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Isolation of the multipotent MSC subpopulation from human gingival fibroblasts by culturing on chitosan membranes

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ABSTRACT

Literature has different opinions regarding the percentage of mesenchymal stem cell (MSC)-like population in human gingival tissue. Isolation of these cells is thus important for clinical applications. In this study, two typical but distinct types of gingival fibroblasts (GF), GF-A and GF-B, were grown from human gingival biopsies. They were characterized for surface markers by flow cytometry as well as the expressions of stemness and neural crest marker genes by RT-PCR. The two types of GF were slightly different in their surface markers; however, they had dramatic difference in the expression levels of stemness marker genes and neural crest marker genes. They also demonstrated distinct differentiation capacity. Upon the appropriate induction, GF-A were capable of osteogenic, adipogenic, chondrogenic, and neurogenic differentiation while GF-B only underwent osteogenic differentiation. By culturing either type of GF on chitosan membranes for 24 h, we were able to isolate two distinct subpopulations in each type of GF, i.e. cells with spheroid-forming ability (GF-AS and GF-BS) or those remained flat and attached (GF-AN and GF-BN). We further characterized these cells, and determined the common properties shared by the spheroid-forming subpopulation "S", as well as those shared by the non-spheroid-forming subpopulation "N". The subpopulation "S" was capable of the multilineage differentiation, while the subpopulation "N" was only efficient in osteogenic differentiation. GF-A and GF-B had different proportions of subpopulations. Chitosan as the cell culture substratum up-regulated the N-cadherin expression of the "S" but not "N" subpopulation, which may account for the cell sorting effect. This study showed that chitosan membranes could be used for isolation of the spheroid forming subpopulation in human GF that contained multipotent adult stem cells of which the number varied among donors and sites.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that can be isolated from many human adult or fetal tissues, including bone marrow [1], adipose tissue [2], umbilical cord blood [3], and placenta [4]. All of these MSCs show adhesion to tissue culture polystyrene dishes with fibroblast-like cell morphology and limited proliferation capacity in vitro. They are normally characterized with their specific lack of hematopoietic and endothelial markers but with variable expressions of several other surface antigens [5]. The most trustworthy definition for MSCs remains to be the capacities for differentiation into cells with the mesenchymal origin, i.e. osteogenic, chondrogenic and adipogenic lineages [6]. Since MSCs are principally collected from tissues, they may contain various subpopulations of cells. The heterogeneous populations of MSCs may impair their multipotent differentiation potentials and self-renewal capacity. To isolate high purity MSCs and prevent their contamination with endothelial cells or fibroblasts is critical for their applications. Various processes have been reported to isolate high purity MSCs. Cell sorting technologies with certain surface markers by flow cytometry or magnetic activated cell sorting (MACS) are the most common and can select a specific subpopulation with increased differentiation potential [7,8]. The expression levels of these markers in MSCs, however, are sometimes similar to those of fibroblasts. Specific selection of MSCs from such heterogeneous cell populations is thus not sufficient enough with these

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methods [9]. Plates coated with 2-methacryloyloxyethyl phosphorylcholine) polymer have been used to select the attached cells from suspended cells in bone marrow aspirates [10].

Other than sorting MSCs, another concept has been developed during recent years to increase their differentiation potentials. Different systems to culture MSCs in three-dimensional (3D) spheroids have been reported. MSC spheroids formed on micropatterned substrates had better efficiency of osteogenesis and adipogenesis differentiation [11,12]. MSC spheroids formed by hanging drops were demonstrated to enhance the antiinflammatory properties [13] and angiogenesis differentiation capacity [14]. We also showed that MSCs from different tissues could form 3D adhesive spheroids with better chondrogenesis differentiation on chitosan membranes [15,16]. MSC spheroids generated by different methods are generally recognized to own better differentiation potential. Nevertheless, isolation of cell subpopulation by spheroid formation has not been reported so far.

Recent studies have identified the existence of stem cells from dental origin. These dental stem cells include human dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth (SHED), periodontal ligament stem cells (PDSC), stem cells from the apical papilla (SCAP) and dental follicle stem cells (DFPC) [17–23]. The accessibility and availability of these dental stem cells for treatment are unfortunately quite limited. The more available human gingival fibroblasts (GF) are mesenchymal-origined and typically elongated and spindle shaped cells that participate in development, maintenance, and repair of gingival connective tissues [24]. GF showed spontaneous osteogenic capacity, probably because they contained some immature mesenchymal cells along with the osteoblastic phenotype in vitro [25]. A few recent studies also demonstrated the multipotent differentiation potentials of GF to become osteoblasts, chondrocytes [26–28], adipocytes [26–29], neurons and endothelial cells [28]. However, the percentages of MSC-like cells in the heterogeneous cell populations of GF vary significantly in literature [29,30] and can be as low as 3–6% [27,28]. It is thus difficult to establish consistent cell sources from GF for clinical applications.

In the present study, the mesenchymal phenotype, neural crest marker gene expression and differentiation properties were analyzed for GF harvested from different donors and sites. Two distinct types of GF were grown from human gingival tissues. The MSC-rich subpopulation was isolated from each type of GF by culturing on chitosan membranes. Proliferation, colony forming ability and differentiation potential of the isolated cells were characterized.

2. Materials and methods

2.1. Cell isolation

GF were isolated and cultured by the following method. Human gingival tissue was obtained from healthy adult gingiva during surgery with informed consent and IRB approval. Tissues were minced into 0.5-mm^3 pieces and explanted into 60-mm tissue culture polystyrene dishes. Tissue explants were cultured in alpha minimum essential medium (α-MEM) (Gibco/BRL) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 μg/ml streptomycin (Gibco), 100 U/ml penicillin (Gibco), and 50 μg/ml gentamicin (Gibco). Cultures were maintained at 37 °C in an incubator with humidified air containing 5% CO_2. When confluent, the primary cells were trypsinized by 0.25% trypsin/EDTA solution (Gibco) and subcultured.

2.2. Analysis of surface markers by flow cytometry

Surface markers for each type of cells were quantified by flow cytometry. The monoclonal anti-bodies included CD29 (BioLegend), CD31 (BioLegend), CD34 (BioLegend), CD44 (BioLegend), CD45 (BioLegend), CD73 (BD Pharmingen), CD90 (Serotec), CD105 (BioLegend), CD146 (BD Pharmingen) and Stro-1 (Santa Cruz). For measurement, 1×10^6 cells were washed twice with phosphate buffered saline (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. A flow cytometer (FACS Caliber, BD) was used for the fluorescence analysis. The non-specific binding was determined using a mouse IgG1-FITC and IgG1-PE negative control. The percentage of positive cells was evaluated based on the fluorescence intensity.

2.3. Doubling time and colony forming ability

Cells of 3rd to 5th passages were used in the following experiments. All cells were checked for the morphology by an inverted phase contrast microscope and for the proliferation (growth curve) by the DNA Hoechst 33528 dye stain assay and a fluorescence spectrophotometer with excitation at 365 nm and emission at 458 nm (Hitachi F2500) [31]. The cell doubling time was calculated from the growth curve. For colony forming assay, cells (1×10^4 cells) within culture medium were seeded into the culture dish. After 14 days of cultivation, the cells were fixed with 4% formalin, and then stained with 1% toluidine blue. Aggregated cells were counted as colonies (CFU-F) under the microscope.

2.4. Preparation of chitosan membranes and cell seeding on chitosan

Chitosan powder was obtained from Fluka. The molecular weight of the chitosan was 510 kDa. The degree of deacetylation measured by NMR was 77.7%. Chitosan was dissolved in 1% acetic acid to obtain 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.

The cell seeding and separation procedures are illustrated in Scheme 1. GF (3×10^4 cells/cm^2, 3rd passage) were seeded on each chitosan membrane in a 24-well tissue culture plate. Cell morphology was observed by an inverted microscope (Leica DMIRB). The dynamic cell movement on chitosan membranes was also recorded by the real-time Cultured Cell Monitoring System (Astec, CCM-Multi). After 24 h, some cells had formed spheroids on the membranes ("S" subpopulation). At this time, the culture plate was removed from the incubator. The slight change in pH caused the spheroids to loosely adhere on the membranes because chitosan was pH-sensitive. These spheroids were soon collected by gentle shaking, flushing with medium and pipetting. The collection of spheroids was confirmed by the microscope. The non-spheroid forming cells that remained attached on the membranes ("N" subpopulation) were then collected by trypsinization. Each tissue sample gave rise to GF with different "S/N" proportions. For the original (pre-sorted) GF with predominant "S" or spheroid-forming subpopulation (typically >80% total cells), they were categorized as GF-A; for the original GF with predominant "N" or non-spheroid forming subpopulation (typically >80% total cells), they were categorized as GF-B. Cells in each subpopulation ("S" and "N") were counted by the Hoechst dye stain assay. The two subpopulations were then replated on T75 flasks for further analysis. The expressions of stemness marker genes (*Oct4* and *Nanog*) and neural crest marker genes (*Sox10* and *Slug*) were analyzed by RT-PCR on cells of the next passage following isolation by membranes. The surface markers were also analyzed by flow cytometry. The doubling time and colony forming ability were evaluated as described. All cells were analyzed on the same passage (5th passage), including those for the differentiation properties stated below.

2.5. Induction of osteogenic differentiation and von Kossa staining

For osteogenic differentiation, cells in a density of 3×10^4 cells/cm^2 were plated (TCPS) in α-MEM supplemented with 10% FBS, 10 mM β-glycerophosphate (Sigma), 0.2 mM ascobate-2-phosphate (Sigma), and 10^{-8} M dexamethasone (Sigma) [32]. The culture was maintained for three weeks. The medium was refreshed twice a week. The expression of *runt-related transcription factor* (*Runx2*) and *osteocalcin* (*OCN*) genes was analyzed by RT-PCR.

Calcium deposition in the cell culture was assessed by von Kossa stain. Cells cultured for three weeks were rinsed in PBS, fixed, stained with 5% silver nitrate (Sigma) and exposed to ultraviolet light for 30 min. They were then rinsed in water, followed by 5% sodium thiosulfate (Sigma) for 3 min, and washed again. Calcium deposition was examined under an optical microscope. The numbers of von Kossa-stained clustered units (bone nodules) were quantified by automated image analysis.

2.6. Induction of adipogenic differentiation and Oil Red O staining

For adipogenic differentiation, cells in a density of 3×10^4 cells/cm^2 were cultured in high glucose DMEM (Gibco) supplemented with 10% FBS, 0.5 mM isobutyl-methylxanthine (Sigma), 200 μM indomethacin (Sigma), 10^{-6} M dexamethasone and 10 μg/ml insulin and cultured for three weeks [33]. The induction medium was refreshed twice a week. The expression of *peroxisome proliferator-activated receptor γ2* (*PPARγ2*) and *lipoprotein lipase* (*LPL*) genes was analyzed by RT-PCR.

Lipid droplets in the cell culture were confirmed by Oil Red O staining. Cells were fixed in 10% formalin, treated with 0.3% Oil red O solution (Sigma) for 15 min, and then repeatedly washed with tap water. Samples were examined under the optical microscope. The numbers of lipid droplets were quantified by automated image analysis [33].