO_2, 21%) and subsequent experiments were performed. For differentiation of mNSCs, 5% FBS and 1uM retinoic acid were added for 7 days.

3. Hypoxia

A hypoxic environment was created by flushing a hypoxia gas mixture using a Forma Series II Water Jacketed CO_2 Incubator (Thermo Fisher Scientific Inc., USA), in which hypoxia (pO_2, 1%) in a gas phase is achieved within 30 minutes after opening and closing the door. More strict controls of hypoxia, such as medium pre-equilibrated under hypoxia, was applied to study the kinetics of hypoxia-responsive GM-CSF expression. Furthermore, a comparative study of hypoxia-responsive GM-CSF expression was also performed using the hypoxia-mimicking reagent, $CoCl_2$.

4. Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA), and 1ug of RNA was used to synthesize cDNA using SuperScript^{TM}Ⅲ First-Strand Synthesis kit (Invitrogen, CA, USA). Briefly, for quantitative PCR, the cDNA was quantified with SYBR green (ABI) using ABZ7500 sequence detection system (ABZ). For traditional

PCR, samples were amplified by PCR machine (ABI) using Taq polymerase (TaKaRa). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene.

5. Enzyme-linked Immunosorbent Assay

Followuing the manufacturer protocol for the enzyme-linked immunosorbent assay (ELISA) kit for GM-CSF (R&D, MN, USA), the designated plate was first rinsed with 100 μl of incubation buffer. The prepared standards or samples (100 μl/well) were added to the plate and incubated at room temperature for 2 hours. Biotin-conjugated (biotinylated anti-GM-CSF, 200 μl/well) solution was added to each well and incubated for 1.5 hours at room temperature. Thirty minutes after the addition of 200 μl of substrate solution, stop solution was added and absorbance at 450 nm was measured using an ELISA reader. Secreted GM-CSF levels from transfected NSCs were calculated using a corresponding standard curve

6. TUNEL Assay

The staining protocol was based on the ApopTag Plus Fluorescenin In Situ Apoptosis Detection Kit (Chemicon, CA, USA). Briefly, cultured NSCs were fixed in

4% paraformaldehyde for 10 minutes, followed by post-fixation with ethanol and acetic acid (2:1, v/v). Working strength enzyme was added for 1 hour incubation and stop/wash buffer was subsequently applied. Samples were then incubated with working strength anti-digoxigenin conjugated mixture for 30 minutes in a dark room. After washing, slides were mounted with DAPI.

7. Fluorescent-activated Cell Sorting Analysis

To confirm the progress of apoptosis, NSCs were incubated with annexin-V FITC in binding buffer (Biobud, Seoul, Republic of Korea). After 15 minutes, NSCs were centrifuged at 1000 g for 5 minutes and washed in cold binding buffer.

8. Spinal Cord Injury

All procedures were approved by the Animal Care and Use Committee of the Yonsei University College of Medicine. Adult male Sprague-Dawley rats (250 g) were anesthetized using Zoletil 50 (Virbac, France), laminectomies were performed at the T9 level, and the intact, exposed spinal cord was compressed with a vascular clip (width, 2mm; occlusion pressure, 30 g) for 10 minutes as previously described. After removing the clip, transfected NSCs (300,000 cells/5 μl) were transplanted into the

injured or non-injured epicenter of the spinal cord using a 5 μl Hamilton syringe. All animals received cyclosporine (10 mg/kg, Chong Kun Dang Pharm, Korea) every day after transplantation. At 2 and 6 weeks after SCI, animals were anesthetized and then transcardially perfused with saline. Spinal cord tissue, including the injury epicenter and transplanted sites, was obtained for further processing.

9. Immunocytochemistry

NSCs were washed three times for 3 minuntes each with PBS, fixed in 4% paraformaldehyde for 15 minutes, and then treated with. 0.3% Triton X-100 with 0.1% BSA for 15 minutes. Subsequently, were NSCs blocked with 10% normal donkey serum for 30 minutes and mouse anti-MAP2 (1:500, Chemicon, CA, USA). After washing completely, NSCs were incubated with anti-mouse secondary antibody conjugated with fluorescein isothiocyanate for 1 hour and then washed. Fluorescence images were collected using an Olympus DP71 camera (Olympus ZX71) or a laser scanning confocal microscope (LSM700, Carl Zeiss).

10. Functional Testing

A subset of animals underwent behavioral analysis to assess the extent of

locomotor recovery. The BBB (Basso, Beattie, and Bresnahan) locomotor rating scale was used to evaluate the quality of hindlimb movement during open field locomotion. BBB scores were assigned from 1 to 6 weeks after spinal cord injury. A total of 48 rats completed all rounds of BBB tests (PBS group, n=12; NSC group, n=12; SV-GM-CSF group, n=12; EpoSV-GM-CSF, n=12).

11. Statistical Analysis

One-way ANOVA was used to compare three experimental groups with a Student-Newman-Keuls test for pairwise comparisons of the subgroups. Data were expressed as mean ± standard deviation. $p < 0.05$ was considered statistically significant.

Ⅲ. RESULTS

1. Increased GM-CSF Expression in Hypoxia

A double promoter system was used to check transfection efficiency, using the DsRed fluorescence gene driven by the EF1 promoter and to induce GM-CSF over-expression in hypoxia. This hypoxia inducible gene expression system showed efficient delivery and significant transcriptional regulation of luciferase as a reporter gene under hypoxia *in vitro*. To prove therapeutic gene over-expression, we inserted GM-CSF following the SV promoter (Fig. 1A). These constructs were confirmed by comparing restriction fragments as well as sequencing analysis. To evaluate the protective effect of GM-CSF over-expression in hypoxia, NSCs were transfected using Lipofectamine 2000. After 4 hours of transfection, followed by stabilization for 24 hours, more than 80% of NSCs showed red fluorescence (Fig. 1B).

To test whether the GM-CSF over-expression is hypoxia-inducible in NSC engineered with the plasmid constructs, RT-PCR was performed after hypoxic chamber (pO_2, 1%) incubation or hypoxia-mimicking reagent (Cobalt chloride, $CoCl_2$, 200 μM) application. EpoSV-GM-CSF transfected NSCs significantly expressed GM-

CSF in response to hypoxia, whereas SV-GM-CSF transfected and control NSCs did not show significantly high levels of GM-CSF due to hypoxia.(Fig. 1C). To check the time pattern for therapeutic gene expression, the time points, 1, 3, 6, 12, and 24 hours after transfection were set. GM-CSF expression in EpoSV-GM-CSF transfected NSCs showed a gradual increase both in hypoxia and $CoCl_2$ in a time dependent manner (Fig. 2A). Compared to the SV-GM-CSF transfected NSCs, EpoSV-GM-CSF NSCs showed a significantly higher expression of GM-CSF at 6 hours in hypoxia or 3 hours in $CoCl_2$. Similarly, ELISA results showed that Epo-GM-CSF NSCs secreted GM-CSF in a time dependent manner, with statistically significance beginning at 12 hours (Fig. 2B).

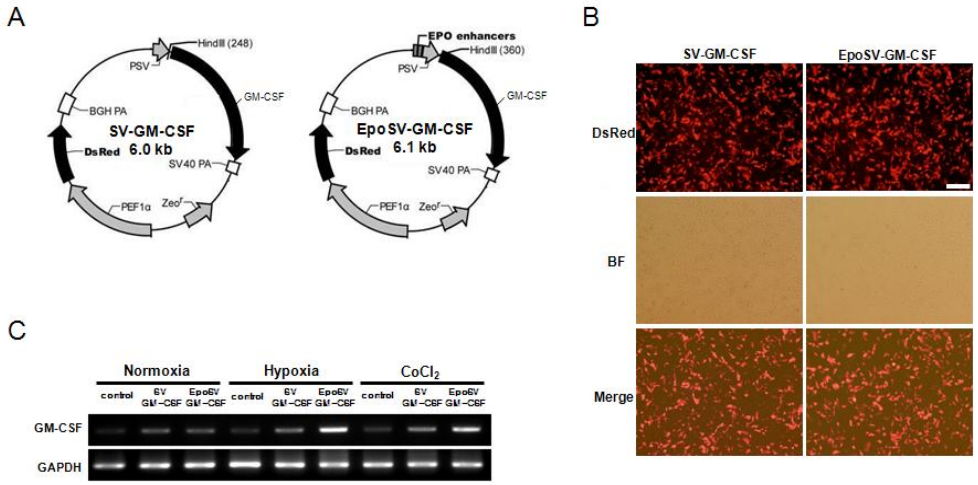

Figure 1. Hypoxia induced GM-CSF plasmid construction and expression. A) The map of SV-GM-CSF and EpoSV-GM-CSF. Two inverted Epo enhancer sequences provide hypoxia specific GM-CSF induction. B) Culture findings of transfected NSC. DsRed detected after transfection with lipofectamine into NSC. Scale bar=200 μm. C) GM-CSF amplification by RT-PCR. GM-CSF expression in EpoSV-GM-CSF was up-regulated in hypoxia and hypoxia mimicking reagent ($CoCl_2$, 200 μM) group.

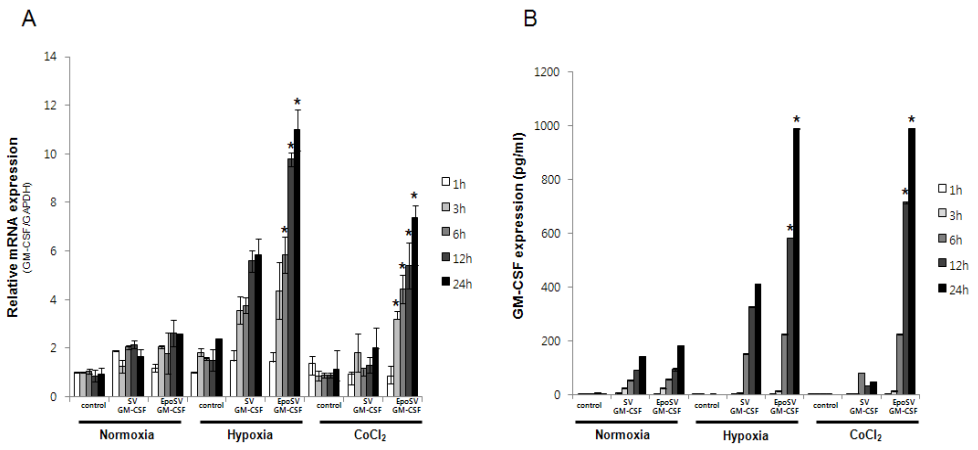

Figure 2. Time dependent GM-CSF gene expression and extracellular expression in hypoxia (1% O_2). One day after transfection with lipofectamine, each NSC group was placed in normoxia, hypoxia or normoxia with $CoCl_2$ (200 μM). Cells and cultured media were harvested for RNA isolation and measurement of secreted GM-CSF at 0, 3, 6, 12 and 24 hours each. A) Real time PCR performed by SYBR. The Epo enhancer effect observed in hypoxia and $CoCl_2$ environment. GM-CSF gene expression increased significantly in a time-dependent manner, especially in EpoSV-GM-CSF group after 12 hours. B) ELISA assay performed using harvested media at each time point. Anti-GM-CSF antibody coated plate was used to detect secreted GM-CSF. GM-CSF secreted by EpoSV-GM-CSF skyrocketed after 12 hours in hypoxia

and $CoCl_2$ compared to SV-GM-CSF. * significant difference between SV-GM-CSF and EpoSV-GM-CSF group at same time point ($P<0.05$). Data are shown as mean ± SD.

2. Over-expression of GM-CSF Counteracts Apoptosis in Hypoxia

To examine the possible neuroprotective effect of hypoxia-induced GM-CSF, the TUNEL assay for apoptotic cell profiles was performed as previously described[24]. NSCs were treated with 500μM of hydrogen peroxide (H_2O_2, 30%) for 24 hours in hypoxia. We counted TUNEL positive cells in 3 random area of each NSCs groups which were 19, 24, 23 in untransfected NSCs, 15, 20, 18 in SV-GM-CSF NSCs, and 4, 7, 5 in EpoSV-GM-CSF NSCs. The number of TUNEL positive cells was significantly higher in the control and SV-GM-CSF group, while the EpoSV-GM-CSF group showed the lowest number of TUNEL positive cells (Fig. 3A). The average numbers were showed as a graph(Fig. 3B).

Phosphatidyl serine(PS) exits in the cytoplasmic cell membrane. When apoptosis occur, PS is disclosed following by alteration of cell membrane phospholipid bilayer. To confirm PS exposure following apoptosis, untransfected NSCs, SV-GM-CSF transfected NSCs, or EpoSV-GM-CSF transfected NSCs were treated with 500 μM of H_2O_2 for 24 hours in hypoxia, and then cells harvested. FACS analysis was performed by annexin-V FITC interaction with PS[7]. Annexin-V positive cells were 28, 31, 32% in untransfected NSCs, 23, 26, 22% in SV-GM-CSF transfected NSCs, and 10, 10, 11%

in EpoSV-GM-CSF NSCs. Annexin-V positive cells clearly decreased in EpoSV-GM-CSF NSCs, showing that hypoxia-inducible GM-CSF expression prevented cell death after H_2O_2 treatment (Fig. 3C). The average percentages were displayed as a graph (Fig. 3D).

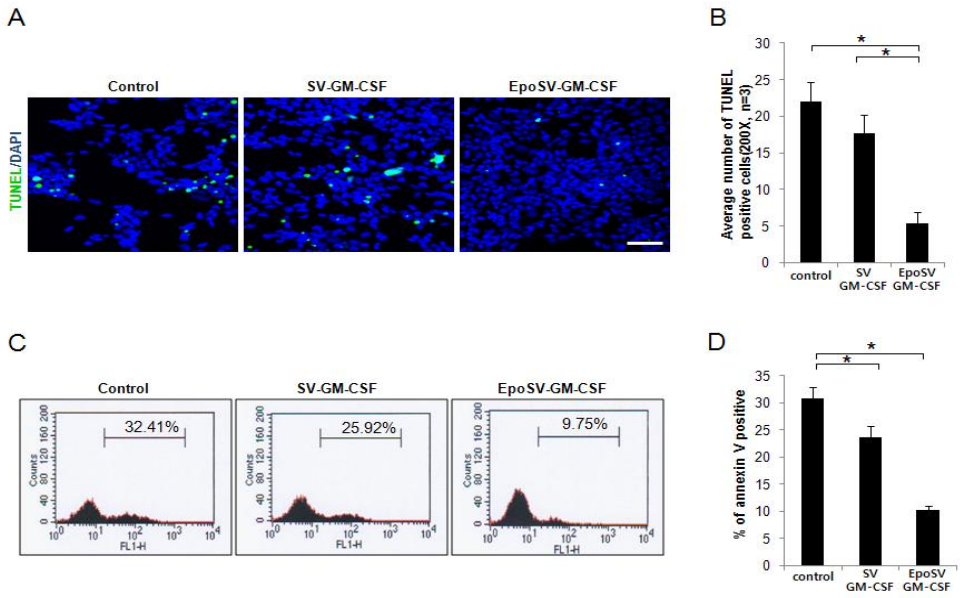

Figure 3. Inhibition of apoptosis by hypoxia-inducible GM-CSF. H_2O_2 (500 μM)-induced apoptosis performed 1 day in hypoxia and TUNEL assay and FACS analysis assessed. A) TUNEL positive cells are green and nuclei are blue. Photographs were taken at 200X magnification. Scale bars= 50um. B) Number of TUNEL positive cells in hypoxia with H_2O_2 treatment (* $P<0.05$). C) FACS analysis. GM-CSF clearly decresed the number of apoptotic annexin-V positive cells in hypoxia treated with H_2O_2. FL1-H indicates FITC annexin-V intensity. D) Graphical representation of the percentage of annexin-V positive cells. GM-CSF over-expression in hypoxia reduced apoptosis by H_2O_2 from 32.41% to 9.75% (* $P<0.05$). Data are shown as mean ± SD.

3. GM-CSF Over-expression in Hypoxia Promotes Neuroprotection

To confirm whether differentiation of the GM-CSF engineered NSCs alters the cells ability to survive the apoptotic conditions of H_2O_2 exposure, we tested differentiated NSCs responses to H_2O_2 injury. To first induce differentiation, NSCs were treated with 1μM retinoic acid (RA) for 7 days[25]. Following the formation of prominent neuronal in vitro networks, NSCs were placed to hypoxia for 24 hrs and harvested. Total mRNA isolated for analysis of β-Ⅲ-tubulin, neuronal marker, and GFAP, astrocyte marker, expression (Fig. 4A). β-Ⅲ-tubulin expression in EpoSV-GM-CSF NSCs increased 2 times more than SV-GM-CSF and control NSCs. In contrast, GFAP expression in EpoSV-GM-CSF NSCs decreased compared to SV-GM-CSF NSCs or untransfected NSCs. After differentiation with RA for 7 days, each group was treated with 500μM H_2O_2 in hypoxia and then TUNEL assay performed (Fig. 4B). We counted 3 random area of each group. The number of TUNEL positive cells are 10, 9, 10 in untransfected NSCs, 6, 9, 9 in SV-GM-CSF NSCs and 1, 3, 4 in EpoSV-GM-CSF NSCs (Fig. 4C). Hypoxia induced over-expression of GM-CSF in EpoSV-GM-CSF group led to a significantly higher amount of neurons protected compared to the SV-GM-CSF and control group.

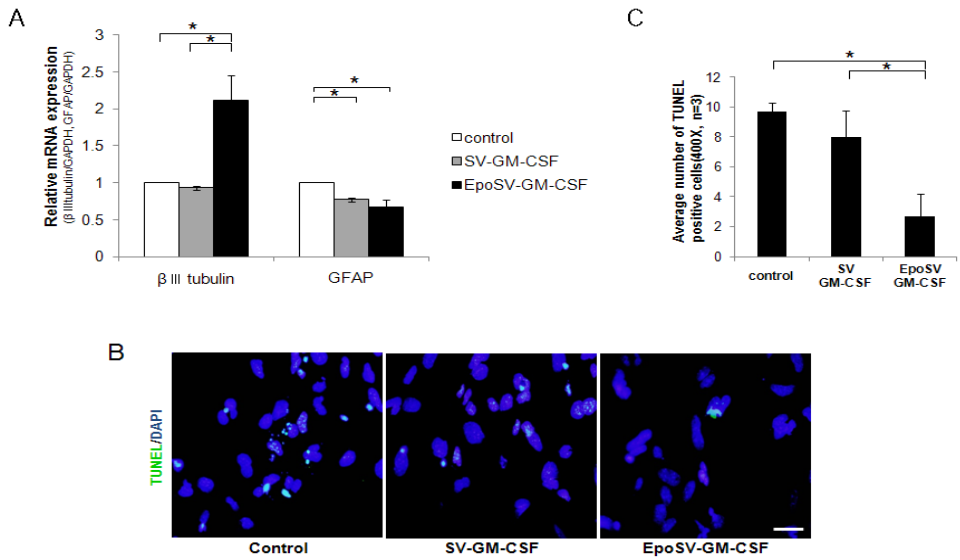

Figure 4. Neuronal protective effect by GM-CSF over-expression in hypoxia. After each plasmid transfection, NSCs were differentiated with RA (1 μM) for 7 days in normoxia or hypoxia. A) Relative amount of mRNA expression for β-Ⅲ tubulin and GFAP measured by qPCR (* $P<0.05$). Although GFAP expression decreased in both SV-GM-CSF and EpoSV-GM-CSF groups, β-Ⅲ tubulin expression was doubled in EpoSV-GM-CSF after differentiation. B) H_2O_2 (500 μM)-induced apoptosis performed 1 day in hypoxia. TUNEL positive NSCs indicated green fluorescence. Scale bar=50 μm. C) Number of TUNEL positive cells significantly decreased in EpoSV-GM-CSF group (* $P<0.05$). Data are shown as mean ± SD.

4. EpoSV-GM-CSF NSC Survival enhancement in SCI model

To demonstrate that GM-CSF decreases apoptosis of the implanted NSCs and enhances NSC survival after transplantation, EpoSV-GM-CSF and SV-GM-CSF transfected NSCs were transplanted into adult male Sprague-Dawley rats. The animals for NSCs transplantation were divided into 6 groups. To compare with controlled induction by Epo enhancer in injured spinal cord, we transplanted SV-GM-CSF transfected NSCs and EpoSV-GM-CSF transfected NSCs in normal spinal cord. Group 1 received SV-GM-CSF transfected NSCs without spinal cord injury (n=3, 300,000 cells/5 μl), group 2 received EpoSV-GM-CSF transfected NSCs without spinal cord injury (n=3, 300,000 cells/5 μl). After spinal cord injury, group 3 received only PBS (n=12, 5μl), group 4 received DsRed transfected NSC (n=12, 300,000 cells/5 μl), group 5 received SV-GM-CSF transfected NSCs (n=12, 300,000 cells/5 μl), and group 6 received EpoSV-GM-CSF transfected NSCs (n=12, 300,000 cells/5 μl). Spinal cord injury was performed by clip compression at the T9 level for 10 mins, and then, NSCs transplantated into the epicenter of the spinal cord injury. All animals received cyclosporine (10 mg/kg) every day after transplantation.

Two weeks after NSCs transplantation, grafted NSCs were detected by DsRed fluorescence. NSCs injected into the normal spinal cord of rats were found clustered

together (Fig. 5A, left). In contrast, NSCs injected into the injured epicenter were found in places, with DsRed positive cells seemingly away from the injury site (Fig. 5A, right). Because DsRed fluorescence intensity stand for gene expression as well as cell survival, optical densities of DsRed fluorescence were measured using MetaMorph software (Fig. 5B). As shown in Fig. 5A and B, GM-CSF over-expressing NSCs had a significantly greater presence in the injured tissue than in the SV-GM-CSF normal or injured tissues, or the EpoSV-GM-CSF normal tissues. To check the cell survival resulting from anti-apoptotic factor, we performed reverse transferase PCR. Anti apoptotic genes, Bcl-2 and Bcl-xL, in the EpoSV-GM-CSF injured group were increased than in any of the other groups, with the remarkable increase of GM-CSF expression (Fig. 5C). Next, to confirm cell viability in rat spinal cord after transplantation, we identified apoptotic cells by TUNEL assay. We compared DsRed transfected NSC received group, SV-GM-CSF transfected NSCs received group, EpoSV-GM-CSF transfected NSCs received group. Fig. 5D shows representative pictures of TUNEL assay and the average number of positive cells were used in the graph(Fig. 5E). Apoptotic cells significantly decreased in EpoSV-GM-CSF transfected NSCs received group rather than DsRed transfected NSCs received group or SV-GM-CSF transfected NSCs received group.

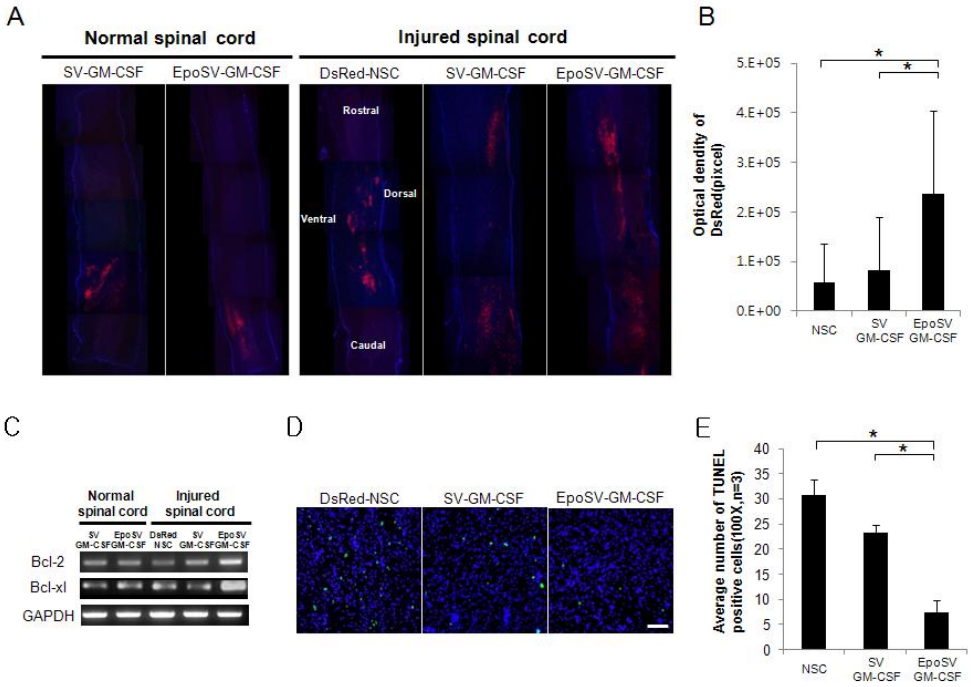

Figure 5. Transplanted NSC survival improvement due to tansfected EpoSV-GM-CSF. At 2 weeks after transplantation into the injured spinal cord, NSC viability was assessed. A) DsRed fluorescence in both SV-GM-CSF and EpoSV-GM-CSF transfected groups were slightly decreased in the normal spinal cord. However, in the injured spinal cord, the intensity was increased in the EpoSV-GM-CSF group compared to the other group. Scale bar=500 μm B) The optical density of DsRed in the injured spinal cord was measured by pixelarea. The density was significantly

increased in EpoSV-GM-CSF treated animals (* $P<0.05$, n=20). C) The anti-apoptosis related genes, *Bcl-2* and *Bcl-xL*, expression of EpoSV-GM-CSF was increased compare with both DsRed-NSC and SV-GM-CSF. The *Bcl-2* and *Bcl-xL* expressions in the normal spinal cord were not changed in both the SV-GM-CSF and EpoSV-GM-CSF groups. D) Apoptosis in tissues obtained 2 weeks after transplantation were analyzed by TUNEL assay. Scale bar=100 μm E) Number of TUNEL positive cells significantly decreased in EpoSV-GM-CSF group (* $P<0.05$). Data are shown as mean ± SD.

5. Transplantation of EpoSV-GM-CSF NSCs Augment Neuronal Differentiation in SCI

At 6 weeks after transplantation, neuronal differentiation of grafted NSCs was examined. Most surviving NSCs were found in the EpoSV-GM-CSF NSC transplanted group. The EpoSV-GM-CSF NSC transplanted group expressed significantly more β-Ⅲ-tubulin, a neuronal marker, than the SV-GM-CSF group (Fig. 6A). However, the increased quantity of GFAP, an astrocyte marker, was not statistically different. Most of the DsRed fluorescent surviving EpoSV-GM-CSF NSCs showed co-localized staining with MAP-2, a neuronal marker, antibody (Fig. 6B). Orange color represents double stained NSCs both green for MAP-2 and red for DsRed and indicates differentiated NSCs after transplantation. All animals were observed until sacrifice and BBB scoring was done by triple-blinded test and evaluated each group. Although all of the animals showed improvement in the BBB locomotor scores, only the EpoSV-GM-CSF NSC group showed significant functional recovery (Fig. 6C).

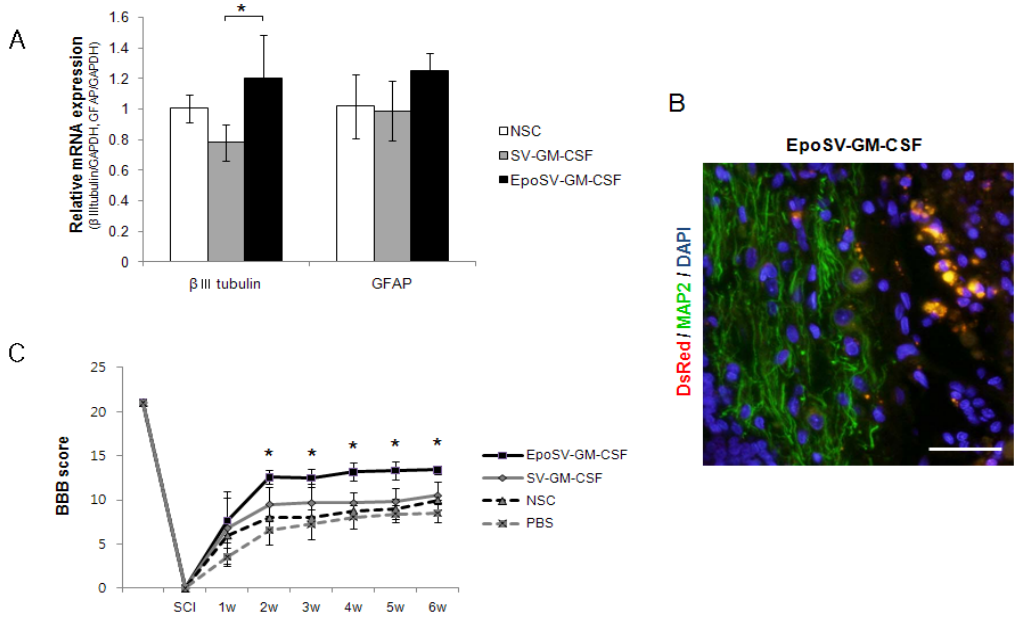

Figure 6. Neuronal differentiation and improvement of locomotor performance in EpoSV-GM-CSF transplanted rat. Injured spinal cords were analyzed 6 weeks after transplantation through RNA expression, immunohistochemistry, and a behavior test. A) β-Ⅲ tubulin and GFAP expression were compared through qPCR. A neuronal differentiation marker, β-Ⅲ tubulin, significantly increased in the EpoSV-GM-CSF group compared to the SV-GM-CSF group (* $P<0.05$). B) Survived transplanted cells detected in EpoSV-GM-CSF group and neuronal differentiation were shown as double positive DsRed and MAP2 using immunohistochemistry. Scale bar=100 μm C)

Functional recovery in EpoSV-GM-CSF groups rapidly and significantly improved from the first week after transplantation. * significant difference between each group ($P < 0.05$). Data are shown as mean ± SD.

IV. DISCUSSION

The unfavorable environment of the injured spinal cord poses challenges for NSC grafts survival, proliferation, migration, and differentiation [2,26,27]. Many cell transplantation studies have reported poor survival rates of NSCs after transplantation after SCI [27,28]. However, recent studies have shown that cell transplantation combined with gene therapy produces more optimistic results [29,30]. This study aimed to increase the viability and potential therapeutic value of transplanted NSCs following SCI. We observed that GM-CSF over-expression by NSCs using a hypoxia-inducible vector enhanced NSC survival both *in vitro* and *in vivo*. Furthermore, transplantation of these engineered NSCs into the hypoxic (ischemic) condition of the injured cord triggers the activation of the EPO vector leading to over-expression of GM-CSF by the NSCs. This not only promotes improved *in vivo* cell survival and differentiation into neuronal phenotypes, but also facilitates improved locomotor recovery by the spinal injured adult rats. EpoSV-GM-CSF NSCs transplantation did not form tumors during the 6 weeks of this study.

GM-CSF and its receptor are found on nervous system cells such as microglia, astrocytes, oligodendrocytes and neurons. Because GM-CSF is able to cross the

blood-brain and blood-spinal cord barriers[31], its role in the nervous system has been explored. As a pro-inflammatory hematopoietic cytokine, GM-CSF has diverse effects on cortical microglia ranging from induction of proliferation to change in morphology which means having migration capacity to areas of cell death[32]. Recently, GM-CSF has been suggested to promote survival of dopaminergic neurons in a murine Parkinson's disease model[18], as well as stimulate axonal outgrowth in paraplegic rats[19]. Although GM-CSF is commercially available and widely used for treating some types of cancer as a vaccine adjuvant[33,34], the therapeutic evaluation of GM-CSF within the hypoxic injured spinal cord has not been previsouly investigated. Here, we used GM-CSF over-expression in hypoxia (Fig. 2) to observe possible neuroprotective effects *in vitro* (Fig. 3 and 4) and *in vivo* (Fig. 5).

Gene expression systems that are more specific for injured tissue are desirable because it may not be beneficial to have a gene activated within surrounding healthy tissue. The current findings suggest that the potentially detrimental hypoxic/ischemic stress that usually accompanies SCI could also be used as a injury specific trigger for gene expression which would then protect transplanted and possibly host cells from the hypoxia[7,8,35,36]. Hypoxia targeting gene regulation is one of the useful strategies for gene therapies. Hypoxia itself is an excellent target, but combination of various

regulatory strategies will be more useful to minimize side effects and maximize efficacy. Several elements, such as the hypoxia-responsive untranslated region (UTR)[37-39], oxygen-dependant degradation (ODD) domain sequence[40,41], RTP801 promoter[42], and Epo enhancer[38,43] have been used to design hypoxia-inducible systems. We have previously confirmed that the Epo enhancer-based system increases gene expression specifically in hypoxic cells. In this study, we verified that Epo enhancer-based GM-CSF expression is tightly regulated in hypoxia , given that EpoSV-GM-CSF transfected NSCs showed GM-CSF expression at basal levels in normoxia, but significantly increased expression only in hypoxic environments (Fig. 2). Using another hypoxic stimulus, $CoCl_2$ (which is used as a hypoxia mimicking reagent to target sterol synthesis[44-46]), GM-CSF over-expression by hypoxia was shown to be a fundamental property of the engineered cells. Similar expression patterns of the secreted GM-CSF in hypoxia were observed by ELISA analysis *in vitro*. Therefore, this gene carrier system specific in hypoxia represents a safe gene therapy tool with a high potential for clinical application.

H_2O_2 is a member of reactive oxidative species (ROS) that participate in development of ischemia including spinal cord injury and neurodegenerative diseases including Parkinsons' disease[47,48]. Several studies reported to use H_2O_2 as an agent to

cause neuronal injury including human neuroblastoma cells, primary cortical astrocytes cultures and adipose tissue-derived mesenchymal stem cell. When EpoSV-GM-CSF NSCs were treated with H_2O_2 injury, we witnessed a protective effect on both un-differentiated and differentiated NSCs in hypoxia *in vitro*. Also in SCI, EpoSV-GM-CSF NSCs protected tissue from apoptosis. GM-CSF was previously reported as having a neuroprotective effect after staurosporin –induced apoptosis in retinal ganglion cells and the vitreous body of Sprague-Dawley rats[49], controlling glial scar composition, and influencing the survival and function of neighboring neurons[50]. Thus, it is possible that over-expressed GM-CSF in EpoSV-GM-CSF NSCs protect themselves by autocrine and paracrine effects. It is not presently known if the EpoSV-GM-CSF NSCs promote enhanced survival of neighboring host tissue cells. This requires further investigation.

The present study also showed that the number of surviving NSCs in the control SV-GM-CSF NSCs and control groups at 2 weeks was much smaller than in the EpoSV-GM-CSF group, suggesting that the death of transplanted NSCs continued after acute transplantation and GM-CSF over-expression in SCI increased transplanted NSC survival. The pattern of anti-apoptotic gene expression in Fig. 5C shows that it is possible that GM-CSF over-expression in EpoSV-GM-CSF NSCs

contributed to NSCs survival within the injured spinal cord through Bcl-2 and Bcl-xL induction. Previous studies have shown that GM-CSF decreased apoptotic activity by induction of Bcl-xL through PI3K-Akt pathway in primary cortical neurons and human neuroblastoma cells *in vitro*[51].

Immunohistochemical findings identified that MAP-2 was expressed in EpoSV-GM-CSF transfected NSCs after transplantation (Fig. 6). We also observed significant locomotor recovery in EpoSV-GM-CSF trasnfected NSCs transplanted goup. Therefore, we demonstrated that increased GM-CSF expression by hypoxic environment in SCI resulted enhancement of grafted NSCs survival and contributed to repair.

Ⅴ. CONCLUSION

In conclusion, our study demonstrates improvement in the survival of transplanted NSCs when they over-express GM-CSF. This suggests that NSCs over-expressing GM-CSF, tightly regulated by a hypoxia-inducible gene expression system, may point toward utility in the clinical treatment of spinal cord injury.

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국문요약

허혈특이적으로 GM-CSF를 과발현하는 mNSC에 의한 척수손상 회복효과

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김 효 진

Axon 의 파괴, Schwann cell 증식, macrophages 회합, 활성화등과 같이 말초신경계 손상시에는 Wallerian degeneration(WD)이 동반된다. 이러한 현상들은 염증유발 혹은 비염증 유발 사이토카인들과 연계되어지며, 손상부위의 fibroblast 에 의한 과립구-대식세포 집락자극인자 (Granulocyte-macrophage colony stimylating factor, GM-CSF)의 분비는 macrophage 를 활성화시켜 myelin phagocytosis 를 유도한다. GM-CSF 수용체는 조혈세포 뿐만 아니라, microglia, astrocyte, oligodendrocyte 등 신경계에서도 발현하는것으로 알려져있다. 말초신경계뿐 아니라, 중추신경계 손상시에도 GM-CSF 는 microglia 증식과 형태변화에 관여하여 myelin 잔해들을 제거하도록 도움으로서

axonal regeneration 을 돕는 중요인자 중의 하나 일 것이라 여겨지고 있다.

최근, 사이토카인 형태로 재조합된 GM-CSF 를 처리하면 신경세포의 apoptosis 가 억제되고 glial scar 형성을 억제하며, 척수손상 회복에 효과적이라는 보고가 있었다. 이에 신경줄기세포에 GM-CSF 를 발현하도록 조작하여 그 효과를 입증하고자 하였다.

유전자 치료의 가장 중요한 요소는 조직 특이 발현기술과 적절한 방법에 의한 전달기술이라고 할 수 있다. Epo enhancer 를 이용하여 허혈 선택적으로 유전자를 발현할 수 있는 시스템을 구축하였다. 이를 신경 줄기 세포에 도입후 1%산소농도 혹은 $CoCl_2$ 처리하에서 GM-CSF 를 발현하도록 유도하고, 유전자 발현과 단백질 발현 증가양상을 Real-Time PCR, ELISA assay 를 통해 관찰하였다. 또한 GM-CSF 가 도입된 줄기세포주에 H_2O_2 로 세포사멸을 유도시킴으로서, 허혈 특이적으로 GM-CSF 를 발현하는 실험군의 생존율이 증가됨을 TUNEL, FACS analysis 를 통해 확인하였으며, Retinoic Acid(RA)를 처리하여 줄기 세포 분화를 유도한후 H_2O_2 를 처리했을때도 허혈특이적인 GM-CSF 발현이 분화된 세포의 생존율을 증가시킴을 관찰하였다. 클립 압박법을 이용하여 흰쥐 척수 손상을 유도하고, GM-CSF 를 허혈특이적으로 발현하는 줄기세포를 이식받은 그룹에서 세포 생존율이 증가되어있음을 Red fluorescence,

TUNEL assay 로 확인하였으며, 행동검사를 통해 GM-CSF 에 의해 척수손상 회복기간이 단축됨을 확인하였다.

이상에서 살펴본 바와 같이, GM-CSF 의 허혈특이적인 발현은 줄기세포주 혹은 분화된 세포의 생존율을 증가시키고, 운동기능 향상에도 효과적임을 확인하였다. 따라서, 척수손상후 GM-CSF 발현 유도는 이식된 줄기세포의 생존율을 증가시켜 치료유전자의 발현을 유지함으로써, 손상에서의 회복을 도울것이며, 이는 척수신경 질환에 대한 새로운 치료 가능성을 제시할 수 있을 것으로 사료된다.

핵심되는 말: 신경줄기세포, 척수손상, 과립구-대식세포 집락자극인자