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Matrix-driven formation of mesenchymal stem cell–extracellular matrix microtissues on soft alginate hydrogels

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ABSTRACT

Mesenchymal stem cells (MSCs) can be made to rearrange into microtissues in response to specific matrix cues, a process that depends on a balance between cell–matrix and cell–cell interactions. The effect of such cues, and especially their interplay, is still not fully understood, particularly in three-dimensional (3-D) systems. Here, the behaviour of human MSCs cultured within hydrogel matrices with tailored stiffness and composition was evaluated. MSC aggregation occurred only in more compliant matrices ($G' \leqslant 120$ Pa), when compared to stiffer ones, both in the presence and in the absence of matrix-bound arginine–glycine–aspartic acid cell–adhesion ligands (RGD; 0, 100 and 200 μM). Fibronectin assembly stabilized cell–cell contacts within aggregates, even in non-adhesive matrices. However, MSCs were able to substantially contract the artificial matrix only when RGD was present. Moreover, compliant matrices facilitated cell proliferation and provided an environment conducive for MSC osteogenic differentiation, even without RGD. Cell interactions with the original matrix became less important as time progressed, while the *de novo*-produced extracellular matrix became a more critical determinant of cell fate. These data provide further insights into the mechanisms by which MSCs sense their microenvironment to organize into tissues, and provide new clues to the design of cell-instructive 3-D matrices.
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1. Introduction

It is recognized that the microenvironment plays an important role in cell behaviour, and it is well established that cells respond to the intrinsic properties of their matrix, namely to mechanical cues [1]. The work of Engler et al. has largely contributed to shedding light on the processes of mechanosensing, notably by demonstrating that matrix elasticity directs stem cell lineage specification [2]. It has also long been known that matrix dimensionality plays a key role in cell signalling events [3,4], affecting, in particular, the way cells experience mechanical stresses and strains [5]. However, there are still many unanswered questions about the intersection between mechanobiology and matrix dimensionality, as most studies have been carried out using two-dimensional or "on top" three-dimensional (3-D) assays that are not representative of the normal environment of most cell types.

The effect of matrix cues in real 3-D systems, where cells are embedded within a hydrogel-like matrix, has been traditionally addressed using extracellular matrix (ECM)-derived hydrogels, such as collagen and fibrin, which do not allow a great level of control over signal presentation. More recently, engineered artificial matrices, with tuneable biochemical and mechanical properties, have also been explored [6,7]. These 3-D models have been vital for systematically deconstructing the role of different signals and their interplay [8,9]. Surprisingly, only a few studies have focused on the use of very soft hydrogels (storage modulus, $G' < 1000$ Pa), although the reported results are quite illuminating. In fact, compliant matrices have been shown to facilitate different cellular activities, including spreading [10,11], proliferation [10,12] and migration [13], among others, in different cell types. Apart from the matrix stiffness, the molecular characteristics of the hydrogels, and their resulting viscoelastic behaviour, also have a direct impact on these processes. Cells in three dimensions must overcome the physical barrier imposed by the polymeric network, and will clearly encounter less resistance in softer and more deformable matrices [14]. For example, Bott et al. [10] have shown that the spreading and proliferation of fibroblasts within poly(ethylene glycol) (PEG) matrices are increased in softer ($G' < 250$ Pa) vs. stiffer ($G' > 1200$ Pa) hydrogels, irrespective of their degradation profile.

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Interestingly, the authors also showed that fibroblasts are able to contract viscous-like collagen gels [15], but not elastic-like PEG hydrogels with a similar G' ($G' \approx 200$ Pa) [10]. Ehrbar et al. have shown that preosteoblastic cells entrapped within soft matrices ($G' < 94$ Pa) can engage in a degradation-independent 3-D migration mode, and overcome the matrix resistance by deforming the network and/or using hydrogel defects [13]. Different studies demonstrated that endothelial cells network assembly and tubulogenesis are favoured in low stiffness matrices [16,17]. Recently, Matyash et al. have shown that soft alginate hydrogels ($G' < 64$ Pa), prepared with sub-stoichiometric concentrations of crosslinking ions, promote extensive neurite growth, even in the absence of cell-adhesion ligands, while protecting neurons against oxidative stress [11]. The communication between cells also seems to be facilitated in compliant matrices. In particular, Reinhart-King et al. have shown that pairs of endothelial cells in compliant substrates ($G' = 500$ Pa) communicate through mechanical signals in a stiffness-dependent manner: when the matrix stiffness is low enough, cells can perceive and react to substrate strains created by the traction stresses of neighbouring cells [14,18]. Altogether, these examples emphasize the interest of further exploring cell behaviour in compliant 3-D matrices. They also suggest that the molecular characteristics and viscoelastic profile of hydrogels, which are generally poorly discussed, are relevant features with a clear impact on the process of mechanosensing.

Here, the polymer dry-mass content of alginate hydrogels with a bimodal molecular weight distribution was modulated to prepare 3-D matrices with very different viscoelastic profiles. While 2 wt.% alginate hydrogels are stiffer and behave predominantly as elastic-like materials, similar to synthetic hydrogels [10], 1 wt.% hydrogels are softer and display a more viscous-like behaviour, typically observed in ECM-derived hydrogels [10]. The hydrogels were also modified with controlled densities of RGD peptides to promote integrin-mediated cell–matrix adhesion. Integrin receptors act as major sensors and integrators of microenvironmental signals, being particularly relevant as mechanotransducers [19]. This versatile system was used to investigate the effect of matrix cues on mesenchymal stem cell (MSC) behaviour in three dimensions, at different levels, and the interplay between viscoelastic properties and ligand presentation was addressed. A new approach was established to foster the formation of human MSC (hMSC) aggregates stabilized by endogenous ECM, which better mimic complex tissue structures and provide interesting 3-D in vitro model systems or building blocks for regenerative therapies [20,21].

2. Materials and methods

2.1. Synthesis and characterization of RGD-alginate

Sodium alginates (Protanal LF 20/40, a gift from FMC Biopolymers) with a high ratio of guluronate to mannuronate content (>60%) with $Mw = 1.5 \times 10^5$ Da and $Mw = 2.5 \times 10^4$ Da were used, respectively, as the high (HMW) and low molecular weight (LMW) components of the hydrogel matrices [22]. Both were purified through dialysis against deionized water for 3 days (molecular weight cutoff (MWCO), 3500 membrane, Spectrum Labs), and with activated charcoal (Sigma, 0.5 g g^{-1} alginate). The HMW alginate was partially oxidized to a theoretical extent of 1% of sugar residues with sodium periodate [23]. Both components were grafted with the cell-adhesion peptide (glycine)4–arginine–glycine–aspartic acid–serine–proline (hereafter abbreviated as RGD), using aqueous carbodiimide chemistry as previously described [24]. Briefly, alginate solutions (1 wt.%) in MES buffer (0.1 M MES, 0.3 M NaCl, pH 6.5) were prepared and stirred overnight (ON) at room temperature (RT). *N*-Hydroxy-sulfosuccinimide (Sulfo-NHS, Pierce) and 1-ethyl-(dimethylaminopropyl)-carbodiimide (EDC, Sigma, 27.4 mg g^{-1} alginate) were sequentially added at a molar ratio of 1:2, followed by 17 mg of RGD peptide (Genscript) per g of alginate. Control samples were prepared without the addition of peptide (hereafter designated as HMW0 or LMW0). After stirring for 20 h at RT, the reaction was quenched with hydroxylamine and the solution was dialyzed against deionized water for 3 days (MWCO 3500). After purification with charcoal, RGD-alginate was lyophilized and stored at −20 °C until further use. The amount of grafted RGD was quantified using the BCA Protein Assay (Pierce). Briefly, samples (1 wt.% RGD-alginate) were incubated in BCA reagent for 60 min at 37 °C in the dark and the absorbance was read at 540 nm in a microplate reader (Power Wave Xs, Biotek). A set of RGD solutions (0 to 1 mg ml^{-1} in 1 wt.% HMW0 or LMW0) were used as standards to produce a calibration curve. Typically, the reaction yield was ~90%, determined by the BCA total protein assay, as described in Ref. [6].

2.2. RGD-alginate 3-D matrices: compositions and in situ hydrogel formation

In situ forming alginate hydrogel matrices were prepared by internal gelation as described previously [16,24,25]. Hydrogel precursor solutions were prepared with binary mixtures (50:50 v/v) of HMW and LMW sodium alginate, at different polymer concentrations (1 and 2 wt.%) and RGD densities (0, 100 or 200 μM: in this paper these formulations are referred to as 1–0, 1–100, 1–200; and 2–0, 2–100, 2–200; where the first number represents the concentration of alginate and the second number denotes the concentration of RGD). The range of RGD density selected herein is comparable to that of commonly used ECM-derived biological matrices [7]. Sodium alginate solutions were sterile-filtered (0.22 μm) and mixed with an aqueous suspension of $CaCO_3$ (Fluka) at a $CaCO_3$/COOH molar ratio of 1.6 [25]. Then, a fresh sterile solution of glucone delta-lactone (GDL, Sigma) was added to trigger gelation. The $CaCO_3$/GDL molar ratio was set at 0.125, and the gelation time was 1 h. For the 3-D culture studies, hydrogel precursor solutions were combined with hMSCs prior to hydrogel formation, as described in Sec. 2.5.

2.3. Dynamic mechanical analysis (DMA) of RGD-alginate hydrogels

Samples were assayed in a TRITEC2000B (Triton Technology). Hydrogel cylinders (5.5 mm diameter; $h = 1.5$ mm) were prepared in a QGel™ 3-D disc caster. After crosslinking, the discs were pre-equilibrated ON at 37 °C in Dulbecco's modified Eagle medium (DMEM) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma, 25 mM) and 0.01% NaN_3 (Sigma), and then loaded onto the compression mode clamp assembly. Measurements were performed under RT conditions (humidity ~44%, 20 °C). A user-defined small compression load was applied to guarantee an adequate contact between the swollen samples and the device. A time-scan (1 Hz and 1% strain, within the linear viscoelastic region (LVR)) was performed for 5 min, and the compressive storage modulus (E', elastic component), loss modulus (E'', viscous component) and damping (E''/E', tan δ) were calculated. The influence of polymer concentration (1 and 2 wt.%) and RGD density (0, 100 and 200 μM) on the hydrogel mechanical properties was analyzed. At least five replicas were tested for each condition, and the experiment was repeated twice. Averages and standard deviations were reported.

2.4. Oscillatory shear rheometry of RGD-alginate hydrogels

Rheological measurements were carried out using a Kinexus Pro rheometer (Malvern). Swollen hydrogel discs were analyzed at day 0 (after swelling to equilibrium in culture media for 2 h) and after incubation (24 h) under standard culture conditions. To guarantee

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