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Synovial stem cells and their responses to the porosity of microfibrous scaffold

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ABSTRACT

Tissue-specific stem cells can be coaxed or harvested for tissue regeneration. In this study, we identified and characterized a new type of stem cells from the synovial membrane of knee joint, named neural crest cell-like synovial stem cells (NCCL-SSCs). NCCL-SSCs showed the characteristics of neural crest stem cells: they expressed markers such as Sox10, Sox17 and S100β, were clonable, and could differentiate into neural lineages as well as mesenchymal lineages, although NCCL-SSCs were not derived from neural crest during the development. When treated with transforming growth factor β1 (TGF-β1), NCCL-SSCs differentiated into mesenchymal stem cells (MSCs), lost the expression of Sox17 and the differentiation potential into neural lineages, but retained the potential of differentiating into mesenchymal lineages. To determine the responses of NCCL-SSCs to microfibrous scaffolds for tissue engineering, electrospun composite scaffolds with various porosities were fabricated by co-electrospinning of structural and sacrificial microfibers. The increase in the porosity in microfibrous scaffolds enhanced cell infiltration in vitro and in vivo, but did not affect the morphology and the proliferation of NCCL-SSCs. Interestingly, microfibrous scaffolds with higher porosity increased the expression of chondrogenic and osteogenic genes but suppressed smooth muscle and adipogenic genes. These results suggest that the differentiation of NCCL-SSCs can be controlled by both soluble chemical factors and biophysical factors such as the porosity of the scaffold. Engineering both NCCL-SSCs and scaffolds will have tremendous potential for tissue regeneration.

1. Introduction

Osteoarthritis represents structural breakdown of the synovial joint, affecting 70 million people in the United States [1]. Thus, there is an urgent need to develop effective treatment for the regeneration of tissues such as cartilage, bone and tendon. Mesenchymal stem cells (MSCs) have been isolated from synovial membrane and show potential for cartilage, bone and tendon tissue engineering [2]. In addition, bioactive scaffolds with transforming growth factor β3 (TGF-β3) or stromal cell derived factor-1 (SDF-1) have been used for in situ cartilage regeneration by recruiting synovial stem cells (SSCs) [3,4]. These results suggest that SSCs are a valuable cell source for both in vitro tissue engineering and in situ knee joint repair. However, the characterization of synovial MSCs is limited to non-specific surface markers such as CD29 and CD44, and it is not clear whether MSCs exist in synovial membranes at an earlier differentiation stage. Here we used explant culture to isolate a precursor of MSCs from the synovial membrane, characterized as neural crest cell-like SSCs (NCCL-SSCs), and investigated how soluble chemical factors and scaffold property could regulate the functions of this MSC precursor.

Scaffolds can be fabricated for tissue engineering by various methods. Electrospinning is a highly versatile method that allows the fabrication of porous, nonwoven and three-dimensional fibrous structures with controllable fiber diameter ranging from nano- to micro-scale [5,6], and thus has been used extensively in bone, cartilage, tendon, adipose tissue and muscle tissue engineering [7–9]. However, the porosity of electrospun scaffolds is generally low as a result of densely packed network of interconnected fibers. In order to increase the porosity of electrospun scaffolds for cell infiltration, many approaches have been investigated, including using a rotating metal-frame cylinder with different rotation speeds [10], tailoring fiber diameter [11], combining nano- and microfibers [9], using NaCl crystals as porogen agents [12], post-processing by laser ablation

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[13] or ultraviolet radiation treatment [14], and incorporation of sacrificial fibers [15]. Here, we utilized co-electrospinning method to create microfibrous scaffold with various numbers of sacrificial fibers and thus varying porosity. In this study, we investigated the effect of scaffold porosity as a biophysical cue of extracellular matrix (ECM) on SSC differentiation, which is not well understood compared to the effects of soluble biochemical stimuli [16].

2. Materials and methods

2.1. Cell isolation

The synovial membrane was isolated from the knee joints of Sprague–Dawley (SD) rats under a dissecting microscope. Tissue segments were washed three times with phosphate-buffered saline (PBS) supplemented with 1% penicillin/streptomycin (P/S), cut into millimeter-sized pieces and placed onto the surface of 6-well plates coated with 1% CellStart (Invitrogen Corp.), and maintained at 37 °C in an incubator with 5% CO_2. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2% chick embryo extract (MP Biomedical, Inc.), 1% FBS, 1% N2 supplement (Invitrogen Corp.), 2% B27 supplement (Invitrogen Corp.), 100 nM retinoic acid (Sigma–Aldrich, Inc.), 50 nM 2-mercaptoethanol (Sigma–Aldrich, Inc.), 1% P/S and 20 ng ml^{-1} bFGF (R&D Systems, Inc). Cells migrated out from the tissues within 3 days. Cells were also isolated from synovial membranes of Wnt1-Cre/LoxP-yellow fluorescence protein (YFP) mouse [17] by using the same method.

2.2. Immunostaining and dye staining

For immunostaining, cells were fixed with 4% paraformaldehyde (PFA), permeabilized with 0.5% Triton X-100 (Sigma–Aldrich, Inc.), and blocked with 1% bovine serum albumin (BSA) (Sigma–Aldrich, Inc.). Samples were incubated with specific primary antibodies against Sox10 (R&D systems), Sox17 (R&D systems), Snail (Santa Cruz Biotechnology, Inc.), Pax-3/7 (Santa Cruz Biotechnology, Inc.), Slug (Santa Cruz Biotechnology, Inc.), vimentin (DAKO), NG2 (Millipore), S100 calcium binding protein B positive (S100β) (Sigma–Aldrich, Inc.), neural filament-medium polypeptide (NFM) (Sigma–Aldrich, Inc.), glial fibrillary acidic protein (GFAP) (Millipore), neuronal class III β-Tubulin (TUJ1) (Chemicon), smooth muscle α-actin (SMA) (Epitomics, Inc), smooth muscle myosin heavy chain (SM-MHC) (Santa Cruz Biotechnology, Inc.), calponin-1 (CNN-1) (Epitomic, Inc.) and Collagen II (Chemicon) for 2 h at room temperature, washed three times with PBS, and incubated with appropriate Alexa 488- and/or Alexa 546-labeled secondary antibodies (Molecular Probes, Inc.). Nuclei were stained with DAPI (Invitrogen Corp.). Fluorescence images were collected using a Zeiss LSM710 confocal microscope (Carl Zeiss MicroImaging). For organic dye staining, cells were fixed with 4% paraformaldehyde for 30 min, washed and stained with alizarin red (Sigma–Aldrich, Inc.), alcian blue (Sigma–Aldrich, Inc.), or oil red (Sigma–Aldrich, Inc.) according to the manufacturer's instructions. Images were collected using a Zeiss Axioskop 2 plus microscope.

2.3. Flow cytometry analysis

For flow cytometry analysis, cells were dissociated after exposure to 0.2% EDTA for 20 min at room temperature. The cells in suspension were blocked with 1% BSA, incubated with specific preconjugated primary antibodies against CD29 (BD Pharmingen) and CD44 (BD Pharmingen). Negative control sample was incubated with a non-specific antibody with the same isotype as the specific primary antibody, and stained with the same secondary antibody. 7-AAD (BD Pharmingen) was used to exclude dead cells. Cells were analyzed by using FACScan flow cytometer (Becton Dickinson, Inc.) and FlowJo software (Tree Star, Inc.).

2.4. Single cell cloning and stem cell differentiation

For the clonal assays, cells were detached, resuspended with maintenance medium, and filtered through membranes with 40 μm pore size to obtain single cells. Filtered cells were seeded onto CellStart-coated 96-well plates at the clonal density (1 cell/well) and cultured for 3 weeks at 37 °C in an incubator with 5% CO_2. For the directed differentiation into peripheral neuron-like cells, Schwann cell-like cells, osteoblasts, adipocytes and chondrocytes, the cells were incubated in specific induction media for 1–3 weeks as described previously [18,19].

2.5. Fabrication of PLLA and PLLA/PGA microfibrous scaffolds

To produce single-polymer poly(L-lactide) (PLLA) (1.09 dl g^{-1} inherent viscosity, MW ~131,000) (Lactel Absorbable Polymers) microfibrous scaffolds (as control scaffolds), we performed electrospinning as described previously [14]. In addition, we used PLLA to make structural fiber and poly(glycolic acid) (PGA) (MW ~100,000) (Polysciences, Inc.) to make fast-degrading sacrificial fibers, and fabricated composite microfibrous scaffolds by co-electrospinning. First, we dissolved PLLA and PGA pellets in hexafluoroisopropanol to prepare PLLA (19% w/v) and PGA (12.5% w/v) solutions, respectively. Two 10 ml syringes (loaded in syringe pumps) were filled with PLLA and PGA, respectively, and fitted with flexible silicon tubing connected to 1.5 in. long stainless steel 23G dispensing needles. The two needles were electrically charged by connecting to a +12 kV high-voltage generator, and were placed 90° apart with the PLLA spinneret facing a grounded collecting drum horizontally. For the three types of scaffolds produced (19% w/v PLLA, 19% w/v PLLA/12.5% w/v PGA (low) and 19% w/v PLLA/12.5% w/v PGA (high)), a random orientation of microfibers was achieved by using a low rotation speed (200 rpm) for the collecting drum. We altered the flow rate and gap distance (distance between needle tip and collecting drum) conditions for PGA to generate two different composite scaffolds with varying numbers of PGA fibers. Briefly, the PGA solution was delivered at a flow rate of 0.4 ml h^{-1} and gap distance of 7 cm for one type of composite scaffold with lower PGA content (referred to as PLLA/PGA (low)) and at a higher flow rate of 1 ml h^{-1} and a shorter gap distance of 4 cm for another type of composite scaffold with higher PGA content (referred to as PLLA/PGA (high)). Depending on the collecting time, finalized microfibrous scaffolds were ~200–350 μm in thickness based on measurements with a thickness gauge (Mitutoyo America).

To degrade the microfibrous scaffolds and selectively remove the PGA sacrificial fibers from co-electrospun composite scaffolds, we prepared 1 cm × 1 cm scaffolds and degraded them in PBS at 37 °C for 2 or 4 weeks. Due to acidic polymer remnants as a result of random hydrolytic degradation, PBS was changed every other day throughout the study. The 2 weeks and 4 weeks post-degraded (PD) scaffolds as well as the as-spun (AS) scaffolds without degradation were examined and imaged by field emission scanning electron microscopy (SEM; TM-1000, Hitachi). Furthermore, to confirm the removal of PGA sacrificial fibers, we performed Fourier transform infrared (FTIR) spectrometry with a FTIR spectrometer (Nicolet Avatar 360, Thermo Fisher Scientific) on the AS and 4 weeks PD scaffolds from the PLLA and PLLA/PGA (high) groups as described previously [20].

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