Adipose-derived Mesenchymal Stem Cells Improve Spontaneous Pain in a Rat Model of Neuropathic Pain

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Introduction

The incidence rate of neuropathic pain is estimated to be 6.9-10% worldwide [1]. Despite its high morbidity, it is unclear whether current treatments such as medication, physical therapy, cognitive therapy, and interventional pain management are sufficient [2]. Multidrug therapy and other treatments are also used, but have limited effects [3]. Against this background, the effects of the transplanting mesenchymal stem cells (MSC) for the treatment of neuropathic pain were initiated and have been verified for the past decade [4,5]. In animal experiments, MSC have been shown to improve stimulus-induced pain in neuropathic pain through their anti-inflammatory and immunomodulatory functions [6]. Various types of MSC exist, including bone marrow derived mesenchymal stem cells (BMMSC), adipose-derived mesenchymal stem cells (ADMSC), and umbilical cord-derived mesenchymal stem cells. ADMSC are advantageous for clinical applications because they can be obtained less invasively and in large quantities compared to other MSC types. While a number of studies have examined the effects of transplanting ADMSC for the treatment of neuropathic pain, these studies evaluated the effects of transplantation by measuring stimulus-induced pain but not spontaneous pain.

Methods for evaluating stimulus-induced pain in animal experiments are similar to those used for stimulus-induced pain and mechanical allodynia in clinical practice. For example, the von-Frey filament test used in animal experiments is also used to assess mechanical allodynia in humans [7]. However, given that only 15 to 50% of patients with neuropathic pain exhibit stimulus-induced pain [8], while almost 100% exhibit spontaneous pain [9], evaluation of spontaneous pain in animal experiments would allow for a more direct translation of the effectiveness of this treatment in clinical practice.

Various methods are used to evaluate spontaneous pain in rodents [10]. One such method was proposed by Kawasaki-Yatsugi et al. [11], who inserted a small magnet into the diseased limb of chronic constriction injury (CCI) rats, placed the rat in a special cage surrounded by a coil, and measured spontaneous pain-related limb movements by detecting changes in the electromagnetic field.

In this study, we hypothesized that the transplantation of ADMSC would improve not only stimulus-induced pain but also spontaneous pain. To verify this hypothesis, we transplanted syngeneic ADMSC into the lesions of CCI model rats and assessed the effectiveness of the treatment by measuring the number of spontaneous pain-related

Abstract

Background: Several studies have investigated the efficacy of transplanting adipose-derived mesenchymal stem cells (ADMSC) in the treatment of neuropathic pain in animals. However, these studies evaluated the effects of transplantation by measuring stimulus-induced pain but not spontaneous pain. Evaluation of spontaneous pain is essential for assessing neuropathic pain in clinical practice, using such measures as the visual analogue scale (VAS). Therefore, spontaneous pain should be evaluated even at the animal experiments stage to verify the efficacy of ADMSC transplantation for treating neuropathic pain. Here, we verify whether ADMSC transplantation improves spontaneous pain in a rat model of neuropathic pain induced by chronic constriction injury of the sciatic nerve.

Methods: ADMSC were isolated from rat adipose tissue and propagated in culture. One week after CCI model rats were generated, ADMSC were transplanted into the epineurium of the area of nerve damage. The effects of transplantation were evaluated by automatically measuring the number of spontaneous pain-related behaviors and quantifying the degree of mechanical allodynia using the von-Frey filament test. A total of 20 F344 rats were used in these experiments.

Results: ADMSC transplantation significantly reduced the number of spontaneous pain-related behaviors and significantly alleviated mechanical allodynia from 21 and 7 days after transplantation, respectively. No animals died during the experiments, and all animals gained weight over the course of the study.

Conclusion: ADMSC transplantation may be an effective treatment for neuropathic pain in clinical practice.
behaviors, using the method described by Kawasaki et al, and the von-Frey filament test.

Materials and Methods

The research protocol was approved by the Animal Research Committee of Dokkyo Medical University (Animal Research Approval Number 1017). Male 9-week-old 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°C and a humidity of 50 ± 10% with a 12-hour light/dark cycle. All animals were weighed once a week and checked for abnormalities. The rats were acclimated in the cages for at least 1 week before the start of the experiments.

A block of adipose tissue was removed from F344 rats that had not undergone the CCI operation. ADMSC were isolated from the adipose tissue and passaged 2 to 4 times to prepare for transplantation. F344 rats were randomly assigned to 2 groups: The CCI surgery group and the control group. CCI surgery was performed on 10 rats according to a previously described method by Bennett and Xie [12]. Another 10 rats injected with phosphate buffered saline (PBS) comprised the control group. One week after the CCI operation, ADMSC were transplanted into the epineurium of the lesions. Pain was evaluated using the 2 methods described below immediately before ADMSC transplantation and then weekly until 6 weeks after transplantation. All experiments were performed between 10:00 to 20:00. The details of each of these steps are provided below.

Isolation and culture of ADMSC

Adipose tissue was removed from the inguinal and abdominal regions of male 9-week-old F344 rats. The tissue was thoroughly washed with PBS and blood vessels and connective tissue were removed. The tissue was subsequently minced and added to a solution containing 0.1% collagenase type 1 (Sigma, St. Louis, MO, USA) and 0.2% dispase (Sigma). The solution was mixed by shaking at 37°C for 60 minutes. Once the tissue was digested, solids were removed by filtering through a 100 µm filter. The filtrate was centrifuged at 1200 rpm for 5 minutes. The resulting cell pellet was washed with PBS and centrifuged again. A cell suspension containing the isolated ADMSC was cultured in basic medium (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum and 2% penicillin-streptomycin at 37°C and 5% CO₂. Cells passaged 2 to 4 times were used as ADMSC for transplantation.

CCI model rats

The CCI operation was performed according to a previously described method by Bennett and Xie [12]. All animals were anesthetized with isoflurane. The fascia between the gluteal muscles and biceps femoris muscle was opened to expose the left sciatic nerve. Four loosely constrictive ligatures of 4/0 chromic gut were tied around the sciatic nerve at 1 mm intervals near the trifurcation. The sciatic nerve at 1 mm intervals near the trifurcation. The sciatic nerve was subsequently minced and added to a solution containing 0.1% collagenase type 1 (Sigma, St. Louis, MO, USA) and 0.2% dispase (Sigma). The solution was mixed by shaking at 37°C for 60 minutes. Once the tissue was digested, solids were removed by filtering through a 100 µm filter. The filtrate was centrifuged at 1200 rpm for 5 minutes. The resulting cell pellet was washed with PBS and centrifuged again. A cell suspension containing the isolated ADMSC was cultured in basic medium (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum and 2% penicillin-streptomycin at 37°C and 5% CO₂. Cells passaged 2 to 4 times were used as ADMSC for transplantation.

Magnet implantation

Magnets were implanted in parallel with the CCI operation. The dorsal skin of the foot of the operated limb was cut open, and a Tellon-coated columnar magnet (1 mm in diameter, 3 mm long; SCT-MAG-TF, Neuroscience Inc., Tokyo, Japan) was implanted under the skin.

ADMSC transplantation

ADMSC were transplanted 7 days after the CCI operation. All animals were anesthetized with isoflurane. To avoid additional nerve damage, the wound from the previous operation was carefully reopened to expose the sciatic nerve. Under microscopic guidance, 1.0 × 10⁶ cells suspended in 50 µL of PBS were transplanted into the epiomneum using 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.

Measurement of spontaneous pain by automatically measuring limb movements including spontaneous pain-related behaviors

A test chamber surrounded by a coil (NS-SCT10R, Neuroscience Inc., Tokyo, Japan) was installed in a dim room at constant temperature (23 ± 2°C). An animal was placed into the chamber and allowed to adapt to the environment for 5 minutes before starting the measurements. Measurements were conducted for 30 minutes per day. CCI model rats displayed abnormal behaviors related to spontaneous pain including lifting and licking the diseased limb, which moved the magnet implanted in the dorsum of the foot. Changes in the electromagnetic field caused by the movement of the magnet 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 following were set as analytical parameters for waveforms formed by movement of the limbs: range of frequency 2.5 to 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 a previously described method by Chaplan et al. [13]. An animal was placed on a metal mesh and in a plastic box, and was adapted to the environment for 30 minutes. A filament was applied perpendicular to the sole of the foot of the diseased limb with pressure such as to cause the filament to bend slightly. If the diseased limb displayed an escape response, this was regarded as a positive response. The up-down method uses 8 filaments (0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0, and 15.0 g). The 2.0 g filament was used first. When a positive response was observed, the next filament of weaker force was used for stimulation; if no response was observed, the next filament of stronger force 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 continuously applied using the up-down method. The 50% reaction threshold was calculated using the reaction pattern from a total of 6 stimulations. When 15.0 g stimulation did not induce a positive reaction, 15.0 g was regarded as the threshold value; when 0.4 g stimulation induced a positive reaction, 0.25 g was regarded as the threshold value.
Statistical analysis

The number of limb movements including abnormal behaviors in the measurement of spontaneous pain 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.

Results

CCI model rats displayed “normal movements” associated with walking and “abnormal movements” including lifting/guarding, flinching/shaking, and licking. No animals died during the experiments. All animals gained weight over time even after the CCI operation and ADMSC transplantation.

Measurement of spontaneous pain

The number of limb movements observed in the ADMSC transplantation group was 344 ± 53 immediately before transplantation, 160 ± 35 at 7 days after transplantation, 147 ± 29 at 14 days after transplantation, 80 ± 15 at 21 days after transplantation, 97 ± 15 at 28 days after transplantation, and 99 ± 25 at 42 days after transplantation. The number of limb movements observed in the control group was 354 ± 36 immediately before transplantation, 234 ± 52 at 7 days after transplantation, 210 ± 42 at 14 days after transplantation, 143 ± 24 at 21 days after transplantation, 197 ± 30 at 28 days after transplantation, 190 ± 38 at 35 days after transplantation, and 153 ± 23 at 42 days after transplantation (Figure 1). Split-plot analysis of variance showed no interaction between measurement time and group but showed a significant difference in the number of limb movements between the ADMSC transplantation and control groups (p=0.036), suggesting that significant differences were observed between control and transplantation groups irrespective of the post-transplantation time. Unpaired t-test was performed as a post-hoc test. This analysis showed significant differences between the ADMSC transplantation and control groups at 21 days (p=0.036), 28 days (p=0.008), and 35 days after transplantation (p=0.031). These results suggest that ADMSC transplantation reduced the number of spontaneous pain-related behaviors from 21 days after transplantation.

Quantification of the degree of mechanical allodynia

The reaction threshold in the ADMSC transplantation group was 3.70 ± 0.62 g immediately before transplantation, 9.77 ± 1.60 g at 7 days after transplantation, 8.45 ± 1.39 g at 14 days after transplantation, 9.34 ± 1.31 g at 21 days after transplantation, 12.24 ± 1.41 g at 28 days after transplantation, 11.74 ± 1.47 g at 35 days after transplantation, and 11.65 ± 1.77 g at 42 days after transplantation. The reaction threshold in the control group was 4.19 ± 0.47 g immediately before transplantation, 3.97 ± 0.63 g at 7 days after transplantation, 3.29 ± 0.39 g at 14 days after transplantation, 2.72 ± 0.45 g at 21 days after transplantation, 2.41 ± 0.28 g at 28 days after transplantation, 2.23 ± 0.18 g at 35 days after transplantation, and 3.11 ± 0.26 g at 42 days after transplantation (Figure 2). Split-plot analysis of variance demonstrated an interaction between measurement time and transplantation group. Unpaired t-test as a post hoc test detected significant differences at 7 days (p=0.006), 14 days (p=0.005), 21 days (p=0.001), 28 days (p=0.00005), 35 days (p=0.0001), and 42 days after transplantation (p=0.001). These results suggest that ADMSC transplantation alleviated mechanical allodynia from 7 days after transplantation.

Discussion

Evidence from several studies has suggested that ADMSC transplantation improves neuropathic pain in animal models [14-17]. However, these studies evaluated the effect of transplantation by measuring stimulus-induced pain but not spontaneous pain. Measurement of stimulus-induced pain is widely used to evaluate pain in rats. In contrast, measurement of spontaneous pain using pain scores such as the visual analogue scale (VAS) is essential for evaluating neuropathic pain in clinical practice. Moreover, Murai et al. noted differences in the effects of drugs on induced reaction and spontaneous pain-related behaviors in rats [18], and suggested that there was a discrepancy between the mechanically-induced scale for reactive pain and that for neuropathic, spontaneous pain-related behaviors.
behaviors. Therefore, we think that to verify the efficacy of ADMSC transplantation against neuropathic pain in clinical practice, it is pertinent to evaluate its efficacy against spontaneous pain, even at the animal experiments stage.

We observed an improvement in stimulus-induced pain by the first week after ADMSC transplantation. This result is similar to that of previous studies in which ADMSC were locally transplanted [15,16]. Spontaneous pain-related behaviors, however, were significantly improved from the third week after transplantation, suggesting that different mechanisms contribute to stimulus-induced pain and spontaneous pain. The mechanisms underlying how ADMSC transplantation improves neuropathic pain are currently unknown. A previous study confirmed that ADMSC administered into the subarachnoid space of CCI model rats engrafted onto the surface of the spinal cord and dorsal root ganglia [16]. These engrafted ADMSC can directly modulate immune cells, which play important roles in nociception and tissue regeneration, and inflammatory reactions. Another mechanism by which MSC may modulate inflammatory and immune processes is through paracrine release of soluble factors such as interleukin-10 (IL-10), leukemia inhibitory factor (LIF), and transforming growth factor-β (TGF-β) [6]. In addition to these mechanisms, MSC is known to induce antihyperalgesic effects through the activation of the endogenous opioid system. It has been 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 is predicted to underlie the antihyperalgesic effects in the late stages of MSC transplantation [5].

Morphine is known to exert its analgesic effects through opioid receptor agonism. While the efficacy of morphine in the treatment of neuropathic pain had been controversial, opioids have recently been used in the management of patients with neuropathic pain [19]. In CCI model rats, morphine reduces the number of spontaneous pain-related behaviors and alleviates mechanical allodynia at a s.c. dose of 3 mg/kg or greater [18]. However, clinical use of high-dose morphine carries a high risk for adverse events including nausea, constipation, sleepiness, sedation, and respiratory depression. Therefore, given that both MSC transplantation and morphine improve neuropathic pain through modulation of the opioid system, our finding that MSC transplantation reduced the number of spontaneous pain-related behaviors in CCI model rats suggests that ADMSC transplantation may be a more effective therapeutic approach for treating neuropathic pain.

Although the safety of autologous MSC transplantation is controversial, the number of clinical studies using autologous MSC transplantation is increasing [20]. Therefore, safety standards for MSC transplantation techniques are warranted to ensure the safe practice of such methods in clinical practice.

All clinical studies using transplantation of autologous ADMSC in the treatment of neuropathic pain [21-25] have demonstrated improvements in spontaneous pain and reported no major complications. However, these studies transplanted the stromal vascular fraction (SVF) and autologous fat instead of ADMSC propagated in culture. We propose that transplantation of ADMSC propagated in culture is more effective for improving pain, although further studies are required to confirm this. This study has some limitations. First, we did not examine the effects of intravenous administration of ADMSC, which has been reported in other studies [14,16,17] to be effective for inhibiting pain. Second, while we verified the inhibitory effect of ADMSC transplantation on pain behaviors, we did not examine the histological or biochemical changes. This study, however, demonstrated that ADMSC transplantation reduced the number of neuropathic spontaneous pain-related behaviors in a CCI rat model. This new evidence helps to resolve the inconsistencies in methods used to evaluate pain between animal experiments and clinical practice, and further confirms the potential of ADMSC transplantation as an effective treatment for neuropathic pain.

Conclusion

We evaluated the effects of ADMSC transplantation on spontaneous and induced pain in CCI model rats. Spontaneous pain was evaluated by automatically measuring the number of spontaneous pain-related behaviors, and stimulus-induced pain was evaluated using the von-Frey filament test. ADMSC transplantation improved spontaneous pain from 21 days, and stimulus-induced pain from 7 days after transplantation. These effects lasted up to at least 6 weeks after transplantation. Our findings suggest that ADMSC transplantation may be an effective and safe treatment for neuropathic pain in clinical practice.

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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.

References


