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The influence of ascorbic acid, TGF-β1, and cell-mediated remodeling on the bulk mechanical properties of 3-D PEG–fibrinogen constructs

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ABSTRACT

Two-dimensional cell culture studies have shown that matrix rigidity can influence cell function, but little is known about how matrix physical properties, and their changes with time, influence cell function in 3-D. Biosynthetic hydrogels based on PEGylated fibrinogen permit the initial decoupling of matrix chemical and mechanical properties, and are thus potentially attractive for addressing this question. However, the mechanical stability of these gels due to passive hydrolysis and cell-mediated remodeling has not previously been addressed. Here, we show that the bulk mechanical properties of acellular PEG–fibrinogen hydrogels significantly decrease over time in PBS regardless of matrix cross-linking density in 7 days. To compensate, smooth muscle cells (SMCs) were encapsulated and stimulated to produce their own matrix using ascorbic acid or TGF-β1. Ascorbic acid treatment improved the mechanical properties of the constructs after 14 days in less cross-linked matrices, but TGF-β1 did not. The increase in matrix modulus of the constructs was not due to an increase in type I collagen deposition, which remained low and pericellular regardless of cross-link density or the soluble factor applied. Instead, ascorbic acid, but not TGF-β1, preferentially enhanced the contractile SMC phenotype in the less cross-linked gels. Inhibition of contractility reduced cell spreading and the expression of contractile markers, and eliminated any beneficial increase in matrix modulus induced by cell-generated contraction of the gels. Together, these data show that PEG–fibrinogen hydrogels are susceptible to both hydrolysis and proteolysis, and suggest that some soluble factors may stimulate matrix remodeling by modulating SMC phenotype instead of inducing ECM synthesis in a 3-D matrix.

1. Introduction

It is now well established that substrate mechanical properties influence cell function in two-dimensional (2-D) cultures. However, it remains unclear if the mechanical properties of three-dimensional (3-D) extracellular matrices (ECMs) similarly influence cell function, due in part to the fact that matrix remodeling may significantly change both the bulk and local mechanical properties over time. Understanding how ECM mechanical properties influence cell function in 3-D is important both in the context of biomaterial design for regenerative medicine, and to improve basic understanding of mechanical influences on pathology. For example, one hallmark of intimal hyperplasia is the gradual stiffening of the arterial wall due to smooth muscle cell (SMC) proliferation and remodeling of the ECM. In normal mature arteries, vascular SMCs adopt and maintain a contractile phenotype to homeostatically control blood flow. However, during the early stages of arteriogenesis, SMCs exist in a synthetic, proliferative phenotype that facilitates growth and stabilization of immature blood vessels [1]. Contractile SMCs often revert to this synthetic phenotype in disease, and this phenotypic switch can be influenced by a variety of local physical and chemical cues from intracellular and extracellular sources [2]. Whether 3-D ECM mechanical properties play a causal role in this phenotypic switch is unclear.

By contrast, the roles of various soluble factors in controlling the phenotypic plasticity of SMCs have been more widely investigated. TGF-β1 is one such factor known to modulate the pathways that control SMC proliferation, differentiation, apoptosis, and migration in a dose-dependent fashion [3]. *In vitro*, TGF-β1 can stimulate ECM synthesis by vascular SMCs [4], and overexpressing TGF-β1 in porcine arteries has been demonstrated to increase procollagen synthesis *in vivo* [5]. Recent work has also shown the effectiveness of TGF-β1 in up-regulating the synthesis of type I collagen in other cell types such as human dermal fibroblasts to improve the mechanical properties of tubular fibrin gels [6]. TGF-β1 is also instrumental to the differentiation

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of fibroblasts into myofibroblasts during wound contraction, primarily due to increased cell contractility and ECM synthesis to the surrounding tissue [7]. Thus, TGF-β1's matrix-promoting effects make it a cytokine of particular interest to strengthen engineered tissue constructs by inducing cells to secrete their own desired ECM.

Ascorbic acid is another soluble factor that has been studied in the context of both matrix remodeling and cardiovascular pathology. Many studies have shown the negative effects of oxidant stress on the vascular wall due to insufficient protection from free radicals, which has been correlated to vascular SMC dedifferentiation and proliferation [8]. Ascorbic acid's antioxidant properties can inhibit the oxidation of lipids and low density lipoproteins, which often occur during the development of atherosclerosis, and thus reduce oxidant stress [9]. Ascorbic acid may also mediate phenotypic changes in vascular SMCs by inhibiting cell proliferation and facilitating differentiation into the contractile phenotype [10]. Additionally, ascorbic acid enhances collagen synthesis and hydroxylation of procollagen [11], and thereby promotes production of types I and IV collagen by vascular SMCs [12].

The effects of these soluble factors on cell phenotype and ECM remodeling have been primarily studied in natural biomaterials such as collagen or fibrin gels. However, the ability to control certain physical and chemical properties independently is not possible in most natural materials, and thus it is difficult to distinguish which factor(s) of the ECM most strongly influences matrix remodeling events. As we have previously described, hydrogels based on PEG–fibrinogen are potentially more suitable alternatives given that this material permits pseudo-independent control over certain material properties [13]. Fibrinogen monomers conjugated to linear PEG chains offer protease-sensitive links that allow for focalized ECM proteolysis, as well as native binding sites recognizable by integrin cell adhesion receptors in most cell types, while cross-linking and hydrogel formation are achieved through the acrylated PEG ends [14].

Recent work has demonstrated comparable viability of SMCs cultured in PEG–fibrinogen gels to pure fibrin gels, the ability to alter the mechanical properties while maintaining a constant protein concentration [13], and the shorter-term remodeling (7 days) of PEG–fibrinogen based on matrix compliance and proteolysis [15]. However, one under-explored question associated with this type of material is the possible hydrolytic degradation of the ester linkage between the cysteine groups on fibrinogen and the acrylate end groups on PEG-diacrylate (PEGDA). Previous studies have demonstrated a rapid hydrolytic degradation of hydrogels composed of PEGDA coupled to cysteines on hydrophilic peptides that were conjugated using the same type of conjugate addition reaction [16]. In the current study, we show that PEG–fibrinogen hydrogels indeed are susceptible to both hydrolytic and proteolytic degradation, which lead to a significant reduction in matrix modulus regardless of matrix cross-link density over a period of 7 days. We supplemented SMC-seeded constructs with ascorbic acid or TGF-β1 in order to stimulate *de novo* ECM synthesis and perhaps circumvent this rapid degradation and improve the mechanical integrity of these hydrogels. Our findings suggest that the interplay of initial matrix cross-link density, cell spreading, and contractility is critical to cell-mediated remodeling of these PEG-based materials in 3-D.

2. Materials and methods

2.1. Cell culture

Primary human aortic smooth muscle cells were purchased through a commercial source (Cascade Biologics, Portland, Oregon). All cultures were maintained in Medium 231 (M231, Cascade Biologics) supplemented with smooth muscle growth supplement (SMGS, Cascade Biologics), 1% penicillin/streptomycin (P/S, MediaTech Inc., Herndon, VA) and 0.5% gentamicin (Sigma, St. Louis, MO) at 37 °C and 5% CO_2. All experiments were performed using Medium 231 with SMGS, 1% P/S, and 0.5% gentamicin. Cells between passages 4–9 were used for all experiments. Fresh media was replenished every other day throughout the culturing period.

2.2. Acrylation of poly(ethylene glycol)

To create cross-linkable acrylate end groups on poly(ethylene glycol), acrylation chemistry was adapted and modified from a previously published protocol [16]. Briefly, 10 kDa PEG-diol (Sigma, St. Louis, MO) was dried in benzene and subjected to azeotropic distillation. Acryloyl chloride (Alfa Aesar, Ward Hill, MA) and triethylamine (Sigma) were added in 4× molar excess with respect to PEG-diol, and reacted under nitrogen overnight away from light. Residual triethylamine salts were removed using vacuum filtration, while the diacrylated PEG product was precipitated in ice-cold diethyl ether and purified with benzene 3×. The product was dried under vacuum in nitrogen at 40 °C overnight. The product was dissolved in deionized water, lyophilized, and stored in −80 °C under nitrogen. The final product had an average acrylation of about 90% as confirmed by ^{1}H NMR.

2.3. Fibrinogen PEGylation

PEGylation of fibrinogen fragments were performed by adapting previously published protocols [14]. Briefly, full-length fibrinogen (Sigma) was dissolved in 8 M Urea in PBS, pH 7.4 to achieve a fibrinogen concentration of 7 mg/ml, and reacted with 2× molar excess Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma) and 4× molar excess PEGDA for 3 h at room temperature in the dark. The resulting product was precipitated with acetone in a separation funnel at room temperature. The product was collected, centrifuged, and the supernatant was removed. The pelleted product was resuspended in 8 M Urea in PBS, pH 7.4, and dialyzed against PBS (pH 7.4, 4 °C) for 24 h in a membrane-dialysis bag with a 12–14 kDa MWCO (Fisher Scientific, Pittsburgh, PA). The PEGylated fibrinogen product (a liquid precursor solution) was tested for polymerization, and the fibrinogen concentration of the liquid product was quantified via bicinchoninic acid assay (Pierce Chemicals, Rockford, IL). The concentration of PEG was quantified by lyophilizing known volumes of the product and the following equation adapted from [13]: $[\mathrm{PEGDA}] = (W_{DP}/V_{LP}) - [\mathrm{PBS}] - [\mathrm{F}]$, where [PEGDA] is the resulting concentration of PEG on PEGylated fibrinogen, W_{DP} is the weight of the dried product, V_{LP} is the initial volume of the liquid PEG–fibrinogen product before lyophilization, [PBS] is the concentration of the salts in PBS, and [F] is the fibrinogen concentration of the product determined from the BCA protein assay. Multiple PEG–fibrinogen batches were characterized and used for the experiments herein to confirm the reproducibility of synthesis.

2.4. PEGDA hydrogel polymerization

Three different concentrations of PEGDA or PEGDA:PEG hydrogels were created for mechanical testing. Briefly, 2.5 wt% PEGDA hydrogels were fabricated by dissolving 25 mg of 10 kDa PEGDA in 1 ml of 50 mM Tris, pH 8.5. The 5 wt% 50:50 PEGDA:PEG and the 2.5 wt% 50:50 PEGDA:PEG hydrogels were generated by varying the relative ratio of cross-linkable 10 kDa PEGDA to inert PEG (Sigma) at 50:50 ratio for each respective weight percent. A free radical solution (2,2-dimethoxy-2-phenylacetophenone in *N*-vinylpyrrolidinone, 600 mg/ml) (Sigma) and 5 mM triethanolamine (Sigma) were added to each precursor solution at a final concentration of 25 μl/ml. The precursor solution was vortexed, centrifuged, and the supernatant liquid was pipetted into the Teflon mold to form cylindrical hydrogels. After UV irradiation, hydrogels were transferred into petri dishes and incubated in PBS for up to 8 days at 37 °C and 5% CO_2.

2.5. Fabrication of acellular and cell-seeded PEG–fibrinogen hydrogels

Acellular PEG–fibrinogen hydrogels were made in cylindrical Teflon wells (5 mm deep and 10 mm in diameter). PEG–fibrinogen precursor solutions (500 μl) with varying amounts of exogenous PEGDA cross-linker (0–2% w/v) and 0.2 wt% photoinitiator (Irgacure 2959, Ciba Specialty Chemicals, Tarrytown, NY) were vortexed, centrifuged, and pipetted into each well. Hydrogels were polymerized with a transparency film over the mold to obtain a smooth surface that facilitated reproducible mechanical testing. Photo-polymerization was performed by UV light irradiation for 5 min. Cylindrical hydrogels were then either mechanically tested immediately or transferred into 12-well plates with PBS or Medium 231 (without serum), and incubated in cell culturing conditions (37 °C, 5% CO_2) for up to 7 days.

For cell culture experiments, PEG–fibrinogen precursor solutions with respective amounts of additional cross-linkable PEGDA (0–2% w/v) and photoinitiator were vortexed, centrifuged, and used to disperse a cell pellet to obtain a final cell seeding density of 1×10^6 cells/ml. The cell suspension in PEG–fibrinogen was pipetted into cylindrical Teflon wells, photo-polymerized, and transferred into 12-well plates with culturing medium. Ascorbic acid (Sigma; 50 μg/ml), TGF-β1 (R&D Systems, Minneapolis, MN; 2.5 ng/ml), or blebbistatin (Calbiochem, Gibbstown, NJ; 50 μg/ml) were refreshed with every media change. All cultures were incubated on an orbital shaker to assist in nutrient perfusion.

2.6. Mechanical testing of hydrogels, gel contraction measurements, and phase microscopy of cell morphology

The bulk mechanical properties of PEG–fibrinogen hydrogels were measured using an MTS Synergie 100 (MTS Systems Co., Eden Prairie, MN) with a 10 N load cell.