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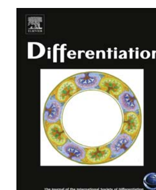

Effect of matrix stiffness on the proliferation and differentiation of umbilical cord mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) are a compatible cellular alternative for regenerative medicine and tissue engineering because of their powerful multipotency. Matrix stiffness plays a profound role on stem cell behavior. Nevertheless, the effect of matrix stiffness on umbilical cordmesenchymal stem cells (UC-MSCs) remains unexplored. To conduct an in-depth exploration, we cultured UC-MSCs on different stiffness (Young's modulus: 13–16, 35–38, 48–53, and 62–68 kPa) polyacrylamide gels coated with fibronectin. We found that the proliferation and adhesion of UC-MSCs varied when cultured on the different matrices, and the spreading capacity was stronger as the stiffness increased (*$P < 0.05$). Real-time quantitative PCR results showed that the soft matrix promoted adipogenic differentiation, with higher expression levels of adipocytic markers like PPARγ and C/EBPα (*$P < 0.05$). In contrast, cells tended to differentiate into muscle when cultured on the 48–53 kPa matrix, which was validated by increased expression of myogenic makers like desminand MOYG (*$P < 0.05$). Moreover, increased expression of osteoblastic makers (*$P < 0.05$), such as ALP, collagen type I, osteocalcin, and Runx2, confirmed that cells differentiated into bone on the high-stiffness matrix.

1. Introduction

Tissue engineering involves functional biomaterial scaffolds and cells for restoring damaged or diseased tissue. Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) (Jin et al., 2015) can differentiate into several lineages, including adipose cells, chondrocytes, osteoblasts, neuronal cells, endothelial cells, cardiomyocytes, hepatocyte-like cells, and pancreatic beta cells (Anzalone et al., 2010, 2011; Wang et al., 2004). hUC-MSCs are derived from an ethically uncontroversial and practically inexhaustible source and may be harvested noninvasively at low cost (Li et al., 2015). Moreover, these cells do not induce teratomas but have anticancer effects (Witkowska-Zimny and Wrobel, 2011). Therefore, hUC-MSCs are attractive cells for a multitude of tissue engineering applications.

It is increasingly clear that cells respond to the mechanical properties of their surrounding matrix, including the effects of mechanics on spreading, morphology, and function (Floren et al., 2016). Many cell types spread more on a stiffer matrix (Lo et al., 2000; Ye et al., 2016; Yeung et al., 2005) or migrate towards regions of higher modulus (Lo et al., 2000; Smith et al., 2006). Tyrosine signaling (Giannone and Sheetz, 2006) and proliferation (Peyton et al., 2006; Subramanian and Lin, 2005) of fibroblasts, smooth muscle cells, and chondrocytes are all regulated by matrix stiffness. Pioneering work on stem cells proved that bone marrow mesenchymal stem cells (MSCs) cultured on a soft matrix (0.1–1 kPa) mimicking the elasticity of the brain became neuronal-like; on a stiffer matrix (8–17 kPa) mimicking the characteristics of muscle, these stem cells developed myogenic properties, whereas on the hardest matrix (25–40 kPa) mimicking pre-mineralized bone, the cells committed to an osteoblast phenotype (Engler et al., 2006). However, the effect of matrices on UC-MSCs still requires research.

Polyacrylamide hydrogels, high-water-absorbency-ratio chemical-crosslink hydrogels obtained by crosslinking and polymerization of acrylamide and bisacrylamide, are widely used in biomaterial research. By adjusting the concentration of acrylamide and bisacrylamide, matrices of varying stiffness ranging from several hundreds to thousands of pascals can be gained, which can truly reflect the cell-survival stiffness in vivo (Lantoine et al., 2016; Trensz et al., 2015). In addition, polyacrylamide hydrogels are used widely as matrices for stem cell culture because of their hydrophilic, stable physicochemical property, less susceptibility to microbial corrosion and enzymatic hydrolysis, and

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non-toxicity to tissue cells (Huang et al., 2015). In this study, we employed fibronectin-coated polyacrylamide hydrogels crosslinked to varying degrees to modify the mechanical microenvironment and determine how hUC-MSCs respond to matrix stiffness in terms of morphology, adhesion, proliferation, and differentiation, which is the basis of application of stiffness on stem cells.

2. Materials and methods

2.1. Cell culture and characterization

Primary MSCs were isolated from the human umbilical cord under ethical approval and maintained in an expansion medium (DMEM-F12, Gibson, USA) consisting of 10% fetal bovine serum (Gibco) supplemented with 1% penicillin/streptomycin (Dalian Meilun Biotech Co., Ltd, China), 10 ng/mL of basic fibroblast growth factor (PeproTech, USA) and incubated in an atmosphere of 5% CO2 at 37 °C. (Witkowska-Zimny et al., 2012). All experimental procedures were approved by the ethics committee of Jilin University and conformed to the regulatory standards. Isolated MSCs were characterized by the expression of surface markers through flow cytometric analysis and immunofluorescence assays. The multipotencyof the MSCs differentiated into mesenchymal lineages, including adipocytes and osteoblasts, was confirmed before the cells were used for the following experiments (Dehkordi et al., 2015; Hou et al., 2009).

The osteogenic differentiation of MSCs was induced in osteogenic medium containing 0.1 μmol/L dexamethasone (Dalian Meilun Biotech Co., Ltd, China), 10 mmol/L b-glycerophosphate, 50 μg/mL ascorbic acid, and 10 nM vitamin D3. The differentiation of MSCs into adipocytes was induced in adipogenic medium containing 1 μM dexamethasone, 10 μg/mL insulin, 100 μg/mL (0.45 mM) 3-isobutyl-1-methylxanthine (IBMX), and 0.1 mM indomethacin. The differentiation-inducing medium was changed every 2 days. MSCs were used at passage 3 for all experiments.

2.2. Flow cytometric analysis

Expression of MSC surface markers was determined using flow cytometry and immunofluorescence staining. Cells were collected, washed thrice with phosphate buffered saline (PBS), and fixed with 4% polyformaldehyde for 20 min. The cells were then blocked with 1% BSA in PBS for 30 min and incubated with 10 μg/mL anti-CD34, anti-CD44, anti-CD45, anti-CD90, and anti-CD105 mAbs (eBioscience, USA) for 1 h.

2.3. Oil red O and alizarin red S staining

For evaluation of lipid droplets, the cells were fixed with 4% paraformaldehyde for 10 min and stained with oil red O for 10 min at room temperature. For characterization of the mineralized matrix, the cells were fixed with 3.7% paraformaldehyde and stained with 1% of alizarin red S solution in water for 10–15 min at room temperature. The cells were observed under an inverted phase contrast microscope.

2.4. Cell cycle detection

Cells were digested by non-enzyme cell detach solutions after 48 and 72 h of plantation, and then rinsed thrice with PBS and fixed with 75% cold ethanol, followed by 4 °C storage overnight. At day 2, the cells were collected by centrifugation. Finally, a cell cycle detection kit (KeyGEN, Nanjing, China) was used to detect the cell cycle.

2.5. Fabrication of polyacrylamide matrices with varying stiffness

Tunable polyacrylamide matrices were prepared as reported previously (Wang and Pelham, 1998). Briefly, glass cover slips were treated with 3-aminopropyltrimethoxysilane and 0.5% glutaraldehyde. Then, 8% acrylamide (sigma, USA) was mixed with varying concentrations of bisacrylamide (0.1%, 0.3%, 0.5%, and 0.7%) (Sigma, USA). Polymerization was initiated with N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium persulfate (sigma, USA). Then, 0.2 mg/mL N-sulfosuccinyimidyl-6-(4′-azido-2′-nitrophenylamino) hexanoate (sulfo-SANPAH) (Thermo, USA) dissolved in 10 mM HEPES (pH 8.5) was applied to cover the polyacrylamide gel and exposed to 365 nm ultraviolet light for 70 min for photo activation in 24-well plates. The polyacrylamide sheet was washed thrice with PBS to remove excess reagent and incubated with fibronectin solution (1 μg/cm^2; Sigma, USA) overnight at 4 °C. Before the cells were plated, the polyacrylamide matrices were soaked in PBS and then in DMEM at 4 °C. The Young's modulus of the polyacrylamide hydrogels was quantified using a biomechanical testing machine under contact load at a strain rate of 0.5 mm/s.

2.6. Microscopy and imaging analysis of cell morphology

The morphologic changes of MSCs were observed and photos were taken using an inverted phase contrast microscope at 8, 48, and 72 h after seeding on polyacrylamide matrices. The major and minor axes of the cells were computed from the thresholded binary image of the cell using NIH ImageJ; the aspect ratio of the cell is the ratio of the major to minor axes.

For scanning electron microscope (SEM) imaging, after being washed thrice in PBS, the cells were fixed with 1% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.2) at 4 °C for 3 days. After removing the glutaraldehyde using PBS, the fixed cells were dehydrated in graded ethanol and then ester exchanged with isoamyl acetate. Finally, the cells were critical point-dried using CO_2.

2.7. Cell adhesion assays

For the analysis of cell adhesion, 1.0 × 10 cells/cm^2 were seeded into each well in a 24-well plate and allowed to attach for 24 h. Then, the cells were washed thrice with PBS to remove non-adherent cells, followed by the addition of 4% paraformaldehyde for 10 min. The cells were then washed thrice with PBS again. After incubation for 5 min with Hoechst, the attached cells were observed using a fluorescent inverted phase contrast microscope.

2.8. EdU cell proliferation assay

Cell proliferation was further analyzed using a Cell-Light™ EdU DNA Cell Proliferation Kit (Ribobio, Guangzhou, China) after 72 h. Briefly, the cells were re-suspended in fresh pre-warmed (37 °C) complete medium, counted, and plated at a density of 3 × 10^4 cells/mL onto a 24-well plate, in which gel slides had been placed. Twenty-four hours later, the cell culture medium was replaced with medium containing EdU, and the cells were incubated for an additional 2 h. Then, the cells were fixed, exposed to an Apollo® reaction cocktail and analyzed using electronic fluorescent microscopy.

2.9. Gene expression analysis

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to determine the relative gene expression of osteogenic specific genes. Total RNA was extracted using the TRI reagent (Sigma-Aldrich, USA). The same amount of total RNA was used to synthesize the first strand cDNA using a HiScript® II 1st Strand cDNA Synthesis Kit (Vazyme Biotech Co.,Ltd, China). The reaction mix contained a 20 ng template of cDNA, ChamQ Universal SYBR qPCR Master Mix (10 μl; Vazyme Biotech Co.,Ltd, China) and 400 nM each of the forward and reverse primers (Sangon, China)in a final volume of 20 μl. The PCR thermal profile consisted of 95 °C for 10 min, followed

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