Biomaterials 35 (2014) 1857–1868

Contents lists available at ScienceDirect

Biomaterials

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

The effect of time-dependent deformation of viscoelastic hydrogels on myogenic induction and Rac1 activity in mesenchymal stem cells

Andrew R. Cameron [a], Jessica E. Frith [a], Guillermo A. Gomez [b], Alpha S. Yap [b], Justin J. Cooper-White [a,c,d,*]

[a] Tissue Engineering and Microfluidics Laboratory, The Australian Institute for Bioengineering and Nanotechnology, University of Queensland, St Lucia, QLD, Australia
[b] Division of Molecular Cell Biology, Institute for Molecular Bioscience, University of Queensland, St Lucia, QLD, Australia
[c] School of Chemical Engineering, University of Queensland, St Lucia, QLD, Australia
[d] CSIRO, Division of Materials Science and Engineering, Clayton, Victoria, Australia

ARTICLE INFO

Article history:
Received 14 October 2013
Accepted 7 November 2013
Available online 9 December 2013

Keywords:
Mesenchymal stem cell
Smooth muscle cell
Creep
Viscoelasticity
Hydrogel
Mechanotransduction

ABSTRACT

Cell behaviours within tissues are influenced by a broad array of physical and biochemical microenvironmental factors. Whilst 'stiffness' is a recognised physical property of substrates and tissue microenvironments that influences many cellular behaviours, tissues and their extracellular matrices are not purely rigid but 'viscoelastic' materials, composed of both rigid-like (elastic) and dissipative (viscous) elements. This viscoelasticity results in materials displaying increased deformation with time under the imposition of a defined force or stress, a phenomenon referred to as time-dependent deformation or 'creep'. Previously, we compared the behaviour of human mesenchymal stem cells (hMSCs) on hydrogels tailored to have a constant stiffness, but to display varying levels of creep in response to an applied force. Using polyacrylamide as a model material, we showed that on high-creep hydrogels (HCHs), hMSCs displayed increased proliferation, spread area and differentiation towards multiple lineages, compared to their purely stiff analogue, with a particular propensity for differentiation towards a smooth muscle cell (SMC) lineage. In this present study, we investigate the mechanisms behind this phenomenon and show that hMSCs adhered to HCHs have increased expression of SMC induction factors, including soluble factors, ECM proteins and the cell–cell adhesion molecule, N-Cadherin. Further, we identify a key role for Rac1 signalling in mediating this increased N-Cadherin expression. Using a real-time Rac1-FRET biosensor, we confirm increased Rac1 activation on HCHs, an observation that is further supported functionally by observed increases in motility and lamellipodial protrusion rates of hMSCs. Increased Rac1 activity in hMSCs on HCHs provides underlying mechanisms for enhanced commitment towards a SMC lineage and the compensatory increase in spread area (isotonic tension) after a creep-induced loss of cytoskeletal tension on viscoelastic substrates, in contrast to previous studies that have consistently demonstrated up-regulation of RhoA activity with increasing substrate stiffness. Tuning substrate viscoelasticity to introduce varying levels of creep thus equips the biomaterial scientist or engineer with a new tool with which to tune and direct stem cell outcomes.

1. Introduction

The intrinsic behaviours of cells, *in vitro* and *in vivo*, are significantly affected by the physical and biochemical microenvironment in which they reside [1,2]. This is especially true for cells having significant plasticity, such as mesenchymal stem cells (MSCs) [3–8]. It has emerged most recently that mechanical cues, derived from various modes of stimulation, can play an important role in influencing the differentiation of MSCs into various tissue cell endpoints, including muscle [3,4]. Externally applied mechanical stimulation of a matrix (through stretching or compression of the matrix) has been shown to induce or up-regulate secretion of cytokines, such as TGF-β_1 [9,10], and ECM molecules, such as laminin [11], in adhered cells, both of which were shown to drive smooth muscle cell (SMC) differentiation of hMSCs [12–16]. In the case of pluripotent stem cells, this was shown to be through autocrine actions [17,18]. However, in

* Corresponding author. Tissue Engineering and Microfluidics Laboratory, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St. Lucia, Queensland 4072, Australia. Tel.: +61 7 3346 3858.
E-mail address: j.cooperwhite@uq.edu.au (J.J. Cooper-White).

0142-9612/$ – see front matter
http://dx.doi.org/10.1016/j.biomaterials.2013.11.023

the absence of such external mechanical stimulation, cells themselves also apply force locally to a matrix through their adhesions. In deforming the surrounding matrix, cells experience a resistive force, the magnitude and time scales of which are defined by the intrinsic mechanical properties of the matrix. This substrate-induced mechanical feedback is also capable of driving cellular behaviours – for example, the stiffness (or compressive modulus) of a substrate, which represents just one mechanical property of a material, has been shown to direct multiple fate choices of hMSCs, including lineage commitment to muscle cells [3].

Most tissues, and indeed many synthetic biomaterials, are not purely elastic but 'viscoelastic' in nature, being composed of both elastic and viscous elements. The resulting spectrum of mechanical properties that cells may sense when attached to a matrix is thus more appropriately described not only by a 'stiffness' value, but instead by its viscoelasticity, in terms of both an elastic component, which can be represented by the storage modulus (G′), and a dissipative or viscous component, represented by the loss modulus (G″). The *higher* the magnitude of the dissipative component (G″) of a matrix, at a constant elastic component contribution (G′), the *more* time dependent deformation incurred under an applied force (such as that applied by an adhered cell), a behaviour that is referred to as 'creep'. One can thus appreciate that as adherent cells begin to exert force on a viscoelastic substrate via their focal adhesions (FAs), in contrast to a purely elastic substrate, substrate creep may result in cells feeling a time dependent reduction in the resistive force they experience when actively pulling on a substrate. This reduction in resistive force, due to the dissipative elements in viscoelastic materials, would be expected to impact not only the size and maturity of the FAs [19], but many other downstream cellular processes. Elucidating the effect of matrix creep on cell behaviour may thus have significant implications in understanding how cells interact with their native 'viscoelastic' microenvironments *in vivo*, as well as predicting how cells will respond to synthetically developed biomaterials.

Indeed, a recent investigation by our group has demonstrated that the dissipative component or loss modulus (G″) of a material's mechanical property slate can significantly influence multiple hMSC behaviours, including differentiation [20]. In stark contrast to the observed capacity of substrates of differing levels of stiffness to *bias* the differentiation of hMSCs into defined tissue endpoints [3], we showed that on a set of viscoelastic polyacrylamide (PAM) hydrogels that displayed increasing levels of creep (but a constant stiffness matched to that of muscle tissue (13.5 kPa)) [20], hMSCs not only showed enhanced differentiation towards a myogenic fate, but also towards osteo- and adipogenic fates when in the presence of inductive factors [20]. However, unlike the effect of creep on osteogenesis or adipogenesis of hMSCs, myogenesis (or more specifically SMC differentiation) was enhanced in *both* basal and myogenic differentiation medium [20]. This was demonstrated by an observed increase in expression of early- and mid-stage SMC markers, αSMC and calponin, on high creep hydrogels (HCHs) compared to low creep hydrogels (LCHs), suggesting an increased propensity for spontaneous induction of SMC differentiation of hMSCs on these hydrogels [20]. This study further confirmed that the enhanced differentiation of hMSCs on substrates of increasing creep was the result of the time dependent decrease in both *passive* and actively generated *isometric* cytoskeletal tension [20], terms previously defined by the cellular tensegrity model [21,22]. In line with this model, it was proposed that hMSCs adhered to these types of substrates would attempt to restore the balance of this lost tension through alternative mechanisms, such as increased spread area (*isotonic* cytoskeletal tension) and increased cell–cell contact (passive tension), behaviours both associated with hMSC differentiation [23,24].

These particular viscoelastic PAM hydrogels more closely mimic *in vivo* tissue mechanics than the purely elastic PAM materials previously utilised, and their use with hMSCs has already lead to new insight into the impacts of the dissipative component of a material's viscoelastic spectrum on stem cell fate choice. However, the exact molecular mechanism by which hMSCs respond to such a mechanical property during and post adhesion is currently unknown.

The current investigation thus aimed to investigate the driver/s behind the observed enhanced smooth muscle myogenesis of hMSCs when adhered to viscoelastic substrates of constant stiffness (approximating muscle tissue) but varying levels of creep. Importantly, we investigate both end point expression (at defined time points) and real time (dynamic) activation of key regulatory proteins within known mechanotransduction pathways, using a range of standard molecular biology techniques and a cellular Rac1 biosensor, exposing the critical role Rac1 (a Rho GTPase) plays in directing the observed behaviours of hMSCs on these tailored viscoelastic substrates.

2. Materials and methods

2.1. Gel fabrication

As previously described [20], PAM gels were formulated from solutions of 30 wt % *Acryl* (Sigma–Aldrich) and 1 wt% *Bis* (Sigma–Aldrich) and crosslinked using tetramethylethylenediamine (TEMED) (Bio-Rad, Regents Park, Australia) and 10 wt% ammonia persulphate (Amresco) in an oxygen free environment (under nitrogen) for approximately 30 min. Thin gels used in the FRET biosensor analysis were attached to the underlying glass by washing the glass with 0.1 M NaOH, silanising the dried surface with aminopropyltriethoxysilane (APTS) for 5 min, washing the surface thoroughly with milli-Q water and then applying 1% glutaraldehyde in PBS for 30 min. The surfaces were washed with milli-Q water and air dried.

2.2. Gel functionalisation for cell seeding

Sulfosuccinimidyl 6(4′-azido-2′-nitrophenyl-amino) hexanoate (SANPAH) (Thermo Fisher) was photocrosslinked to the surface of the gels at a concentration of 1 mg/mL using UV light for 30 min. Gels were thoroughly washed in PBS and functionalised with 50 μg/mL type I collagen (bovine derived) (Devro Medical) by soaking the SANPAH treated gels in collagen solution overnight at 4 °C. Gels were again washed with PBS before cell seeding.

2.3. Differentiation, TGF-β_1, ECM protein and N-Cad analysis

2.3.1. Cell culture conditions

hMSCs were obtained from the Mater Medical Research Institute, Brisbane and used under approval from the Medical Research Ethics Committee at the University of Queensland (#2010001069). For analysis, hMSCs were seeded at 4000 cells/cm^2 and cultured in growth or differentiation medium for 7 days before samples were collected for analysis. Growth medium (GM) consisted of low-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Myogenic medium consisted of GM supplemented with 10 ng/mL TGF-β_1 and 30 μM ascorbic acid. Osteogenic medium consisted of GM supplemented with 2 mM L-glutamine, 0.1 μM dexamethasone, 50 μM ascorbic acid, and 10 mM β-glycerophosphate. Adipogenic medium consisted of high-glucose DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 0.2 mM indomethacin, 1 μM dexamethasone, 0.5 mM IBMX, and 10 μg/mL insulin. Mixed osteogenic/adipogenic medium contained 1:1 adipogenic:osteogenic differentiation media.

2.3.2. Quantitative real-time reverse transcription polymerase chain reaction (qPCR)

2.3.2.1. RNA isolation and cDNA synthesis. Total RNA was isolated using an RNeasy Mini Kit with on-column DNase treatment (QIAGEN VWR, Stockholm, Sweeden) according to the protocol given by the manufacturer. The concentration and purity of RNA were determined by using a Nano-Drop spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE). cDNA was synthesised from up to 200 ng of RNA (with equal amounts for all samples in one experiment) using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) in a total volume of 21 μL, as per manufacturer's instructions. An equivalent volume of DNase and RNase-free water was used in place of RT Enzyme Mix for no-RT controls.

2.3.2.2. Quantitative real-time reverse transcription polymerase chain reaction (qPCR). qPCR reactions were set up in triplicates with each reaction having a total volume of 10 μL containing 1X Platinum SYBR Green qPCR SuperMix-UGD (Invitrogen), 0.2 μM forward and reverse primers (Table 1) and 1 μL cDNA. A 7500 Fast Real-Time PCR System (Applied Biosystems) was used at standard cycling parameters of 50 °C for 2 min, 95 °C for 2 min and then 95 °C for 15 s and 60 °C for 30 s for a total of 40