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Influence of the stiffness of three-dimensional alginate/collagen-I interpenetrating networks on fibroblast biology

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ABSTRACT

Wound dressing biomaterials are increasingly being designed to incorporate bioactive molecules to promote healing, but the impact of matrix mechanical properties on the biology of resident cells orchestrating skin repair and regeneration remains to be fully understood. This study investigated whether tuning the stiffness of a model wound dressing biomaterial could control the behavior of dermal fibroblasts. Fully interpenetrating networks (IPNs) of collagen-I and alginate were fabricated to enable gel stiffness to be tuned independently of gel architecture, polymer concentration or adhesion ligand density. Three-dimensional cultures of dermal fibroblasts encapsulated within matrices of different stiffness were shown to promote dramatically different cell morphologies, and enhanced stiffness resulted in upregulation of key-mediators of inflammation such as IL-10 and COX-2. These findings suggest that simply modulating the matrix mechanical properties of a given wound dressing biomaterial deposited at the wound site could regulate the progression of wound healing.

1. Introduction

Wound healing is a complex physiological process orchestrated by multiple cell types, soluble factors and extracellular matrix (ECM) components [1]. Most cutaneous injuries heal rapidly within a week or two, though often leading to the formation of fibrotic scar tissue which is neither aesthetically desirable nor functional [2]. However, several pathogenic abnormalities, ranging from diabetic ulcers to infection or continued trauma, contribute to failure to heal [3]. Chronic non-healing wounds are a cause of significant morbidity and mortality, and constitute a huge burden in public health care with estimated costs of more than $3 billion per year in the United States [4]. The goal of wound care therapies is to regenerate tissues such that the structural and functional properties are restored to the levels prior to injury [5]. The use of biomaterials as scaffolds for skin healing is a successful long-lasting concept [6], as demonstrated by the use of porous crosslinked networks of collagen and glycosaminoglycans (GAG) which induced full regeneration of functional skin within 4 weeks [7,8]. Currently, wound dressing biomaterials often incorporate antimicrobial, antibacterial, and anti-inflammatory agents [9] to further aid and enhance natural skin healing. The wound dressing market is expanding rapidly as it becomes clear that no single dressing is suitable for all wounds, and that their physiochemical properties can be manipulated to target the different stages of the healing process [10].

Following a skin injury, disruption of the tissue architecture leads to a dramatically altered mechanical context at the site of the wound [11]. Mechanical cues in the wound microenvironment can

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guide the behavior of a milieu of infiltrating cells such as recruited immune cells [12] and fibroblasts [14]. Mechanical cues are also known to sponsor or hinder different stages of the wound repair response, from epithelial morphogenesis [15] to blood vessel formation [16]. However, the importance of mechanical forces in the context of wound dressing design has been often overlooked.

In this study we investigated if one could control the behavior of dermal fibroblasts involved in the wound healing response by simply tuning the storage modulus of a model wound dressing biomaterial. Numerous material systems have been developed to help understand how ECM mechanics regulate cell behaviors, from migration [17,18] to differentiation [19,20]. However, these material systems do not always allow one to perform three-dimensional cell cultures where matrix stiffness is decoupled from scaffold architecture, polymer concentration or adhesion ligand density. One way to achieve this separation is through the design of interpenetrating network (IPN) hydrogels, which consist of two or more polymer networks that are not covalently bonded but at least partially interlaced [21]. We developed a biomaterial system composed of IPNs of collagen-I and alginate; both of these components are widely used in the tissue engineering field. The sodium alginate polymeric backbone presents no intrinsic cell-binding domains, but can be used to regulate gel mechanical properties. The collagen-I presents specific peptide sequences recognized by cell surface receptors, and provides a substrate for cell adhesion that better recreates many *in vivo* contexts. Encapsulated cells sense, adhere and pull on the collagen-I fibrils and depending on the degree of crosslinking of the intercalated alginate mesh, it is expected they will feel more or less resistance to deformation from the matrix. The alginate backbone is ionically crosslinked by divalent cations (in this case, Ca^{2+}), thus solely changing the concentration of calcium modulates the stiffness of the IPN. *In vivo*, fibroblasts are recruited to the wound site for the synthesis, deposition and remodeling of the new ECM [1], being one of the most important cell mediators of the wound healing response. Hence in this study we assessed the *in vitro* behavior of primary dermal fibroblasts isolated from the dermis of healthy non-diabetic donors when encapsulated within IPNs of varying stiffness, to partially mimic the effects of mechanical cues on the response of fibroblasts migrating into a wound site *in vivo*.

2. Materials and methods

2.1. Cell culture

Human dermal fibroblasts (ZenBio) were cultured according to the manufacturer's protocol, and used between passages 6 and 11. For routine cell culture, cells were cultured in dermal fibroblasts culture medium (ZenBio), a DMEM-based culture medium containing fetal bovine serum, 4.15 g/L D-glucose, penicillin, streptomycin and amphotericin B. The manufacturer also reports the addition of specific growth factors necessary for optimal expansion of human dermal fibroblasts. Cells were maintained at sub-confluence in the incubator at 37 °C and 5% CO_2. The culture medium was refreshed every three days.

2.2. Alginate preparation

High molecular weight (LF20/40) sodium alginate was purchased from FMC Biopolymer. Alginate was dialyzed against deionized water for 2–3 days (molecular weight cutoff of 3500 Da), treated with activated charcoal to remove any contaminants, sterile filtered (0.22 μm), lyophilized, and then reconstituted in serum-free DMEM medium at 2.5% w/v. RGD-decorated alginate was prepared using carbodiimide chemistry to couple the oligopeptide GGGGRGDSP (Peptides International) to the alginate, such that on average 20 RGD peptides were coupled to each alginate polymer [22].

2.3. IPNs preparation

All IPNs in this study consisted of 1.5 mg/ml rat-tail collagen-I (BD Biosciences), and 5 mg/ml high molecular weight alginate. The IPN matrix formation process consisted of two steps. In the first step, reconstituted alginate (2.5% w/v in serum-free DMEM medium) was delivered into a centrifuge tube and put on ice. Rat-tail collagen-I was mixed with a 10× DMEM solution in a 1:10 ratio to the amount of collagen-I needed and the pH was adjusted to 7.4 using a 1 M NaOH solution. The final concentration of rat-tail collagen-I in the IPN was adjusted to 1.5 mg/ml using serum-free DMEM. The rat-tail collagen-I solution was then thoroughly mixed with the alginate solution. Once the collagen-alginate mixture was prepared, the human dermal fibroblasts were washed, trypsinized (0.05% trypsin/EDTA, Invitrogen), counted using a Z2 Coulter Counter (Beckman Coulter), resuspended at a concentration of 3×10^6 cells per ml in cell culture medium and mixed with the collagen-alginate mixture. The collagen-alginate-cells mixture was then transferred into a pre-cooled 1 ml luer lock syringe (Cole-Parmer).

In the second step, a solution containing calcium sulfate dihydrate ($CaSO_4.2H_2O$, Sigma), used to crosslink the alginate network, was prepared as follows. Calcium sulfate dihydrate was reconstituted in water at 1.22 M and autoclaved. For each IPN, 100 μl of DMEM containing the appropriate amount of the calcium sulfate slurry was added to a 1 ml luer lock syringe. The syringe with the calcium sulfate solution was agitated to mix the calcium sulfate uniformly, and then the two syringes were connected together with a female–female luer lock coupler (Value Plastics). The two solutions were mixed and immediately deposited into a well of a 48-well plate. The plate was then transferred to the incubator at 37 °C and 5% CO_2 for 60 min to allow gelation, after which culture medium was added to each gel. Culture medium was refreshed every two days for the duration of each experiment.

2.4. Scanning electron microscopy

For scanning electron microscopy (SEM), IPNs were fixed in 4% paraformaldehyde (PFA), washed several times in PBS, and serially transitioned from dH_2O into absolute ethanol with 30 min incubations in 30, 50, 70, 90, and 100% ethanol solutions. Ethanol dehydrated IPNs were dried in a critical point dryer and adhered onto sample stubs using carbon tape. Samples were sputter coated with 5 nm of platinum–palladium and imaged using secondary electron detection on a Carl Zeiss Supra 55 VP field emission scanning electron microscope.

2.5. Elemental analysis

For elemental analysis, IPNs were fixed in 4% paraformaldehyde (PFA), washed several times in PBS, quickly washed with dH_2O, froze overnight at −20 °C and lyophilized. Elemental analysis, via Energy Dispersive Spectroscopy (EDS), was performed using a Tescan Vega3 scanning electron microscope equipped with a Bruker Nano XFlash 5030 silicon drift detector.

2.6. Mechanical characterization of IPNs

The mechanical properties of the IPNs were characterized with an AR-G2 stress controlled rheometer (TA Instruments). IPNs and collagen-I hydrogels without cells were formed as described above, and directly deposited onto the pre-cooled surface plate of the rheometer. A 20 mm plate was immediately brought into contact before the IPN started to gel, forming a 20 mm disk of IPN. The plate was warmed to 37 °C, and the mechanical properties were then measured over time as described previously [23]. Briefly, the storage modulus at 0.5% strain and at 1 Hz was recorded periodically until it reached its equilibrium value (30–40 min, Fig. S2). A strain sweep was performed to confirm that this value was within the linear elastic regime, followed by a frequency sweep. Further quantitative analysis on the viscoelastic properties of these IPNs was performed as follows. Phase angle δ, defined as $\delta = \tan^{-1}(G''/G')$, was calculated based on the measured G' and G'' at 0.1 Hz. The frequency dependent rheology of the gels followed a weak power law of the form $G' \sim f^{\alpha}$. The power law exponent α was calculated by fitting the frequency dependence of G' of each measurement to this equation. This power law relationship is characteristic of many biological materials, including cells and reconstituted actin networks [24,25], and provides a useful parameter with which to characterize the viscoelasticity of the gels.

2.7. Analysis of macromolecular transport in IPNs

The diffusion coefficient of 70 kDa fluorescently labeled anionic dextran (Invitrogen) through IPNs used in this study was determined. For these studies, IPNs of varying mechanical properties encapsulating 0.2 mg/ml fluorescein-labeled dextran were prepared in a standard tissue culture 48-well plate. IPNs were allowed to equilibrate at 37 °C for 1 h, before serum-free phenol red-free DMEM medium was added to the well. Aliquots of this media were taken periodically to measure the molecular diffusion of dextran from the hydrogels into the media. Samples were continuously agitated using an orbital shaker, and fluorescein-labeled dextran concentration was measured using a fluorescence plate reader (Biotek). The measurements were interpreted using the semi-infinite slab approximation as described previously [26].

2.8. Immunohistochemistry

The IPNs were fixed in 4% paraformaldehyde for 1 h at room temperature and washed in PBS overnight at 4 °C. The IPNs were embedded in 2.5% low gelling temperature agarose (Lonza) by placing the gels in liquid agarose in a 40 °C water bath for several hours and subsequent gelling at 4 °C. A Leica vibratome was used to cut 200 μm sections. To visualize the general protein content and distribution