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Rejuvenation of chondrogenic potential in a young stem cell microenvironment

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ABSTRACT

Autologous cells suffer from limited cell number and senescence during *ex vivo* expansion for cartilage repair. Here we found that expansion on extracellular matrix (ECM) deposited by fetal synovium-derived stem cells (SDSCs) (FE) was superior to ECM deposited by adult SDSCs (AE) in promoting cell proliferation and chondrogenic potential. Unique proteins in FE might be responsible for the rejuvenation effect of FE while advantageous proteins in AE might contribute to differentiation more than to proliferation. Compared to AE, the lower elasticity of FE yielded expanded adult SDSCs with lower elasticity which could be responsible for the enhancement of chondrogenic and adipogenic differentiation. MAPK and noncanonical Wnt signals were actively involved in ECM-mediated adult SDSC rejuvenation.

1. Introduction

Cartilage regeneration is a primary focus in the tissue engineering field due to the limited regenerative abilities of cartilage. Presently, the most effective treatment for repairing cartilage defects is autologous chondrocyte implantation (ACI). Though this technique has developed through generations and achieved short-term success, it is still mostly restricted by availability of a large quantity of high quality autologous chondrocytes. Despite the fact that bone marrow-derived stem cells (BMSCs) and adipose-derived stem cells (ASCs) have been investigated, there are increasing concerns of donor site morbidity due to harvesting, chondrogenic hypertrophy, and unstable chondrogenic phenotype [1,2]. Fortunately, mesenchymal stem cells (MSCs) derived from synovial tissue (SDSC) are currently attractive solutions due to their characteristic as a tissue-specific stem cell for chondrogenesis as well as the ease of harvesting through a small punch biopsy during arthroscopic surgery [3]. However, challenges remain in lengthened culturing time for preparing a sufficient number of such autologous cells, especially when elderly patients are considered [4].

To optimize the application of adult stem cells, an efficient expansion system that can rejuvenate or at least maintain the self-renewal and differentiation potentials of adult SDSCs (ASDSCs) is urgently needed. Recent work from our laboratory suggested that decellularized extracellular matrix (ECM) derived from 3-month-old porcine SDSCs enhanced proliferation and subsequent chondrogenic differentiation of both stem cells [5–11] not only *in vitro* [12] but also *in vivo* [13]. Different from the above reports using young porcine cells, in clinics, patients with cartilage defects are usually middle-aged. Adult MSCs lack telomerase activity resulting in telomere shortening after serial passaging *in vitro* [14]. Studies have shown that decellularized ECM from human adult stem cells, such as SDSCs [15] or BMSCs [16], exhibited a limited capacity to rejuvenate expanded stem cells' chondrogenic potential. Compared to adult MSCs, fetal MSCs maintained longer telomeres and higher telomerase activity [17]. Further, our recent studies demonstrated that human fetal SDSCs (FSDSCs) possessed multi-differentiation

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capacities, including chondrogenesis, osteogenesis, and adipogenesis [18], suggesting SDSCs from a fetal source could be a cell source for deposition of a decellularized ECM, which provides a young and healthy microenvironment for ASDSC rejuvenation.

In this study, we hypothesized that ECM deposited by FSDSCs provided a better *in vitro* microenvironment for ASDSC expansion and retention of chondrogenic potential. We explored potential mechanisms underlying ASDSC rejuvenation by using proteomics and immunofluorescent staining for chemical composition of ECM, atomic force microscopy (AFM) for elastic modulus of both ECM and expanded ASDSCs, and Western blot for potential involvement of the mitogen-activated protein kinase (MAPK) and Wnt signaling pathways. We also evaluated ECM expanded cells in adipogenic and osteogenic potentials to determine whether this rejuvenation only favored a tissue-specific lineage.

2. Materials and methods

2.1. DSCM preparation

Human fetal source SDSCs (FSDSCs) were obtained from ScienCell™, Research Laboratories (Carlsbad, CA) and adult source SDSCs (ASDSCs) were obtained from Asterand (North America Laboratories, Detroit, MI). Both cell types were used to prepare decellularized ECM, termed FE and AE, respectively, as described previously [15,18]. Briefly, plastic flasks (PL) were precoated with 0.2% gelatin (Sigma, St. Louis, MO) at 37 °C for 1 h and seeded with passage 3 SDSCs at 6000 cells per cm^2. After cells reached 90% confluence, 250 μM of L-ascorbic acid phosphate (Wako Chemicals USA Inc., Richmond, VA) was added for 10 days. The deposited ECMs were incubated with 0.5% Triton X-100 containing 20 mM ammonium hydroxide at 37 °C for 5 min to remove the cells; they were stored at 4 °C in phosphate-buffered saline (PBS) containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone until use.

2.2. Evaluation of cell proliferation and apoptosis

2.2.1. Cell counting and morphology

PL expanded passage 3 ASDSCs (PL3) were plated at 3000 cells per cm^2 on FE, AE, or PL for one passage with growth medium containing alpha-minimum essential medium (αMEM), 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone. Expanded ASDSCs were termed FE4, AE4, and PL4. Cell number was counted in 175 cm^2 flasks ($n = 6$) using a hemocytometer. To observe cell morphology, FE4, AE4, and PL4 were fixed in 4% paraformaldehyde; the cell membrane was labeled with Vybrant® Dil Cell-labeling solution (Life Technologies, Grand Island, NY) and mounted with Prolong® Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies). Both FE and AE were immunostained using monoclonal antibody for type I collagen (Sigma) conjugated with fluorescein isothiocyanate (FITC) and visualized with a Nikon TE2000-S Eclipse inverted microscope (Melville, NY).

2.2.2. Proliferation index

Before cell expansion, passage 3 ASDSCs were labeled with CellVue® Claret (Sigma) at 2×10^{-6} M for 5 min according to the manufacturer's protocol. After eight days, expanded cells were collected and measured using a BD FACS Calibur™ 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).

2.2.3. Flow cytometry analysis

The following primary antibodies were used to detect expanded SDSC surface immunophenotype profiles: CD29 (Abcam, Cambridge, MA), CD90 (BD Pharmingen, San Jose, CA), CD105 (BioLegend, San Diego, CA), stage-specific embryonic antigen 4 (SSEA4) (BioLegend), integrin b5 (Cell Signaling, Danvers, MA), and isotype-matched IgGs (Beckman Coulter, Fullerton, CA). The secondary antibody was goat anti-mouse IgG (H + L) R-phycoerythrin conjugated (Life Technologies). Samples ($n = 3$) of each 2×10^5 expanded cells were incubated on ice in cold PBS containing 0.1% Chrom-Pure Human IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% NaN_3 (Sigma) for 30 min. The cells were then sequentially incubated in the dark in the primary and secondary antibodies for 30 min. Fluorescence was analyzed by a FACS Calibur (BD Biosciences) using FCS Express software package (De Novo Software, Los Angeles, CA).

2.2.4. Apoptosis assay

Apoptosis of expanded cells was detected using the Annexin V-FITC Apoptosis Detection Kit (BioVision Inc., Milpitas, CA). Briefly, 2×10^5 detached cells from each group ($n = 3$) were labeled with FITC conjugated annexin V and propidium iodide for 15 min. Samples were measured using FACS Calibur (BD Biosciences) and analyzed using the FCS Express software package (De Novo Software).

2.2.5. Resistance to oxidative stress

Expanded cells were incubated with 1 mM hydrogen peroxide (H_2O_2) at 37 °C for 1 h. To measure intracellular reactive oxygen species (ROS), cells were incubated with 1 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Life Technologies) for 15 min. The plates were read on a FlUOstar OPTIMA (BMG Labtech Inc., Cary, NC) with an excitation wavelength of 485 nm and emission of 530 nm. Samples were assayed in triplicate.

2.3. Cell and ECM interaction: evidence in morphology, chemistry, and elasticity

2.3.1. Scanning electron microscope (SEM)

Representative samples ($n = 2$) were primarily fixed in 2.5% glutaraldehyde (Sigma) for 2 h, followed by secondary fixation in 2% osmium tetroxide (Sigma) for another 2 h. The samples were then dehydrated in a gradient ethanol series, in hexamethyldisilazane (HMDS, Sigma) 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).

2.3.2. Proteomics analysis of AE and FE

Decellularized ECMs were collected in 25 mM Tris–HCl (pH 7.6)/150 mM NaCl/0.5% SDS buffer solution containing protease inhibitors. Samples were precipitated with cold acetone and the pellet was solubilized with ProteaseMax (Promega, Madison, WI) detergent prepared in 50 mM NH_4HCO_3 buffer (pH 7.9). Protein samples were reduced with dithiothreitol (DTT) at 56 °C for 45 min and alkylated with 20 mM iodoacetamide in the dark at room temperature for 0.5 h. Trypsin digestion was performed at 37 °C overnight. Digested peptide mixtures were each separated by high pH reversed phase chromatography on a C18 column and 40 fractions (600 μL each) were collected and pooled into eight larger fractions due to the preliminary nature of the experiment. Samples were concentrated under vacuum to approximately 50 μL, and 8 μL of sample was analyzed on an LTQ-FT Ultra hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). In addition, we