Cytotherapy, 2016; 18: 229–241

International Society for Cellular Therapy
ISCT®

Neurosphere formation enhances the neurogenic differentiation potential and migratory ability of umbilical cord-mesenchymal stromal cells

TAKEO MUKAI[1,2], TOKIKO NAGAMURA-INOUE[2], TAKAHISA SHIMAZU[2], YUKA MORI[2], ATSUKO TAKAHASHI[2], HAJIME TSUNODA[3], SATORU YAMAGUCHI[4] & ARINOBU TOJO[1,2]

[1]Division of Molecular of Therapy, Center for Advanced Medical Research, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [2]Department of Cell Processing and Transfusion, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [3]Department of Obstetrics, NTT Medical Center Tokyo Hospital, Tokyo, Japan, and [4]Department of Obstetrics, Yamaguchi Hospital, Chiba, Japan

Abstract

Background aims. The human umbilical cord (UC) is a rich source of mesenchymal stromal cells (MSCs), which have been reported to have multi-lineage potential. The objectives of this study were to investigate the characteristics and capacity of UC-MSC neurosphere formation and whether this event enhances the propensity of UC-MSCs to undergo neural differentiation. *Methods.* UC-MSCs were collected by the improved explant method. UC-MSCs and neurosphere-forming UC-MSCs (UC-MSC-neurospheres) were induced to undergo neurogenic differentiation, the latter of which were induced by suspension culturing in the presence of epidermal growth factor and basic fibroblast growth factor. The differentiation and migratory capacities of the individual cultures were then compared on the basis of the expression of neural markers, as measured by immunocytochemistry, immunoblotting and quantitative real-time polymerase chain reaction and transwell assays, respectively. *Results.* Both UC-MSCs and UC-MSC-neurospheres were capable of differentiating into neurogenic cells when cultured in neurogenic differentiation medium. However, pre-conditioned UC-MSC-neurospheres exhibited significantly higher expression of neural markers—including microtubule-associated protein (*MAP2*), *MUSASHI1*, glial fibrillary acidic protein (*GFAP*), and *NESTIN*—compared with those derived from UC-MSCs directly. Moreover, UC-MSC-neurospheres expressed significantly higher levels of the stemness markers *NANOG*, *KLF4* and *OCT4* than did UC-MSCs. Migration assays also revealed that both UC-MSCs and UC-MSC-neurospheres actively migrate toward glucose-depleted cells. *Conclusions.* Neurogenic differentiation potential probably is greater in UC-MSC-neurospheres than in UC-MSCs. Thus, UC-MSC-neurospheres may serve as a better source of cells for neurogenic regenerative medicine.

Key Words: *mesenchymal stromal cell, neural differentiation, neurosphere, umbilical cord*

Introduction

Mesenchymal stromal cells (MSCs)—also known as mesenchymal stem cells—can be harvested from several sources, including the bone marrow (BM), cord blood (CB), umbilical cord (UC), placenta, and adipose tissue [1–5]. MSCs are reported have self-renewal capacity, multi-lineage differentiation potential and the ability to migrate toward sites of inflammation or injury [6,7]. This potential for transdifferentiating into not only the mesoderm lineage but also the endoderm and ectoderm, including the neural lineage, has raised the possibility of using MSCs in regenerative medicine to treat various neurological disorders [8]. Several studies have been conducted to assess whether MSC therapy can protect against neuronal injury such as cerebral ischemia and traumatic brain injury [9,10]. MSC neuronal differentiation protocols have been under investigation for several years, and many methods for neural differentiation have been reported, and include the use of chemical compounds, trophic factors, neurosphere formation, co-culture systems and genetic manipulation, among others [11,12]. Of these protocols, the neurosphere culture system remains the most frequently adopted method to enrich and expand neural stem cells (NSCs) [13].

There have been a few reports on neurospheres formed of MSCs isolated from Wharton's jelly (WJ)

Correspondence: **Tokiko Nagamura-Inoue**, MD, PhD, Department of Cell Processing and Transfusion, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. E-mail: tokikoni@ims.u-tokyo.ac.jp

(Received 9 May 2015; accepted 24 October 2015)

http://dx.doi.org/10.1016/j.jcyt.2015.10.012

within the UC, the main component of umbilical cord extracellular matrix [12,14]. However, the significance of neurosphere formation of MSCs derived from whole UC—comprised of an umbilical artery, two veins and WJ—has not yet been examined. Additionally, whether UC-MSC-neurospheres and their dissociated counterparts retain the ability to migrate to injured, glucose-depleted neurogenic cells remains unknown. In this study, we characterized the cellular functions of UC-MSCs and UC-MSC-neurospheres—both intact and dissociated, with respect to their propensity to undergo neurogenic differentiation and migrate toward injured cells.

Methods

Improved explant method for isolation and expansion of UC-MSCs

The present study was approved by the Ethics Committee of the Institute of Medical Science, University of Tokyo, and the NTT Medical Center Hospital, Japan. UCs were collected after informed consent was obtained from pregnant women planning to undergo cesarean sections. The fresh and frozen-thawed UC tissues were minced into 1- to 2-mm^3 fragments with Cellamigo (Tsubakimoto Co) for improved explant isolation [15]. Tissue fragments were cultured with α-minimal essential medium (αMEM; Wako Pure Chemical Industries, Ltd) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich) and antibiotics-antimycotics (Antibiotic-Antimycotic, 100×; Life Technologies) at 37°C with 5% CO_2. The fibroblast-like adherent cells that migrated from the tissue fragments were harvested with the use of 0.05 wt/vol% Trypsin-5.3 mmol/L ethylene diamine tetraacetic acid/4Na Solution (Wako Pure Chemical Industries) and the fragments removed by filtering with a 100-μm cell strainer (BD Falcon) [16]. These cells were defined as passage 1 (P1) cells. The harvested P1 cells were counted with the use of trypan blue staining and plated at a density of 2.5×10^5 cells per 10-cm dish (BD Falcon) in αMEM supplemented with 10% FBS and antibiotics-antimycotics until passage 3 (P3), when cells were used for UC-MSC experimental analyses. UC-MSCs used in each experiment were derived from the same umbilical cord donor, and the experiments were performed repeatedly with MSCs derived from different umbilical cords.

Neurogenic differentiation of UC-MSCs

UC-MSCs were plated at a density of 4×10^3 cells/cm^2 in 24-well plates (BD Falcon) with Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Wako Pure Chemical Industries, Ltd) supplemented with 10% FBS and antibiotics-antimycotics, and cultured until 80% to 90% confluency. MSCs were rinsed with phosphate-buffered saline (PBS), induced with MSC Neurogenic Differentiation Medium (PromoCell GmbH) and incubated for at least 3 days, with the medium being changed every 2 days.

Neurosphere formation and neural differentiation

UC-MSCs were plated at a density of 1×10^4 cells/well in 96-well Nunclon Sphera microplates (Thermo Fisher Scientific Inc), using DMEM/F12 medium (Wako Pure Chemical Industries, Ltd) supplemented with 20 ng/mL of epidermal growth factor (EGF, Sigma-Aldrich), 20 ng/mL of basic fibroblast growth factor (bFGF, Sigma-Aldrich), and antibiotics-antimycotics. Medium was changed every 2 days for 3 to 7 days to generate neurospheres. For neurogenic differentiation, the formed spheres were transferred to 24-well plates (BD Falcon), allowed to adhere, cultured with MSC Neurogenic Differentiation Medium (PromoCell GmbH) and incubated for at least 3 days, with medium changes every 2 days. Some spheres were used for immunostaining or RNA extraction, and the remaining ones were dissociated by use of Accutase (Innovative Cell Technologies, Inc) for cell counting, cell viability testing and migration assays. Briefly, neurospheres were transferred to a 15-mL conical tube and centrifuged at 100 *g* for 1 min. The supernatant was discarded and 1 mL of Accutase was added to the pelleted spheroids and incubated at room temperature for 10 min. Neurospheres were then dissociated by pipetting to form a single-cell suspension before cell quantification by means of trypan blue exclusion assay.

Adipogenic, osteogenic and chondrogenic differentiation

UC-MSCs were plated at a density of 2×10^4 cells/well in 12-well plates and induced to differentiate into adipocytes with culture medium supplemented with 100 μmol/L indomethacin (Sigma-Aldrich), 0.5 mmol/L 3-isobutyl-1-methylxanthine (Sigma-Aldrich) and 10 μg/mL insulin (Sigma-Aldrich) [16]. We performed adipogenic differentiation by treating the UC-MSCs with cocktail medium for 3 weeks, and the medium was replenished every 3 days, followed by staining with oil red O (Sigma-Aldrich) to detect lipids.

To investigate the osteogenic differentiation capacity of UC-MSCs, cells were cultured in 24-well plates in αMEM supplemented with 10% FBS. On the following day, the medium was replaced with osteogenic induction medium containing 10 nmol/L dexamethasone (Sigma-Aldrich), 10 mmol/L β-glycerol phosphate (Sigma-Aldrich), 100 mmol/L ascorbic acid (Sigma-Aldrich) and 50 ng/mL recombinant human bone morphogenic protein 2 (rhBMP2; Peprotech)