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Original

Adipose-derived Mesenchymal Stem Cells Improve Both Spontaneous Pain and Allodynia in a Rat Neuropathic Pain Model

Masaya Imanishi[1], Hirotaka Asato[1], Kohei Umekawa[1],
Shoichi Sasaki[1], Tetsuji Terashima[2]

[1] *Department of Plastic and Reconstructive Surgery, Dokkyo Medical University, Shimotsuga, Tochigi, Japan*
[2] *Department of Anesthesiology, Dokkyo Medical University, Shimotsuga, Tochigi, Japan*

SUMMARY

Purpose : Several studies investigated the efficacy of transplanting adipose-derived mesenchymal stem cells (ADMSCs) in the treatment of neuropathic pain in animals. However, these studies evaluated the effects of ADMSCs transplantation by assessing the mechanical allodynia but not spontaneous pain. Here, we verify whether ADMSCs transplantation improves spontaneous pain in a rat model of neuropathic pain.

Methods : ADMSCs were isolated from rat adipose tissue and cultured. Chronic constriction injury (CCI) model rats were created by surgical maneuver. A total of 20 F344 rats underwent surgery and were divided into 2 groups, the ADMSCs group and the control group. One week after CCI surgery, ADMSCs were transplanted into the epineurium of the damaged nerve. The effects of ADMSCs transplantation were evaluated by the number of spontaneous pain-related behaviors and the degree of mechanical allodynia. The degree of mechanical allodynia was assessed with the von-Frey filament test.

Results : No rats died during the experiments and all CCI model rats were established successfully. ADMSCs transplantation improved mechanical allodynia on and after 7-day post-transplantation and spontaneous pain on and after 21-day post-transplantation with the statistically significant differences. These improvement effects were observed until 6-week post-transplantation in mechanical allodynia and 5-week post-transplantation in spontaneous pain.

Conclusion : ADMSCs transplantation improved not only mechanical allodynia but also spontaneous pain. ADMSCs transplantation may be an effective treatment for neuropathic pain in clinical practice.

Key Words : adipose-derived mesenchymal stem cells, neuropathic pain, allodynia, spontaneous pain, chronic constriction injury model

INTRODUCTION

The prevalence of neuropathic pain is estimated to be 6.9-10% worldwide[1)]. Only 15-50% of patients with neuropathic pain exhibit mechanical allodynia[2)], while almost 100% of patients struggle with spontaneous pain[3)]. Despite that the neuropathic pain can be a major burden, the current treatments include pharmacotherapy, physical therapy, cognitive therapy, inter-

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Reprint requests to : Masaya Imanishi, MD
Department of Plastic and Reconstructive Surgery, Dokkyo Medical University 880 Kitakobayashi, Mibu-Machi, Shimotsuga-Gun, Tochigi, 321-0293, Japan.

ventional pain management, and other treatments that are still insufficient[4,5]. Recently, the effects of the mesenchymal stem cells (MSCs) transplantation as the treatment for neuropathic pain were examined and verified[6,7]. In animal experiments, MSCs transplantation was shown to improve mechanical allodynia in neuropathic pain through their anti-inflammatory and immunomodulatory properties[8]. Various types of MSCs exist such as bone marrow derived MSCs, adipose-derived MSCs (ADMSCs), and umbilical cord-derived MSCs. ADMSCs are advantageous for clinical applications because they can be obtained less invasively and in large quantities compared to other MSCs types. While several studies examined the effects of ADMSCs transplantation for the treatment of neuropathic pain[9~12], these studies evaluated that effects by assessing mechanical allodynia but not spontaneous pain. Methods to evaluate mechanical allodynia in animal experiments are similar to those used for mechanical allodynia evaluation in clinical practice. For example, the von-Frey filament test used in animal experiments is also used to assess mechanical allodynia in humans[13].

As mentioned above, almost all patients with neuropathic pain have spontaneous pain. Evaluating spontaneous pain improvement by the treatment in animal experiments would be more directly applicable to the judgement of the treatments effectiveness for neuropathic pain in human than evaluating mechanical allodynia improvement.

In this study, we hypothesized that the transplantation of ADMSCs would improve not only mechanical allodynia but also spontaneous pain. To verify this hypothesis, we transplanted syngeneic ADMSCs into the lesions of chronic constriction injury (CCI) model rats and assessed its efficacy to the spontaneous pain improvement with the method described by Kawasaki Yatsugi et al[14] and to the mechanical allodynia improvement with the von-Frey filament test.

METHODS

The research protocol was approved by the Animal Research Committee of Dokkyo Medical University (Animal Research Approval Number 1017). 9-week-old Male F344 rats weighing 180-220 g (Japan SLC, Shizuoka, Japan) were used. The rats were kept individually in cages under standard laboratory conditions at 23±2℃ and a humidity of 50±10% with a 12-hour light/dark cycle. All animals were weighed once a week and checked for health status. The rats were acclimated in the cages for at least 1 week before the beginning of the experiment.

A block of adipose tissue was removed from F344 rats those that had not undergone the CCI surgery. ADMSCs were isolated from the adipose tissue and passaged 2-4 times to prepare for transplantation. CCI surgery was performed on 20 rats according to a previously described method by Bennett and Xie[15]. Rats were randomly assigned to 2 groups : the ADMSCs transplantation group and the control group (10 for each group). One week after the CCI surgery, the ADMSCs transplantation group rats were transplanted with ADMSCs into the epineurium of the lesions. The control group rats were only injected with phosphate buffered saline (PBS) into the epineurium of the lesions. Spontaneous pain and mechanical allodynia were evaluated before ADMSC transplantation and then weekly until week 6 of post-transplantation. All experiments were performed between 10 : 00 and 20 : 00. The details of each of these steps are described below.

Isolation and culture of ADMSCs

Adipose tissue was removed from the inguinal and abdominal regions of 9-week-old male F344 rats. The tissue was thoroughly washed with PBS and then blood vessels and connective tissue were removed. The tissue was subsequently minced. The minced tissue was added to a solution containing 0.1% collagenase type 1 (Sigma, St. Louis, MO, USA) and 0.2% dispase (Sigma) and mixed by shaking at 37℃ for 60 minutes. Digested tissue was filtered through a 100 μm filter. The filtrate was centrifuged at 1200 rpm for 5 minutes. The isolated cell pellet was washed with PBS and centrifuged again. A cell suspension containing the isolated ADMSCs was cultured in basic medium (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum and 2% penicillin-streptomycin at 37℃ and 5% CO_2. Cells at passages 2-4 were used as ADMSCs for transplantation.

CCI model rats

The CCI surgery was performed as described by Bennett and Xie[15]. All animals were anesthetized with isoflurane. The fascia between the gluteal muscles and biceps femoris muscle was opened to expose the left sciatic nerve. Proximal to the trifurcation of the sciatic nerve, four 4/0 chromic gut ligatures were loosely tied around the nerve with the intervals of 1 mm. The ligatures were constricted to approximately a third of the diameter of the nerve. The fascia and skin were closed with 4/0 nylon thread.

ADMSCs transplantation

ADMSCs were transplanted 7 days after the CCI surgery. All animals were anesthetized with isoflurane. To avoid additional nerve damage, the previous scar was carefully reopened to expose the sciatic nerve. Under microscopic magnification, 1.0×10^6 cells mixed with 50 μL of PBS were transplanted into the epineurium with a 32G needle. In the control group, 50 μL of PBS was injected into the epineurium. The fascia and skin were closed with 4-0 nylon thread.

Assessment of spontaneous pain by automatically measuring the number of limb movements including spontaneous pain-related behaviors[14]

This assessment was performed according to the method previously proposed by Kawasaki-Yatsugi et al[14]. Magnets were implanted at the timing of CCI surgery. A Teflon-coated columnar magnet (1 mm in diameter, 3 mm long ; SCT-MAG-TF, Neuroscience Inc., Tokyo, Japan) was implanted under the dorsal skin of the ipsilateral foot. A test chamber surrounded by a coil (NS-SCT10R, Neuroscience Inc., Tokyo, Japan) was installed in a dim room at constant temperature (23±2℃). An animal was placed into the chamber and given acclimation period of 5 minutes. Measurements were conducted for 30 minutes per day. Any movements of the magnet-implanted limb including abnormal behaviors related to spontaneous pain (e.g. lifting and licking the CCI limb) caused the changes in the electromagnetic field and generated a voltage, the amount of which was dependent on the speed and direction of magnet movement. The voltage generated in the coil was amplified and digitized via an interface unit (NS-SCTB16, Neuroscience Inc., Tokyo, Japan). Abnormal behaviors were automatically detected as spike waveforms, and counted by analytical software (MicroAct® ; NS-SC-S100, Neuroscience Inc., Tokyo, Japan). The followings were set as analytical parameters for waveforms formed by movement of the limbs : range of frequency 2.5-20 Hz, threshold 0.01 V, minimum duration 0.09 seconds, shortest duration gap 0.03 seconds.

Quantification of the degree of mechanical allodynia using the von-Frey filament test

Mechanical allodynia was evaluated by the up-down method using von-Frey filaments according to the previously described method by Chaplan et al[16]. An animal was placed in a plastic box with a metal mesh floor. After the adaptation period of 30 minutes, a filament was applied perpendicular to the sole of the ipsilateral limb with the pressure by which the filament was slightly bent. Withdrawal of CCI limb upon stimuli was regarded as a positive response. Eight filaments (0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, and 15.0 g) were used. The 2.0 g filament was used first. When a positive response was observed, the next weaker filament was used for stimulation ; if no response was observed, the next stronger filament was used. Two consecutive stimulations in which an animal's reaction changed from negative to positive or from positive to negative was defined as the first 2 reactions, after which 4 stimulations were consecutively applied based on the animal response. In cases where continuous positive and negative response were observed to the exhaustion of the filament set, values of 0.25 g and 15.0 g were applied respectively. According to the reaction pattern to these 6 stimulations, the 50% reaction threshold was calculated by the formula[16].

Statistical analysis

The number of limb movements including abnormal behaviors and the reaction threshold in the von-Frey filament test are reported as the mean ± standard error of the mean (SEM). Statistical analyses were performed using split-plot analysis of variance. Unpaired *t*-test was used as a post-hoc test. SPSS version 24 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all analyses. P values less than 0.05 were considered statistically significant.

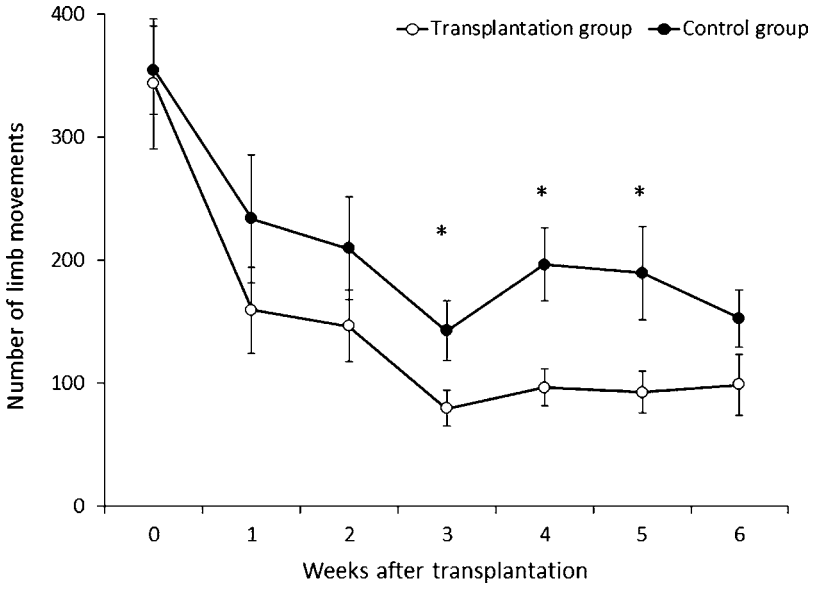

Figure 1 Effect of adipose-derived mesenchymal stem cells transplantation on limb movements including spontaneous pain-related behaviors
All data indicate mean ± standard error of the mean. Asterisk (*) indicates statistically different from the control group.

RESULTS

All the CCI model rats displayed "normal movements" associated with walking and "abnormal movements" including lifting/guarding, flinching/shaking, and licking. The number of limb movements in the ADMSCs transplantation group (344 ± 53) and in the control group (354 ± 36), after CCI surgery and before ADMSCs transplantation or PBS injection, increased compared to that of 10 syngeneic rats without CCI surgery (80.5 ± 11.6) with the statistical significance. The reaction threshold in the ADMSCs transplantation group (3.70 ± 0.62) and in the control group (4.19 ± 0.4), after CCI surgery and before ADMSCs transplantation or PBS injection, decreased compared to that of 10 syngeneic rats without CCI surgery (14.19 ± 0.54) with the statistical significance. Unpaired *t*-test was used to check the result of CCI surgery.

Those changes indicated that the CCI model rats were successfully established. No animals died during the experiments. All animals kept gaining weight over time even after the CCI surgery and ADMSCs transplantation.

Assessment of spontaneous pain

The number of limb movements in the ADMSCs transplantation group and the control group were as shown in Fig. 1. Measurements were performed before ADMSCs transplantation and on 7-, 14-, 21-, 28-, 35-, and 42-day post-transplantation. Split-plot analysis of variance showed no interaction between measurement timing and groups and showed a statistically significant difference in the number of limb movements between the ADMSCs transplantation group and the control group ($p = 0.036$). It suggested that statistically significant differences were observed between transplantation group and control group, irrespective of measurement timing. Unpaired *t*-test was performed as a post-hoc test. This analysis showed significant differences between the ADMSCs transplantation group and the control group on 21-, 28-, and 35-day post-transplantation (all of them, $p < 0.05$). These results suggest that ADMSCs transplantation reduced the number of spontaneous pain-related behaviors on and after 21-day post-transplantation.

Quantification of the degree of mechanical allodynia

The reaction threshold in the ADMSCs transplantation group and the control group were shown in Fig. 2. The reaction threshold was evaluated before ADMSCs transplantation and on 7-, 14-, 21-, 28-,

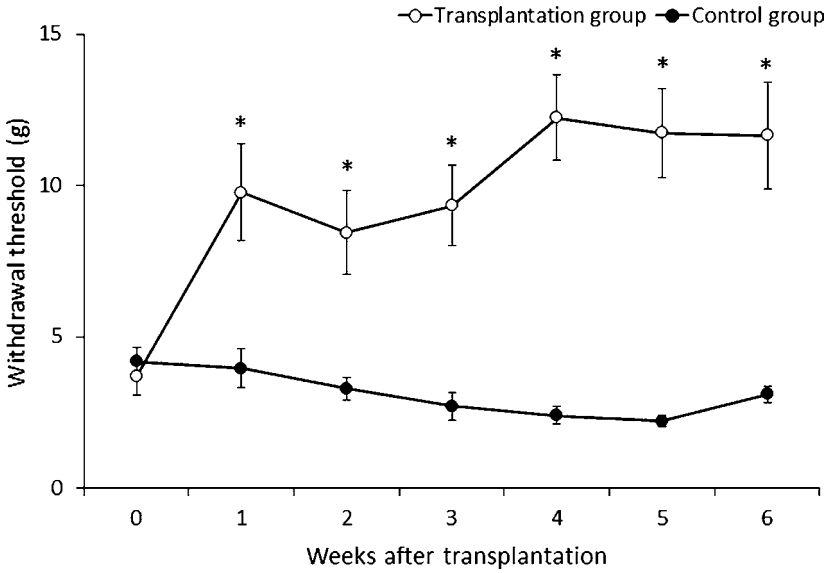

Figure 2 Effect of adipose-derived mesenchymal stem cells transplantation on mechanical allodynia

All data indicate mean±standard error of the mean. Asterisk (*) indicates statistically different from the control group.

35-, and 42-day post-transplantation. Split-plot analysis of variance demonstrated an interaction between measurement timing and groups. Unpaired *t*-test as a post hoc test detected significant differences on 7-, 14-, 21-, 28-, 35-, and 42-day post-transplantation (all of them, $p<0.05$). These results suggested that ADMSCs transplantation alleviated mechanical allodynia on and after 7-day post-transplantation.

DISCUSSION

Several studies suggested that ADMSCs transplantation improves neuropathic pain in animal models[9~12]. However, these studies evaluated the efficacy of ADMSCs transplantation by assessing only mechanical allodynia but not spontaneous pain. Murai et al.[17] noted differences in the effects of drugs on spontaneous and evoked pain-related behaviors in rats, and suggested that there are different mechanisms in spontaneous and evoked pain symptoms in neuropathic pain condition. Therefore, we think that to verify the efficacy of ADMSCs transplantation against neuropathic pain in clinical practice, it is pertinent to evaluate its efficacy against spontaneous pain, even in the animal experiments stage.

The experimental models for neuropathic pain have been developed : (1) CCI model of Bennett and Xie[15] ; (2) the tight ligation of the partial sciatic nerve model of the Seltzer et al[18] ; and (3) the tight ligation of spinal nerves model of Kim and Chung[19]. All three models mentioned above produce spontaneous pain and mechanical allodynia. In this study, we used the CCI model as a rat neuropathic pain model, because, in the previous studies, the CCI model was used to evaluate ADMSCs transplantation effects on neuropathic pain[9,11,12].

We observed an improvement in mechanical allodynia on and after 7-day post-transplantation. This result is similar to that of previous studies in which ADMSCs were locally transplanted[10,11]. Spontaneous pain-related behaviors, however, were significantly improved on and after 21-day post-transplantation, suggesting that different mechanisms contribute to mechanical allodynia and spontaneous pain. It is currently unknown how ADMSCs improves neuropathic pain. In the previous study, ADMSCs administered into the subarachnoid space of CCI model rats were histologically found on the surface of the spinal cord and dorsal root ganglia[11]. These ADMSCs can directly modulate the inflammatory reactions and immune cells, which play important roles in nociception of pain and tissue regeneration. It is also suggested that MSCs modulate inflammatory and immune processes

through paracrine release of soluble factors such as interleukin 10, leukemia inhibitory factor, and transforming growth factor-β[8]. In addition to these mechanisms, MSCs are known to induce antihyperalgesic effects through the activation of the endogenous opioid system. Guo W et al. hypothesized that the activation of peripheral opioid receptors plays a main role in inducing antihyperalgesic effects in the early stages of MSC transplantation, while the activation of central opioid receptors underlie the antihyperalgesic effects in the late stages of MSC transplantation[7].

While the effectiveness of morphine, an opioid agonist, in the treatment of neuropathic pain had been controversial, opioids have been recently used in the management of patients with neuropathic pain[20]. In CCI model rats, morphine reduced the number of spontaneous pain-related behaviors and alleviated mechanical allodynia at an s.c. dose of 3 mg/kg or greater[17]. However, clinical use of high-dose morphine carries a risk of adverse events including nausea, constipation, sleepiness, sedation, and respiratory depression. Given that both MSCs transplantation and morphine improve neuropathic pain via modulation of the opioid system, our finding that MSCs transplantation reduced the number of spontaneous pain-related behaviors in CCI model rats is not inconsistent with theories suggested previously. ADMSCs transplantation may be a safer therapeutic approach for neuropathic pain than morphine.

Although the safety of autologous MSCs transplantation is controversial, the fact that the number of clinical studies using autologous MSCs transplantation is increasing[21], indicating that MSCs transplantation techniques satisfy the safety standard in clinical practice.

All clinical studies using transplantation of the stromal vascular fraction (SVF) and autologous fat for the treatment of neuropathic pain[22~26] have demonstrated improvements in spontaneous pain without any major complications. We propose that transplantation of cultured ADMSCs, ADMSCs purity of which is higher than that of SVF and autologous fat, may be more effective for improving pain than transplantation of SVF and autologous fat.

This study has some limitations. First, we did not examine the effects of intravenous administration of ADMSCs for neuropathic pain. The intravenous administration of ADMSCs was reported as effective to alleviate neuropathic pain[9,11,12]. Second, while we verified the inhibitory effect of ADMSCs transplantation on pain behaviors, we did not examine the histological or biochemical changes. This study, however, demonstrated that ADMSCs transplantation reduced the number of spontaneous neuropathic pain-related behaviors in a CCI rat model. This new finding may suggest that ADMSCs transplantation is effective treatment to spontaneous neuropathic pain, which is seen in almost all cases of neuropathic pain, in clinical practice.

CONCLUSION

The sufficiently effective treatment for the neuropathic pain is not established yet. ADMSCs transplantation emerges as a therapeutic option for the neuropathic pain. We evaluated the effects of ADMSCs transplantation on spontaneous pain and mechanical allodynia in CCI model rats. In this study, ADMSCs transplantation improved not only mechanical allodynia but also spontaneous pain. A possibility is suggested that ADMSCs transplantation is effective to mechanical allodynia and spontaneous pain of neuropathic pain in clinical practice. Further researches are necessary to confirm this possibility.

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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