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Fast isolation and expansion of multipotent cells from adipose tissue based on chitosan-selected primary culture

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ABSTRACT

Adipose-derived adult stem cells (ASCs) have gained much attention because of their multipotency and easy access. Here we describe a novel chitosan-based selection (CS) system instead of the conventional plastic adherence (PA) to obtain the primary ASCs. The minimal amount of adipose tissue for consistent isolation of ASCs is reduced from 10 mL to 5 mL. The selection is based on the specific interaction between cells and chitosan materials, which separate ASCs by forming spheroids during primary culture. The primary culture period was reduced from 4 days to one day and more ASCs (ten-fold expansion) were achieved in a week. The average duration for obtaining 1×10^7 cells takes about seven days from 5 mL of adipose tissue, compared to 14 days using the conventional PA method from 10 mL of adipose tissue. The replicative senescence of CS-ASCs is not evident until the fifteenth passage (vs. eighth for the PA-ASCs). The obtained ASCs (CS-ASCs) have less doubling time for the same passage of cells and show greater stemness than those obtained from the conventional PA method (PA-ASCs). Moreover, CS-ASCs undergo trilineage differentiation more effectively than PA-ASCs. The greater differentiation potential of CS-ASCs may be associated with the enrichment and maintenance of CD271 positive cells by chitosan selection of primary culture.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that can be isolated from many tissues, including bone marrow [1], adipose tissue [2], cord blood [3], and placenta [4]. They show adhesion to tissue culture polystyrene dish with fibroblast-like morphology and replicative senescence after a few passages *in vitro*. MSCs are characterized with the lack of hematopoietic and endothelial cell markers and presence of several other surface antigens, typically CD73, CD90, and CD105 [5]. The most reliable definition for MSCs remains to be their differentiation capacities into cells of osteogenic, chondrogenic, and adipogenic lineages [6]. Since MSCs are principally collected from tissues, they may be contaminated with various types of cells. The heterogeneous cell populations may impair the self-renewal capacity and differentiation potentials of MSCs. Therefore, isolation of high purity MSCs without contamination with endothelial cells, fibroblasts, or other cells is of critical importance for their applications. Fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS) are the most common sorting technologies, which can select a population enriched with certain surface markers and enhance the differentiation properties [7,8]. These markers, however, are frequently similar to those existing in fibroblasts. Besides, the methods are not sufficient enough for the specific selection of MSCs [9]. Materials technology for isolation of MSCs is rarely reported, though there was one report in isolating embryonic stem cells by selective adherence to hyaluronan [10] and a recent report in isolating induced pluripotent stem cells by adhesion against shear using microfluidics [11]. On the other hand, plates coated

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with 2-methacryloyloxyethyl phosphorylcholine have been employed to increase the sensitivity for adhesion-dependent cells and separate the attached cells from suspended cells in human bone marrow aspirates [12]. In addition, a membrane filtration unit made of polyurethane-epoxy has been developed for obtaining CD34 positive ASCs [13].

Culturing the isolated MSCs as three-dimensional (3D) multicellular spheroids has recently become a new trend because of the better differentiation capacities in general. 3D MSC spheroids may form on micropatterned substrates [14,15], nonadherent and concave culture surfaces [16], or by hanging drops [17,18]. These 3D techniques, however, cannot be applied to isolate MSCs. Chitosan is a positively charged polysaccharide derived from chitin, which is the second abundant polysaccharide in nature and structural component in the exoskeleton of crustaceans such as crabs and shrimps. Formation of 3D adhesive spheroids on chitosan-based materials was reported to separate more potent and MSC-like cells from a heterogeneous population of gingival fibroblasts from the third passage even when the subpopulation accounted for less than 20% [19]. The selection principle was based on separating the attached OB-cadherin expressing cells from the spheroid-forming N-cadherin expressing cells after the heterogeneous subcultured cells were seeded on chitosan membranes for 24 h. On the other hand, generating spheroids from hanging drops or suspension culture did not select the more potent MSC-like population from gingival fibroblasts. The specific interaction between MSCs and chitosan-based materials may be associated with the adsorption and intracellular transport of calcium by the chitosan substrate [20]. Not only for gingival fibroblasts, many types of MSCs from human as well as animals tend to form spheroids on chitosan-based substrates, which include the bone marrow stromal cells (BMSCs), adipose-derived adult stem cells (ASCs), and placenta-derived multipotent cells (PDMCs) [21–23].

ASCs have attracted much interest because of their therapeutic potential. Compared to BMSCs that may often require painful spinal anesthesia and have low yield upon processing, ASCs possess the advantages of less invasive access and are available in greater quantities [24–26]. They are capable of differentiation into mesodermal tissues such as bone, cartilage, tendons, skeletal muscle, and fat as well as non-mesodermal tissues such as neurons and endocrine pancreatic cells [27,28]. Abundant ASCs can be easily obtained from the lipoaspirate, a waste product of surgery [29,30]. However, a small part of contaminating cells such as hematopoietic stem cells could be the source of differentiation in ASC experiments that may cause unpredicted risk in some clinical use [31]. As mentioned, the affinity-based separation methods such as FACS and MACS can provide high purity of stem cell populations (≥75% purity) [32]. Nevertheless, there is still no unique identifier to distinguish ASCs from other cell populations in the routine culture of the stromal-vascular fraction (SVF) from homogenized adipose tissue. Besides, these methods need more initial cell numbers (greater than 1×10^7) for further separation and the isolation period is prolonged (about 2 weeks) for gaining sufficient cells. Long-term *in vitro* culture of MSCs may alter the differentiation potential or have the problem of cellular senescence [33,34]. Therefore, the rapid isolation of a sufficient amount of homogenous stem cells while maintaining their stemness is of critical importance for further applications.

The traditional culture method of MSCs is the plastic adherence (PA). It is highly desirable to select MSCs from the harvested tissue, use them directly, or to amplify them within very early passages. Here we describe a novel method to quickly select more potent ASCs from a small amount of the subcutaneous adipose tissue or lipoaspirates based on the specific interaction between ASCs and chitosan.

2. Materials and methods

2.1. Preparation of the chitosan-based matrix

Chitosan (from crab shell, middle-viscous, Sigma, cat. no. 28191) was dissolved in 1% (v/v) acetic acid to prepare 2% (w/v) chitosan solution. The chitosan solution (3 mL) was poured into the lumen of a 5 mL plastic syringe, with both ends of the syringe sealed with parafilm. The syringe was placed in a −20 °C refrigerator for 24 h, moved to a freeze-dryer, and lyophilized for 72 h. The dry chitosan-based matrix was removed from the syringe and immersed in 50/50 (v/v) 1 N NaOH/absolute ethanol for one day at −20 °C for solvent exchange, matrix gelatinization, and disinfection. The chitosan-based matrix was washed extensively with deionized water to remove NaOH residue and salts. The chitosan-based matrix was dried in laminar hood, packed back into the 5 mL syringe, and reserved till use.

2.2. Preparation of the chitosan-based membrane

Chitosan was dissolved in 1% (v/v) acetic acid to prepare 1% (w/v) chitosan solution. An aliquot of 300 μL 1% (w/v) chitosan solution was spread onto each 1.5 cm round coverslip glass. The membrane was air-dried and immerse in 1 N NaOH for 5 min. The membrane was washed in phosphate-buffer saline (PBS) for three times, dried, and disinfected with 70% ethanol before use.

2.3. ASC isolation by the conventional PA method

All tissues were obtained with approval from the Institutional Animal Care and Use Committee (for rabbit tissue) or Institutional Review Board (for human tissue). The fat tissue was washed in Hank's Balanced Salt Solution (HBSS, Gibco) to remove clots. The tissue was then cut into several pieces. The pieces were treated with 3 mg/mL (200 U/mL) collagenase (Type I, Sigma) in HBSS for 30 min at 37 °C, with gentle agitation. The cell pellet was obtained by centrifugation at 3500 rpm for 10 min. The cell pellet was washed by PBS at 1500 rpm for 10 min and re-suspended in PBS. The cells were passed through a 70-μm mesh filter (BD Falcon). The cells were washed by centrifugation at 1500 rpm for 10 min and re-suspended in PBS. The cell suspension was moved to a T-150 flask in a 37°C/5% CO_2 incubator. The culture medium was Dulbecco's modified Eagle medium (DMEM)-low glucose/F12 (Gibco) supplemented by 10% (v/v) fetal bovine serum (FBS, HyClone), 1% antibiotics (penicillin–streptomycin, Gibco), and 10 mg/L L-glutamine.

2.4. Fast cell selection by the novel chitosan-based system

The cell suspension was passed through the chitosan-based matrix packed syringe by gravity. The final 25% portion of the cell suspension was pushed out the syringe by the piston (about 1 mL/min). The cell filtrate was collected, plated on the chitosan-based membrane at a density of 3.5×10^4 cells/cm^2 approximately, and put in the 37°C/5% CO_2 incubator. Cells were cultured on chitosan membranes for 24 h, some cells formed spheroids and the others attached on the membranes. When the culture plate was removed from the incubator, the slight pH change of the culture medium made the spheroids only loosely adhere or suspend on the membranes. These spheroids were soon collected by gentle shaking and pipetting. The spheroid suspension was centrifuged at 500 rpm for 5 min and plated on a T-150 flask using the culture medium described for the conventional culture. Cells on the surface of chitosan including those in the form of spheroids and those in the form of attached cells were individually collected by pipetting and trypsin, counted by the Hoechst dye stain assay, and analyzed the