Published in final edited form as:

Acta Biomater. 2007 January ; 3(1): 59–67.

Influence of Thrombin Concentration on the Mechanical and Morphological Properties of Cell-seeded Fibrin Hydrogels

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Abstract

Fibrin is a biopolymer that has been used in a variety of biomaterial, cell delivery and tissue engineering applications. The enzyme thrombin catalyzes the formation of fibrin microfibrils, which form a three-dimensional mesh in which cells can be directly embedded at the time of gel formation. In this study, fibrin hydrogels containing vascular smooth muscle cells were created using varying concentrations of thrombin. Over 7 days in culture, all gels decreased in volume as the fibrin matrix compacted, and the degree of gel compaction increased as thrombin concentration decreased. The material modulus and ultimate tensile stress of the gels also increased with decreasing thrombin concentration. Addition of thrombin to similar constructs made using collagen Type I did not show an effect on gel compaction or mechanical properties, suggesting that these effects were a result of thrombin's action on fibrin polymerization, and not cellular functions. Cell proliferation in fibrin hydrogels was not significantly affected by thrombin addition. Matrix examination using scanning electron microscopy showed increasing fibrin fiber diameters as thrombin concentration decreased. Confocal microscopic imaging of the actin cytoskeleton showed that cell morphology on two-dimensional substrates of fibrin showed marked changes, with higher thrombin concentrations producing cells with longer cellular projections. However, these morphological changes were not as apparent in cells embedded in three-dimensional (3-D) matrices, in which cells exhibited a similar morphology independent of thrombin concentration. These results relate features of the matrix and cellular components of 3-D fibrin constructs to mechanical properties, and contribute to the understanding of structure-function relationships in cell-seeded, 3-D protein hydrogels.

Keywords

Fibrin; Thrombin; Smooth muscle cells; Tissue engineering

1. Introduction

Polymerized fibrin is a major component of blood clots and a key regulator of wound healing. In vivo, formation of fibrin clots is initiated by vascular injury, which causes the release of the enzyme thrombin, a serine protease that activates many constituents of the coagulation cascade. Thrombin cleaves peptide fragments from the soluble plasma protein fibrinogen, yielding insoluble fibrin peptides that aggregate to form fibrils. A fibrin meshwork is formed, which entraps platelets and other blood-borne components to create a clot that is stabilized through

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crosslinking by the transglutaminase Factor XIII. The initial clot prevents bleeding, and the fibrin mesh provides a provisional matrix to initiate the wound healing response.

The structural and biochemical properties of the fibrin polymer make it a promising candidate as a scaffold in tissue engineering and regenerative medicine. Modification and functionalization of fibrin matrices has been used to provide controlled release of genes [1] and growth factors [2]. In addition, fibrin naturally contains sites for cell binding, and therefore has also been investigated as a substrate for cell adhesion, spreading, migration and proliferation. Because of its established effects on vascular cells, fibrin is of particular interest as a scaffold in vascular tissue engineering [3-5]. In this context, smooth muscle cells are directly suspended in a fibrinogen solution, which is injected into an annular mold and subsequently polymerized by the addition of thrombin. The result is a tubular, cell-seeded, three-dimensional (3-D) hydrogel construct that has been proposed as a model of the vascular media. However, the engineered tissues created in this way generally lack the mechanical properties of the native blood vessel, and therefore a number of strategies to strengthen such tissues have been investigated.

The components used to create fibrin-based engineered vascular tissues have been investigated, including cell source [6], fibrinogen concentration [7] and inclusion of other structural proteins [8]. Culture parameters used to grow these engineered tissues also have been explored. For example, it has been shown that the addition of growth factors [9] and longer culture times [10] tend to produce stronger constructs with more developed extracellular matrix. Although the structure and properties of fibrin clots have been studied extensively [11-14], little is known about how the fibrin microstructure affects the macroscopic mechanical properties of cell-seeded engineered tissues.

Fibrin gel structure is determined to an important extent by kinetic factors. The self-assembly of fibrin fibers is influenced by the concentration of fibrinogen, calcium and thrombin as well as other proteins [15]. While ionic strength and hydrogen ion concentration are important, clotting time is a dominant parameter in the determination of structure [16-17]. Increases in thrombin concentration are associated with faster gelation times [18-19], as well as characteristics of the gel microstructure such as fiber size and porosity. At high thrombin concentrations, tight networks are formed with more fiber bundles with finer and thinner fibers. As the thrombin concentration is decreased, the average fiber bundle size increases and the gel becomes more porous [17,19].

In the study presented here, we examined how changes in thrombin concentration at the time of gelation influence the morphology of 3-D, cell-seeded fibrin matrices, and in turn how matrix morphology affect the macroscopic material properties of these fibrin scaffolds. In addition, it is known that changes in fibrin microstructure can modulate cellular behavior [20]. We therefore also characterized cell number and morphology in fibrin constructs, as well as the influence of thrombin concentration on these parameters. Since the compaction of fibrin gels is in part a cell-mediated process, and since thrombin can be a potent regulator of smooth muscle cell activity [21-23] we also added thrombin to control constructs made of Type I collagen in order to determine whether property changes were due to an effect on the matrix or cellular component. Our overall goal was to determine what features of gel preparation and matrix morphology were desirable in producing robust fibrin scaffolds.

2. Materials and methods

2.1 Cell isolation and culture

Rat aortic smooth muscle cells (RASMC) were isolated from adult Sprague-Dawley rats using collagenase digestion. Cells were cultured on T-75 flasks in complete medium, which

contained Dulbecco's Eagle Modified Media with F-12 (DMEM/F-12) supplemented with fetal bovine serum (FBS, 10% v/v) and penicillin/streptomycin/L-glutamine (PS-LG, 1% v/v). Cells were grown until confluence in a 37°C incubator at 5% CO₂. Cells were detached by brief exposure to trypsin-EDTA solution followed by the addition of complete media, and then centrifuged for 5 min at 1000 rpm. RASMC used in construct preparation were at passages 4-10. All cell culture reagents were obtained from Mediatech (Herndon, VA).

2.2 3-D construct preparation

Fibrinogen solution at a concentration of 4.0 mg/ml was prepared using lyophilized bovine fibrinogen (Sigma Chemical, St Louis, MO) and cold complete media supplemented with ϵ -amino-caproic acid (ACA, Sigma), an inhibitor of the fibrinolytic enzyme plasmin [24]. This mixture was allowed to dissolve on a rotating/shaker apparatus at 4°C. RASMC at a concentration of 1.0×10^6 cells/ml were combined with bovine fibrinogen solution (50% v/v), FBS (10% v/v) and bovine thrombin (Sigma) diluted in complete medium (40% v/v) to make fibrin gels at a final protein concentration of 2.0 mg/ml. Thrombin concentration was varied to produce four different sets of constructs: 1.0, 0.1, 0.01 and 0.001 units of thrombin/mg of fibrinogen (U T/mg F). The protein-cell solution was poured into a test tube and an inner mandrel was inserted to produce a tubular geometry. The constructs were allowed to gel for 3 h at 37°C. It was observed that lower thrombin concentrations required longer incubation times to ensure gelation. After gelling, the constructs were removed from the test tube and statically cultured for 7 days. The construct culture medium was also supplemented with ACA, to inhibit construct degradation. At day 1, construct ends were freed from the mandrel ends, allowing compaction in the axial direction.

Collagen constructs were prepared with the addition of thrombin enzyme to determine thrombin's effect on the cellular component of the matrix. Collagen solution was prepared at 4.0 mg/ml by dissolving bovine collagen (Sigma) in 0.02 N acetic acid using a magnetic stir bar at 4°C. Control constructs were prepared by mixing collected cells with FBS (10% v/v), 5 $\times$ DMEM (20% v/v), 0.1 N NaOH (10% v/v) and collagen solution (50% v/v) for a final protein concentration of 2.0 mg/ml. Collagen with thrombin constructs were prepared as described above; however, thrombin at a concentration of 0.001 UT/mg collagen (UT/mg C) was added during preparation. Collagen constructs were also molded into a tubular geometry and subjected to compaction analysis, mechanical testing and DNA analysis on day 7.

2.3 2-D substrate preparation

2-D fibrin gels with varying amounts of thrombin were prepared as described above, and 2.0 ml of the mixture was cast onto polystyrene tissue culture plastic dishes (T-25 flasks). After polymerization for 30 min at 37°C, 1.0×10^4 cells/cm² were seeded evenly on top of the fibrin gel. These constructs were subjected to confocal microscopy for the observation of cell morphology after a 72 h culture period.

2.4 Compaction analysis

Changes in gel volume (compaction) were assessed at days 1, 3 and 7 using digital image analysis. Dimensions of the tubular 3-D constructs were determined using ImageTool software, and gel volume was computed from this data, assuming the shape of the gel to be a hollow cylinder. Gel compaction was determined as a percentage of the original volume on day 0.

2.5 Mechanical testing and analysis

Uniaxial tensile testing was performed on day 7 using a mechanical testing system (EnduraTEC, Eden Prairie, MN) equipped with a 250 g load cell. The day 7 time point was chosen because at this point gel compaction had leveled off, but the embedded smooth muscle cells had not

had time to produce mechanically significant levels of new extracellular matrix proteins. Tubular constructs were segmented into ~5 mm long sections. Segment dimensions were obtained from image analysis. Ring segments were placed on a ring-test apparatus, preconditioned with three cycles of 20% strain and subsequently strained to failure at a rate of 0.3 mm/s.

Stress and strain were calculated from the force-displacement data, using the equations for engineering stress and strain and the initial cross-sectional area. Material modulus and ultimate tensile stress (UTS) were determined from the stress-strain profile. The material modulus was computed as the slope of the linear region of the stress-strain curve while the UTS was determined as the peak stress attained. Maximum load attained prior to failure was also determined as a sample property.

2.6 Cell quantification

Cell number was assessed at day 7. Hoechst 33258 dye (Molecular Probes Inc., Eugene, OR) was used to determine sample DNA content for 3-D gels. Constructs were dehydrated, and then digested in Proteinase K (Promega Inc., Madison, WI) at 55°C for 16-20 h. Next, samples were diluted and loaded into a 96-well plate, which also contained standards and a series of blanks. DNA-binding dye was added to each well, and the plate was protected from light for 20 min. Fluorescence was quantified using a fluorescence microplate reader (BioTek Inc, Winooksi, VT) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The DNA content of each construct was converted to a cell number based on cell and DNA standards.

2.7 Scanning Electron Microscopy (SEM)

SEM was used as a tool for the observation of the fibrin scaffold microstructure. SEM examination of constructs was performed using critical point drying and gold coating of samples. Constructs were first washed in PBS, then fixed in a 4% glutaraldehyde solution for 1 h. Fixation was followed by a dehydration series in 30, 50, 70, 90, 95 ($\times 2$) and 100% ($\times 2$) ethanol solutions for 45 min each. Dehydrated samples were placed in a Samdri 795 critical point dryer (Tousimis Corp., Rockville, MD), where ethanol was replaced with CO₂ and subsequently removed. The dried samples were mounted on aluminum stubs, sputter-coated with gold, and viewed with a LEO 1550 VP Field Emission SEM (LEO Electron Microscopy Inc., Thornwood, NY).

2.8 Confocal microscopy

Confocal microscopy was used to assess cell morphology within the fibrin meshwork. Cell morphology was visualized by staining the actin cytoskeleton of the cells. Constructs were first washed, then fixed in a 3.7% formaldehyde solution, followed by permeabilization with a 0.1% Triton X-100 solution. The constructs were incubated with Alexafluor 488-conjugated phalloidin (Molecular Probes) in 1% bovine serum albumin, which was used to block non-specific binding. Ethidium homodimer (Molecular Probes) was added in the final incubation step, for the visualization of the cellular nuclei. Confocal images were obtained using a Zeiss 510 META laser scanning microscope (Carl Zeiss) and related software.

2.9 Data analysis

All numerical data were collected from 3-5 independent experiments and analyzed using ANOVA with Tukey's test. Values of $p < 0.05$ were considered significant.

3. Results

Construct volume in 3-D fibrin constructs decreased progressively from day 0 to day 7. Images of fibrin constructs at day 7 are shown in Fig. 1 and gel volume over time is shown in Fig. 2 (A). All samples compacted to less than 10% of their original volume by day 7. Constructs made with 0.001 UT/mg F samples compacted significantly more than all other matrices on all days. Similarly, the 0.01 UT/mg F constructs compacted significantly more than 1.0 UT/mg F (days 3 and 7) and 0.1 UT/mg F (days 1, 3 and 7). Collagen gels also compacted progressively over time, though to a lesser degree than fibrin constructs (Fig. 2(B)). Thrombin at a concentration of 0.001 UT/mg C was added to the collagen constructs in order to examine whether increased gel compaction was the result of stimulation of cells, rather than an effect on the matrix. Collagen constructs compacted to around 15-20% of their original volume by day 7, and there was no significant difference between constructs with and without added thrombin.

Tensile mechanical testing data are shown in Fig. 3. Both the material modulus and ultimate tensile stress (UTS) increased as the concentration of thrombin decreased in fibrin gels (Fig. 3(A)). The 0.01 UT/mg F constructs had modulus and UTS values that were approximately 2-fold greater (18.9 and 24.0 kPa, respectively) than both 1.0 and 0.1 UT/mg F constructs (9.93 and 13.4 kPa, 8.86 and 11.3 kPa, respectively). The 0.001 UT/mg F constructs showed an increase of about 3-fold (28.2 and 38.6 kPa, respectively) when compared to 1.0 and 0.1 UT/mg F constructs. There was no significant difference between the mechanical properties of 1.0 and 0.1 UT/mg F constructs. Both the material modulus ($R = -0.91$) and UTS ($R = -0.90$) were highly correlated to the degree of compaction in fibrin constructs. In collagen gels (Fig. 3(B)), addition of thrombin did not significantly affect the mechanical properties of the constructs. The maximum force at failure for fibrin constructs, shown in Fig. 4, was also determined to be a sample property. The value for 1.0 UT/mg F constructs (87.7 mN) was significantly lower than the 0.01 (126 mN) and 0.001 UT/mg F (133 mN) constructs. There was also a difference between 0.1 UT/mg F (98.4 mN) and the lower thrombin concentrations. There were no significant differences between 1 and 0.1 UT/mg F or 0.01 and 0.001 UT/mg F constructs.

Cell number as determined by DNA quantitation was not statistically different between any of the 3-D fibrin constructs at day 7, as shown in Fig. 5(A). The average cell number in fibrin constructs at day 7 was 4.3 million cells per construct, which was an increase of approximately 24% over the initial cell number of 3.7 million cells per construct at day 0. In collagen constructs, cell proliferation was somewhat higher, with an increase to 4.7 million cells per construct by day 7 in collagen gels with no thrombin, and to 5.6 million cells per construct in collagen gels treated with 0.001 UT/mg C, as shown in Fig. 5(B). Although cell numbers were higher in the presence of thrombin, this difference was not statistically significant at day 7.

SEM examination showed that changing thrombin concentration affected the morphology of fibers in 3-D fibrin constructs, as shown in Fig. 6. Higher thrombin concentrations resulted in more highly interconnected fiber meshes with finer fiber structure and smaller pores between fibers. As thrombin concentration decreased, fiber size increased and more fiber bundling was evident. The features of the network at lower thrombin concentrations were less uniform.

Visualization of the actin cytoskeleton of smooth muscle cells embedded in 3-D fibrin matrices using confocal microscopy is shown in Fig. 7. Cells were generally aligned along the long axis of the tubular constructs (indicated by arrow in panels in Fig. 7). The extent of cell spreading was not noticeably affected by thrombin concentration in the 3-D fibrin matrix, and the cells displayed a characteristic spindle-shaped appearance. In contrast, cell morphology on 2-D fibrin gels was markedly influenced by changing the concentration of thrombin, as shown in Fig. 8. Higher thrombin concentrations (e.g. 1.0 and 0.1 UT/mg F, Fig. 8(A) and (B)) produced

cells with long projections, whereas lower concentrations of thrombin (0.01 and 0.001 UT/mg F) resulted in more compact cells with multiple shorter projections. Control cells, cultured on tissue culture plastic in the absence of thrombin (Fig. 8(E)) were even more cuboidal in shape, with very short cellular projections.

4. Discussion

The goal of this study was to examine the effect of thrombin concentration on the formation and mechanical properties of cell-seeded fibrin hydrogels. The relationship between thrombin concentration and fiber morphology has long been established [11,18] and early work in the field noted a greater inherent stiffness in fibrin clots with larger structural units [11]. Our study extends this work more directly to situations in which fibrin gels are constructed as scaffolds for tissue engineering, in particular in situations where cells are embedded directly inside hydrogel constructs. A more thorough mechanical characterization of such scaffolds, as well as an examination of the effects of thrombin on the cellular component, is important in developing fibrin-based engineered tissues and cell delivery vehicles.

Decreasing the concentration of exogenous thrombin added at the time of fibrin gelation resulted in both increased gel compaction as well as thickening of the fibrin microfibers. It is not clear that these phenomena are related; however, decreasing thrombin concentrations also resulted in markedly improved material properties. Increased gel compaction in such constructs leads to increased mechanical strength because it produces a denser material with a higher amount of protein per unit volume. Gel compaction was highly correlated with the mechanical properties of constructs. Increasing fiber diameter may also contribute to higher material properties, since the load carried per fiber is larger; however, we were not able to reliably quantify both the diameter and number of fibers from the SEM images obtained in this study. This information would be required to associate increased fibril diameters at the microscopic level with increased material properties, and we are developing methods to extract these data. However, our results that show increased maximum force at failure at lower thrombin concentrations support the idea that thicker protein fibers produce constructs with more robust properties.

In order to determine whether thrombin's effects on vascular smooth muscle cells was responsible for the increased gel compaction and resulting mechanical properties, we added thrombin to protein constructs made of Type I collagen, which gels via a pH-mediated fibril aggregation mechanism that does not require thrombin. In this case, neither gel compaction nor construct mechanical properties were affected by the addition of thrombin. This suggests that the effects of thrombin that we observed in fibrin gels are a result of changes in the matrix structure, rather than altered cell function.

Changes in matrix morphology can regulate cellular processes such as migration [25] and morphology [26], and can also influence the early wound healing response [27]. We therefore assessed cell morphology and proliferation in response to varying thrombin concentrations and the associated changes in matrix structure. In 2-D cultures of smooth muscle cells on top of fibrin substrates, cell morphology was clearly altered. At lower thrombin concentrations (larger fibers) and on tissue culture plastic, cells exhibited a cuboidal morphology typical of moderate passage smooth muscle cells in culture, and as thrombin concentration increased (leading to smaller fibers) cells produced more projections. In general, cellular morphology in 3-D was less stellate than in 2-D, but there were no clear differences in cell morphology as thrombin concentration varied. Alignment of cells in 3-D gels along the longitudinal axis of the constructs was evident. This was presumably a response to the stresses built up in the matrix soon after gel formation, before the constructs were freed from the mandrel, since constrained compaction has been shown to cause cell alignment [28]. It is interesting that changes in matrix morphology

caused altered cell shape on 2-D substrates, but that these changes were not evident in 3-D matrices in which the matrix morphology was similarly altered. From the data presented in this study, it is difficult to decouple the effects of matrix structure and mechanical influence. In 3-D matrices, the cells can remodel and compact the matrix, whereas 2-D matrices are immobilized by adhesion to the underlying plastic substrate. Although it appears that the ability to freely remodel the matrix allows cells to adopt a morphology that is less dependent on of the microstructure of the matrix, a different system must be used in order to separate these confounding influences.

These data relate important interconnected features of fibrin hydrogels, and show how these features can be controlled by altering the thrombin concentration used to form the gels. The degree of gel compaction and fibrin microfibril structure play a role in determining the macroscopic material properties of fibrin constructs. Addition of thrombin to Type I collagen constructs did not cause a similar increase in mechanical properties and cell morphology in 3-D fibrin gels was not affected by thrombin concentration, suggesting that increases in mechanical properties were a result of thrombin's effect on matrix structure. These results contribute to the understanding of structure-function relationships in cell-seeded protein hydrogels, and can be applied to developing improved scaffolds for tissue engineering.

Acknowledgments

This work was funded by NIH grant number R21-EB003978 from the National Institute of Biomedical Imaging and Bioengineering (NIBIB). We also thank Dr William Samsonoff of the Wadsworth Center of the New York State Department of Health for his assistance with SEM imaging.

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Fig. 1. Fibrin constructs made with varying amounts of thrombin (1.0, 0.1, 0.01 and 0.001 UT/mg F, as labeled) after 7 days in culture. Scale bar is 1 cm.

Fig. 2.
Gel compaction as a percentage of the original volume for (A) fibrin matrices and (B) collagen matrices on days 1, 3 and 7.

Fig. 3. Material properties: material modulus and ultimate tensile strength (UTS) of (A) fibrin matrices and (B) collagen matrices, as determined by uniaxial tensile testing. * = significantly different from 0.01 UT/mg F, # = significantly different from 0.001 UT/mg F.

Fig. 4. Sample properties: maximum force at failure for fibrin matrices, as determined by uniaxial tensile testing. * = significantly different from 0.01 UT/mg F, # = significantly different from 0.001 UT/mg F.

Fig. 5.
Cell number in (A) fibrin matrices and (B) collagen matrices at day 7 in culture.

Fig. 6. Scanning electron micrographs of 3-D fibrin matrices with varying thrombin concentrations: (A) 1.0 UT/mg F, (B) 0.1 UT/mg F, (C) 0.01 UT/mg F, (D) 0.001 UT/mg F.

Fig. 7. Confocal laser scanning microscopy images of the actin cytoskeleton of RASMC in 3-D fibrin matrices: (A) 1.0 UT/mg F, (B) 0.1 UT/mg F, (C) 0.01 UT/mg F, (D) 0.001 UT/mg F.

Fig. 8. Fluorescence microscopy of the actin cytoskeleton of RASMC on 2-D fibrin substrates: (A) 1.0 UT/mg F, (B) 0.1 UT/mg F, (C) 0.01 UT/mg F, (D) 0.001 UT/mg F, (E) control (tissue culture plastic, no thrombin).