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Time-dependent cellular morphogenesis and matrix stiffening in proteolytically responsive hydrogels

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ABSTRACT

Mesenchymal stromal cells residing in proteolytically responsive hydrogel scaffolds were subjected to changes in mechanical properties associated with their own three-dimensional (3-D) morphogenesis. In order to investigate this relationship the current study documents the transient degradation and restructuring of fibroblasts seeded in hydrogel scaffolds undergoing active cell-mediated reorganization over 7 days in culture. A semi-synthetic proteolytically degradable polyethylene glycol–fibrinogen (PF) hydrogel matrix and neonatal human dermal fibroblasts (NHDF) were used. Rheology (in situ and ex situ) measured stiffening of the gels and confocal laser scanning microscopy (CLSM) measured cell morphogenesis within the gels. The assumption that the matrix modulus systematically decreases as cells locally begin to enzymatically disassemble the PF hydrogel to become spindled in the material was not supported by the bulk mechanical property measurements. Instead, the PF hydrogels exhibited cell-mediated stiffening concurrent with their dynamic morphogenesis, as indicated by a four-fold increase in storage modulus after 1 week in culture. Fibrin hydrogels, which were used as the control biomaterial, proved similarly adaptive to cell-mediated remodeling only in the presence of the exogenous serine protease inhibitor aprotinin. Acellular and non-viable hydrogels also served as control groups to verify that transient matrix remodeling was entirely associated with cell-mediated events, including collagen deposition, cell-mediated proteolysis, and the formation of multicellular networks within the hydrogel constructs. The fact that cell network formation and collagen deposition both paralleled transient stiffening of the PF hydrogels, further reinforces the notion that cells actively balance between proteolysis and ECM synthesis when remodeling proteolytically responsive hydrogel scaffolds.

1. Introduction

Hydrogel biomaterials are becoming more prevalent as scaffolds in cell therapy and tissue engineering [1]. Among the advantages that hydrogels provide in these applications are their tissue-like water content and controllable mechanical properties [2]. Moreover, recent advances in hydrogel design have introduced semi-synthetic materials that are capable of inductive cell signaling and biodegradation via cell-mediated proteolysis [3]. Bioactive features can be incorporated into the polymeric backbone of the hydrogel network by conventional conjugation chemistry, which provides important growth signals to resident cells via molecular interactions at the cell–biomaterial interface [4]. Inductive cell signaling may also include agonists that stimulate the production of naturally secreted extracellular matrix (ECM) proteins that are essential as part of any tissue repair process [5]. Concurrently, proteolytic degradation of the material ensures the timely removal of the basic constituents of the scaffold in favor of ECM laid down by resident cells [6]. Mechanical properties are emerging as important inductive features of hydrogel scaffolds in tissue engineering, mainly through mechano-transduction [7,8]. Most tissue regeneration applications that utilize hydrogel scaffolds consider the mechanical interface between resident cells and the scaffold within the context of the adaptive cellular response to microenvironments [9]. Sometimes this interface is transient, as some cell-compatible hydrogels are specifically designed to undergo biodegradation in order to accommodate cellular remodeling [10]. In this context the transient events that occur during this remodeling process involve proteolysis, hydrolysis and ECM production. Proteolysis and ECM synthesis are both controlled by biomolecular cell signaling, whereas material hydrolysis is mediated based on external factors not controlled by cells [11]. Previous attempts to control scaffold remodeling have focused primarily on regulating hydrolysis using specific water-soluble synthetic polymers with known hydrolytic rates of biodegradation [12]. These strategies rely on bulk degradation of the material rather than locally mediated proteolysis associated with cell-secreted enzymes. Alternative approaches now include preparing proteolytically responsive

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hydrogel scaffolds containing protease-sensitive crosslinkers or other semi-synthetic constituents [13]. The advantage of this strategy includes better control over local cell-mediated disassembly of the scaffold in tandem with local matrix deposition, without bulk degradation of the material. The disadvantage of this approach is that little is known about how cell-responsive hydrogel biomaterials are degraded and restructured during cellular morphogenesis. In order to take advantage of mechano-transduction using three-dimensional (3-D) hydrogel scaffolds that are proteolytically responsive, further understanding of how cells regulate matrix reorganization at the microenvironment level is required for these systems [14]. A few tissue engineering studies have recently attempted to document the process of local proteolysis in cell-responsive hydrogels using sophisticated new microscopy techniques, with some degree of success [15–17]. Nevertheless, the spatial and temporal changes in local mechanical properties of a hydrogel during cellular remodeling are still poorly understood. Moreover, the traditional instrumentation used to map the local mechanical properties of gels, including microrheology and atomic force microscopy, have been difficult to apply with cell-seeded 3-D hydrogel scaffolds. Given the importance of mechanical signaling in tissue engineering, more studies will be required to understand and predict the mechanical properties of the scaffold as cells reorganize the material over the course of several days in culture.

In this study we set out to investigate the transient mechanical and physical properties of a semi-synthetic material composed of polyethylene glycol (PEG) and fibrinopeptide crosslinkers (PEG–fibrinogen), using bulk properties measurements. Previous studies in our group have documented cellular remodeling within these hydrogel scaffolds [18,19], showing the respective roles of mechanics, proteolysis and structural features [20–25]. We now build upon these earlier findings by measuring the transient modulus of the cell-seeded PF hydrogel scaffolds using ex situ measurements with a strain rate controlled rheometer. Cellular morphogenesis in the hydrogels was also documented using confocal laser scanning microscopy (CLSM). The transient mechanical properties measured using this approach were correlated with specific patterns of morphogenesis observed by CLSM. The relationships that are uncovered in this work can further contribute to investigations of 3-D cell culture in cell-responsive hydrogel scaffolds.

2. Methods

2.1. Fibrinogen PEGylation

PEG-diacrylate (PEG-DA) was prepared as described elsewhere [26]. Briefly, linear PEG-OH with an average molecular weight of 10 kDa (Aldrich, Sleeze, Germany) was reacted with acryloyl chloride (Merck, Darstadt, Germany) at a molar ratio of 1.75:1 relative to –OH groups in dichloromethane in the presence of triethylamine (Fluka). The final product was precipitated in ice cold diethyl ether, dried under vacuum, and end group conversion of the product was confirmed by H^1 NMR to be 99% (data not shown). Conjugation of the linear PEG-DA to denatured bovine fibrinogen was performed as described elsewhere [27]. The fibrinogen concentration in the product was determined using the bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL) and the degree of PEG substitution was calculated according to published protocols [20]. Polymerization of the PF solution was performed by exposure to UV light (365 nm, 5 mW cm^{-2}) for 5 min in presence of 0.1% w/v Irgacure™ 2959 photoinitiator (Ciba Specialty Chemicals, Tarrytown, NY).

2.2. Cell culture in 3-D PF hydrogels

Neonatal human dermal fibroblasts (NHDF) were cultured according to standard protocols between passages 8 and 14. The NHDFs were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, UK) containing 10% fetal bovine serum (FBS) (Biological Industries, Israel), 1% penicillin–streptomycin (Biological Industries), 1% L-glutamine (Gibco), 0.2% 2-mercaptoethanol (Gibco), and 1% non-essential amino acid solution (Biological Industries). Cell-seeded PF constructs were prepared with NHDFs (5×10^6 cells ml^{-1}) by dispersing the cells in 1 ml of PF precursor solution (7–8 mg ml^{-1} fibrinogen). The cell solution was then placed in a custom-made 24 mm diameter Teflon mold (Supplementary Fig. S1b) and cross-linked by exposure to long wavelength UV light (365 nm, 5 mW cm^{-2}) in the presence of 0.1% w/v Irgacure™ 2959 photoinitiator for 4 min. The constructs were then incubated at 37 °C in 5% CO_2 for 1, 4, and 7 days or taken immediately for rheometry measurements (day 0) as detailed below. Constructs with the same concentration of non-viable NHDFs and acellular constructs were also used as controls (incubation was performed under the same conditions). Non-viable cells were obtained by trypsinizing NHDFs and incubating them in DMEM containing 20% DMSO for 30 min; total loss of cell viability was confirmed using trypan blue staining (Sigma) and a Countess® automated cell counter (Invitrogen Life Technologies, USA). Under certain conditions the PF constructs were incubated in the presence of 20 μg ml^{-1} aprotinin (bovine lung origin, Sigma) in the culture medium.

2.3. Cell culture in 3-D fibrin hydrogels

NHDFs (5×10^6 cells ml^{-1}) were dispersed in a 1 ml solution of 8 mg ml^{-1} bovine fibrinogen (Sigma) and placed in the same Teflon molds (Supplementary Fig. 1b). The fibrin constructs were cross-linked as before using 0.75 NIH U ml^{-1} thrombin (Sigma) for 45 min at 37 °C in 5% CO_2. Following this incubation period the constructs were either taken for immediate rheological testing (day 0) or incubated for similar time periods and under similar conditions to those described for the PF constructs. Under certain conditions the fibrin constructs were incubated in culture medium supplemented with 20 μg ml^{-1} aprotinin.

2.4. In situ rheological characterization

PF precursor solution (200 μl) containing photoinitiator was placed in a strain rate-controlled shear rheometer with a 20 mm parallel plate geometry (AR-G2, TA Instruments, New Castle, DE), as shown in Supplementary Fig. 1a. A time sweep oscillatory test was performed at 25 °C for 5 min with a 2% sinusoidal strain and 3 rad s^{-1} frequency in order to monitor the in situ liquid to solid transition (gelation) of the solutions during the photo-polymerization reaction. To determine the optimal strain amplitude and oscillatory frequency used for the time–sweep test, prior strain sweep and frequency sweep measurements were performed [20]. All in situ rheometry testing was performed with a 60 s preconditioning cycle followed by 4 min of UV curing (365 nm, 5 mW cm^{-2}) while the storage and loss modulus values (G' and G'') were continuously recorded using Rheology Advantage Instrument Control AR Software. The reported storage modulus was taken as the real part of the complex shear modulus $G^* = G' + iG''$ at the conclusion of the time sweep test. All oscillatory tests were also performed for fibrin hydrogels. Fibrin cross-linking was achieved by mixing a fibrinogen precursor solution with 0.75 NIH U ml^{-1} thrombin. The fibrinogen/thrombin solution was immediately placed on the parallel plates of the rheometer and cross-linked for 45 min under constant monitoring at 25 °C.

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