International Journal of Biological Macromolecules 79 (2015) 133–143

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

The effect of hyaluronan on the motility of skin dermal fibroblasts in nanofibrous scaffolds

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ARTICLE INFO

Article history:
Received 14 January 2015
Received in revised form 20 April 2015
Accepted 23 April 2015
Available online 2 May 2015

Keywords:
Hyaluronan
Electrospun
Cell migration

ABSTRACT

Nanofibrous scaffolds that use the native extracellular matrix are promising developments in skin tissue regeneration because they provide the proper environment for the adhesion, migration and growth of skin dermal fibroblasts, important during wound healing. In this study, we focus on hyaluronan as a native ECM that regulates cellular motility in nanofibrous scaffolds. PCL/HA nanofibrous scaffolds were generated by electrospinning and assessed for various physicochemical properties. HA-based scaffolds significantly enhanced cell infiltration *in vitro* and *in vivo*. The observation of movements in living cells revealed that HA-based scaffolds regulated cell migration speed and direction. This phenomenon may influences by the variation in cell adhesion receptors-integrin β1, and vinculin formation and distribution. Furthermore, we confirmed that HA/CD44 interactions can activate the TGF-β/MMP-2 signaling pathway that promotes cell motility. These findings suggest HA functions in the cell motility of nanofibrous scaffolds and have potential implications for the use of HA-based scaffolds in skin tissue regeneration applications.

1. Introduction

Cell motility is critical in many physiological and pathological processes, as well as in tissue-engineering applications. Cell motility is moderated by a complex, spatiotemporally integrated set of biophysical mechanisms that are influenced by intracellular signaling and the biochemical and biophysical properties of the surrounding extracellular matrix (ECM) [1,2]. Understanding how ECM can influence cell motility within physiological microenvironments will improve the future design of biofunctional scaffolds [3], since one of the main challenges to successful grafting is to rapidly cellularize the initially acellular scaffolds [4].

As one of the key ECMs involved in cell motility, hyaluronan (HA) has been well studied [5]. HA is a ubiquitously distributed linear glycosaminoglycan composed of repeating disaccharides of (1–3) and (1–4)-linked β-D-glucuronic acids and *N*-acetyl β-D-glucosamine monomers [6,7]. HA is also a ligand for cluster determinant 44 (CD44), a cell-surface receptor found on most cell types, and upregulation of HA has been reported following tissue injury and cellular motility [8]. Along with a potential role in cellular migration, the extracellular portion of CD44 can function as a specialized platform for growth factors and matrix metalloproteinases (MMPs), the interactions between CD44 and these functional proteins of which are subject to proteolytic cleavage [9,10]. The cytoplasmic tail of CD44 can interact with proteins that bind to the actin cytoskeleton and previous studies have implicated CD44 as a co-receptor for Integrin family mediated cell adhesion and migration [11].

Due to its excellent biocompatibility and biodegradability, HA has been extensively used in tissue engineering scaffolds, including cartilage tissue engineering, skin wound healing, revascularization, *etc.* [7,12,13]. There has also been interest in electrospun HA in order to mimic the architecture of the natural ECM. HA is difficult to electrospin because the unusually high viscosity and surface tension of the aqueous HA solution hinders the electrospinning process [14]. Hence, a blend of HA and a carrier polymer, such as poly(ethylene oxide) (PEO) [15] or poly(ε-caprolactone) (PCL) can improve the electrospinning process [16].

In previous studies we found poly(ε-caprolactone) (PCL), silk fibroin (SF) and HA nanofibers could be processed to form porous structures that support tissue ingrowth, and correctly oriented pore structures could be induced by swelling [17]. In this study, we tested PCL as a carrier polymer and successfully fabricated PCL/HA blend nanofibrous scaffolds to investigate the influence of HA on the formation of cell adhesion receptors and the expression of cell biochemical factors. PCL has been widely used as the synthetic polymer of choice in electrospun fibers owing to its excellent electrospinnability, favorable mechanical properties, good blend-compatibility, and slow degradation [18]. PCL blending can be used to avoid chemical cross-linking and maintain the stability of

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http://dx.doi.org/10.1016/j.ijbiomac.2015.04.059
0141-8130/

electrospun nanofibers. Thus, we hypothesize that PCL acts as the main structural component of the scaffold, while HA provides an ECM biochemical surface to promote cell migration. We investigated the contribution of HA-CD44 interactions in integrin-β1, the distribution of vinculin, and MMP-2 production mediated by transforming growth factor-β (TGF-β). This study may help to develop the next generation of bioactive tissue engineering scaffolds that can control cell motility by engineering the local extracellular environment.

2. Materials and methods

2.1. Materials

HA (high molecular weight, sodium salt, 2.5×10^6 Da, from *Streptococcus equi*) and PCL (*Mw* = 90 kDa) were obtained from Sigma–Aldrich, Co. (USA). Hexafluoroisopropanol (HFIP) and formic acid (FA) were purchased from Dupont Chemical Co. (USA). Fibroblasts media: Modified Eagle's Media (MEM), fetal bovine serum (FBS) and antibiotics were purchased from Gibco LifeTechnology, Co. (USA). CellTiter 96-Aqueous One Solution Reagent (MTS), used in the cell proliferation assay, was purchased from Promega Co. (USA). Enzyme-linked immunosorbent assay (ELISA) kits for human transforming growth factor (TGF)-β1 was purchased from NeoBioscience Technology, Co. (China). Fluorescein isothiocyanate (FITC)-Phalloidin and 4′-6-diamidino-2-phenylindole (DAPI) were purchased from Enzo Life Sciences International, Inc. (USA) and Roche Applied Science (Germany), respectively. Mouse monoclonal anti-CD44, anti-vinculin and anti-intergrin β1 antibodies were purchased from Abcam, Inc. (USA). Alexa Fluor 488 and 555-labeled secondary antibody were obtained from Invitrogen (USA). Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich.

2.2. Preparation of electrospinning nanofibers

Nanofibers were fabricated using a conventional electrospinning setup reported earlier. PCL solution was prepared by dissolving 0.5 g of PCL in 5 ml HFIP to form 10% (w/v) solution. HA solution was prepared by dissolving 0.06 g of HA in 3 ml FA to form 2% (w/v) solution. Two different solutions were prepared by mixing PCL and HA in ratios of 5:0, 5:1, and 5:2 (v/v).

Electrospinning was performed in a fume hood using open cage target to collect fibers. Relative humidity was adjusted by flushing the hood with dry air. A volume flow rate of 1.5 ml/h of the electrospun solutions through a blunted stainless steel needle (ID = 0.8 mm) was maintained using a syringe pump (Longer Precision Pump, Baoding, China). For electrospinning, a range of voltage of 15–20 kV was applied to the capillary tube using a high voltage power supply (Dongwen High Voltage Power Supply Plant, Tianjin, China). The distance between the capillary tube and the grounded target was 12–15 cm. Random oriented fibrous scaffolds were collected on the open cage target of d = 15 cm when rotating at 300 r/min.

2.3. Characterization of nanofibrous scaffolds

2.3.1. Field emission scanning electron microscopy (FESEM)

The morphology of nanofibrous scaffolds was characterized by FESEM (Zeiss Auriga crossbeam system, Germany) with an accelerating voltage of 5 kV after coating with gold. The diameter of the fibers was measured from the SEM images using image analysis software (ImageJ, National Institutes of Health, USA) and calculated by selecting 100–150 fibers SEM images randomly.

2.3.2. Transmission electron microscopy (TEM)

In order to detect internal structure properties, the fibers were prepared for TEM (FEI, Holland) by directly depositing the samples onto a copper grid, which was coated in advance with a supportive Formvar film followed by a carbon coating. It was operated at 120 kV.

2.3.3. Fourier-transform infrared spectroscopy (FTIR)

Chemical analysis of HA powder, PCL nanofibrous and PCL/HA nanofibrous were performed by FTIR spectroscopy over a range of 4000–400 cm^{-1}. FTIR spectra of different samples were obtained by a Nicolet spectrometer system (System 2000, Perkin-Elmer) with a KBr detector. Around 1 mg of dried sample was mixed with 100–120 mg of KBr and compressed into pellets.

2.3.4. Mechanical testing

Mechanical properties of different scaffolds were determined using a tabletop uniaxial testing instrument (Instron 5567, USA) using a 50-N load cell under a cross-head speed of 10 mm/min at ambient conditions (relative humidity ~70%). All samples were prepared in the form of rectangular shape with dimensions of 40 mm × 20 mm × 200 μm (long × wide × thick) from the electrospun nanofibrous scaffolds. At least four samples were tested for each type scaffold. From the Young's modulus, tensile strength, and elongation at break were obtained.

2.3.5. Water contact angle

For determination of hydrophilicity of scaffolds, water contact angles of electrospun scaffolds were measured by a Model 200 video based optical system (Future Scientific Ltd. Co., Taiwan, China). The images of water drops on the sample surface were recorded by a camera, and then analyzed with software supplied by the manufacturer. Six samples were measured in each group. Three different points were measured for each sample. The initial distilled water volume of 5 μl was used in each measurement after 3 s exposure at ambient temperature.

2.4. Cell culture

The normal human skin primary fibroblast cells FEK4 (kindly supplied from Dr. RM Tyrrell's Lab, University of Bath, UK) were derived from a newborn foreskin explants. FEK4 cells were cultured in 10% fetal bovine serum (FBS)/modified eagle medium (MEM) supplemented, with 1% penicillin and streptomycin. Experiments were carried out with cells from passage 8 to passage 12. The nanofibrous scaffolds were immersed in 70% ethanol for 30 min, dried under sterile conditions and exposed to UV radiation for 1 h, washed 3 times with PBS for 20 min each and incubated with MEM (free serum) for 24 h before cell seeding. Cells were further seeded onto nanofibrous scaffolds, placed in a 24-well plate at a density of 1×10^5 cells/cm^2 and cultured with MEM and 10% FBS mixture at 37 °C, 5% CO_2 and 95% humidity.

2.5. Cell proliferation assay

To study the cell proliferation on different scaffolds, viable cells were determined by using the One Solution Cell Proliferation Assay (MTS). After 1 and 3 days of cell seeding in 24-well plate, cells were washed with PBS and incubated with 20% of MTS reagent containing serum free medium. After 3 h of incubation at 37 °C in 5% CO_2, aliquots of supernatant were pipetted into a 96-well plate. The absorbance of the content of each well was measured at 490 nm using a spectrophotometric microplate reader (Model 680, Bio-Rad).