Biomaterials xxx (2014) 1–12

Contents lists available at ScienceDirect

Biomaterials

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

The role of physiological mechanical cues on mesenchymal stem cell differentiation in an airway tract-like dense collagen–silk fibroin construct

Chiara E. Ghezzi [a], Benedetto Marelli [a], Ilaria Donelli [b], Antonio Alessandrino [b], Giuliano Freddi [b], Showan N. Nazhat [a,*]

[a] Department of Mining and Materials Engineering, McGill University, Montreal, Quebec, Canada H3A 2B2
[b] Innovhub – Stazioni Sperimentali per l'Industria, Div. Stazione Sperimentale per la Seta, Milan, Italy

ARTICLE INFO

Article history:
Received 17 March 2014
Accepted 14 April 2014
Available online xxx

Keywords:
Dense collagen gel
Silk fibroin
Trachea
Airway smooth muscle cells
Cartilage

ABSTRACT

Airway tracts serve as a conduit of transport in the respiratory system. Architecturally, these are composed of cartilage rings that offer flexibility and prevent collapse during normal breathing. To this end, the successful regeneration of an airway tract requires the presence of differentiated chondrocytes and airway smooth muscle cells. This study investigated the role of physiological dynamic mechanical stimulation, *in vitro*, on the differentiation of mesenchymal stem cells (MSCs), three-dimensionally seeded within a tubular dense collagen matrix construct-reinforced with rings of electrospun silk fibroin mat (TDC–SFC). In particular, the role of either shear stress supplied by laminar fluid flow or cyclic shear stress in combination with circumferential strain, provided by pulsatile flow, on the chondrogenic differentiation, and contractile lineage of MSCs, and their effects on TDC–SFC morphology and mechanical properties were analysed. Chondrogenic differentiation of MSCs was observed in the presence of chondrogenic supplements under both static and laminar flow cultures. In contrast, physiological pulsatile flow resulted in preferential cellular orientation within TDC–SFC, as dictated by dynamic circumferential strain, and induced MSC contractile phenotype expression. In addition, pulsatile flow decreased MSC-mediated collagen matrix remodelling and increased construct circumferential strength. Therefore, TDC–SFC demonstrated the central role of a matrix in the delivery of mechanical stimuli over chemical factors, by providing an *in vitro* niche to control MSC differentiation, alignment and its capacity to remodel the matrix.

1. Introduction

Tissue engineering aims to replace damaged or diseased tissues by creating functional neo-tissues *in vitro* through the use of committed cells and dedicated scaffolds. In addition to providing clinical therapies for replacing organs and hierarchical tissues, engineered organ and tissue replica have also been developed to create *in vitro* tissue models [1]. These models are designed to study complex physiological and pathological mechanisms *in vitro*; supporting the development of therapies to prevent or cure underlying diseases [2]. In the case of tubular tissues, these constructs also present an increased level of complexity in terms of geometry and architecture, together with a mixed cell population, and constant cyclic mechanical stimulation that modulate cellular responses and ultimately the functionality of the tissues. In particular, airway tracts, which represent the conduit of transport in the respiratory system, are affected by diverse medical conditions ranging from neoplasms and trauma injuries to inflammatory and degenerative diseases (*e.g.* asthma and cystic fibrosis), which require functional three-dimensional (3D) airway tissue substitutes and models to be used in clinical settings [3].

Native airway tracts should be flexible and compliant to accommodate the respiratory movements and structurally stable to prevent collapse during normal breathing. Therefore, airway tissue is reinforced by cartilage rings to maintain conduit flexibility without structural instability during inspiration [4]. Several 3D tissue-engineered airways have been proposed, which are mainly based on biodegradable synthetic polymers and in combination

* Corresponding author. Department of Mining and Materials Engineering, McGill University, 3610 University Street, Montreal, Quebec, Canada H3A 2B2.
Tel.: +1 514 398 5524; fax: +1 514 398 4492.
E-mail address: showan.nazhat@mcgill.ca (S.N. Nazhat).

http://dx.doi.org/10.1016/j.biomaterials.2014.04.040
0142-9612/

with helical elastomeric templates to mimic tracheal geometrical features [5–7]. However, the use of non-biologically derived constructs has been shown to illicit an acute inflammatory reaction and discourage cell invasion, growth, and organization [8]. Therefore, natural polymers (*e.g.* type I collagen gels and sponges) have been implemented in combination with synthetic polymers to increase the biocompatibility of the engineered airway tissues [9–12]. Yet, the inherent complexity of processing composite, multilayered, and hybrid materials limits the translation of these scaffolds into clinical use [3]. In contrast, tissue-engineered airway constructs based purely on natural polymers have not been largely investigated, due to the limited mechanical properties of naturally derived hydrogels, *e.g.* hyaluronic acid, type I collagen and fibrin [13] and, although silk fibroin displays high strength and stiffness, it has only been used as a coating in rabbit tracheal defect reconstruction [14].

Along with a construct, a suitable cell source should be readily available in large volumes to be effectively used in tissue engineering approaches. In this view, mesenchymal stem cells (MSCs) represent a potential candidate for generating mesenchyme cells in bone, vascular, cartilage, and nerve tissue substitutes and models. In addition, since MSCs have the ability to differentiate into a variety of cell types; including chondrocytes and smooth muscle cells (SMCs) [15], they can also be considered for the regeneration of the airway tract. MSC differentiation has been shown to be affected by soluble factors, substrate stiffness and topography, as well as dynamic mechanical stimulation, such as shear stress and cyclic strain [16–19]. In particular, it has recently been demonstrated that the *in vitro* mechanotransduction of external mechanical stimulation regulates MSC growth [20], cytoskeleton organization [21], and its osteogenic, chondrogenic and contractile phenotypes [21–23]. Optimized and balanced culture profiles have been shown to significantly improve *in vitro* engineered tissues (*e.g.* ligament), where sequential biochemical and mechanical stimulations have been alternated [24]. However, systematic studies focused on segregating the effects of chemical and mechanical cues on MSC differentiation when seeded in a 3D physiologically relevant construct has not been extensively reported.

To this end, this study investigated the role of physiological mechanical stimulation, *in vitro*, on the differentiation of MSCs, seeded within an airway tract-like electrospun silk fibroin ring-reinforced tubular dense collagen construct (TDC–SFC). The effect of shear stress supplied by laminar flow of complete medium with or without growth factor supplements (chondrogenic medium) or shear stress in combination with cyclic circumferential strain, provided by pulsatile flow, on the chondrogenic differentiation, and contractile lineage of MSCs, orientation, and their effects on TDC–SFC morphology and mechanical properties were analysed.

2. Materials and methods

2.1. MSC culture

MSCs, extracted from bone marrow isolated from C57BL/6 mice at < 8 weeks of gestation through mechanical and enzymatic digestion were purchased from Invitrogen (Carlsbad, CA, USA). According to the manufacturer's instructions, C57BL/6 MSCs below passage 10 express a flow-cytometry cell-surface protein profile positive for CD29, CD34, and Sca-1 (>70%), and negative for CD117 (<5%). The cells were expanded and cultured in complete growth medium, prepared from Dulbecco's Modified Eagle Medium F-12 (DMEM/F-12, Gibco®, Invitrogen, Carlsbad, CA, USA) with GlutaMAX™-I containing 10% MSC-Qualified Foetal Bovine Serum (FBS, Gibco®, Invitrogen, Carlsbad, CA, USA) and 5 μg/ml Gentamicin (Gibco®, Invitrogen, Carlsbad, CA, USA). MSCs were expanded up to passage 8 to prevent dedifferentiation.

2.2. Preparation of electrospun silk fibroin mat

Bombyx mori cocoons were purchased from the Unità di ricerca di apicultura e bachicoltura (CRA-API), Sezione Bachicoltura, Padova, Italy. Sericin extraction was carried out by autoclaving at 120 °C for 15 min and rinsing in deionized water until complete removal of the globular protein [25]. The resulting SF fibres were neutralized and dissolved in a saturated lithium bromide solution (Sigma–Aldrich, Italy) at 60 °C. The 10% (w/v) solution was then dialysed against deionized water with D9402 dialysis tubing cellulose membrane (Sigma–Aldrich, Italy), obtaining a final SF aqueous solution concentration of ~2% (w/v). SF films were prepared by solvent casting at room temperature.

As previously described [26], electrospun SF mats were prepared by dissolving SF films in formic acid (98 vol.%, Sigma–Aldrich, Italy) at room temperature under gentle stirring. SF solution (final concentration of 7.5%) was then transferred into a 50 ml syringe and electrospun using an *ad-hoc* electrospinning apparatus [27] using a flow rate of 1.1 ml/h, an electric field of 24 kV, and an electrode distance of 10 cm for a deposition time of 2 h [27]. The SF mats were then treated in methanol for 15 min to increase SF crystallinity [28].

2.3. Preparation of MSC-seeded TDC–SFCs

A tubular construct was prepared using a previously established methodology based on dense collagen [29], which was reinforced with electrospun SF insertion in order to mimic the natural airway architecture and increase the structural and mechanical properties (Fig. 1A). SF mats were cut into 3 mm-wide strips with a surgical scalpel, and then sterilized by immersion in 70% ethanol overnight and subsequently rinsed in sterile water [30] (Fig. 1Ai). Neutralized collagen solution was prepared by adding 12 ml of solubilized bovine dermis type I collagen solution (4.8 mg/ml in acidic solution, Collagen Solutions LLC, San Jose, CA, USA) to 3 ml of 10× DMEM (Sigma–Aldrich, Canada), and aliquots of 5 M NaOH (Sigma–Aldrich, Canada). MSCs were incorporated into the neutralized collagen solution at a cell density of 2.11×10^5 cells/ml (Fig. 1Aii). The cellular collagen solution was initially poured into the bottom part of vertically stacking sub-mould ($45 \times 50 \times 7$ mm^3), designed to stabilize four SF electrospun strips inserted in the middle of collagen solution equidistant from the mould extremities (Fig. 1Aiii), as previously described with a rectangular SF mat in a planar geometry [26]. Post collagen gelation at 37 °C for 30 min, the highly hydrated collagen gel–SF hybrid (Fig. 1Aiv) was transferred onto blotting paper, supported by stainless steel mesh. Plastic compression was applied with an unconfined compressive stress equivalent to 1 kPa for 5 min, resulting in the multilayered hybrid assembly composed of two DC sheets reinforced with unidirectionally aligned electrospun SF strips (Fig. 1Av). TDC–SFC was generated by rolling the DC–SF–DC hybrid assembly along the long axis of a cylindrical polytetrafluoroethylene mandrel (3.4 mm diameter) into three concentric layers of approximately 550 μm wall thickness (Fig. 1Avi, B) [29].

2.4. Dynamic culturing

Acellular and MSC-seeded TDC–SFCs were dynamically stimulated via either pulsatile or laminar flow and compared to static culturing for 7 days (Table 1). In particular, the samples investigated were named as follows: acellular as made sample (A_{made}), acellular sample cultured in static condition (A_{static}), acellular sample cultured under pulsatile flow (A_{pulse}), cell-seeded sample cultured in static condition (C_{static}), cell-seeded sample cultured under laminar flow ($C_{laminar}$), and cell-seeded sample cultured under pulsatile flow (C_{pulse}). Immediately after preparation, TDC–SFCs were gently removed from the cylindrical mandrel, inserted on both sides of plastic barbed fittings attached to the anchor shafts of the bioreactor chamber and securely tightened with 4-0 suture threads (Perma-Hand, Ethicon Inc., USA) to prevent fluid leakage, as previously reported [31]. The assembly was placed in an incubator (Forma Environmental Chamber 3920, Thermo Scientific, Canada) for precise environmental control (*i.e.* 37 °C and CO_2 at 5%). Pulsatile flow (C_{pulse}) was generated by an ElectroForce® Biodynamic Test Instrument 5160 (Bose Corp., USA) equipped in vascular configuration. A gear pump (Micropump Inc. of Index Corporation, USA) was used to supply a steady flow (75 ml/min) in series with a pulsatile manifold to generate a waveform of 1 Hz frequency and 25 ml/min of dynamic flow amplitude. The pulsatile flow parameters resulted in transmural tracheal pressure oscillations of 20/30 cmH_2O and <5% circumferential strain measured with an intraluminal Mikro-Tip® Catheter Pressure Transducer (Millar Instruments, Inc., USA). For comparison, TDC–SFCs were also cultured under laminar flow ($C_{laminar}$) of steady flow of 75 ml/min. In all tests, an additional TDC–SFC was also cultured in the chamber freely floating as static control (C_{static}) and the culturing medium (300 ml) was replaced at day 4. MSC-seeded TDC–SFCs were cultured for 7 days in either chondrogenic (CD) or control growth (ND) media for comparison. CD medium consisted of complete growth medium further supplemented with 200 ng/ml of insulin-like growth factor-I (IGF-I, R&D Systems, Inc., MN, USA) and 1 ng/ml of transforming growth factor-beta (TGF-beta, R&D Systems, Inc., MN, USA).

Mean wall shear stress, τ_{mean} (dyn/cm^2), was calculated using the Hagen–Poiseuille equation [32] as $\tau_{mean} = 4\mu Q/\pi R_0^3$, where μ is the dynamic viscosity of the culture medium with 10% FBS (1 cP [33]), Q is the volumetric flow rate (ml/min), and R_0 is the TDC–SFC internal radius. The mean shear stress applied during the cycle was calculated as $\tau_{mean} = 3.2 \pm 1$ dyne/cm^2, in the range of luminal shear stress generated by airflow at rest breathing (0.5–3 dyne/cm^2) [34].

2.5. MSC viability and distribution

MSC viability within C_{pulse} and C_{static} was assessed at day 7 in CD. DC–SF–DC ring-shaped specimens (5 mm in length) were incubated for 60 min in complete

Please cite this article in press as: Ghezzi CE, et al., The role of physiological mechanical cues on mesenchymal stem cell differentiation in an airway tract-like dense collagen–silk fibroin construct, Biomaterials (2014), http://dx.doi.org/10.1016/j.biomaterials.2014.04.040