Available online at www.sciencedirect.com
ScienceDirect
Acta Biomaterialia 3 (2007) 59–67
Acta BIOMATERIALIA
www.actamat-journals.com

Influence of thrombin concentration on the mechanical and morphological properties of cell-seeded fibrin hydrogels

Shaneen L. Rowe, SungYun Lee, Jan P. Stegemann *

Department of Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180-3590, USA

Received 13 March 2006; received in revised form 2 August 2006; accepted 7 August 2006

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 cross-linking 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,

* Corresponding author.
E-mail address: stegemann@rpi.edu (J.P. Stegemann).

1742-7061/$ - see front matter
doi:10.1016/j.actbio.2006.08.006

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% CO_2. 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 (UT/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× 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

ID	Title	Pages
2567	Influence of thrombin concentration on the mechanical and morphological properties of cell-seeded fibrin hydrogels	9