Acta Biomaterialia 31 (2016) 156–166

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Human mesenchymal stem cells cultured on silk hydrogels with variable stiffness and growth factor differentiate into mature smooth muscle cell phenotype

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ARTICLE INFO

Article history:
Received 18 August 2015
Received in revised form 4 November 2015
Accepted 23 November 2015
Available online 24 November 2015

Keywords:
Silk fibroin
Tunable hydrogel
Stem cell differentiation
Growth factor

ABSTRACT

Cell-matrix and cell-biomolecule interactions play critical roles in a diversity of biological events including cell adhesion, growth, differentiation, and apoptosis. Evidence suggests that a concise crosstalk of these environmental factors may be required to direct stem cell differentiation toward matured cell type and function. However, the culmination of these complex interactions to direct stem cells into highly specific phenotypes *in vitro* is still widely unknown, particularly in the context of implantable biomaterials. In this study, we utilized tunable hydrogels based on a simple high pressure CO_2 method and silk fibroin (SF) the structural protein of *Bombyx mori* silk fibers. Modification of SF protein starting water solution concentration results in hydrogels of variable stiffness while retaining key structural parameters such as matrix pore size and β-sheet crystallinity. To further resolve the complex crosstalk of chemical signals with matrix properties, we chose to investigate the role of 3D hydrogel stiffness and transforming growth factor (TGF-β1), with the aim of correlating the effects on the vascular commitment of human mesenchymal stem cells. Our data revealed the potential to upregulate matured vascular smooth muscle cell phenotype (myosin heavy chain expression) of hMSCs by employing appropriate matrix stiffness and growth factor (within 72 h). Overall, our observations suggest that chemical and physical stimuli within the cellular microenvironment are tightly coupled systems involved in the fate decisions of hMSCs. The production of tunable scaffold materials that are biocompatible and further specialized to mimic tissue-specific niche environments will be of considerable value to future tissue engineering platforms.

Statement of Significance

This article investigates the role of silk fibroin hydrogel stiffness and transforming growth factor (TGF-β1), with the aim of correlating the effects on the vascular commitment of human mesenchymal stem cells. Specifically, we demonstrate the upregulation of mature vascular smooth muscle cell phenotype (myosin heavy chain expression) of hMSCs by employing appropriate matrix stiffness and growth factor (within 72 h). Moreover, we demonstrate the potential to direct specialized hMSC differentiation by modulating stiffness and growth factor using silk fibroin, a well-tolerated and -defined biomaterial with an impressive portfolio of tissue engineering applications. Altogether, our study reinforce the fact that complex differentiation protocols may be simplified by engineering the cellular microenvironment on multiple scales, i.e. matrix stiffness with growth factor.

1. Introduction

Loss of vascular function associated with cardiovascular disease, such as atherosclerosis, represents the leading medical epidemic in the United States and typically requires surgical intervention through synthetic or autologous vascular grafts [1].

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http://dx.doi.org/10.1016/j.actbio.2015.11.051

To overcome the limitations associated with adult cell sources, which are often restricted by source or compromised by disease, mesenchymal stem cells (MSCs) have emerged as potential candidates for vascular tissue engineering [2]. However, despite their growing application, several MSC-based vascular regeneration strategies have been met with inconsistent results, and in some cases resulted in abridged vascular function. For instance, direct bolus delivery of MSCs to injured vasculature can lead to a dysfunctional endothelium, resulting in a higher incidence of vessel occlusion compromising vessel mechanics [3]. Likewise, MSCs injected at the site of infarcted hearts resulted in increased levels of calcification and ossification of the surrounding cardiac tissue [4]. These findings suggest a lack of fundamental understanding of the complex vascular niche environment and mechanisms accredited to MSC differentiation toward mature, functional vascular tissues.

Cell interactions with the local microenvironment are recognized in several important biological events including cell adhesion, growth, differentiation, and apoptosis [5,6]. In particular, substrate biophysical properties such as rigidity [7,8], geometry [9,10] biological ligand [11,12], soluble factor [13], or combination thereof [14] have been revealed to influence MSC differentiation events. Evidence suggests that a concise crosstalk of these environmental factors may be required to direct MSC differentiation toward desired cell type and function. These findings are supported by recent reports which reveal MSCs acquiring tissue-specific characteristics when co-cultured with mature cells types or exposed to preformed biological matrices *in vitro*, highlighting the important regulatory role elicited by the specific signals of the microenvironment toward stem cell differentiation [15]. However, current methods to regulate stem cell differentiation are often executed independently of other factors, i.e. stiffness or growth factor individually, or lack the amenity to integrate these parameters into a tailorable milieu. A biomimetic approach, incorporating several environmental signals, such as cell-matrix and cell-biomolecule interactions, will help to establish more robust and specific MSC differentiation protocols.

Integration of complex cellular signaling environments into biomaterial scaffolds presents a considerable challenge to the tissue engineering community [16]. A variety of synthetically-formulated as well as natural materials have been evaluated for 3D biomaterial scaffolds [17]. Synthetic materials are attractive for their cost, reproducible fabrication and facile manufacturing yet their lack of cell-recognition sites as well as potential for toxic degradation products causing undesirable inflammation are often disadvantageous [18]. On the contrary, natural materials represent an attractive scaffold platform as they have excellent biological properties, such as cellular recognition, biocompatibility and the potential to degrade through known metabolic processes [19,20].

Silk fibroin (SF), a natural protein extracted from *Bombyx mori* silkworms, is an attractive material for tissue engineering due to its excellent mechanical properties, biocompatibility, tunable degradation rate, and mild inflammatory response *in vivo* [21]. A diversity of regenerative tissues has been reported using SF-based constructs including bone [22,23], cartilage [24], vascular [25–27], skin [28], nervous [29], hepatic [30] and ocular [31] among others [21]. We previously developed a technique to produce porous, SF hydrogels with tunable stiffness and morphology using the green solvent, carbon dioxide (CO_2) [32]. Hydrogel elastic moduli approaching soft tissues (E = 6–30 kPa), combined with ease of fabrication and biocompatibility, motivated us to use these SF materials as a platform to instruct stem cell differentiation toward the vascular smooth muscle cell (SMC) lineage in a precise manner. In addition to substrate rigidity, chemical signals are important for vascular development, maintenance, and regeneration and collectively constitute a complex process involving the interactions of many cellular features *in vivo* [33]. However, the effects of stiffness and its interaction with growth factors have yet to be sufficiently studied, the resolution of which may provide new insights into processes of cellular regeneration and tissue maintenance as they pertain to the cellular microenvironment.

In the present study, we address cellular differentiation on tunable SF hydrogels prepared from a solvent-free CO_2 processing method. The transforming growth factor β (TGF-β) family is a potent regulator of several cell functions such as proliferation, spreading [34] and is strongly associated with vascular smooth muscle cell (vSMC) differentiation of stem cells [13]. Therefore, the focus of this work is on exploiting the combined use of substrate stiffness and growth factor (TGF-β1) on SF matrices, with the aim of correlating the effects on the vascular commitment of human mesenchymal stem cells (hMSCs). The production of tunable scaffold materials that are biocompatible and further specialized to mimic vascular niche environments will be of considerable value to future tissue engineering platforms.

2. Materials and methods

2.1. SF hydrogel preparation

Aqueous silk fibroin (SF) solutions were prepared from degummed cocoons of *B. mori* and subsequently dissolving the obtained fibers in 9.3 M LiBr (Fluka Chemicals, Buchs, Switzerland) aqueous solution (10% w/v) at 65 °C for 4 h and filtered to eliminate impurities. The obtained SF solution was then placed in Slide-A-Lyzer cassette 3500 Da MWCO (Pierce, Thermo Scientific) and dialyzed against distilled water for 3 days at room temperature to remove residual salts. Following dialysis SF solution volume was adjusted with distilled water to reach the desired concentration. Silk hydrogels were prepared from a previously published protocol using high pressure CO_2 [32]. Briefly, SF solutions at different concentrations (1.5, 2, 3 and 4 wt%) were syringed into a custom Teflon mold consisting of 12 cylindrical specimens (h: 3 mm, Ø 10 mm) and placed within a stainless steel high pressure reaction vessel (BR-300, Berghof Products + Instruments, Eningen, Germany). The temperature of the reactor was controlled through an electrical heating jacket run by a BDL-3000 temperature controller (Berghof). Once the system had been sealed and thermal equilibrium established (40 °C), CO_2 gas was introduced in the reactor and pressurized at a working pressure of 60 bar through a high-performance liquid chromatography (HPLC) pump (Model 426, Alltech, Deerfield, IL, USA) and isolated for specific gelation times (0–8 h). Following the set gelation period, the system was depressurized slowly (approximately 30 min) to avoid sample rupture due to the high pressure release. Collected hydrogel specimens were immediately placed in PBS and stored at 4 °C for future characterization.

2.2. Hydrogel structural characterization

2.2.1. Thermal analysis by differential scanning calorimetry

Following SF hydrogel formation, specimens were shock frozen in liquid nitrogen and subsequently lyophilized to prepare dry samples for thermal analysis. Silk hydrogel thermal properties were acquired using a differential scanning calorimeter (DSC) (Mettler, Model DSC30, Columbus, OH, USA) with N_2 gas flow, at a heating rate of 10 °C/min from 0 °C to 350 °C.

2.2.2. Fourier-Transform Infrared Spectroscopy (FTIR) analysis

Silk protein structural characteristics were investigated before and after high pressure CO_2 treatment using FTIR Spectrum One (Perking Elmer, Waltham, MA, USA) with a zinc selenide crystal.