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Delineation of *in vitro* chondrogenesis of human synovial stem cells following preconditioning using decellularized matrix

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ABSTRACT

As a tissue-specific stem cell for chondrogenesis, synovium-derived stem cells (SDSCs) are a promising cell source for cartilage repair. However, a small biopsy can only provide a limited number of cells. Cell senescence from both *in vitro* expansion and donor age presents a big challenge for stem cell based cartilage regeneration. Here we found that expansion on decellularized extracellular matrix (dECM) full of three-dimensional nanostructured fibers provided SDSCs with unique surface profiles, low elasticity but large volume as well as a fibroblast-like shape. dECM expanded SDSCs yielded larger pellets with intensive staining of type II collagen and sulfated glycosaminoglycans compared to those grown on plastic flasks while SDSCs grown in ECM yielded 28-day pellets with minimal matrix as evidenced by pellet size and chondrogenic marker staining, which was confirmed by both biochemical data and real-time PCR data. Our results also found lower levels of inflammatory genes in dECM expanded SDSCs that might be responsible for enhanced chondrogenic differentiation. Despite an increase in type X collagen in chondrogenically induced cells, dECM expanded cells had significantly lower potential for endochondral bone formation. Wnt and MAPK signals were actively involved in both expansion and chondrogenic induction of dECM expanded cells. Since young and healthy people can be potential donors for this matrix expansion system and decellularization can minimize immune concerns, human SDSCs expanded on this future commercially available dECM could be a potential cell source for autologous cartilage repair.

1. Introduction

Articular cartilage has a limited capacity for self-repair. Once damaged, the injured cartilage will develop defects. Despite promising results from autologous chondrocyte implantation [1], donor tissue availability is a challenge. Recent advances make adult stem cells an attractive cell source for cartilage regeneration, especially tissue-specific stem cells such as synovium-derived stem cells (SDSCs) [2,3]. Since *in vitro* expansion is a necessary step before *in vivo* application, accompanying cell senescence and dedifferentiation represent a formidable challenge for stem cell-based cartilage repair [4].

We found that decellularized extracellular matrix (dECM) deposited by mesenchymal stem cells could rejuvenate stem cells [5–11] and primary cells [12–14] in both proliferation and differentiation capacity. For instance, dECM deposited by SDSCs significantly promoted expanded porcine SDSCs (pSDSCs) in both proliferation and chondrogenic potential [5]. *In vivo* transplantation of dECM expanded pSDSCs demonstrated efficacy in promoting cartilage regeneration in a partial thickness cartilage defect porcine model [15].

Our recent reports suggested that this *in vitro* cell expansion system also benefits human SDSC (hSDSC) expansion and rejuvenation of chondrogenic potential [16,17], which brings hope for the potential use of this approach in clinical treatment [18,19]. However, a concomitant up-regulation of type X collagen (*COL10A1*) was also observed in chondrogenically differentiated hSDSCs that have undergone dECM expansion, indicating a tendency toward chondrogenic hypertrophy.

In this study, we fully characterized cell morphology, volume, elasticity, and surface phenotypes in hSDSCs following dECM expansion. We not only defined proliferation and chondrogenic potential in dECM expanded hSDSCs but also examined whether

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increased expression of *COL10A1* could be a sign of endochondral bone formation. Since both the mitogen-activated protein kinase (MAPK) and Wnt signals are critical pathways for chondrogenesis and have crosstalk in stem cell mediated cartilage regeneration [20], these two signals were evaluated for their changes in both cell expansion and chondrogenic induction of hSDSCs after preconditioning using dECM and conventional plastic flasks, which might provide evidence for further investigation of potential mechanisms underlying the rejuvenation of hSDSCs by dECM expansion.

2. Materials and methods

2.1. SDSC culture

Adult human synovial fibroblasts (4 donors, two male and two female, average 43 years old, all had no known joint disease), referred to as hSDSCs [16,17], were obtained from Asterand (North America Laboratories, Detroit, MI). Human SDSCs were plated and cultured in a growth medium [alpha minimum essential medium (αMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone (Invitrogen, Carlsbad, CA)] at 37 °C in a humidified 5% CO_2 and 21% O_2 incubator. The medium was changed every three days.

2.2. dECM preparation

The preparation of dECM was described in our previous study [16,17]. Briefly, plastic flasks (Plastic) were precoated with 0.2% gelatin (Sigma–Aldrich, St. Louis, MO) at 37 °C for 1 h and seeded with passage 3 (P3) hSDSCs at 6000 cells/cm^2. After the cells reached 90% confluence, 50 μM L-ascorbic acid phosphate (Wako Chemicals USA, Inc., Richmond, VA) was added for 8 days. The medium was changed every other day. The deposited matrix was incubated with 0.5% Triton X-100 containing 20 mM ammonium hydroxide at 37 °C for 5 min and stored at 4 °C in phosphate-buffered saline (PBS) containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone.

2.3. SDSC expansion

P3 hSDSCs were cultured at 3000 cells/cm^2 for one passage on two substrates: dECM or Plastic. The cell number was counted using a hemocytometer. Expanded cells were also evaluated for cell morphology using scanning electronic microscopy (SEM) and atomic force microscopy (AFM), cell number using a hemocytometer, and proliferation index and surface markers using flow cytometry.

2.4. Morphological observation using the SEM and AFM

Representative samples (n = 2) were primarily fixed in 2.5% glutaraldehyde (Sigma–Aldrich) for 2 h, followed by secondary fixation in 2% osmium tetroxide (Sigma–Aldrich) for another 2 h. The samples were then dehydrated in a gradient ethanol series, in hexamethyldisilazane (HMDS, Sigma–Aldrich) at a ratio of 1:1 with ethanol twice for 1 h each time, in HMDS at a ratio of 1:2 with ethanol overnight, and in HMDS three times for 4 h each time. The samples were air-dried for 24 h and gold sputter was added. The images were recorded by an SEM (Hitachi, Model S 2400).

Morphology of culture substrates (dECM and Plastic) and both morphology and elasticity of expanded hSDSCs were performed using an MFP-3D-BIO AFM (Asylum Research, TE2000-U, Santa Barbara, CA) integrated with an inverted fluorescence microscope (Nikon Eclipse, Ti-U, Nikon Instruments Inc., Melville, NY) and Olympus TR400-PB cantilevers with manufacturer spring constant of 0.09 N/m. The samples were imaged in Petri dishes after they were fixed with 1% glutaraldehyde and washed once with PBS. The location of the cantilever on the sample was confirmed using a 10× microscopy objective. For the morphology imaging, each sample was mapped in PBS buffer using contact mode. An area of 90 μm by 90 μm was imaged with the pixel resolutions of 512 and a scan rate at 0.2 Hz. For the quantitative nanomechanical analysis, Sneddon's modification of the Hertz model developed for a four-sided pyramid was employed. The fixed cell sample elasticity (Young's modulus, E) was corrected with the indentation of the tip, δ, through the following equation: $E = \frac{\pi}{2}\frac{1-\nu^2}{\tan\alpha}\frac{F}{\delta^2}$, where E is the elastic modulus, ν is Poisson's ratio with a value of 0.5 for ECM and cells, F is the force given by the cantilever deflection multiplied with the cantilever spring constant, α is the open angle used in this study which had a value of 36°, and lastly δ is the indentation depth [21]. The average height, projected cell area and the volume of the fixed cells were calculated using the MFP-3D Bio dedicated *Igor Pro* software (Asylum Research). The diameter of matrix fibers was determined by the measurement of full width of half maximum (FWHM) using ImageJ software.

2.5. Measurement of expanded cell proliferation index using flow cytometry

Before cell expansion, passage 3 hSDSCs were labeled with CellVue® Claret (Sigma–Aldrich) at 2×10^{-6} M for 5 min according to the manufacturer's protocol. Expanded cells were collected and measured using a BD FACS (fluorescence activated cell sorting) Calibu™ flow cytometer (dual laser) (BD Biosciences, San Jose, CA). Twenty thousand events of each sample were collected using CellQuest Pro software (BD Biosciences) and cell proliferation index was analyzed by ModFit LT™ version 3.1 (Verity Software House, Topsham, ME).

2.6. Evaluation of cell surface phenotypes using flow cytometry

The following primary antibodies were used in flow cytometry to detect hSDSC surface phenotypes: integrin β1 (CD29) was purchased from Abcam (Abcam, Cambridge, MA); CD105 and the stage-specific embryonic antigen-4 (SSEA-4) were from Santa Cruz Biotechnology (Santa Cruz, CA); and CD90 was from BD Pharmingen (BD Biosciences). IgG1 and IgG2a (Beckman Coulter, Fullerton, CA) were used as the isotype controls. Samples (n = 3) of each 0.2×10^6 expanded cells were incubated on ice in cold PBS containing 0.1% ChromPure Human IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% NaN_3 (Sigma–Aldrich) for 30 min. Then, the cells were sequentially incubated in the dark in the primary antibodies for 30 min. The fluorescence was analyzed by a FACS Calibur (BD Biosciences) using FCS Express software package (De Novo Software, Glendale, CA).

2.7. Chondrogenic induction and evaluation of expanded hSDSCs

After *in vitro* expansion, 0.25×10^6 of hSDSCs from each group were centrifuged at 500*g* for 5 min in a 15-mL polypropylene tube to form a pellet. After overnight incubation (day 0), the pellets were cultured for 35 days in a serum-free chondrogenic medium consisting of high-glucose Dulbecco's Modified Eagle's Medium (DMEM), 40 μg/mL proline, 100 nM dexamethasone, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM ascorbic acid-2-phosphate, and 1 × ITS™ Premix [6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenousacid, 5.35 μg/mL linoleic acid, and 1.25 μg/mL bovine serum albumin (BSA), from BD Biosciences]