Biomaterials 49 (2015) 68–76

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Matrix stiffness determines the fate of nucleus pulposus–derived stem cells

Yosi Navaro [a], Nadav Bleich-Kimelman [a], Lena Hazanov [b], Iris Mironi-Harpaz [b], Yonatan Shachaf [b], Shai Garty [c, g], Yoav Smith [d], Gadi Pelled [a, e, f], Dan Gazit [a, e, f], Dror Seliktar [b], Zulma Gazit [a, e, f, *]

[a] Skeletal Biotech Laboratory, The Hebrew University–Hadassah Faculty of Dental Medicine, Ein Kerem, Jerusalem 91120, Israel
[b] Department of Biomedical Engineering, Technion–Israel Institute of Technology, Haifa 32000, Israel
[c] Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel
[d] Genomic Data Analysis Unit, The Hebrew University–Hadassah Medical School, The Hebrew University of Jerusalem, Jerusalem 91120, Israel
[e] Department of Surgery, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA
[f] Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA
[g] Max Planck Institute for Intelligent Systems, Stuttgart, Germany

ARTICLE INFO

Article history:
Received 17 September 2014
Accepted 20 January 2015
Available online

Keywords:
Elasticity
Matrix stiffness
Fibrinogen
Hydrogel
Stem cells
Intervertebral disc

ABSTRACT

Intervertebral disc (IVD) degeneration and consequent low-back pain present a major medical challenge. Nucleus pulposus–derived stem cells (NP–SCs) may lead to a novel therapy for this severe disease. It was recently shown that survival and function of mature NP cells are regulated in part by tissue stiffness. We hypothesized that modification of matrix stiffness will influence the ability of cultured NP-SCs to proliferate, survive, and differentiate into mature NP cells. NP-SCs were subcultured in three-dimensional matrices of varying degrees of stiffness as measured by the material's shear storage modulus. Cell survival, activity, and rate of differentiation toward the chondrogenic or osteogenic lineage were analyzed. NP-SCs were found to proliferate and differentiate in all matrices, irrespective of matrix stiffness. However, matrices with a low shear storage modulus ($G' = 1$ kPa) promoted significantly more proliferation and chondrogenic differentiation, whereas matrices with a high modulus ($G' = 2$ kPa) promoted osteogenic differentiation. Imaging performed via confocal and scanning electron microscopes validated cell survival and highlighted stiffness-dependent cell-matrix interactions. These results underscore the effect of the matrix modulus on the fate of NP-SCs. This research may facilitate elucidation of the complex cross-talk between NP-SCs and their surrounding matrix in healthy as well as pathological conditions.

1. Introduction

Intervertebral disc (IVD) degeneration and consequent low-back pain present a major medical challenge with no optimal solution in sight. In Western society, this pathological condition is prevalent among people younger than 45 years of age and percentage of the work force affected varied from 2% to 8% with days of absence from work per patient [1].

The IVD consists of three major anatomic zones: the nucleus pulposus (NP), the annulus fibrosus (AF), and cartilage endplates. These three anatomic zones are distinct but uniquely attached, contributing to the mechanical function of the IVD [2,3]. The NP is a gel-like substance that contributes to the load-bearing capacity of the IVD and sustains the flexion/extension and lateral bending are spine movements required for many daily activities. This gelatinous substance plays an important role in the IVD's mechanical function by redistributing spinal compressive loads [4]. During IVD degeneration, the disc's biophysical and biochemical properties are altered. The pathogenesis of IVD degeneration is complex and principally relies on resident NP cells to revitalize tissue [3,4].

The NP is originally derived from the notochord [5–7]; the NP cells in the immature nucleus are smaller and contain more

* Corresponding author. Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA. Fax: +1 (310) 248 8066.
E-mail address: zulmag@ekmd.huji.ac.il (Z. Gazit).

http://dx.doi.org/10.1016/j.biomaterials.2015.01.021
0142-9612/

condensed and smaller nuclei than the notochordal cells [8]. By early adulthood, the NP becomes populated by chondrocyte-like cells [2,5,6]. It is well established that the NP contains NP-derived stem cells (NP–SCs), which maintain homeostasis in this tissue [2,7,8] and have been shown to commit to osteogenic and chondrogenic lineages in vitro [9].

With respect to the biochemical composition of NP tissue, it is highly hydrated and contains negatively charged sulfated glycosaminoglycans (sGAGs), collagens, and other noncollagenous proteins [3,6]. On initiation of IVD degeneration, the biosynthesis of proteoglycans and collagen is inhibited [1]. Specifically, the NP cell population alters its biochemical and secretory function, resulting in decreases in proteoglycan content and in *aggrecan* and *type II collagen* gene expression, as well as biophysical changes such as decreased osmotic pressure, cell volume, and fixed charge density [4]. These alterations initiate a catabolic modification in the structure of the extracellular matrix (ECM), which normally maintains IVD functionality [5]. Evidently, these alterations also affect the cell's proliferation rate and differentiation potential [9]. Recent studies have shown that interactions between NP cells and the ECM depend not only on matrix protein composition, but also on matrix stiffness; thus, changes in cell fate and phenotype are likely to be modulated by tissue stiffness and the mechanical microenvironment of the cells [10]. These observations are in complete agreement with those of others who demonstrated earlier that the mechanical stiffness of the ECM has a profound effect on the fate of mesenchymal stem cells (MSCs) [11–14]. For example, Choi et al. [15] documented that the fate of MSCs is directed down an osteogenic lineage when these cells are exposed to certain ECM mechanical properties. Moreover, the secretion of calcified ECM by osteoblast-like cells was shown to be a mechanosensitive response to substrate stiffness [16]. Similarly, others have demonstrated that ECM stiffness modulates the proliferation and differentiation of mature NP cells in vitro [5,6,11,12,17].

Recently, we demonstrated that cultures of NP-SCs from degenerate discs differ in their proliferation and differentiation capacities when compared to cells from healthy discs [9]. We proposed that ECM stiffness and changes in the elasticity of the degenerated disc matrix are associated with the impaired function of resident NP-SCs, which probably contributes to the onset of the IVD degeneration process. However, to the best of our knowledge, there have been no studies to date that document the effect of matrix stiffness on the fate of NP-SCs.

There is a growing consent that 3D models recreate significant characteristics of the microenvironment providing more relevant biological information than 2D models. In the conventional 2D cultures, cells propagate in monolayers on a solid substrate; they grow flat and reach a short height, relatively fixed. Cells that are grown in a 3D model keep a 3D structure, more versatile, with measurable dimensions all round; furthermore, the interactions between close cells cultured in 3D are all around, not restricted to the boundaries of the cells in a particular plane, as in 2D cultures [18]. In the current investigation, we set out to explore the role of matrix stiffness on the fate of NP-SCs in culture. To do this, we developed a hydrogel biomaterial system that can encapsulate NP-SCs in a three-dimensional (3D) culture, sustain the cells' survival, and provide mechanical cues to the cells based on variations in the storage shear modulus of the encapsulating milieu. This tunable hydrogel is a semi-synthetic material made from adducts of fibrinogen and poloxameric block copolymers called Tetronic 1307. We took advantage of the distinct physical properties of the fibrinogen–Tetronic adducts to control the physical properties of the resulting hydrogel in a manner independent from other critical material properties such as ligand density (i.e. fibrinogen concentration). These materials enabled us to investigate the in vitro fate of NP-SCs in a highly controlled mechanical environment and provided us with a potential scaffold material for NP regeneration. Accordingly, we addressed the hypothesis that modification of matrix stiffness can be used to enhance the ability of NP-SCs to proliferate, survive, and differentiate into mature NP cell populations.

2. Materials and methods

2.1. Hydrogel manufacturing

2.1.1. Tetronic 1307–tetraacrylate synthesis

The acrylation of Tetronic 1307 (T1307, also known as Pluracare1307, O-BASF MW = 18 kDa) was conducted in the same way as the deacrylation of polyethylene glycol [19]. Briefly, the process was carried out under an Argon atmosphere by reacting T1307–tetraol in a solution of dichloromethane (Aldrich, Sleeze, Germany) and toluene (Bio-Lab, Jerusalem, Israel) with acryloyl–chloride (Merck KGaA, Darmstadt, Germany) and triethylamine (TEA) (Fluka, Buchs, Switzerland) at a molar ratio of 150% relative to the hydroxyl groups. The final product was precipitated out in petroleum ether (40°–60°C) (Bio-Lab). The solid polymer was dried under vacuum conditions for 48 h. The product was characterized by proton nuclear magnetic resonance (NMR) to determine the average number of acryl groups on the T1307 molecule.

2.1.2. Conjugation of T1307 to fibrinogen

Fibrinogen was conjugated to Tetronic-tetraacrylate (T1307-TA) by a Michael-type addition reaction. To conjugate fibrinogen to the synthetic polymers, an 8.3-mg/ml solution of fibrinogen in PBS (150 mM) with 8 M urea was supplemented with tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Sigma, St. Louis, MO, USA) at a molar ratio of 1.5:1 TCEP to fibrinogen cysteines. After dissolution of the fibrinogen, the functionalized polymer (T1307-TA) in a solution of PBS and 8 M urea (260 mg/ml) was added at a molar ratio of 4:1 synthetic polymer to fibrinogen cysteines (T1307-TA). The reaction was incubated for 3 h at room temperature, after which the volume of reaction was doubled by adding PBS and 8 M urea. The conjugated protein was precipitated out by adding 6 volumes of acetone (Bio-Lab) to the final solution. The precipitate was dissolved in PBS containing 8 M urea at a protein concentration of 7–9 mg/ml and then dialyzed against PBS at 4°C for 2 days with two changes of PBS per day (Spectrum, MW cutoff 12–14 kDa). The net fibrinogen concentration in the Tetronic1307–fibrinogen (TF) precursor was determined using a Nano-drop ND-2000 spectrophotometer. The final product was characterized according to previously published protocols [20].

2.2. Biomechanical analysis of the hydrogel

2.2.1. In situ rheological characterization

The *in situ* hydrogel formation, mechanical properties, and cross-linking kinetics were characterized using an AR-G2 shear rheometer (TA Instruments, New Castle, DE, USA) equipped with a Peltier plate temperature-controlled base. Time-sweep oscillatory tests were performed in 50-mm parallel-plate quartz geometry using 600 μl of acellular TF precursor solution containing 0.1% w/v Irgacure 2959 initiator (Ciba, Basel, Switzerland). To monitor the in situ liquid-to-solid transition (gelation), the acellular precursor solution was first held at 4°C for 1 min to allow the liquid to equilibrate, followed by a 7-min exposure to UV light (365 nm, 2 mW/cm^2). Then the temperature was raised to 37°C where it was held for an additional 5 min without UV light. To find the linear viscoelastic region of the time-sweep tests, oscillatory strain (0.1–10%) and frequency sweeps (0.1–10 Hz) were conducted in two separate samples at 4°C (following exposure to UV light) and again at 37°C (the temperature was raised to 37°C after exposure to UV light at 4°C, as before). The linear viscoelastic region was found to be in the range of 2% strain and 1 Hz frequency (data not shown). Using time-sweep experiments, the viscoelastic material properties, including the storage and loss modulus values (G′ and G″), as well as the phase angle, were continuously recorded [21]. The plateau storage modulus, G′ was reported as the characteristic measure of the elastic properties of the hydrogels. The reported G′ was taken as real part of complex shear modulus, $G^* = G' + iG''$ at the conclusion of the time-sweep test. Consequently, the G″ values were one to two orders of magnitude lower then the G′ values. Two acellular hydrogel compositions were characterized for the experimental design in the proceeding sections: a low modulus (G′ = 1 kPa) and a high modulus (G′ = 2 kPa) material. The two test group materials were created by adding increasing amounts of Tetronic 1307-TA to the TF, and they were identified by their initial in situ measurements of storage shear modulus. The inclusion of cells into the precursor solutions did not alter their in-situ rheological properties (data not shown).

2.2.2. Ex situ rheological characterization

Ex situ measurements of hydrogel modulus were designed to measure how the hydrogel modulus was affected by cultured cells after certain durations in vitro. For this, precast hydrogels cultured at various time points were placed in an AR-G2 shear rheometer (TA Instruments, New Castle, DE, USA) equipped with 20-mm parallel-plate geometry. To ensure that the *ex situ* rheological characterization results were valid, the sample diameter was always matched to the diameter of the