Hypoxic Preconditioning Increases the Neuroprotective Effects of Mesenchymal Stem Cells in a Rat Model of Spinal Cord Injury

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Abstract

The functional deficit caused by Spinal Cord Injury (SCI) is clinically incurable and current treatments have limited effects. Previous studies have suggested that cell-based therapy using Mesenchymal Stem Cells (MSCs) pre-treated with drugs or gene transfection have possible therapeutic effects. Hypoxic preconditioning is one of the most likely treatments of cell-based therapy without altering genes; however, few reports are available about Hypoxia-Preconditioned MSCs (H-MSC) transplantation for SCI. Here we demonstrate the therapeutic potential of H-MSC transplantation using SCI model rats. H-MSC expressed significantly higher mRNA levels of vascular endothelial growth factor-1 and carbonic anhydrase IX, hypoxia inducible genes. H-MSC transplantation resulted in remarkable functional improvement in the SCI model rats compared to no transplantation. Expression of brain-derived neurotrophic factor and the autophagy-associated marker beclin1 mRNA was significantly upregulated in rat spinal cord that underwent H-MSC transplantation. Furthermore, conditioned medium of the H-MSC significantly prevented cell death of NG108-15 cells exposed to oxidative or inflammatory stress. These results suggest that hypoxia preconditioning is an effective strategy for SCI in cell-based therapy using MSCs.

Keywords: Cell-based therapy; Spinal cord injury; Mesenchymal stem cells; Hypoxic culture; Preconditioning; Neuroprotection; Vegf

Introduction

Although current therapies, such as novel drugs [1] or neuro-rehabilitation [2] have been developed to treat Spinal Cord Injury (SCI), these therapies could not achieve radical cure. Cell-based therapy using Mesenchymal Stem Cells (MSCs) has been gaining attention as a novel approach to treat the damage caused by SCI. MSCs can be isolated from various tissues, such as bone marrow [3] or adipose tissue [4], and have self-renewal and multi-lineage differentiation potential. Animal experiments [5] and clinical trials [6,7] have revealed that MSCs transplantation reduces lesion volume and promotes functional improvement of central nervous system disorders including SCI.

The mechanisms of functional recovery after MSCs transplantation to treat SCI were suggested as 1) homing and neural differentiation of transplanted MSCs in the lesion site [8], 2) modulation of the inflammatory reaction by transplanted MSCs [9], 3) paracrine effects mediated by chemokines and growth factors released from transplanted MSCs, such as Neurotrophins (NT) 1 or 2, Brain-Derived Neurotrophic Factor (BDNF), Grail cell line-Derived Neurotrophic Factor (GDNF), and Vascular Endothelial Growth Factor (VEGF) [10-12]. However, the differentiation potential of MSCs in vivo remains unclear because very few transplanted MSCs are detected at the lesion site [11]. Thus, it has been proposed and commonly accepted that the functional benefits of MSCs transplantation are due to a paracrine effect [11,13]. The pathology of traumatic SCI results from primary damage (initial mechanical damage) and secondary damage due to vascular and biochemical effects [14]. MSCs transplantation to treat traumatic SCI is expected to reduce secondary damage of the spinal cord through a paracrine neuroprotective effect.

Transplanting pre-treated MSCs using drugs [15] or gene transfection [16] has highly therapeutic effects in disease models. Culturing cells under hypoxic conditions is a less invasive, alternative method to pre-condition of transplanted cells [17-19]. Although MSCs are usually cultured under normoxic conditions, MSCs exist in low oxygen (hypoxic) conditions in vivo [20] and the oxygen tensions are an important factor during MSC culture because stem cells are particularly sensitive to their microenvironment [21]. VEGF is known as a key regulator of vasculogenesis and angiogenesis [22]. In addition, VEGF has neurotrophic and neuroprotective effects in vitro and in vivo [23,24]. Although several studies have demonstrated that VEGF mRNA or protein expression is upregulated in hypoxia-preconditioned MSCs [25-27], few reports have demonstrated the neuroprotective effects of hypoxia-preconditioned MSCs transplantation to treat SCI. Therefore, the present study aimed to determine whether hypoxia-preconditioned MSCs have a neuroprotective effect in vitro and in vivo and improve functional defects in a rat model of SCI.

Materials and Methods

All protocols in this study were approved by the Animal Testing Committee Guidelines at Hiroshima University. Animal care and...
handling procedures were in accordance with National Institutes of Health guidelines.

Isolation and differentiation of rMSCs

Rat MSCs (rMSCs) were harvested from the femurs and tibias of 3-week-old Sprague-Dawley (SD) rats. The cells were seeded onto a culture dish (Sumitomo Bakelite Co., Tokyo, Japan) and cultured in Dulbecco’s Modified Eagle Medium with low glucose (DMEM-L) (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 units/ml), and streptomycin (100 µg/ml; both from Sigma-Aldrich). The cells were maintained at 37°C in 5% CO₂, and the medium was changed every 3 days.

The cell differentiation assay was performed using the Rat Mesenchymal Stem Cell Functional Identification Kit (R & D Systems, Inc., Minneapolis, MN, USA), according to the manufacturer’s protocol. The cells were finally stained with Oil Red-O solution (Wako Pure Chemical Industries, Osaka, Japan) for 15 min or with Alizarin Red-S solution (Sigma-Aldrich) for 30 min.

Cell culture under hypoxic conditions

rMSCs were seeded (2 × 10⁴ cells/cm²) onto a culture dish for 24 h. Then, the cells were cultured under normoxic (21% partial pressure of oxygen (pO₂)) or hypoxic (10%, 5%, 2%, or 1% pO₂) conditions in a hypoxic chamber. After a 24 h culture, the cells were collected in phosphate-buffered saline (PBS).

Reverse transcription and real-time polymerase chain reaction (PCR)

Total RNA was extracted with Nucleospin® RNA (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol. cDNA was synthesized with ReverTra Ace-α (Toyobo Co., Ltd., Osaka, Japan). Real-time PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) and the Fast Start Universal Probe Master (Roche, Basel, Switzerland) according to the manufacturer's protocol. The cells were finally stained with Oil Red-O solution (Wako Pure Chemical Industries, Osaka, Japan) for 15 min or with Alizarin Red-S solution (Sigma-Aldrich) for 30 min.

Preparation of rMSC conditioned medium and NG108-15 cell culture

rMSCs were seeded onto a culture dish and maintained in growth medium. After reaching 80% confluent, medium was changed to fresh growth medium and cells were cultured in normoxic (21% pO₂) or hypoxic (1% pO₂) condition. 24 h after exposed to normoxic or hypoxic conditions, the cell culture medium were collected from both culture conditions as Conditioned Medium (CM).

Preparation of rMSC conditioned medium and NG108-15 cell culture

Motor functional analysis

The inclined plane test and the Basso-Beattie-Bresnahan locomotor rating scale (BBB scale) were used to evaluate hind limb function. As reported previously, the inclined plane test assesses the maximum angle at which the animal can maintain its position for 5 s on an inclined plane [29]. The BBB scale is a 22-point scale that systematically and logically follows recovery of hind limb function, and ranges from a score of 0, indicative of no observed hind limb movement, to a score of 21, representative of a normal ambulating rodent [30]. Motor functional analyses were performed before SCI and on days 0-7, 10, 14, and 21 after SCI.

Spinal cord tissue sampling and mRNA expression analysis of the spinal cord lesion site

The rats were anesthetized 24 h after MSC transplantation. Spinal cord tissues were removed and soaked in RNA Later (Sigma-Aldrich). Total RNA was extracted from injured spinal cord segments (2 mm centered on the lesion site). RNA extraction and reverse transcription were performed as described above. Real-time PCR was performed using oligonucleotide primer sets corresponding to the cDNA sequences of rat Bdnf, Gdnf, Bcl2, Bax, beclin 1 (Becn1), interleukin-1 beta (Iliib), interleukin-10 (Ili0), tumor necrosis factor alpha (Tnfa), and tumor necrosis factor receptor superfamily, member 1A (Tnfrsf1a). Actb was used as an endogenous control.

Oxidative or inflammatory stress exposure to NG108-15 and cell death assay

NG108-15 cells were exposed to oxidative or inflammatory stress to evaluate the neuroprotective effect of N-MSC-CM or H-MSC-CM. Growth medium of rMSCs (absence of rMSCs culture) was used as a control. H₂O₂ (Santoku Chemical Industries, Tokyo, Japan) was used to mimic an oxidative stimulus to cells [31]. Lipopolysaccharide (LPS) (Wako pure chemical industries, Osaka, Japan) was used to mimic an inflammatory stimulus to cells [9]. As preliminary experiment, NG108-15 cells were cultured in different concentrations of H₂O₂ or LPS to determine optimal condition, and 500 µM H₂O₂ and 200 ng/ml LPS were determined as optimal condition for stress exposed experiment (Supplementary Figure S1A and S1B). NG108-15 cells were seeded onto culture dish (Sumitomo Bakelite Co.) and maintained in growth medium.
medium. The medium was changed to fresh rMSCs growth medium (Ctrl), N-MSC-CM, or H-MSC-CM (with 500 µM H2O2 or 200 ng/ml LPS) 48 h after seeding. The cells were collected and centrifuged 24 h after exposure to stress. The cells were centrifuged, suspended in PBS, and cell survival rate was determined with a counting chamber (Sunlead Glass Corp., Saitama, Japan) using trypan blue stain, and the remaining cells were collected for the mRNA expression analysis.

**mRNA expression analysis of stress exposed NG108-15 cells**

Total RNA was extracted from the NG108-15 cells samples and reverse transcription was performed as described above. Real-time PCR was performed using oligonucleotide primer sets corresponding to the cDNA sequences of rat Bcl2, Bax, Becn1, and Tnfrsf1a. Actb was used as an endogenous control.

**Statistical analysis**

Data were evaluated using one-way analysis of variance (ANOVA) with the Bonferroni test for mRNA expression analysis. Two-way ANOVA with the Bonferroni test was used for the motor functional analysis. Statistical analyses were performed using the JSTAT software (Sato, Japan). A p-value <0.05 was considered significant.

**Results**

**Differentiation potential of rMSCs**

The differentiation potential of the isolated rMSCs into adipocytes and osteoblasts was estimated to identify as the MSCs. Before differentiation, rMSCs were negative for specific staining (Oil Red-O and Arizarin Red-S staining), but positive cells were observed after differentiation (data not shown).

**Effects of hypoxic culture conditions on growth factor or apoptotic-associated mRNA expression**

rMSCs were cultured under five different conditions to determine the optimal oxygen level. Expression of the known hypoxia inducible genes, Car9 and Vegf increased significantly only under the 1% pO2 condition compared with that under the normoxic condition (Figure 1A and 1B). Adm also showed tendency to increase under the 1% pO2 condition, although this was not significant (Figure 1C). No differences in expression levels of Bdnf, Gdnf, and the Bax/Bcl2 ratio, an apoptotic indicator, were observed among those conditions (Figure 1D-1F).

**Behavioral recovery of spinal cord injured rats**

We assessed motor function using the inclined plane test and the BBB scale to compare the functional benefits of N-MSC and H-MSC transplantation after SCI. As a result, rats in the H-MSC group demonstrated more significant improvements on the inclined plane test than those in the Ctrl group at 14 and 28 days after injury (Figure 2A). The BBB scale also showed that the H-MSC group rats seemed to improve more compared with those in the other groups, although the differences were not significant (Figure 2B). Rats in the N-MSC group seemed to improve motor functions, but the differences were not significant.

**Effects of hypoxia-preconditioned MSCs transplantation on mRNA expression of the spinal cord lesion site**

In order to clarify the role of transplanted H-MSC in spinal cord lesion site, mRNA levels of neurotrophic factor, inflammatory, or apoptotic genes were evaluated. The Bdnf mRNA expression level was significantly higher in the spinal cords of rats in the H-MSC group than those in the Ctrl group (Figure 3A). Gdnf mRNA expression tended to be higher in the H-MSC group, but not significant (Figure 3B). The autophagy-associated marker, Becn1 was also significantly higher in...
Discussion

In the present study, we investigated whether hypoxia-preconditioned MSCs had a neuroprotective effect in vitro and in vivo, and demonstrated that hypoxia preconditioning was an effective strategy for SCI in cell-based therapy using MSCs.

Although previous studies have reported the hypoxic culture conditions for preconditioning of cell-based therapy [17-19], the optimal culture conditions remain unknown. In this study, Car9 and Vegf expression increased significantly in rMSCs under the 1% O2 hypoxic condition compared with that under the normoxic condition. Car9 is a hypoxia-induced protein involved in pH regulation and a target gene of hypoxia-inducible factor-1 (HIF-1), which is one of the master regulators in hypoxic response [32]. Vegf is also a well-known hypoxia-inducible gene, and important for signalling migration of endothelial cells and induction of angiogenesis [25]. Our results suggest...
that hypoxic preconditioning in this study effectively activated adaptive signaling in hMSCs against hypoxia-relating stress stimuli. On the other hand, no differences in the Bax/Bcl2 ratio were detected among the five different pO2 conditions for 24 h. Potier et al. reported that exposure to hypoxia over 72 h can lead to massive MSC death [33], but no remarkable cell mortality was observed under our study conditions, suggesting that 1% pO2 for 24 h was enough condition for expressing neurotrophic/angiogenesis genes.

Rats in the H-MSC group showed significant functional recovery compared to those in the Ctrl group. Bdnf and Becn1 mRNA expression levels at the spinal cord lesion site of rats in the H-MSC group were significantly higher than those in the Ctrl group. BDNF plays an important role in survival and differentiation of various neural cells types during neural development and after brain injury, which affect functional recovery [34-36]. Although BDNF was reported to play a role in regulating VEGF expression [37,38], it has been recently confirmed that BDNF expression and action are dependent on the vascular endothelial growth factor receptor-2 identifying BDNF as a downstream signaling partner of VEGF [39,40]. Moreover, VEGF together with BDNF accelerate brain plasticity after stroke [41]. In this study, H-MSC expressed a high level of Vegf, which may affect Bdnf expression at the spinal cord lesion site. Becn1 is an autophagy-related gene and has been described as an essential autophagy effector [42]. Chen et al. reported that autophagy is essential for maintaining cellular homeostasis during the stress response, and they concluded that BDNF may be a novel neuroprotective candidate for upregulating autophagy [43]. In addition, it has been suggested that VEGF attenuates SCI by increasing autophagy [44]. Taken together, our results suggest that Vegf expression of transplanted H-MSCs and/or Bdnf expression at the spinal cord lesion site promote autophagy for neuroprotective remodeling, resulting motor functional recovery. Although hypoxic preconditioning is able to improve the therapeutic potential of H-MSC, previous researches reported the epigenetic changes due to hypoxia culture [45-48]. Therefore, it is still essential to investigate the epigenetic changes in the MSCs cultured in the hypoxia condition.

The neuroprotective paracrine effect of hypoxia-preconditioned MSCs were analyzed in vitro using NG108-15 cells in this study. Various secondary injuries such as oxidative stress and inflammation contribute to cell death and functional disability after SCI [49,50]. H2O2 and LPS are used to mimic this environment in vitro [9,31]. In the present study, the survival rate of NG108-15 cells exposed to oxidative stress or inflammatory stress was significantly higher in cells maintained in the H-MSC-CM than in the N-MSC-CM or Ctrl. The Bax/Bcl2 ratio was significantly lower and Becn1 expression was significantly higher in H-MSC-CM when NG108-15 cells were exposed oxidative stress. Reactive oxygen species-induced activation of apoptosis signal-regulating kinase 1 (ASK1) plays crucial roles in oxidative stress-mediated cell death [51]. Nako et al. reported that VEGF treatment prevents oxidative stress-induced endothelial apoptosis by inhibiting activation of ASK [52]. In addition, VEGF upregulates the expression of Becn1, which is an autophagy-related gene [44]. In this study, we assumed that H-MSC-CM included VEGF secreted from cultured H-MSCs, which inhibited ASK activation and upregulated Becn1 expression, resulting inhibition of the cell death of NG108-15 cells exposed to oxidative stress. Regarding the inflammatory stress exposure, Tnfrsf1a expression was significantly lower and Becn1 expression was significantly higher in cells maintained in H-MSC-CM than those in N-MSC-CM or the Ctrl. LPS is a well-known trigger of tumor necrosis factor (TNF) expression in inflammation and neural cell death. TNF is a proinflammatory cytokine that exerts its action through the TNF receptor superfamily [53]. Previous studies have shown that both TNF and the TNF receptor are upregulated after nerve injury [54,55]. Moreover, Wang et al. reported that MSCs inhibit LPS-stimulated signaling in hepatic stellate cells [56]. In this study, H-MSC-CM may suppress inflammatory signal and upregulate Becn1 expression, which prevented death of NG108-15 cells exposed to inflammatory stress.

Although previous studies reported that MSCs transplantation reduces lesion volume and promotes functional improvement of central nervous system disorders including SCI [5-7], we did not observe significant motor functional improvement with N-MSC transplantation in this study. We have also reported therapeutic effect of neural stem cell transplantation in brain injury model [34]. In this study, we could find effective improvement of motor functions with half number of H-MSC transplantation compared to our previous study using mice model, but did not that with N-MSC. Optimization of MSCs transplantation must be necessary in next step to reach future clinical trial.

Conclusion

We here provide novel evidence that hypoxia preconditioning of MSCs accelerated functional recovery in SCI model rats, at least through secretion of protective cytokines and induction of autophagy for tissue remodeling. Although optimization of transplantation protocol is needed, our results suggest that hypoxia preconditioning of MSCs is a useful strategy for cell-based therapy.

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References


