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The effects of spheroid formation of adipose-derived stem cells in a microgravity bioreactor on stemness properties and therapeutic potential

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ABSTRACT

Adipose-derived stem cells (ADSCs) represent a valuable source of stem cells for regenerative medicine, but the loss of their stemness during *in vitro* expansion remains a major roadblock. We employed a microgravity bioreactor (MB) to develop a method for biomaterial-free-mediated spheroid formation to maintain the stemness properties of ADSCs. ADSCs spontaneously formed three-dimensional spheroids in the MB. Compared with monolayer culture, the expression levels of E-cadherin and pluripotent markers were significantly upregulated in ADSC spheroids. Spheroid-derived ADSCs exhibited increased proliferative ability and colony-forming efficiency. By culturing the spheroid-derived ADSCs in an appropriate induction medium, we found that the multipotency differentiation capacities of ADSCs were significantly improved by spheroid culture in the MB. Furthermore, when ADSCs were administered to mice with carbon tetrachloride-induced acute liver failure, spheroid-derived ADSCs showed more effective potentials to rescue liver failure than ADSCs derived from constant monolayer culture. Our results suggest that spheroid formation of ADSCs in an MB enhances their stemness properties and increases their therapeutic potential. Therefore, spheroid culture in an MB can be an efficient method to maintain stemness properties, without the involvement of any biomaterials for clinical applications of *in vitro* cultured ADSCs.

1. Introduction

Stemness properties of mesenchymal stem cells (MSCs), which are capable of self-renewal and multi-lineage differentiation, endow them with great potential for tissue engineering and regenerative medicine [1]. Adipose-derived stem cells (ADSCs) represent an abundant source of MSCs that are easily accessible from subcutaneous adipose tissue via liposuction [2]. MSCs comprise only a small proportion of the bone marrow or adipose tissue, and they must be extensively expanded in culture to achieve the numbers required for any therapeutic strategy [3,4]. During *in vitro* expansion of common monolayer cultures, however, MSCs quickly lose their primitive stemness properties, such as replicative ability, colony-forming efficiency, and differentiation capacity [5,6]. Therefore, maintaining stemness properties has become a crucial issue for future clinical applications of *in vitro*-cultured ADSCs.

The stemness properties of MSCs are retained in the *in vivo* microenvironment, which comprises soluble growth factors, cell–cell interactions and cell–matrix interactions [7,8]. Accumulating evidence has suggested that the cellular microenvironment plays an important role in determining stemness properties [9,10]. Compared with conventional monolayer cultures, three-dimensional (3D) culture methods offer a cellular niche that is more similar to the *in vivo* microenvironment [11]. Several 3D cell culture methods, including the use of hydrogels, porous scaffolds or polymers, as well as cellular aggregates, have been developed to maintain the stemness properties of stem cells [12–14]. Among these methods, spheroid culture is a typical scaffold-free 3D cell culture system that takes advantage of the natural self-assembly tendency of numerous cell types [15]. Spheroid formation enables

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cells to assemble and interact under native forces and allows them to generate their own extracellular matrix [16]. When cells contact, interact and communicate with other cells rather than with artificial scaffolds, the cell culture more closely replicates the *in vivo* environment [11]. Previous studies have demonstrated that spheroid formation of MSCs cultured on chitosan films or using hanging drop facilitated their stemness maintenance [16,17]. Furthermore, a recent study demonstrated that differentiated cells were reprogrammed by and acquired stemness properties from spheroid culture [18]. Therefore, spheroid culture may be an efficient method to maintain the stemness properties of MSCs.

Spheroids can be generally obtained via three approaches: hanging drop, low-attachment culture conditions, and dynamic culture [19–21]. Although the two former methods are convenient for generating homogeneous aggregates and do not cause shear stress damage, these techniques are not suitable for high-activity and large-scale production of spheroids. Dynamic cultures involve spheroids that are grown in a spinner flask or a microgravity bioreactor (MB) [16,22]. In addition to the obvious advantages of large-scale and high-density cell culture in a dynamic culture system, the MB also offers the benefits of reduced shear stress and increased mass transfer compared with spinner flask [22]. Cell damage caused by mechanical agitation is reduced by gentle shear stress [16]. Increased mass transfer promotes the transport of nutrients and the removal of metabolites from spheroids growing in an MB [23]. Thus, these advantages of MB potentially increase cell viability of MSCs. Moreover, increasing evidence has demonstrated that MBs are useful in the scale-up of stem cells [24,25]. Our previous studies have shown that MBs promote spheroid formation and large-scale expansion of stem cells [23]. Therefore, spheroid culture of MSCs in MB has a promise to not only meet fully the requirements of large-scale production as well as high-activity and high-density cell culture for clinical application, but also preserve their stemness properties. However, it is unclear whether the stemness properties of MSCs can be maintained or improved by spheroid culture in MB.

In this study, we employed an MB to develop a straightforward and effective method of biomaterial-free-mediated spheroid formation to improve the stemness properties of ADSCs for clinical application. The stemness properties of spheroid-derived ADSCs, including proliferative ability, pluripotent maker expression, colony-forming efficiency, and multipotency differentiation capacity, were investigated. We further examined the therapeutic potential of spheroid-derived ADSCs for carbon tetrachloride (CCl_4)-induced acute liver failure (ALF) in non-obese diabetic severe combined immunodeficient (NOD-SCID) mice. Possible mechanisms for the improvement of stemness properties are discussed based on spheroid formation of ADSCs in the MB.

2. Materials and methods

2.1. Isolation and culture of ADSCs

Subcutaneous adipose tissue was obtained from patients undergoing elective surgical procedures at the Department of Plastic Surgery. This study was approved by the Research Ethics Committee of the Daping Hospital and Third Military Medical University, and all participants provided informed written consent. ADSCs were isolated as described previously [26]. Freshly isolated cells were resuspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin–streptomycin (Invitrogen, Carlsbad, CA). The cell suspensions were seeded into tissue culture flasks or MBs (Synthecon, Inc., Houston, TX) at a density of 10^6 cells/ml and incubated at 37 °C in humidified atmosphere with 5% CO_2. The MB was set to rotate at a speed of 25 rpm. Every 2–3 days, 80% of the culture medium was replaced with fresh medium. After 5 days of culture, ADSC spheroids aggregated in the MB were dissociated with trypsin/ethylenediaminetetraacetate (EDTA) solution (Gibco) and then transferred to a tissue culture flask for further experiments (spheroid-derived ADSCs). We used ADSCs that were continuously cultured in tissue culture flasks as the control (monolayer ADSCs).

2.2. Characteristics of ADSC spheroids

To calculate the size distribution of spheroids, spheroid suspensions were taken from the MB on days 1, 3 and 5. Images of spheroids were photographed using digital microscopy. The diameters of the spheroids were measured using Image Pro software (Media Cybernetics, Silver Spring, MD). For the apoptosis assay, spheroids were stained using a propidium iodide (PI, Sigma–Aldrich, St Louis, MO)/4,6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich) apoptosis detection kit according to the manufacturer's instructions. The expression levels of stemness marker genes and E-cadherin in ADSC spheroids were analyzed by quantitative reverse transcription-polymerase chain reaction (RT-PCR) or immunofluorescence staining.

2.3. Morphology and proliferation viability of spheroid-derived ADSCs

After 5 days of culture, the spheroids were removed from the MB and placed directly into tissue culture flasks for culture. Cell morphology was observed using a phase-contrast microscopy. To investigate the proliferation viability of spheroid-derived ADSCs, spheroids in the MB were dissociated by trypsin/EDTA and then cultured at a density of 5×10^4 cells/ml under the same monolayer culture conditions as described above. Cells were lifted with trypsin/EDTA and then counted using a hemocytometer at various times during the 7-day period.

2.4. Colony-forming unit-fibroblast (CFU-F) assay

The CFU-F assay was performed using modified techniques as described previously [17]. ADSCs derived from monolayer and spheroids were cultured in culture medium at a density of 1000 cells per-100 mm dish. The medium was changed every 3 days. After 14 days of culture, the cells were fixed with 4% paraformaldehyde and stained with 3% crystal violet solution (Sigma–Aldrich). The number of colonies (diameter $\geq$ 2 mm) was counted.

2.5. In vitro multipotency differentiation assay

The spheroids were collected at 5 days, dissociated into single cells, and induced with different media for differentiation of the three germ layers. Adipogenic, osteogenic and chondrogenic differentiation of ADSCs derived from spheroids or monolayer cultures were performed as described previously [27]. After 4 weeks of induction, the adipogenic-specific genes peroxisome proliferator-activated receptor-γ (PPAR-γ) and lipoprotein lipase (LPL), the osteogenic-specific genes osteocalcin and osteopontin, and the chondrogenic-specific genes collagen II and aggrecan were detected by quantitative RT-PCR as described below. The cells were stained using the Oil red O procedure, Alizarin red or Alcian blue solution to further observe the presence of neutral lipid vacuoles in adipocytes, calcium deposition in osteocytes or collagen II in chondrocytes, respectively.

Hepatogenic and neurogenic differentiation of spheroid or monolayer ADSCs were selected to test the representative transdifferentiation capacities of endodermal and ectodermal lineages. Hepatogenic and neurogenic differentiation were induced as described in a previous study [17]. After 21 days of culture, the expression levels of hepatogenic (albumin, CK-18 and CYP3A4) or neurogenic (β-III tubulin and nestin) markers were analyzed by quantitative RT-PCR and immunofluorescence.

2.6. Quantitative RT-PCR

Total RNA was extracted from ADSCs derived from spheroids and monolayer cultures using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The total RNA concentration was determined by optical density at 260 nm using a spectrophotometer. After removing residual DNA with DNase I, equal amounts of RNA (1 μg) were added to a reverse transcriptase reaction mixture with oligo-dT primers (Invitrogen). The Power SYBR Green RT-PCR Kit (Applied Biosystems, Foster City, CA) was used to perform quantitative RT-PCR using the Bio-Rad CFX96 Real-Time system (Bio-Rad Laboratories, Inc., Hercules, CA). The expression level was analyzed and normalized to GAPDH in the cDNA samples. The fold change of gene expression was calculated using the 2-ΔΔCT method. Primer sequences are provided in Table S1.

2.7. Immunofluorescence

Spheroids were taken from the MB after 5 days and seeded directly onto slides for 2 h of culture. Spheroids or cells on slides were fixed in 4% paraformaldehyde and washed three times with PBS, and they were then immersed in PBS containing 0.1% Triton X-100 (Sigma–Aldrich) and 1% normal serum for 30 min at room temperature. Samples were then incubated overnight at 4 °C with the following primary antibodies: rabbit anti-E-cadherin and goat anti-Oct4 (1:200, Santa Cruz Biotech, Inc., Santa Cruz, CA), rabbit anti-Nanog (1:400, Abcam, Cambridge, UK), goat anti-Sox2 (1:300, Invitrogen), rabbit anti-Rex-1 (1:400, Invitrogen), rabbit anti-ALB (1:500, Bethyl Laboratories, Montgomery, TX), rabbit anti-CK18 (1:200, Invitrogen), goat anti-CYP3A4 (1:200, Invitrogen), rabbit anti-β-III tubulin (1:400, Invitrogen), goat anti-nestin (1:400, Invitrogen). After incubation with primary antibodies, cells were washed with PBS and then incubated with FITC-conjugated goat anti-rabbit IgG or Cy5-conjugated donkey anti-goat IgG (1:400, Invitrogen) for 1 h at room temperature. Nuclear DNA was dyed with DAPI. Images were captured using the Leica confocal microscopy system.