Isolation and Culture of Primary Rat Adipose Derived Stem Cells Using Porous Biopolymer Microcarriers

Guang-Zhen Jin^{1,2}, Jeong-Hui Park^{1,2}, Ivan Wall^{1,3*}, Hae-Won Kim^{1,2,4*}

¹Institute of Tissue Regeneration Engineering (ITREN), Dankook University, Cheonan, Korea

²Department of Nanobiomedical Science & BK21 PLUS NBM Global Research Center for Regenerative Medicine, Dankook University, Cheonan, Korea

³Department of Biochemical Engineering, University College London, London, UK

⁴Department of Biomaterials Science, Dankook University Dental College, Cheonan, Korea

Adipose-derived stem cells (ADSCs) are an attractive source of material for mesenchymal stem cell research due to the abundance of adipose and relative ease of access compared with bone marrow. A key consideration for research is whether cell isolation methods can be improved, to reduce the process steps needed to isolate and expand cell material. In the current study, we used macroporous biopolymer microcarriers to isolate primary ADSCs. We found that the method was capable of isolating ADSCs that were subsequently capable of being transferred to culture dishes and expanded *in vitro*. Moreover, flow cytometry revealed that they expressed typical stem cell markers and were capable of undergoing tri-lineage differentiation. In summary, it is feasible to use biopolymer microcarriers for retrieval of viable ADSCs that retain identity markers of stem cell function.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are still a widely studied source of cells for regenerative medicine and are anticipated to be the key cellular component for tissue engineering components of the musculoskeletal system. Whilst bone marrow was traditionally the preferred source of MSCs, an increasing number of studies have reported the benefits of MSC derived from adipose tissue, so called adipose-derived stem cells (ADSCs). ADSCs share the multipotent characteristics reported for bone marrow-derived MSCs and are capable of differentiating into adipocytes, chondrocytes, osteoblasts, myoblasts, and possibly neural cells [1-7]. Therefore, these cells have wide potential application for tissue engineering strategies [8-11].

Retrieving cells from human subjects is an area of constant

consideration due to the need for ethical approval and to convince patients to give informed consent for tissue donation, which typically involves needles, biopsies or even larger excision methods. Therefore thought to which methods yield greatest cell number and best 'quality' cells, yet with minimally invasive tissue retrieval techniques is continually evolving and critical for meeting the wider needs of research and pre-clinical development.

Subcutaneous adipose tissue is a readily available and abundant source of ADSCs. Currently, basic enzymatic digestion is a widely used method for isolation of the ADSCs from adipose tissue biopsy samples obtained by liposuction or excision [12,13]. This procedure can produce high-quality ADSCs but a major disadvantage is the risk of culture contamination because of multi-step handling. Another traditional method is an explant culture [14]. Explant culture actively selects the subset of cells from the biopsy that can migrate out into the culture dish and so confers the advantage of an intrinsic quality control measure. There are still issues associated with sterility during transport of biopsy material and a multistep process using passaging techniques is needed to expand cells that were isolated in this way.

A potential alternative method could use microcarriers as a means to retrieve cells and as a cell expansion tool. Microcar-

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***Corresponding author:** Ivan Wall, Department of Biochemical Engineering, University College London, Gordon Street, London WC1H 0AH, UK.

Tel: 44-207-679-9586, Fax: 44-207-209-0703, E-mail: i.wall@ucl.ac.uk

***Corresponding author:** Hae-Won Kim, Institute of Tissue Regeneration Engineering (ITREN), Dankook University, 119 Dandae-ro, Dongnam-gu, Cheonan 31116, Korea.

Tel: 82-41-550-3081, Fax: 82-41-550-3085, E-mail: kimhw@dku.edu

riers that support cell attachment and growth have enormous potential for achieving scalable cell expansion that is required to meet the clinical demand for cell therapies [15-17]. In our previous study, we cultured successfully rat thoracic aorta-derived endothelial cells using dense PCL microcarriers [18]. In this study we assessed the suitability of macroporous PCL microspheres to culture rat ADSCs to overcome the shortcomings of explant and enzyme digestion methods (Fig. 1).

MATERIALS AND METHODS

Animals

This study was approved by the Animal Care and Use Committee of Dankook University. Three male Sprague-Dawley rats (weighing 450–550 g) were used.

Materials

Polymer and solvents used for the microsphere preparation

Poly(ϵ -caprolactone) (PCL; Sigma, USA, Mw=80000); camphene ($C_{10}H_{16}$, Sigma-Aldrich, USA); chloroform (Sigma-Aldrich, USA); poly(vinyl alcohol) (PVA, Sigma-Aldrich); 70% ethanol

Figure 1. Schematic illustration of the multistep process for the isolation of primary culture of rat adipose-derived stem cells using porous microcarriers.

Reagents used for the primary culture

Dulbecco's phosphate-buffered saline (DPBS; Gibco, USA); 0.25% trypsin/1 mM EDTA (Gibco-BRL, USA), dimethyl sulfoxide (DMSO; Sigma, USA); Dulbecco's modified Eagle's high-glucose medium (DMEM, 4.5 g/L glucose; Gibco-BRL, USA); minimal essential medium (α -MEM; Gibco); fetal bovine serum (FBS; Gibco-BRL, USA); penicillin/streptomycin (Sigma, USA)

Tools used for surgery

Scissors; forceps; needle holders; scalpel blade; sutures

Cell culture vessels

60 mm dishes; T75 flasks, T150 flasks

Imaging devices

Dissecting microscope; Samsung digital camera; inverted microscope (Olympus IX71, Tokyo, Japan); scanning electron microscopy (SEM; Hitachi S-3000H, Japan).

Fabrication of PCL microspheres

The PCL was dissolved in chloroform within a vial at 5 wt.-%. As a porogen, a kind of oligomeric polyester, camphene was dissolved in a vial at 20% (w/v) concentrations. The PCL solution was mixed with the camphene solution while stirring for 4 h. The mixture solution was dropped within a water pool containing 2 wt.-% PVA while stirring at 430 rpm at a temperature of 4°C. The ratio of the mixture solution to the water bath was determined as 30. The mixture solution became solid rapidly on dropping into the ice-cooled bath, and after the solidification, the solution was further stirred for an additional 6 h in order to harden the microspheres and to induce pore channels. The hardened porous microspheres were washed with ice-cooled distilled water five times through filtration with CHM[®] Ashless Filter papers (Weight: 85 g/m², Thickness: 180 μ m). The obtained PCL microspheres were further freeze-dried and kept at 4°C for further characterization and use. The morphology of the microspheres was examined with the SEM at an accelerating voltage of 15–20 kV (Fig. 2).

In vivo implantation of PCL microspheres

Total sixty mg of microspheres were sterilized with 70% ethanol for 2 h and washed with PBS solution three times and then dried under the laminar flow clean bench for further use. The rats were anesthetized by an intramuscular injection using ketamine (80 mg kg⁻¹; Ketara[®], Yuhan Corp., Seoul, Korea) and xylazine (10 mg kg⁻¹; Rompun[®], Bayer Korea Ltd., Seoul, Korea). Small incisions were made on the dorsal skin. Two pouches per animal were created by blunt dissection of subcu-

taneous sites, and thirty mg sterile PCL microspheres were immediately implanted into each pouch. The grafts were harvested 7 days after surgery.

Cell culture procedure

The adipose tissue-microsphere constructs obtained 7 days after surgery were wrapped by a dense fibrous tissue capsule. The specimens had a dimension of $15 \times 10 \times 3$ mm (Fig. 3). They were washed with PBS solution three times and the dense fibrous tissues were removed using the scalpel blade under the $10\times$ magnification dissection microscope. And then the harvest-

ed microspheres were washed with PBS solution three times for further characterization and cell culture.

To confirm the presence of adipose tissue within the scaffolds, the specimens were stained using Oil Red O (0.3% in isopropanol; Sigma, USA) and Alexa Fluor 488 phalloidin (A12379; Invitrogen, USA) (Fig. 4). The Oil Red O detailed staining procedure is described in the sections of the adipogenic differentiation of the ADSCs. For phalloidin staining, the specimens were fixed with 4% paraformaldehyde (PFA), treated with 0.2% Triton X-100, blocked for 30 min in PBS containing 1% BSA to prevent non-specific protein binding and then incu-

Figure 2. (A and B) Morphologies and (C) size distribution of porous microcarrier.

Figure 3. Preparation of samples at 7 days after surgery. (A) Rat after surgery, (B) subcutaneous tissue specimen, and (C) harvested specimen.

Figure 4. Morphology of cell-microcarrier constructs after sample collection. (A and B) Oil Red O staining, (C) phalloidin staining, and (D) DAPI staining.

bated for 30 min at room temperature (RT) with 20 nM phalloidin diluted in PBS. The specimens were washed with PBS three times for 5 min each at RT and then covered with mounting medium. A fluorescence image was obtained using an Olympus IX 71 inverted microscope equipped with a DP-72 digital camera.

For cell culture, the adipose tissue-microsphere constructs were placed into the 60 mm dish and cultured in DMEM containing 10% FBS and penicillin (100 U/mL)/streptomycin (100 µg/mL) at 37°C with 5% CO₂ in air in a humidified incubator for 12 days. On day 13, the cells were trypsinized and transferred into T75 culture flask and cultured continuously for 7 days. On day 20, the cells were trypsinized and transferred into T150 culture flask and cultured continuously for another 7 days. The media was changed twice weekly.

For cell cryopreservation, the cells were harvested with 0.25% trypsin/1 mM EDTA and resuspended in DMEM containing 40% (v/v) FBS and 10% DMSO at a concentration of 1×10^6 cells/mL and loaded into cryopreservation ampoules in 1 mL aliquots. A total of 5 vials were frozen in liquid nitrogen at -196°C.

Cell growth observation and proliferation assay

The optical images of the cell growth state at all time points was obtained using an Olympus IX71 inverted microscope equipped with a DP-72 digital camera. To quantify the proliferation of the cells, the cell counting was performed using the trypan blue exclusion test. Briefly, a uniform suspension containing the cells was added to the trypan blue dye by a 1:1 dilution. The number of viable cells was obtained by counting the cells of four 1×1 mm² squares of a hemocytometer and averaging. Total cell number of viable (unstained) cells was calculated directly as mean values of viable cells. Each experiment

was repeated three times.

Characteristic analysis of the primary ADSCs culture

The characteristic of the ADSCs were confirmed by immunofluorescence staining for vimentin [19] and flow cytometry analysis [20]. For vimentin staining, 1×10^4 ADSCs were directly seeded in 24-well plates with coverglass. After 3 days, the medium was removed and the cells were fixed by incubating each well with 1 mL of 4% PFA for 10 min at RT. The PFA was aspirated and the wells were washed once with 1 mL of $1 \times$ PBS at RT. The $1 \times$ PBS was discarded, the cells were incubated for 30 min at RT in blocking buffer (1% bovine serum albumin in $1 \times$ PBS) and permeabilization was achieved by incubation with 0.1% Triton X-100 for 5 min. The blocking buffer was removed and primary antibody vimentin solution (1:5000 dilution in $1 \times$ PBS, polyclonal; Millipore, USA) was added. The mixture was incubated in the dark for 2 h at RT. The primary antibody solution was aspirated and the wells were washed with 1 mL of $1 \times$ PBS three times for 5 min each at RT. The secondary antibody fluorescein isothiocyanate (FITC)-conjugated anti-chicken IgG solution (1:320 dilution in $1 \times$ PBS, Sigma, USA) was added and incubated for 30 min at RT in the dark. The cells were washed with 1 mL of $1 \times$ PBS three times for 5 min each at RT. For nuclei counterstaining, the wells were incubated with 0.5 mg/mL 4',6-diamidino-2-phenylindole (DAPI; Sigma, USA) for 5 min at RT. The DAPI was removed and the coverglass containing the cells was mounted on the slide with two drops of mounting medium. The borders of each slide were covered with nail polish to fix the coverglass to the slide. A fluorescence image was taken on the Olympus IX71 inverted microscope equipped with a DP-72 digital camera.

For flow cytometry analysis, the cells were suspended in 1 mL $1 \times$ PBS at approximately 1×10^6 cells/mL. To detect ADSCs-surface antigens, a 100 µL of the cell suspension was put into a flow cytometry tube, primary antibody against CD11b, CD29, CD44H, or CD45 antibodies (BD Pharmingen, San Diego, CA, USA) was added into the tube at 1 µg/mL and incubated for 30 min at 4°C. The cells were washed with 2 mL of $1 \times$ PBS and pelleted by centrifugation at 1500 rpm for 5 min at RT. The supernatant was discarded and the cell pellet was resuspended with 100 µL of $1 \times$ PBS. FITC-conjugated secondary antibody (cat# sc2010; Santa Cruz Biotechnology Inc., USA) at 1 µg/mL was added into the tube and incubated for 30 min at 4°C in the dark. The cells were washed with 2 mL of $1 \times$ PBS and pelleted by centrifugation at 1500 rpm for 5 min at RT. The supernatant was discarded and the cell pellet was resuspended with 0.5 mL of $1 \times$ PBS. Labeled cells were analyzed by Flow Cytometer (FACS Calibur: Becton Dickinson, USA) and Cell Quest software (Becton Dickinson, USA).

Osteogenic, chondrogenic and adipogenic potential of ADSCs

Osteogenesis

Osteogenic phenotype was induced in monolayer culture by seeding cells at 20000 cells/well in a 24-well plate and cultured until confluent. The osteogenic induction medium consisted of α -MEM containing 10% FBS, antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin), 100 nM dexamethasone (Sigma, USA), 10 mM β -glycerophosphate (Sigma, USA), USA and 50 μ g L-ascorbic acid (Sigma, USA). Differentiation proceeded for 3 weeks with media changes twice weekly.

To confirm the osteogenic differentiation of the ADSCs, the cells were stained for with Alizarin Red S (1%, pH 4.2; Sigma, USA). Briefly, the induced cells fixed with 4% PFA for 10 min, then stained with 1% Alizarin Red S for 30 min. Mineralized nodules stained red were imaged by the inverted microscope.

Chondrogenesis

Chondrogenic differentiation was induced in monolayer culture by seeding cells at 20000 cells/well in a 24-well plate and cultured until confluent. The chondrogenic medium consisted of DMEM containing 10% FBS, antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin), 1% insulin-transferrin-selenium (ITS; PAA Laboratories, Inc.), 37.5 μ g/mL L-ascorbic acid, and 100 nM dexamethasone. 10 ng/mL transforming growth factor- β 1 (TGF- β 1; PeproTech, USA) was added fresh to the

medium before each medium change. Induction proceeded for 3 weeks with media changes once every 2 days.

To confirm the chondrogenic differentiation of the ADSCs, the cells were stained for with Alcian Blue (1%, pH 2.5; Sigma, USA). Briefly, the induced cells fixed with 4% PFA for 10 min, then stained with Alcian Blue for 30 min. Extracellular glycosaminoglycans stained blue were imaged by the inverted microscope.

Adipogenesis

Adipogenic differentiation was induced in monolayer culture by seeding cells at 20000 cells/well in a 24-well plate and cultured until confluent. The cells were then treated for 72 h with the adipogenic induction medium consisted of DMEM containing 10% FBS, antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin), 1 μ M dexamethasone (Sigma, USA), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma Aldrich, USA), 100 μ M indomethacin (Sigma Aldrich, USA), and 1% ITS and afterwards for 24 h with adipogenic maintenance medium consisting of DMEM containing 10% FBS, antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin), and 1% ITS. This cycle was repeated four times. Afterwards the cells were cultured for one more week in adipogenic maintenance medium.

To confirm the adipogenic differentiation of the ADSCs, the cells were stained for with Oil Red O solution to visualize the formation of lipid droplets. Briefly, the induced cells fixed with 4% PFA for 10 min, then stained with Oil Red O for 50 min.

Figure 5. Phase contrast micrograph images of primary adipose-derived stem cells migrating from microcarriers onto tissue culture 60 mm dish over time at (A) 1, (B) 3, (C) 7, and (D) 12 days.

The lipid droplets were stained red and observed by the inverted microscope.

Statistical analysis

Data are shown as the mean $\pm$ one standard deviation. Statistical comparisons were made using the one-way ANOVA. $p < 0.05$ was considered to be statistically significant.

RESULTS

Macroporous microcarrier scaffold characterization

The macroporous PCL microcarriers were produced as described previously [21] and characterized using SEM to demonstrate their morphology (Fig. 2A and B). They had a relatively broad size distribution (Fig. 2C) and those within the range 200–650 μm (average diameter = 454 μm) were used for

cell culture. Regular-sized large pores ($\sim 100 \mu\text{m}$) and small pores ($\sim 50 \mu\text{m}$) between the large pores were produced on the microspheres, which is regarded as adequate pore structure for cell penetration and growth [22,23].

Cell growth and phenotype

The adipose tissue-microsphere constructs were obtained 7 days after surgery (Fig. 3). At this time, the specimens were transferred to a 60 mm dish and cultured. Cells on microcarriers stained positive for Oil Red O (Fig. 4A and B) and phalloidin (Fig. 4C and D). The ADSCs started to migrate from the microcarriers onto the plastic substrate of the culture dish from 3 days post incubation and the culture was maintained for 12 days (Fig. 5). The resulting cell population was then transferred to a T75 flask and cultured continuously for 7 days until they reached 80–90% confluence (Fig. 6A and B). Thereafter, the

Figure 6. (A-D) Phase contrast micrograph images of primary adipose-derived stem cells growth onto tissue culture flasks (T75 and T150) at day 1 and 7, and (E) cell number counted. Statistically significant difference noted (* $p < 0.01$).

cells were transferred to a T150 flask and cultured continuously for another 7 days (Fig. 6C and D). The cell population grew in size to form a monolayer with cells displaying morphology typical of fibroblast-like cells and tightly attached to the cell culture vessels 3 days after initial culturing. Cell expansion was also assessed (Fig. 6E). Following the initial period of cell re-

trieval in 60 mm dishes, a total of $32.50 \pm 1.95 \times 10^4$ (left pouch) and $35.75 \pm 1.52 \times 10^4$ (right pouch) cells were harvested. In T75 flasks, $93.75 \pm 1.09 \times 10^4$ (left pouch) and $89.50 \pm 2.22 \times 10^4$ (right pouch) were produced respectively 7 days after culturing. Finally, $250.00 \pm 2.54 \times 10^4$ (left pouch) and $272.25 \pm 4.56 \times 10^4$ (right pouch) cells were produced respectively in T150 flasks following 7 days culture.

The phenotype of the cells was then assessed to determine whether the expanded cell population was indeed the ADSCs. The cells in culture were immunoreactive by immunocytochemical staining for the MSC marker vimentin protein (Fig. 7A and B). Finally the cell population resulting from the overall culture period was assessed for MSC surface antigen profiles by flow cytometry (Fig. 7C-F). The ADSCs were strongly positive for CD29 and CD44H, while negative for CD11b and CD45.

The tri-lineage differentiation potential of ADSCs was tested by culturing them under multiple differentiation conditions. To evaluate mineral deposition, osteogenic differentiation was performed in monolayer culture for 3 weeks. The induced cells were stained positive with Alizarin Red S stain for mineral deposition in their newly formed matrix (Fig. 8A). To evaluate the content of cartilage specific proteoglycans in induced cultures, ADSCs were cultured in chondrogenic induction medium containing dexamethasone and TGF β 1 for 3 weeks. Alcian blue staining revealed a high content of cartilage matrix in the induced cells (Fig. 8B). To evaluate adipogenic differentiation of the ADSCs, cells were treated with adipogenic media for 3 weeks and stained with Oil Red O for lipid droplets, that these droplets contain noticeable neutral lipids (Fig. 8C).

DISCUSSION

The present study demonstrates that ADSCs can be rapidly harvested from rat tissue using our simple procedure by placing macroporous microcarriers that support their migration and proliferation. This procedure may satisfy the requirements of many MSC studies. One of the advantages of our method is that

Figure 7. Phenotypic characterization of primary adipose-derived stem cells. (A) ADSCs positive for vimentin, and (B) counter-stained with DAPI. (C-F) Flow-cytometric determination of cell-surface antigen profiles of ADSCs. The cells were positive for MSC markers CD29 (D) and CD44H (E) and negative for non-MSC markers CD11b (C) and CD45 (F). Arrows indicate control sample for each marker analysis.

Figure 8. (A) Osteocyte, (B) chondrocyte, and (C) adipocyte differentiation of primary adipose-derived stem cells.

retrieval on microcarriers may have utility for large scale cell expansion on the microcarrier substrate using bioreactors. This makes it possible for the donor to be the recipient of autologous ADSCs. Moreover, the procedure is completed within a few minutes after the anesthetization. This may reduce the burden on the donor and in the case of animal studies, reduce the need to sacrifice laboratory animals.

The method of preparing porous microspheres used in this study is unique; the process is relatively simple while the porosity and pore size can be controlled effectively. Generally, porogen is needed to generate pores in the solid spheres and it is removed during the process mainly through evaporation or extraction with solvents. In this study, the camphene used as a porogen easily solidifies and sublimates at mild conditions. The pore channel generation in the microspheres is through the process of phase separation and camphene sublimation. The camphene, PCL and chloroform mixture becomes phase-separated when a temperature is reduced and a solvent is evaporated. Camphene grows to form a connected channel and then sublimates during the stirring step. In this way, highly open-channeled porous microspheres can be produced.

Our previous report showed that the cells populated the macroporous microspheres more efficiently than on the dense microspheres after periods of cell expansion [21]. Because the scaffolding conditions of the porous microspheres will contribute to the migration of cells through the pores and proliferation of cells in the internal-spaced areas, we chose porous microspheres rather than dense ones. Biomaterial scaffolds used in the previous study included PCL and also poly(lactic acid), poly(glycolic acid) and poly(lactide-co-glycolide), amongst others. However, we utilized PCL in the current study, as PCL is the least degradable amongst them and hence is thought to provide the most stable scaffold to support cells without concern of degradation-related issues during cell isolation. The cell type isolated with the PCL microspheres can be variable initially, however, when sub-cultured over a couple of passages, ADSCs become the major population; thus, the sub-culture step is needed to achieve a high purity stem cell population.

From the perspective of clinical application, recent reports have evaluated the possibility of using cell-coated sutures to deliver cells. For instance, Reckhenrich et al. [24] showed that surgical sutures filled with ADSCs promoted wound healing. Guyette et al. [25] reported that suture-based cell engraftment could deliver cells to the heart more efficiently than conventional methods such as intramuscular injection. Moreover, the as-isolated cells can also be used clinically, but the cell number is low that may not have therapeutic effects; thus the post-culture of cells after the isolation with microspheres can secure enough population of cells that can be better used in clinical

settings. Based on these aspects, we believe that our procedure could be used for the ultimate application of the ADSC to human health problems.

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Conflicts of Interest

The authors have no financial conflicts of interest.

Ethical Statement

The protocol of the housing, care and experimental protocol were approved by the Institutional Animal Care and Use Committee of Dankook University, Republic of Korea.

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