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Mesenchymal stem cells transmigrate across brain microvascular endothelial cell monolayers through transiently formed inter-endothelial gaps

Takashi Matsushita[a,b,1], Tatsuya Kibayashi[b,1], Takahiro Katayama[b], Yuuki Yamashita[b], Syuuichirou Suzuki[a], Jun Kawamata[a], Osamu Honmou[c], Masabumi Minami[b], Shun Shimohama[a,*]

[a] Department of Neurology, School of Medicine, Sapporo Medical University, South 1, West 16, Chuo-ku, Sapporo 060-8543, Japan
[b] Department of Pharmacology, Graduate School of Pharmaceutical Sciences, Hokkaido University, North 12, West 6, Kita-ku, Sapporo 060-0812, Japan
[c] Department of Neural Repair and Therapeutics, School of Medicine, Sapporo Medical University, South 1, West 16, Chuo-ku, Sapporo 060-8543, Japan

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ABSTRACT

Mesenchymal stem cells (MSCs) hold much promise for cell therapy for neurological diseases such as cerebral ischemia and Parkinson's disease. Intravenously administered MSCs accumulate in lesions within the brain parenchyma, but little is known of the details of MSC transmigration across the blood–brain barrier (BBB). To study MSC transmigration across the BBB, we developed an *in vitro* culture system consisting of rat brain microvascular endothelial cells (BMECs) and bone marrow-derived MSCs using Transwell or Millicell culture inserts. Using this system, we first investigated the influence of the number of MSCs added to the upper chamber on BMEC barrier integrity. The addition of MSCs at a density of 1.5×10^5 cells/cm^2 led to disruption of the BMEC monolayer structure and decreased barrier function as measured by the transendothelial electrical resistance (TEER). When applied at a density of 1.5×10^4 cells/cm^2, neither remarkable disruption of the BMEC monolayers nor a significant decrease in TEER was observed until at least 12 h. After cultivation for 24 h under this condition, MSCs were found in the subendothelial space or beneath the insert membrane, suggesting that MSCs transmigrate across BMEC monolayers. Time-lapse imaging revealed that MSCs transmigrated across the BMEC monolayers through transiently formed intercellular gaps between the BMECs. These results show that our *in vitro* culture system consisting of BMECs and MSCs is useful for investigating the molecular and cellular mechanisms underlying MSC transmigration across the BBB.

Mesenchymal stem cells (MSCs) were originally reported as fibroblastoid cells with clonogenic potential *in vitro* by Friedenstein et al. [9]. This cell population can be isolated from bone marrow cells by adhesion to the culture dishes. MSCs have a potential to differentiate into mesenchymal lineage cells, including osteocytes and adipocytes. They are also reported to differentiate into cells of other lineages, such as neurons and cardiomyocytes [13,22]. MSCs hold great promise for cell therapy in neurological diseases such as cerebral ischemia and Parkinson's disease because intravenously administrated MSCs accumulate in damaged tissues and ameliorate the disease process [10,18]. The advantages of using MSCs for cell therapy are not only that they are obtainable with relative ease from host tissues such as bone marrow but also that the use of autologous cells enables us to diminish the risk of rejection. In addition, intravenous administration is generally less invasive than local administration into the lesion. The migratory potential toward the damaged tissues is one of the most remarkable features of MSCs. Integrin-mediated adhesion (*e.g.*, vascular cell adhesion molecule-1/very late antigen-4 axis) or matrix metalloproteinase-dependent extracellular matrix degradation are reported to be involved in the transmigration of MSCs across the endothelium in peripheral tissues [8,19], but the detailed mechanisms remain to be elucidated.

The blood–brain barrier (BBB), which is primarily formed by brain microvascular endothelial cells (BMECs), severely restricts the passage of immune cells and molecules into and out of the brain. The strong barrier properties are primary characteristics of brain endothelium and are not seen in any peripheral endothelia. Studies using animal models of cerebral ischemia and prion disease have shown that intravenously injected MSCs migrate into the lesions in the brain [12,18]. However, very little is known of the mechanism of MSC transmigration across the BBB.

Abbreviations: MSC, mesenchymal stem cell; BBB, blood–brain barrier; BMEC, brain microvascular endothelial cell; EGFP, enhanced green fluorescent protein; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; FBS, fetal bovine serum; TEER, transendothelial electrical resistance; TJ, tight junction.

* Corresponding author at: Department of Neurology, School of Medicine, Sapporo Medical University, South 1, West 16, Chuo-ku, Sapporo 060-8543, Japan.
Tel.: +81 11 611 2111; fax: +81 11 622 7668.
E-mail address: shimoha@sapmed.ac.jp (S. Shimohama).

[1] These authors contributed equally to this work.

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Leukocyte recruitment into the brain has been observed in patients and experimental animals with cerebral ischemia or multiple sclerosis [1,17]. *In vitro* BBB models that are composed of BMECs have been widely exploited to elucidate the mechanisms underlying the leukocyte infiltration into the CNS. MSC transmigration across BMEC monolayers has not previously been examined using an *in vitro* model composed of primary BMECs; however, the interactions of MSCs with endothelial cells isolated from peripheral blood vessels have been investigated [19].

In this study, we developed an *in vitro* culture system consisting of rat BMECs expressing the *enhanced green fluorescent protein* (*EGFP*) gene and fluorescently labeled MSCs. Time-lapse imaging using this *in vitro* culture system revealed that MSCs migrate through BMEC monolayers via transiently formed intercellular gaps (the paracellular route).

All of the experimental procedures using animals were performed in accordance with the guidelines of the Japanese Pharmacological Society and the study was approved by the Hokkaido University Animal Experimentation Committee. All rats used in this study were purchased from Japan SLC (Hamamatsu, Japan). BMECs were prepared from 4- to 6-week-old male and female Sprague-Dawley transgenic rats, which express the *EGFP* gene under the control of the cytomegalovirus enhancer and the chicken β-actin promoter [11], according to the method of Calabria et al. [3], with some modifications. After decapitation, the brains were isolated and stored in ice-cold Dulbecco's modified Eagle's medium (DMEM). Each brain was cut into two hemispheres and rolled on filter paper to remove the meninges. The cerebral cortices were separated from the surrounding tissues, minced with scissors, and homogenated gently. The homogenates were suspended in DMEM containing 0.7 mg/mL Type II collagenase (Worthington Biochemical, Lakewood, NJ, USA) and 39 U/mL DNase I (Worthington Biochemical), and then the suspensions were digested with vigorous shaking for 1.25 h at 37 °C. After adding ice-cold DMEM, the suspensions were centrifuged at 1000 × *g* for 8 min at 4 °C. The supernatant was aspirated carefully, and the pellet was resuspended in DMEM containing 20% bovine serum albumin (Sigma, St. Louis, MO, USA), and then centrifuged at 1000 × *g* for 20 min at 4 °C. The pellet was resuspended in DMEM containing 1 mg/mL Collagenase/Dispase (Roche Diagnostics, Mannheim, Germany) and 39 U/mL DNase I, and then further digested with shaking for 1 h at 37 °C. After adding ice-cold DMEM, the suspended cells were centrifuged at 700 × *g* for 10 min at 4 °C. The pellet was resuspended in ice-cold DMEM and layered over a 33% continuous Percoll gradient and then centrifuged at 1000 × *g* for 10 min at 4 °C. The Percoll gradient was prepared by mixing 9 mL of Percoll (GE Healthcare UK, Buckinghamshire, UK) with 1 mL 10 × phosphate-buffered saline (PBS), 1 mL fetal bovine serum (FBS), and 19 mL PBS and then by centrifuging at 30,000 × *g* for 1 h at 4 °C. The layer containing microvessel cells was collected, resuspended in ice-cold DMEM, and centrifuged at 700 × *g* for 10 min at 4 °C. The pellet was resuspended with culture medium and plated on a 60-mm dish coated with Collagen Type IV (Sigma). The culture medium for BMECs was composed of DMEM with 20% heat-inactivated FBS, 1 ng/mL basic fibroblast growth factor (Roche Diagnostics), 100 μg/mL heparin (Sigma), 1.4 μM hydrocortisone (Sigma), and 1 × antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA, USA). The cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere in the presence of 10 μg/mL puromycin (Sigma) for the initial 48 h, and then in the presence of 4 μg/mL puromycin. After 5–6 days of cultivation, the cultures became confluent and the purity of the BMECs exceeded 95%, as determined by immunostaining for the rat BMEC marker RECA1.

BMECs in the 60-mm dish were detached and reseeded at 2.5×10^5 cells/cm^2 on the upper side of a Transwell (pore size 8.0 μm; membrane surface area 0.33 cm^2; Cat No. 3422; Corning, Corning, NY, USA) or Millicell (pore size 8.0 μm; membrane surface area 0.6 cm^2; Cat No. PI8P01250; Millipore, Bedford, MA, USA) membrane coated with Collagen Type IV. The Transwell and Millicell inserts were placed in 24-well plates and 35-mm glass bottom dishes (No.1-S; Matsunami Glass Industries, Kishiwada, Japan), respectively. The respective volume of culture medium was 0.1 and 0.6 mL in the upper and lower chambers for the Transwell inserts, and 0.2 and 2 mL in the upper and lower chambers for the Millicell inserts. The cultures were maintained for 2 days in culture medium for BMECs in the absence of puromycin.

MSCs were prepared from bone marrow as described previously [14]. Briefly, 4- to 6-week-old male Wistar/ST rats were sacrificed by decapitation. The femurs were dissected free from soft tissue, and the epiphyses were removed with a rongeur. Femur midshaft bone marrow tissue was flushed into culture medium consisting of DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin sulfate, and 2 mM L-glutamine, and the bone marrow cells from both femurs were seeded in a 100-mm culture dish. The next day, the medium was replaced with fresh culture medium to remove non-adherent cells, and then the MSCs were maintained by exchanging with fresh medium at 4-day intervals at 37 °C in a humidified 5% CO_2 atmosphere. MSCs at passage 2–4 were used for the experiments.

The MSCs were fluorescently labeled with PKH26 (Sigma) at least 24 h before the transendothelial migration assay. PKH26-labeled MSCs suspended in serum-free DMEM were added to the upper chamber of the BMECs precultured Transwell inserts at densities of 1.5×10^4, 3.0×10^4, or 1.5×10^5 cells/cm^2 concurrently with the medium change. The medium in the lower chamber was exchanged with fresh culture medium for MSCs. The cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. During the migration assay, MSCs and BMECs were observed with an inverted fluorescence microscope (IX-70; Olympus, Tokyo, Japan) before and 6, 12, and 24 h after adding the MSCs. Images of both cells with the focal point adjusted for the BMEC monolayers were obtained using a cooled charge-coupled device camera (VB-6010; KEYENCE, Osaka, Japan). The transendothelial electrical resistance (TEER) was measured using a Millicell-ERS volt-ohm meter (Millipore) equipped with Endohm-6 chamber electrodes (World Precision Instruments, Berlin, Germany). The TEER was measured before and 3, 6, 9, 12, and 24 h after adding the MSCs. The TEER ($\Omega \times$ cm^2) was calculated by subtracting the resistance of a blank membrane from the measured resistance and then multiplying this by the membrane surface area.

Following the 24-h migration assay, the cultures were fixed with 4% paraformaldehyde in PBS containing 4% sucrose for 30 min at 4 °C. The fixed cultures were rinsed with PBS, mounted on glass slides and coverslipped with VECTASHIELD (Vector Laboratories, Burlingame, CA, USA). To analyze the localization of MSCs, a confocal fluorescence microscope (A1R; Nikon, Tokyo, Japan) equipped with a Plan Fluor VC 60 × water-immersion objective lens (NA 1.2) was used. Thirty serial sections in the *z*-plane were acquired at 0.8-μm intervals and images of the *xz*- and *yz*-planes were created. Numbers of MSCs at three different localizations (MSCs on BMECs, in the subendothelial space, and beneath the Transwell membrane) were counted in an area of 1 mm^2 and the data were acquired as the mean ± SEM from three independent experiments. In addition, some samples were embedded in 4% carboxymethylcellulose gels, frozen with dry ice powder. To obtain cross-sectional images of the cultures, 20-μm-thick frozen sections were prepared by cutting perpendicularly to the insert membrane using a cryostat and mounted on microscope slides.

Time-lapse imaging was performed under a Nikon A1R confocal fluorescence microscope equipped with an S Plan Fluor 40× objective lens (NA 0.6) with an extra-long working distance (2.8–3.6 mm). Two days after plating the BMECs on the Milli-