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MSCs can be differentially isolated from maternal, middle and fetal segments of the human umbilical cord

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Abstract

Background aims. Human Wharton's jelly–derived mesenchymal stromal cells (hWJMSCs) are possibly the most suitable allogeneic cell source for stromal cell therapy and tissue engineering applications because of their hypo-immunogenic and non-tumorigenic properties, easy availability and minimal ethical concerns. Furthermore, hWJMSCs possess unique properties of both adult mesenchymal stromal cells and embryonic stromal cells. The human umbilical cord (UC) is approximately 50–60 cm long and the existing studies in the literature have not provided information on which segment of the UC was studied. In this study, hWJMSCs derived from three anatomical segments of the UC are compared. *Methods.* Three segments of the whole UC, each 3 cm in length, were identified anatomically as the maternal, middle and fetal segments. The hWJMSCs from the different segments were analyzed via trypan blue exclusion assay to determine the growth kinetics and cell viability, flow cytometry for immunophenotyping and immunofluorescence and reverse transcriptase polymerase chain reaction (RT-PCR) for expression of stromal cell transcriptional factors. Furthermore, the trilineage differentiation potential (osteogenic, adipogenic and chondrogenic) of these cells was also assessed. *Results.* hWJMSCs isolated from the maternal and fetal segments displayed greater viability and possessed a significantly higher proliferation rate compared with cells from the middle segment. Immunophenotyping revealed that hWJMSCs derived from all three segments expressed the MSC markers CD105, CD73, CD90, CD44, CD13 and CD29, as well as HLA-ABC and HLA-DR, but were negative for hematopoietic markers CD14, CD34 and CD45. Analysis of the embryonic markers showed that all three segments expressed Nanog and Oct 3/4, but only the maternal and fetal segments expressed SSEA 4 and TRA-160. Cells from all three segments were able to differentiate into chondrogenic, osteogenic and adipogenic lineages with the middle segments showing much lower differentiation potential compared with the other two segments. *Conclusions.* hWJMSCs derived from the maternal and fetal segments of the UC are a good source of MSCs compared with cells from the middle segment because of their higher proliferation rate and viability. Fetal and maternal segments are the preferred cell source for bone regeneration.

Key Words: *regenerative medicine, stromal cells, tissue engineering, umbilical cord, Wharton's jelly*

Introduction

Mesenchymal stromal cells (MSCs) and tissue engineering have tremendous potential for applications in regenerative medicine and as possible cures for degenerative diseases. Stromal cells are undifferentiated cells that reside within tissues and organs and possess the potential to self-renew through cell division under physiological and in vitro conditions. They can be induced to become tissue or organ specific cells with special functions. In general, human stromal cells are classified as hematopoietic stromal cells (hHSC), adult mesenchymal stromal cells (hMSC), embryonic stromal cells (hESC) and tissue-specific stromal cells, depending on their source and differentiation potential [1]. Human bone marrow is the most common source of MSCs, and it has been widely explored in clinical studies for cell-based therapies and tissue engineering [2,3]. Nevertheless, these cells have limitations. Only a limited number of MSCs can be harvested through an invasive and painful method, and the quality of nucleated cells tends to decrease with age. There is also a possible risk of infection at the site of bone marrow aspiration, especially in patients with diabetes

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mellitus and hypertension. Therefore, an alternative source of MSCs is required for therapeutic applications.

The mucous connective tissue derived from the human umbilical cord (UC), Wharton's jelly, is a unique source of stromal cells because the derived cells exhibit characteristics of both adult hMSC and hESC to a certain extent. Human Wharton's jelly stromal cells (hWJMSC) display the characteristic MSCs CD29 + , CD73 + , CD90 + , CD146 + , CD14– and CD34–) and express ESC markers (SSEA3, SSEA4, TRA-181, OCT-4, SOX2 and Nanog), although at lower levels compared with ESCs [4]. Irrespective of the expression of the ESC markers, they have been shown to be non-tumorigenic [5] and are known to secrete cytokines, hyaluronic acid, collagen, glycosaminoglycans and anti-inflammatory factors in abundance; many of these factors are essential for the formation of cartilage and bone and may have anti-cancer properties [6]. Taken together, hWJMSCs are considered a primitive MSCs population with high plasticity that are flexible for use in tissue engineering and during development [7,8]. In addition, Wharton's jelly is preferred as a source for stromal cells because of its easy availability, painless harvesting and minimal associated ethical issues.

Studies had shown that hWJMSC are less immunogenic compared with MSCs from other sources [1,7,9–11]. A detailed study performed by Troyer and Weiss on the immunological properties of hWJMSC concluded that there was no evidence of immunorejection of allogeneic hWJMSCs by the host, thus making these cells a possible candidate for allogeneic transplant [4]. hWJMSCs can also be stored cryogenically, thawed, revived and expanded for therapeutic use [12].

Many studies have isolated and characterized hWJMSCs from various compartments of the UC such as the perivascular compartment, endothelial compartment, umbilical arteries, umbilical vein, Wharton's jelly, mixed cord and a few non-standardised compartments (amnion, subamnion and intervascular) [12,13]. However, the exact demarcation of compartments and profiling are unclear. Indeed, the normal physiological length of an UC is 50–60 cm, and in previous studies, there has been no mention of which specific segment of the UC was used. In this study, we have, for the first time, divided the whole UC into three distinct anatomic segments: the maternal, middle and fetal segments (Figure 1).

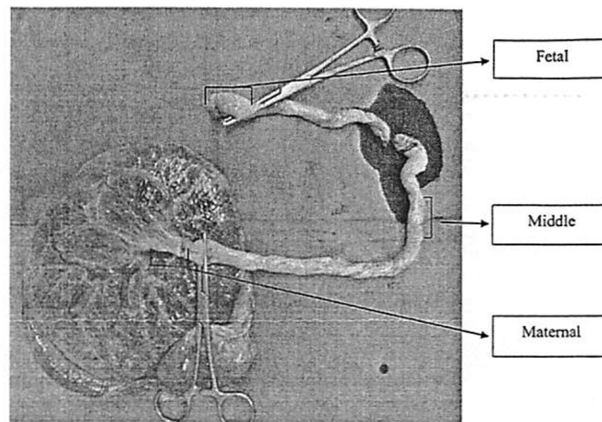


Figure 1. Anatomic division of the human UC into three distinct segments (3 cm in length: maternal, middle and fetal segments).

Methods

hWJMSC isolation and subculture

All samples were collected at the UKM Medical Centre, with informed consent from patients and institutional ethical approval (no. FF-2014-066). All samples were obtained from healthy patients (n = 6) who delivered full term (38–40 weeks) infants by elective cesarean delivery. In the operation theater, the three segments of the same human UC were identified (3 cm from the maternal placenta end, 3 cm of the middle segment and 3 cm from the fetal end). To prevent contamination of hWJMSCs culture, the cord segments were thoroughly rinsed and washed with Dulbecco's phosphate-buffered saline (DPBS; Gibco-Invitrogen) within 3 h of sample collection. The UC samples were dissected with a sterile scalpel after stripping off both umbilical arteries and the vein. Wharton's jelly was then minced into 2 mm^2 pieces and digested with 20 mL of 0.6% (w/v) collagenase type II (Worthington) in Dulbecco's Modified Eagle's Medium (DMEM) low glucose medium + 1% antibiotic-antimycotic in an incubator shaker (250 rpm/h) at 37°C for 1 h. The digested tissue was then centrifuged at 5000 rpm for 5 min. The pelleted cells were suspended in low-glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% Glutamax (Gibco), 1%, vitamin C (Sigma Aldrich), 1% antibiotic-antimycotic (Gibco) and 1% HEPES buffer solution (Gibco) and cultured in six-well plates (Corning).

All cultures were maintained in a CO_2 incubator (GalaxyR) at 5% CO_2 and 37°C. Culture medium was changed every 3 days. When primary cells at passage 0 reached 90% confluency, cells were trypsinized with 0.05% trypsin-ethylenediaminetetraacetic acid (BioWhittaker) and replated.

Growth kinetic analysis

Cells of the three segments were trypsinized at 90% confluency. The percentage of viable cells and the total number of cells were determined by manual cell counting using the trypan blue exclusion method on a hemocytometer. Cells were then reseeded in a T175 tissue culture flask (Corning) with a constant seeding

density of 5000 cells/cm^2 for three passages. The medium was changed every 3 days. The population doubling time, growth rate and percentage of cell viability were determined as follows:

Number of cell doubling = D, where D = Log C/Log 2, and C = no of cells (final)/no of cells (initial);
Population doubling time = Number of days/D; and
Percentage viability = Number of live cells/Total number of cells × 100%.

Immunophenotypic analysis by flow cytometry

hWJMSCs at passage 3 (n = 6) were incubated with conjugated antibodies against CD105, CD34, CD29, CD73, CD44, HLA-ABC, CD14, CD45, CD90 and CD14 (BD Biosciences). Cells were then fixed with 4% paraformaldehyde, kept at 4°C and analyzed within 24 h. Cell analysis was performed using a FACSCalibur (BD Biosciences) and BD CellQuest Pro software (BD Biosciences). For each sample, 1 × 10^4 cells were analyzed.

Quantitative embryonic stromal cell gene expression by reverse transcriptase polymerase chain reaction

RNA extraction was performed using the RNeasy Plus Mini kit (Qiagen, Germany) according to the manufacturer's recommendations. All three segments of hWJMSCs at passage 3 (n = 6) were lysed with RLT Plus Buffer and integrated before passing through the gDNA Eliminator spin column to remove genomic DNA. RNA was precipitated by adding an equal volume of 70% ethanol to the filtered lysate. This was followed by centrifugation though an RNeasy spin column. The RNA pellet was collected and washed with RW1 Buffer and RPE Buffer, then resuspended in 40 μL of RNase-free water. cDNA synthesis was performed. Primers targeting the extracellular matrix and nuclear genes (i.e., Nanog, Sox2, Rex-1 and OCT-4) were designed using Primer 3 software according to the NIH GenBank database. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The analysis was performed using the QuantiTect Reverse Transcription kit (Qiagen). Briefly, the reverse transcription reaction was performed by the addition of Quantiscript Reverse Transcriptase, Quantiscript RT Buffer and RT Primer Mix and incubating at 42°C for 20 min. Subsequently, a PCR mix composed of the Quantified SyBR Green Master mix (Qiagen), forward and reverse primers and RNAase-free water was prepared. Quantitative PCR was performed using a Bio-Rad iCycler PCR machine for 40 cycles with the following program: denaturation at 94°C for 15 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 30 seconds. Melt curves were analyzed to confirm the specificity of the amplified product.

Immunofluorescent staining for embryonic stromal cell markers

hWJMSCs at P1 (n = 6) were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 for 10 min and blocked in 10% goat serum in PBS for 1 h. Cells were incubated with the primary antibody overnight at 4°C followed by washing and incubation with secondary antibodies (i.e., goat anti-mouse AlexaFluor 488 or goat anti-rabbit AlexaFluor 594; 1:150, Sigma), as appropriate, for 1 h at 37°C. The unconjugated primary antibodies were against TRA-1-60, Nanog, SSEA-4, SSEA-3 and Oct 3/4 (1:80, Stromalgent). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 0.1 μg/mL, Sigma). A Nikon A1R confocal laser scanning microscope (Nikon) was used to evaluate stained cells and capture images.

Multilineage differentiation assays

hWJMSCs from the three segments at passage 3 (n = 3) were cultured to be differentiated into adipocytes, chondrocytes and osteocytes for 3 weeks. Adipogenic medium consisted of DMEM/F12 supplemented with 3-isobutyl-3-methylxaanthine (Sigma), dexamethasone (Sigma) and insulin (Sigma). Medium was changed twice weekly. On day 21, intracellular lipid droplets were stained with fresh oil red O. Images were captured using Image Pro software (Nikon). The optical density was measured using a maximum wavelength of 490 nm on a spectrophotometer.

Osteogenic medium consisted of alpha-MEM supplemented with dexamethasone (Sigma), β-glycerol phosphate (Sigma) and ascorbic acid (AsA; Sigma). Osteogenic medium was changed twice weekly. Alizarin red staining was done according to the manufacturer's recommendations. For chondrogenic differentiation, hWJMSCs at passage 3 were trypsinized and centrifuged (n = 3) to form a micromass. The pellet was cultured with medium supplemented with TGF-β3 for 3 weeks. The proteoglycan-rich extracellular matrix of cartilage tissue was detected by safranin O staining. Quantification of the positively stained area was performed using Image Pro software.

Statistical analysis

Data were analyzed using SPSS version 20. Data are presented as mean ± SEM. Descriptive analysis was performed, and one-way analysis of variance (two-tailed) was used to compare means across the maternal, middle and foetal segments. The Bonferroni post hoc

test was used to compare means between groups. A P value <0.05 was considered statistically significant.

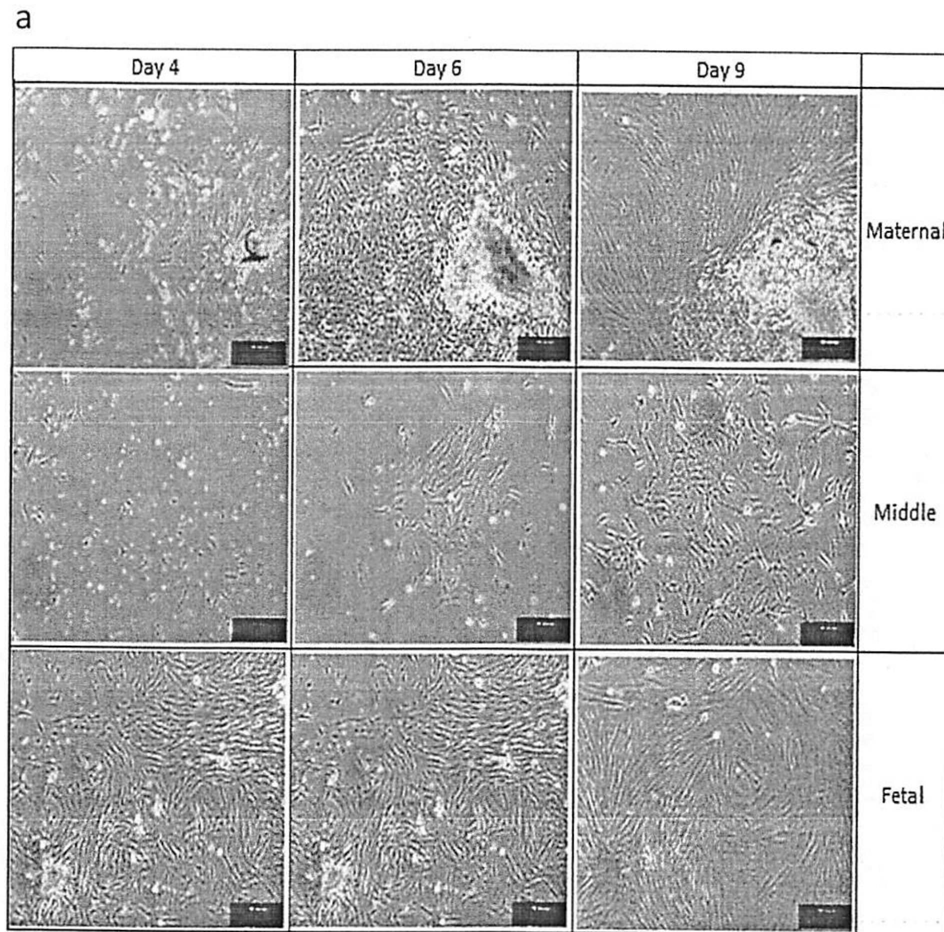
Results

hWJMSC isolation and subculture

The results reported were obtained from eight samples (n = 8). hWJMSCs were successfully isolated from three segments of human UC and cultured for three passages with a constant seeding density of 5000 cells/cm^2 at passages 1 to 3 in T175 culture flasks. Figure 2 shows homogenous, plastic adherent, spindle-shaped cells in monolayer cultures.

Growth kinetic analysis

A remarkable difference in cell attachment and proliferation rate was observed among cells from the three segments. Cells from maternal and fetal segments started to attach the next day while cells from the



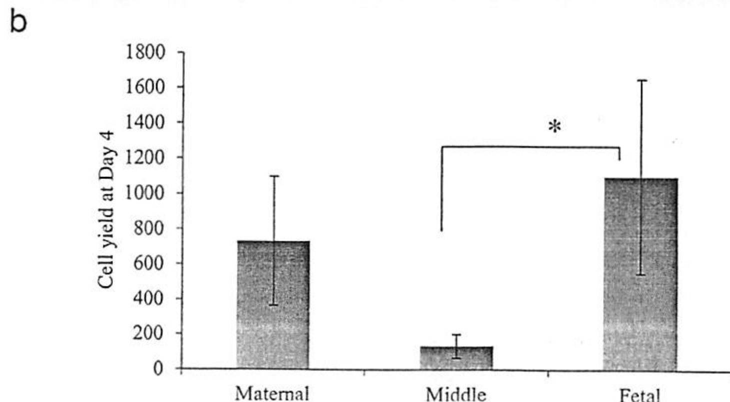


Figure 2. Comparison of hWJMSCs (passage 0) obtained from maternal, middle and fetal segments. (A) Microscopic observation of cultures at day 4, 6 and 9. (B) Cell yield at day 4 culture. * $P < .05$.