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Spheroid formation of mesenchymal stem cells on chitosan and chitosan-hyaluronan membranes

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ABSTRACT

Stem cells can lose their primitive properties during in vitro culture. The culture substrate may affect the behavior of stem cells as a result of cell–substrate interaction. The maintenance of self-renewal for adult human mesenchymal stem cells (MSCs) by a biomaterial substrate, however, has not been reported in literature. In this study, MSCs isolated from human adipose (hADAS) and placenta (hPDMC) were cultured on chitosan membranes and those further modified by hyaluronan (chitosan-HA). It was observed that the MSCs of either origin formed three-dimensional spheroids that kept attached on the membranes. Spheroid formation was associated with the increased MMP-2 expression. Cells on chitosan-HA formed spheroids more quickly and the size of spheroids were larger than on chitosan alone. The expression of stemness marker genes (*Oct4*, *Sox2*, and *Nanog*) for MSCs on the materials was analyzed by the real-time RT-PCR. It was found that formation of spheroids on chitosan and chitosan-HA membranes helped to maintain the expression of stemness marker genes of MSCs compared to culturing cells on polystyrene dish. The maintenance of stemness marker gene expression was especially remarkable in hPDMC spheroids (vs. hADAS spheroids). Blocking CD44 by antibodies prevented the spheroid formation and decreased the stemness gene expression moderately; while treatment by Y-27632 compound inhibited the spheroid formation and significantly decreased the stemness gene expression. Upon chondrogenic induction, the MSC spheroids showed higher levels of *Sox9*, *aggrecan*, and *collagen type II* gene expression and were stained positive for glycosaminoglycan and collagen type II. hPDMC had better chondrogenic differentiation potential than hADAS upon induction. Our study suggested that the formation of adhered spheroids on chitosan and chitosan-HA membranes may sustain the expression of stemness marker genes of MSCs and increase their chondrogenic differentiation capacity. The Rho/Rho-associated kinase (ROCK) signaling pathway may be involved in spheroid formation.

1. Introduction

Stem cells are able to self-renew and differentiate into various types of cells for therapeutic purposes. They may lose the capabilities to differentiate or self-renew after several passages in vitro. The difficulty in maintaining the self-renewal of stem cells is due to the insufficient microenvironment [1]. The microenvironment niche could determine the fate of stem cells to self-renew or differentiate [2]. Various biomaterials have recently been developed to maintain the self-renewal of embryonic stem cells [3–7]. However, using biomaterials to maintain the characteristics of adult stem cells has not been reported so far.

Mesenchymal stem cells (MSCs) from various sources are capable of differentiating into cells of different lineages under proper culture conditions. Human adipose-derived adult stem cells (hADAS) are multipotent cells taken from human adipose tissue. Adipose tissue, like bone marrow, is derived from the embryonic mesenchyme and contains a stroma that is easily isolated [8]. Recent reports have demonstrated that hADAS can be induced to differentiate into bone, chondrocytes, adipose tissue, muscle,

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neurons and endothelial cells. Specifically, hADAS have chondrogenic potential for development into the engineered cartilage [9–14], and biomaterial scaffolds can play a significant role in the process [15–18]. On the other hand, human placenta derived multipotent cells (hPDMC) have been discovered to be a new source of MSCs [19]. The successful formation of placenta is a critical process in embryogenesis, and hPDMC can be differentiated into bone, adipose tissue, hepatocytes, muscle, neurons and endothelial cells [19–23]. hPDMC resemble the other MSCs in multilineage differentiation potential and cell-surface antigen expression [24]. hPDMC have been shown to possess chondrogenic potential [25,26]; therefore, they could be a new cell source for cartilage tissue engineering.

Chitosan is the deacetylated derivative of chitin, the second most abundant natural polysaccharide in the world. The biocompatibility of chitosan has been attributed to its structural and functional similarity to glycosaminoglycans (GAGs), making it a biomaterial candidate for cartilage engineering [27,28]. Hyaluronan (HA) on the other hand is a natural GAG that carries negative charge. HA can enhance cell migration, proliferation, and matrix secretion [29]. HA is also a major component of the articular cartilage matrix and that of the synovial fluid. HA has been reported to provide a suitable niche for stem cells to differentiate into the chondrogenic lineage [18,30–32].

In the present study, hADAS and hPDMC were cultured on chitosan membranes or those further modified by HA at a proper seeding density. Both of these cells quickly formed three-dimensional (3D) spheroids on either membranes. Spheroid formation had a significant impact on the stemness marker gene expression of these stem cells. The effect of blocking CD44 or Rho/Rho-associated kinase (ROCK) on the spheroid formation was investigated. Possible mechanisms for the maintenance of MSC self-renewal were discussed based on spheroid formation on these membranes.

2. Materials and methods

2.1. Isolation and culture of hPDMC

Term (38–40 weeks after gestation) placentas from healthy donor mothers were obtained with informed consent according to the procedures approved by the institutional review board. The harvested pieces of tissue were washed several times in phosphate buffered saline (PBS) and then mechanically minced and enzymatically treated with 0.25% trypsin for approximately 10 min at 37 °C [33]. After mechanical and enzymatic treatment, the homogenate was cultured in complete medium for cell culture consisting of Dulbecco's modified Eagle medium-low glucose (DMEM-LG) (Gibco) supplemented by 10% (v/v) fetal bovine serum (FBS) (Gibco), 10 mg/l penicillin–streptomycin, and 10 mg/l L-glutamine (Invitrogen). Cell cultures were maintained in an incubator at 37 °C with a water-saturated atmosphere and 5% CO_2. The medium was refreshed twice every week. Cells of the seventh to the twelfth passages were used in this study.

2.2. Isolation and culture of hADAS

hADAS were obtained from the subcutaneous adipose tissue discarded during surgery following the procedures approved by the institutional review board. Cells were enzymatically isolated from adipose tissue. The adipose tissue was minced into several pieces and treated with 200 U/ml type I collagenase (Sigma–Aldrich) in PBS at 37 °C for 30 min with gentle agitation [8]. The homogenate was cultured in complete medium for cell culture. The medium consisted of DMEM-LG/F12 (1:1) (Gibco) supplemented by 10% FBS (Gibco) and 10 mg/l penicillin–streptomycin and 10 mg/l L-glutamine (Invitrogen). Cultures were maintained in the 37 °C/5% CO_2 incubator. The medium was refreshed one to two times every week. Cells of the third to the eighth passages were used in this study.

2.3. Analyses of surface markers for the progenitor populations and the stemness marker gene expression

Surface markers for each type of cells were quantified by flow cytometry using CD29, CD31, CD34, CD44, CD45, CD105, CD106 (all from BioLegend), CD73 (BD Pharmingen), and CD90 (Serotec) antibodies. 5×10^5 cells were washed twice with PBS, resuspended in 100 μl of PBS containing monoclonal antibodies and incubated for 30 min at 4 °C. The cells were then washed twice and resuspended in 500 μl of PBS. Fluorescence analysis was performed with a flow cytometer (FACS Caliber, BD). The non-specific binding of the fluorescein isothiocyanate (FITC) and phosphatidyl ethanolamine (PE) conjugates were determined in control samples using a mouse IgG1-FITC and IgG1-PE negative control (Serotec). The expression levels of stemness marker genes (*Oct4*, *Sox2* and *Nanog*) were analyzed by the real-time RT-PCR with details described in a separate section (Section 2.12).

2.4. Preparation of chitosan and chitosan-HA membranes

Chitosan powder was obtained from Fluka (USA). The molecular weight of the chitosan was 510 kDa. The degree of deacetylation measured by NMR was 77.7%. Hyaluronan (sodium salt) was obtained from SciVision Biotech Inc. (Kaohsiung, Taiwan). The molecular weight was about 2500 kDa. Chitosan was dissolved in 1% acetic acid to obtain a 1% chitosan solution. The solution (300 μl) was coated on 1.5 cm-diameter coverslip glass placed in a petri dish. Chitosan membranes formed when the solvent evaporated in a laminar cabinet after 24 h. Sodium hydroxide (0.5 N) was added to the petri dish for 30 min. The membranes were then washed three times with PBS. To prepare chitosan-HA membranes, 300 μl of HA solution containing different amounts of HA was further added on each chitosan-coated coverslip so the amount of HA was 0.1, 0.5 or 2.5 mg per cm^2 of chitosan membranes. HA has strong negative charge and can form complex with the positively charged chitosan immediately. The chitosan and chitosan-HA membranes with different doses of HA were abbreviated as C, CH0.1, CH0.5 and CH2.5 respectively. The chitosan-HA membranes with different amounts of HA were washed fives times wish PBS to remove the unbound HA and were later lyophilized. Dry chitosan and chitosan-HA membranes (on coverslip glass) were placed in each well of a 24-well tissue plate for cell studies.

2.5. Gluronic acid assay for HA and analysis for the release of HA from chitosan-HA

The amount of HA was quantified by the gluronic acid assay described in literature [27]. Chitosan-HA membranes were washed with 0.1 N HCl and centrifuged at 1500 rpm for 10 min to detach all HA from chitosan. After neutralization, the HA-containing samples in 100 μl were hydrolyzed by 500 μl of 120 mM sodium tetraborate in concentrated sulfuric acid at 100 °C for 1 h. 40 μl of 2 mg/l m-hydroxydiphenyl reagent was then added to the reaction mixture. After 15 min of incubation, the absorbance of the resulting solution was measured at 520 nm. The amount of HA (mg) was determined from the absorbance.

The release of HA from chitosan-HA was measured in a period of 10 days in the basal medium by the gluronic acid assay as described above. Chitosan-HA membranes were placed in each well of a 24-well tissue culture plate. 500 μl of medium was removed from each well at different time points during the culture period. The amount (mg) of HA released from a membrane was calculated and normalized to the initial amount (adsorbed). The results in basal medium were shown as the cumulative release in percentage. Data were based on the average of three independent cultures at each time point. To mimic the real culture environment, the medium was changed after 3 and 7 days during the experimental period. Additional experiments were conducted in parallel by switching the basal to chondrogenic medium after three days.

2.6. Cell seeding

hADAS and hPDMC (3×10^4 cells) were seeded on each membrane in 24-well tissue culture plates. Cell morphology was observed by an inverted microscope (Leica DMIRB). Cell grouping and spheroid formation were examined at 3 days. The dynamics of spheroid formation from cells cultured on chitosan and chitosan-HA membranes were also recorded by the real-time Cultured Cell Monitoring System (Astec, CCM-Multi, Japan). The expression of stemness marker genes (*Oct4*, *Sox2* and *Nanog*) was analyzed at 1, 3, 7, and 10 days by the real-time RT-PCR. Cells seeded in the culture well (tissue culture polystyrene, TCPS) served as the control.

2.7. Proliferation rate and viability of MSCs cultured on materials

The number of cells cultured on TCPS, chitosan or chitosan-HA membranes in basal medium was determined by Hoechst 33528 fluorescent dye assay at 1, 3, 7 and 10 days. Cells and cell spheroids were digested in papain (Sigma) solution and reacted with 0.1 mg/ml Hoechst 33528 dye (Sigma). The fluorescence was measured at room temperature by a fluorescence spectrophotometer (Hitachi F2500, Japan), with excitation at 365 nm and emission at 458 nm. The cell numbers were calculated against a standard curve obtained from known amount of cells.

Cell viability was determined using propidium iodide (PI) (Sigma) staining and flow cytometry for cells cultured in basal medium for 3 and 7 days. The solution of PI (concentration 2 mg/ml) was added to cell suspension before the analysis by the flow cytometer. The percentage of cells without being stained by PI was defined as the cell viability.