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Histological Examination of Engineered Mesenchymal Stem Cells Improve Bladder Function in Rat

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랫드에서 방광기능 향상의 엔지니어링 중간엽 줄기세포의 조직학적 소견

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ABSTRACT

This study was undertaken to examine the effects and to investigate the relevant mechanisms of overexpressing stromal cell-derived factor-1 (SDF-1) produced by engineered mesenchymal stem cells, in a neurogenic bladder (NB) rat model. Sprague-Dawley (SD) rats (N=48) were randomly divided into 4 groups comprising 12 rats each: control group, Injury group, Injury+imMSC group, and Injury+SDF-1 eMSC group. Rats in the Injury+imMSC group were treated with imMSCs, whereas the Injury+SDF-1 eMSC group were administered SDF-1 eMSCs. After 4-weeks therapy, the bladder and pelvic nerve (PN) tissues were examined by subjecting to Masson's trichrome staining and immunofluorescence. Administration of SDF-1 eMSC resulted in improved smooth muscle content in the bladder tissue, significantly increased β -III tubulin expression of the PN, and enhanced SDF-1 expression ($P<0.05$). The bladder wall repair can be attributed to the overexpression of SDF-1 by SDF-1 eMSCs. Significantly increased SDF-1 expression was obtained in the Injury+SDF-1 eMSC group ($P<0.05$). The crushed PN also showed significant recovery in the Injury+SDF-1 eMSC group ($P<0.05$). In conclusion, our results indicate that SDF-1 eMSCs express more SDF-1 in vivo, thereby facilitating the repair of injured nerve and recovery of NB in rats.

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INTRODUCTION

The number of men with neurogenic bladder (NB) is continually growing [1], which was induced by various neurogenic conditions [2]. Spinal cord injury was the main reason, but nerve injury during pelvic surgery also contributed to NB dysfunction [3]. As a neurological

disease, NB decreased quality of life (QoL) [4]. However, the therapy for NB are suboptimal with the aim to protect the upper urinary function and recover bladder dysfunction [5].

Stem cells therapy as a novel and promising treatment has been used in many area, which take effect by differentiation and secretion [6]. Engrafted stem cells differentiated various cells, participated in tissue repair and impacted disorder. Meanwhile, engrafted stem cells secreted bio-factors, such as VEGF, nNOS and SDF-1 [7], which took effect in target tissue. In injured tissue,

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Ang 1 stimulated angiogenesis and restored injured tissue. nNOS has been proved to improved nerve damage in vivo [8]. SDF-1 as a chemotactic factor drove immune cells and tissue repair cell to target [9]. Researchers found injured tissue highly expressed SDF-1 and homed engrafted stem cells to target. Gathered stem cells repaired damaged tissue and continually overexpressed SDF-1 which looped a positive feedback. In NB, bladder dysfunction because of denervation atrophy contributed to frequent urination, urinary incontinence, voiding and urinary retention. So repair injured nerve, recover atrophic bladder and rebuild bladder function were the key to improve NB.

In this experiment, we established a NB rat model by bilateral pelvic nerve (PN) injury, and explored the effect of stem cells treatment on NB using an overexpressed SDF-1 engineered imMSCs. We made a hypothesis that stem cells treatment would develop NB function and recover atrophic bladder by promoting angiogenesis, tissue regeneration and nerve recovery.

MATERIALS AND METHOD

1. Cell preparation

BM-MSCs were obtained from the Catholic Institute of Cell Therapy (CIC) (Catholic MASTER Cells, CIC, Korea). Human bone marrow aspirates were obtained from the iliac crest of healthy donors aged 20 to 55 years with approval from the Institutional Review Board of Seoul St. Mary's Hospital (approval numbers KIRB-00344-009 and KIRB-00362-006). The bone marrow aspirate from each donor who consented was collected and sent to the Good Manufacturing Practice (GMP). BM-MSCs (Catholic MASTER Cells) were obtained from the CIC (Seoul, Korea).

Primary bone marrow mesenchymal stem cells (BM-MSCs) were cultured in low glucose-containing Dulbecco's modified Eagle's medium (DMEM, Gibco, US) supplemented with 20% fetal bovine serum (FBS, Gibco, US) and 5 ng/mL basic fibroblast growth factor (bFGF, Cell

Signaling Technology, Danvers, US) at 37°C at 5% CO₂, but engineered BM-MSCs were cultured with 10% FBS.

To generate engineered BM-MSCs, c-myc, hTERT and tetracycline transactivator (tTA) and SDF-1 genes were synthesized and transfected with pBD lentiviral vector (SL BIGEN, Seongnam, Korea). Immortalized upregulated SDF-1 engineered BM-MSCs (SDF-1 eMSCs) were selected monoclonal by antibiotics. Selected SDF-1 eMSCs were isolated cell population by limiting dilution method. Meanwhile vectors without SDF-1 gene were administered into BM-MSCs (imMSCs) as a control. Final monoclonal cell was selected by SDF-1 protein expression, proliferation rate and other MSC phenotypes. Before in vivo injection, engineered imMSCs was irradiated.

2. Experimental animal and study design

Fifty 8-week-old male Sprague-Dawley rats weighing about 270~300 g were purchased from a Korean company (Orient Bio Co., Seongnam, Korea). All animal experiments in this study were approved by the CUMC-2016-0218-01. 36 rats were separated into 3 experimental groups: a Injury group, an Injury +imMSCs group and an Injury +SDF-1 eMSCs group with 12 rats in each group. 12 normal rats was belonged to control group. Bilateral pelvic nerve (PN) of all rats were identified under anesthesia. And then rats in 3 experimental groups were administered by a PN crush. Rats in normal group were administered by sham surgery. After surgery, rats were housed individually with freely available food and water.

3. MSCs treatment

One weeks after NB model established, rats in Injury+imMSCs group and Injury+SDF-1 eMSCs group were treated with imMSCs or SDF-1 eMSCs, through around PN and intra-bladder wall injection under anesthesia (1×10^6 MSCs diluted in phosphate-buffered saline). Rats in NB and control group were injected to equal saline. To track the location of engrafted stem cells, we labeled them with a fluorescent dye (Cell

Tracker™ CM-DiI; Molecular Probes, Eugene, OR) before injection according to the manufacturer's protocol.

4. Histology and immunofluorescence staining

The collected PN and bladder were fixed in 4% paraformaldehyde for 24 hours at 4°C before creating a paraffin block. The primary antibodies were used as following: β III-tubulin (diluted 1:200; Abcam, Cambridge, UK), α smooth muscle actin (α -SMA, diluted 1:500; Abcam), stromal cell-derived factor-1 (SDF-1 diluted 1:200; Abcam, Cambridge, UK) and 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc., Burlingame, CA) was used to stain the nuclei. Digital images were obtained using a Zeiss LSM 800 Meta confocal microscope (Zeiss, Oberkochen, Germany), and the mean intensity was calculated using ZEN 2012 (Zeiss).

5. Masson's trichrome staining

The proportion of collagen and smooth muscle for rats was evaluated by Masson trichrome staining. In brief, Bladder wall sections were deparaffinized, rehydrated with graded alcohols, immersed in warm Bouin's solution (Sigma) (55~60°C) for 2 hours and

washed in running tap water for 2 min followed by distilled water for 30~60 seconds, and then stained with Weigert Hematoxylin (Merck, Darmstadt, Germany) for 10 min and washed out. Subsequently, Smooth muscle was stained red with Biebrich Scarlet-Acid-Fuchsin (Sigma) for 10 min, rinsed and immersed in phosphomolybdic phosphotungstic acid (Sigma) for 15 min. And collagen was stained blue with Aniline Blue (Sigma) for 10 min and rinsed in distilled water followed by immersion in 1% acetic acid for 5 min. Finally, the tissues were rehydrated with 100% ethanol, left to air dry and mounted. All sections of bladder tissue were stained at the same time.

6. Statistical analysis

All data are presented as mean $\pm$ standard error (SD) and were analyzed by SPSS version 22.0 software (IBM, Armonk, NY). Student's t-test and one-way ANOVA as appropriate were used to evaluate whether differences among groups were significant. $P < 0.05$ was considered statistically significant.

Figure 1. Representative images of Masson Trichrome stain in bladder. (A) Red is smooth muscle, and blue is collagen. Scale bar, 200 μ m. Original magnification: $\times 100$ (B) smooth muscle/collagen for each group. Each bar shows the mean values (standard deviation). * $P < 0.05$, # $P < 0.01$.

RESULTS

1. SDF-1 eMSCs repaired bladder wall by overexpressing SDF-1

The contents of smooth muscle and collagen in the bladder wall were observed by Masson's trichrome staining. The result (Figure 1A) showed that smooth muscle ratios of the Injury+SDF eMSC group was obviously superior compared to Injury group. The ratios of smooth muscle (Figure 1B) in the normal, Injury, Injury+imMSC, and Injury+SDF eMSC groups are, in order, 1.31, 0.43, 0.73, and 1.19. Meanwhile, we detected the content of α -SMA in bladder wall. The result (Figure 2) showed that after stem cells injection, there are more α -SMA existing in the bladder wall which meant there are more smooth muscle and angiogenesis producing in injured tissues. Then we detected the number of engrafted stem cells and SDF-1 expression in each bladder. The results (Figure 3) showed that there were more SDF-1 expression in Injury+SDF eMSC group ($P<0.05$).

2. Crushed pelvic nerve were recovered by SDF-1 eMSCs

After MSCs treatment, MSCs around PN were evaluated. Figure 4 showed that nerve recovery was significantly improved by SDF eMSCs, and β -III-tubulin expression in Injury+SDF eMSC group was significantly higher compared to other experimental groups. For displaying the results more accurately, a quantitative analysis was carried out. And the figure 4B show nerve in Injury+SDF eMSC group is more than in imMSC group ($P<0.05$), which means more SDF-1 expression accelerated damaged nerve recovering.

DISCUSSION

As the requirement of people for a higher QoL, NB therapy has been needed widely [10]. NB symptoms including frequent urination, urinary incontinence, voiding dysfunction and urinary retention were confused patients in daily life [11]. However, by now the reason of causing NB was still unclear [12]. So most urologists focused on relieving NB symptoms to improve the QoL.

Figure 2. Representative images of α -SMA used to immunofluorescence staining. (A) Representative images of bladder for each group. Green is α -SMA, and blue is nuclei. Scale bar, 100 μ m. Original magnification: $\times 200$ (B) Mean intensity of α -SMA for each group. Each bar shows the mean values (standard deviation). * $P<0.05$, ** $P<0.01$.

Figure 3. Representative images of SDF-1 used to immunofluorescence staining. (A) Representative images of bladder for each group. Green is SDF-1, and red is MSCs. Scale bar, 50 μ m. Original magnification: $\times 200$ (B) Mean intensity of SDF-1 for each group. Each bar shows the mean values (standard deviation). * $P < 0.05$, # $P < 0.01$.

Figure 4. Representative images of β -III tubulin used to immunofluorescence staining. (A) Representative images of PN for each group. Green is β -III tubulin, and red is MSCs. Scale bar, 500 μ m. Original magnification: $\times 30$ (B) Mean intensity of β -III-tubulin for each group. Each bar shows the mean values (standard deviation). * $P < 0.05$, # $P < 0.01$.

In this study, we devoted ourselves to researching how to improve NB by decreasing the appearance of NB symptoms. And we also explored the associated mechanisms by establishing a PN-crushed NB rat

model. In this experiment, we found engrafted SDF-1 eMSCs expressed more SDF-1 in vivo compared to imMSCs. By stem cells transplantation, smooth muscle in bladder wall was increasing. Furthermore, cystometry,

contractility and voiding frequency were significantly improved after stem cells treatment. These results illuminated that stem cells could relieve NB by expressing more SDF-1. Meanwhile, for exploring the reason why stem cells promoted proliferation of smooth muscle, we detected the expression of SDF-1. The immunofluorescence results showed that with stem cells injection, SDF-1 increased. The expression of SDF-1 was upregulated, which told us a reason of injured PN improved [7].

SDF-1 as a kind of chemotactic factor can promote directional migration of cells [13]. In a recent study, Carbajal KS et al. [14] found SDF-1 regulated homing of engrafted stem cells to target tissue in mice. But in this study we found in a higher SDF-1 microenvironment, there were not more engrafted stem cells migrating to the target tissue, which was different from our previous study [7]. In this study, we did inject stem cells around the PN and bladder wall directly. Therefore, cells restored the bladder dysfunction directly. But SDF-1 secreted by injured PN could not drive engrafted stem cell efficiently because of longer distance. A number of engrafted stem cell did not migrate around injured PN, which led to a similar quality of stem cells in Injured+imMSC group and Injured+SDF eMSC group. However, in our results we found as engrafted stem cells moved along with circulatory system, there were still some stem cells migrating around the injured PN. And these stem cells expressed abundant SDF-1 to stimulate the PN recovery. Meanwhile, we found under a similar numbers of engrafted stem cells, but SDF-1 eMSCs expressed more SDF-1 compared to imMSCs and the injured bladder was improved better by SDF-1 eMSCs.

In our study, we proved engrafted stem cells recovered bladder wall and restored bladder function in a NB rat. There are some defects in this study. First, this study was focus on the animal experiment, but as for mechanism exploring, a cell experiment might be a good choice. In vitro study, signaling pathways and bio-factors were easier to study. So in next study, we will block some key bio-factors in associated signaling

pathways to explore the behind mechanism. Second, we used 8-weeks rats in this study. However, in clinical patients with NB usually suffered for a longer time than 8 weeks. NB treatment at an early stage of NB development may cause a selection bias. It was proved that SDF-1 eMSCs expressed more SDF-1 in vivo for NB improvement. These results may show a new basis for NB treatment.

요약

이 연구의 목표는 엔지니어링 중간엽 줄기세포에 의해 발현된 SDF-1의 효과를 규명하고 신경인성방광 랫 모델에서 관련 메커니즘을 조사하는 것이다. Sprague-Dawley 랫(N=48)을 대조군, 신경인성방광군, 신경인성방광군+imMSC군 및 신경인성방광군+SDF-1 eMSC 군으로 무작위 선정하였다. 신경인성방광 랫 모델은 양측 골반 신경 손상으로 유도하였으며 골수 유래 중간엽 줄기세포를 immortalized한 MSC (empty vector)와 upregulated SDF-1한 MSC (immortalized+SDF-1 치료유전자 발현)로 엔지니어링 하였다. 엔지니어링 중간엽줄기세포를 양측 골반 신경 손상부위와 방광에 주사하여 생착시켰다. 주사 4주 후 치료 효과를 양측골반신경 및 방광 조직을 마운트, 염색 및 면역 염색으로 분석하였다. 신경인성방광군+SDF-1 eMSC 군에서 방광 평활근이 유의하게 증가하였다($P<0.05$). 신경마커 베타-III 튜블린 및 SDF-1 발현 또한 유의하게 증가하였으며($P<0.05$), 이를 통해 손상된 신경을 복구하고, 신경인성 방광 랫 모델의 방광조직을 회복시켰다.

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