*Cytotherapy*, 2017; 19: 839–848

International Society for Cellular Therapy
ISCT®



# Intravenous infusion of adipose-derived stem/stromal cells improves functional recovery of rats with spinal cord injury

YUKI OHTA[1], AKEMI HAMAGUCHI[2], MASANORI OOTAKI[1], MINORU WATANABE[3], YUKO TAKEBA[1], TAROH IIRI[1], NAOKI MATSUMOTO[1] & MITSUKO TAKENAGA[2]

[1]*Department of Pharmacology, St. Marianna University School of Medicine, Kawasaki, Kanagawa, Japan,* [2]*Institute of Medical Science, St. Marianna University Graduate School of Medicine, Kawasaki, Kanagawa, Japan, and* [3]*Institute for Animal Experimentation, St. Marianna University Graduate School of Medicine, Kawasaki, Kanagawa, Japan*

**Abstract**

*Background aims.* Adipose tissue has therapeutic potential for spinal cord injury (SCI) because it contains multipotent cells known as adipose-derived stem/stromal cells (ASCs). In this study, we attempted intravenous ASC transplantation in rats with SCI to examine the effect on functional recovery. *Methods.* ASCs ($2.5 \times 10^6$) were intravenously infused into SCI rats, after which hindlimb motor function was evaluated. Distribution of transplanted ASCs was investigated and growth factor/cytokine levels were determined. *Results.* Intravenous transplantation of ASCs promoted the functional recovery in SCI rats and reduced the area of spinal cord cavitation. A distribution study revealed that ASCs gradually accumulated at the site of injury, but long-term survival of these cells was not achieved. Levels of growth factors increased only slightly in the spinal cord after ASC transplantation. Unexpectedly, cytokine-induced neutrophil chemoattractant (CINC)-1 showed a transient but substantial increase in the spinal cord tissue and blood of the ASC group. CINC-1 was secreted by ASCs *in vitro*, and the sponge implantation assay showed that CINC-1 and ASCs induced angiogenesis. CINC-1 promoted functional recovery in SCI rats, which was similar to the ASCs. Expression of glial cell line-derived neurotrophic factor was greater in the ASC group than in the CINC-1 group, although both promoted extracellular signal-regulated kinase (ERK)1/2 phosphorylation; Akt phosphorylation was enhanced in the spinal cord after ASC transplantation. *Conclusions.* Our findings indicated that intravenously transplanted ASCs gradually accumulated in the injured spinal cord, where cytokines such as CINC-1 activated ERK1/2 and Akt, leading to functional recovery.

**Key Words:** *adipose-derived stem/stromal cells, cytokine-induced neutrophil chemoattractant-1, intravenous infusion, spinal cord injury*

## Introduction

Spinal cord injury (SCI) can result in partial or complete paralysis and disability. Currently, there is no effective treatment for SCI. Cell transplantation is being explored as a strategy to promote tissue regeneration, and a variety of cells, including embryonic stem cells, have been tested for their therapeutic potential for SCI [1–4]. However, there are some limitations to their clinical application, which include ethical concerns, problems with immunogenicity and safety issues related to the potential for tumorigenicity [5]. Somatic stem cells, such as bone marrow–derived mesenchymal stromal cells (BM-MSCs), may offset these problems because they are capable of self-renewal and can differentiate into mesodermal lineages as well as neuronal and glial cells [6–8]. MSCs (variously referred to as mesenchymal stromal cells or multipotent stromal cells) have been found in many tissues other than BM [9–11]. Adipose tissues are an attractive source of MSCs because of their ubiquitous distribution, accessibility and the ease with which they can be harvested. Adipose tissue–derived MSCs, called adipose-derived stem/stromal cells (ASCs), are obtained from adherent cultures of the stromal-vascular fraction separated from fatty tissues [9]. ASCs and BM-MSCs have similar characteristics [12] and can undergo multilineage differentiation into fat, bone, cartilage, smooth muscle, and neuronal, endothelial or hepatic cells [9,13–15]. ASCs also secrete a variety of growth factors, such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), which might mediate regeneration of the injured spinal cord [16]. Furthermore, adipose tissue contains larger

Correspondence: **Yuki Ohta**, PhD, Department of Pharmacology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan. E-mail: yuki-o@marianna-u.ac.jp

*(Received 28 December 2016; accepted 7 April 2017)*

ISSN 1465-3249 
http://dx.doi.org/10.1016/j.jcyt.2017.04.002

populations of MSCs than does BM [17], thus making ASCs a promising target for regenerative medicine.

One important problem is the delivery of stem cells to the site of injury. In most animal experiments as well as in our previous study [4], cell transplantation was performed by direct injection into the damaged parenchyma, providing effective recovery of motor function and spinal cord preservation after SCI [1–4]. However, direct injection into the lesion is too invasive for human SCI. Intravenous administration has been chosen as the method of transplantation in patients with central nervous system disorders [18,19], with its possible recent report for its effectiveness [20].

In this study, we attempted intravenous ASC transplantation in rats with SCI to examine the effect on functional recovery and to investigate the role of ASCs.

## Methods

### *Animals*

Female Sprague-Dawley (SD) (Charles River Laboratories Japan) and green fluorescent protein (GFP) transgenic rats (SD, CAG-EGFP, Japan SLC) were housed in an animal room at a constant temperature (23 ± 1°C) and humidity (50–60%) with a 12-h light/dark cycle and were allowed free access to a standard diet and water. Experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of St. Marianna University Graduate School of Medicine. The experimental protocol was approved by the Animal Research Committee, Institute for Animal Experimentation, St. Marianna University Graduate School of Medicine (approval numbers 1210002 and 1310005).

### *ASCs*

Dorsal fat pads were obtained from 9-week-old rats under anesthesia. The adipose tissue was minced and digested for 1 h in 0.1% collagenase (Thermo Fisher Scientific) at 37°C. The digestion process was stopped by adding Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific) containing 20% fetal bovine serum (Thermo Fisher Scientific) and 1% antibiotic-antimycotic (Thermo Fisher Scientific). The digested suspension was filtered through a 70-μm nylon mesh cell strainer (BD Biosciences) to remove tissue debris and centrifuged at 1000 rpm for 5 min. The pellet (stromal-vascular fraction) was suspended in medium and cultured in a 10-cm culture dish at 37°C in humidified atmosphere 5% $CO_2$ and 95% room air. After 24 h, nonadherent cells were gently removed. Adherent cells reached 80% confluence within 5–6 days (passage 0 [P0]). Confluent cells (ASCs) were detached with 0.25% trypsin/1 mmol/L ethylenediamine tetraacetic acid (EDTA; Thermo Fisher Scientific) and plated in 75-$cm^2$ flasks (P1). The doubling time (DT) was determined by manually counting the number of cells from P1 to P7.

### *Real-time reverse transcriptase-polymerase chain reaction*

Total RNA was extracted from ASCs with TRIzol reagent (Thermo Fisher Scientific). After reverse transcription of 1 μg of total RNA, an aliquot of the cDNA was amplified in a LightCycler system (Roche) with LightCycler FastStart DNA Master SYBR Green I (Roche) according to manufacturer's instructions. Transcript expression of nestin (neural stem cells), βIII tubulin (neurons) and glial fibrillary acidic protein (GFAP; astrocytes) was investigated with hypoxanthine phosphoribosyltransferase as an internal control. Primer sequences were described previously [4].

### *SCI model, intravenous ASC transplantation and functional evaluation*

Spinal cord injury was induced by the weight-drop method [21]. Ten-week-old rats weighing 210–230 g were anesthetized, and dorsal laminectomy was performed at the T10 vertebra. A 10-g weight was then dropped onto the spinal cord from a height of 25 mm. Postoperatively, animals were kept warm on highly absorbent bedding and injected with antibiotics; they received manual bladder expression twice daily until reflex bladder emptying returned.

On day 8 after SCI, the rats were randomly assigned to two groups, and cell transplantation was carried out. ASCs were incubated in growth medium containing 10 ng/mL basic fibroblast growth factor (bFGF; Thermo Fisher Scientific) for 24 h before transplantation. The cells were detached with trypsin/EDTA and resuspended in physiological saline; $2.5 \times 10^6$ cells (in 500 μL) were infused via the tail vein at a rate of 25 μL/min using a stereotaxic microinjector (model 310, Muromachi Kikai). SCI control animals received the same volume (500 μL) of physiological saline alone.

Hindlimb motor function was evaluated in SCI rats by using the Basso-Beattie-Bresnahan (BBB) score, a locomotor rating scale developed by Basso et al. [22]. A score from 0 (complete paralysis) to 21 (normal gait) was recorded before and after SCI.

### *Spinal cord specimens*

Under anesthesia, animals were perfused with physiological saline and then with 4% paraformaldehyde in phosphate-buffered saline (PBS) (Wako Pure Chemical Industries) via intracardiac injection.

For hematoxylin and eosin staining, spinal cord specimens were embedded in paraffin, cut into 4-μm

sections and placed on SUPERFROST MAS-coated glass slides (Matsunami Glass). The area of cavitation was measured by using WinROOF software (Mitani Corporation).

For detection of GFP-expressing cells, spinal cord specimens were fixed by immersion for 1 h, followed by cryoprotection in 10%, 20% and 30% sucrose PBS at 4°C, and finally frozen in Tissue-Tek O.T.C. compound (Sakura FineTechnical). Then 10-μm sections were cut and placed on SUPERFROST MAS-coated glass slides. Fluorescence was acquired under a conventional microscope equipped with a CCD camera (IX70, Olympus).

*Investigation of ASC distribution*

ASCs were labeled with [$^3$H]-thymidine ([methyl, 1′, 2′-$^3$H]thymidine, PerkinElmer Japan). On day 8 after SCI, [$^3$H]-labeled ASCs (1247092 dpm/2.5 × $10^6$ cells) were intravenously infused into rats by the same protocol as mentioned earlier. After 3, 24 and 48 h, a 15-mm spinal cord segment containing the damaged region, blood and target organs were harvested, followed by solubilization with Soluene-350 (PerkinElmer Japan). Aliquots of each solution were then used for the determination of radioactivity with a β counter (LSC-6100, ALOKA).

*Measurement of cytokines*

Medium with or without bFGF (10 ng/mL) was replaced when ASCs grew to approximately 80% confluence. After culture for 24 h, the supernatant was collected for the measurement of cytokine expression and the number of cells was counted with a hemocytometer.

Serum or plasma (EDTA) was prepared from whole blood collected under anesthesia. Spinal cord (15 mm length) lysate was prepared in buffer containing 20 mmol/L tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl; pH 7.5), 2 mol/L NaCl, 0.1% Tween-80, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich), and 1 mmol/L EDTA, allowing them to swell on ice before centrifugation at 15000 rpm for 30 min at 4°C.

Levels of HGF (Institute of Immunology), VEGF (Immuno-Biological Laboratories), and cytokine-induced neutrophil chemoattractant (CINC)-1 (Immuno-Biological Laboratories) were measured with enzyme-linked immunosorbent assay kits according to manufacturer's instructions.

*Cytokine array analysis*

A RayBio rat cytokine antibody array (RayBiotech) was used to assess cytokine expression in the spinal cord lysate and serum according to the manufacturer's instructions. Signals were directly detected with a chemiluminescence imaging system.

*Sponge implantation*

Sterilized 8-mm sponges (Astellas Pharma) were subcutaneously implanted into the backs of normal rats under anesthesia. The sponges were infused with 100 μL of ASCs (2.5 × $10^6$ cells), CINC-1 (R&D Systems), or saline just before implantation. Four days later, each sponge was harvested for measurement of the hemoglobin content with a hemoglobin determination kit (Wako).

*Comparison of ASCs and CINC-1 in SCI rats*

On day 8 after SCI, the rats were randomly assigned to three groups, and treatment of ASCs (2.5 × $10^6$ cells in 500 μL saline), CINC-1 (5 μg/kg in 500 μL saline) or saline (500 μL) was carried out. Hindlimb motor function was evaluated by using the BBB score.

*Western blot analysis*

Spinal cord lysate was prepared in buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1(v/v)% Triton X-100, 5 mmol/L EDTA, 1 mmol/L PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, Phosphatase Inhibitor Cocktail Set II (Calbiochem, Merck KGaA) or the same buffer as described earlier.

Aliquots of lysate (20–70 μg) were separated by electrophoresis on 5–20% gradient SDS polyacrylamide gels. The protein concentration was determined by using a BCA protein assay kit (Thermo Fisher Scientific). Proteins were transferred to polyvinylidene difluoride membranes (Clearblot P, ATTO Corporation) and incubated with anti-Akt (pan) (C67E7; rabbit, Cell Signaling Technology), phosphor-Akt (Thr308) (C31E5E; rabbit, Cell Signaling), p44/42 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK)1/2; rabbit, Cell Signaling), phosphor-p44/42 MAPK (Thr202/Thr204; rabbit, Cell Signaling), glial cell line–derived neurotrophic factor (GDNF) (D-20; sc-328, rabbit, Santa Cruz Biotechnology), GFAP (clone G-A-5; mouse, Sigma-Aldrich), myelin basic protein (MBP) (D-18; sc-13912, goat; Santa Cruz Biotechnology), or anti-β-actin (clone AC-15; mouse, Sigma-Aldrich). The membranes were then incubated with electrochemiluminescence (ECL) horseradish peroxidase (HRP)-labeled anti-goat immunoglobulin (Ig)G (sc-2020; Santa Cruz Biotechnology), HRP-labeled anti-mouse IgG (GE Healthcare UK), or HRP-labeled anti-rabbit IgG (GE Healthcare UK). Finally, each membrane was incubated with ECL Prime Western blotting detection system (GE Healthcare UK) and exposed to a Polaroid