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Fibrin-based 3D matrices induce angiogenic behavior of adipose-derived stem cells

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ABSTRACT

Engineered three-dimensional biomaterials are known to affect the regenerative capacity of stem cells. The extent to which these materials can modify cellular activities is still poorly understood, particularly for adipose-derived stem cells (ASCs). This study evaluates PEGylated fibrin (P-fibrin) gels as an ASC-carrying scaffold for encouraging local angiogenesis by comparing with two commonly used hydrogels (i.e., collagen and fibrin) in the tissue-engineering field. Human ASCs in P-fibrin were compared to cultures in collagen and fibrin under basic growth media without any additional soluble factors. ASCs proliferated similarly in all gel scaffolds but showed significantly elongated morphologies in the P-fibrin gels relative to other gels. P-fibrin elicited higher von Willebrand factor expression in ASCs than either collagen or fibrin while cells in collagen expressed more smooth muscle alpha actin than in other gels. VEGF was secreted more at 7 days in fibrin and P-fibrin than in collagen and several other angiogenic and immunomodulatory cytokines were similarly enhanced. Fibrin-based matrices appear to activate angiogenic signaling in ASCs while P-fibrin matrices are uniquely able to also drive a vessel-like ASC phenotype. Collectively, these results suggest that P-fibrin promotes the angiogenic potential of ASC-based therapeutic applications.

1. Introduction

Regenerative medicine strategies that rely on the therapeutic potential of stem cells employ additional components such as scaffolds [1], growth factors [2], stress conditioning [2,3], and genetic modifications [4]. For example, adult stem cells isolated from patients' mature tissues, such as bone marrow or fat, can be modulated for specific tissue regeneration with the correct chemical cue and/or physical stress [2,3]. Furthermore, engineered substrates can be targeted to optimize cellular activities including changes in morphology, proliferation, differentiation, and production of extracellular matrix and angiogenic molecules [5–7]. Conditioned media collected from stem cells cultured under normal or stimulated conditions can be a potential therapy to treat damaged tissues, suggesting that the paracrine action of stem cells is an important mechanism in healing tissues [8–11].

Adipose-derived stem cells (ASCs) have been highlighted as a potential adult stem cell source for regenerative medicine [12]. They have demonstrated multi-lineage differentiation potential into bone, fat, and muscle and elaborate abundant extracellular matrix proteins such as collagen type I [8,15]. In addition, a number of studies have demonstrated their vasculogenic properties as well as mesenchymal-like identities, *e.g.,* surface characteristics such as CD29 and CD90 [13,14]. ASCs are also known to secrete pivotal angiogenic growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), matrix-degrading enzymes (i.e., matrix metalloproteinases), and proinflammatory cytokines (*e.g.,* interleukins) [8,10,16–18]. ASCs can regulate the activity of fibroblasts, monocytes/macrophages, and endothelial cells (ECs), which are key effectors of wound healing and tissue regeneration. Based on the regenerative potential of ASCs, ASCs may be able to contribute to accelerated neovascularization in a wound bed [1,7].

The appropriate hydrogel delivery system may drive one or more of these mechanisms to facilitate restoration of oxygen and nutrients to the wound environment. In the tissue-engineering field, natural polymer hydrogels and synthetic–natural blends based on FDA-approved materials, such as collagen and fibrin, are promising stem cell scaffolds; these materials have desirable biodegradability, biocompatibility, and are minimally invasive to deliver. Mechanical, spatial (pore architecture and surface topography), and chemical properties of 3D cell-adherent materials mediate stem cell activation through membrane receptor-triggered signaling pathways. Understanding ASC behaviors in collagen and

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fibrin gels is critical to improving ASC-mediated tissue regeneration. Moreover, materials that enhance the angiogenic potential of ASCs can promote the formation of well-organized vascular networks within the scaffold itself and bridge gaps between the scaffold material and injured local tissues [1,7].

Collagen-based materials show low-level immunogenicity and have been widely researched and clinically tested in numerous tissue engineering applications, such as bone, cartilage, and vascular tissues [19–21]. Furthermore, collagen gels containing ASCs showed therapeutic potential for both rigid (*e.g.,* bone and tendon) as well as soft tissues (*e.g.,* blood vessels and skin) [22–24]. In contrast, fibrin, a polymeric component of blood clots, is more angiogenic than collagen and is used clinically as a surgical sealant [25–27]. Fibrin gels have also shown promise as a skin dressing, providing hemostasis while simultaneously minimizing wound exposure to the external environment [25,27]. However, fibrin gels have relatively weak mechanical properties and degrade rapidly in the body, losing their utility as a scaffold.

To overcome the disadvantages of unmodified natural polymers, several studies have sought to develop copolymers that integrate natural and synthetic polymers and their properties, including PEGylated fibrin (P-fibrin). Fibrin can be covalently modified with amine-reactive polyethylene glycol (PEG). According to our prior work, P-fibrin gels showed more stable mechanical properties and increased the vascular morphology and phenotype of embedded bone marrow mesenchymal stem cells (BMSCs) relative to fibrin [6,28,29]. ASCs also demonstrated endothelial-like phenotypes in the P-fibrin gels, although this phenomenon was not directly compared to fibrin and collagen [7]. The aim of the current study is to expand the field's understanding of how 3D hydrogel systems can modify the regenerative potential of ASCs by a direct comparison of three hydrogel systems: (1) collagen, (2) fibrin, and (3) P-fibrin gels, in identical *in vitro* culture conditions. The intent of this study is to compare both the phenotype of the cultured stem cells as well as the potential for paracrine secretion; two major potential mechanisms for tissue regeneration. Here we investigated the morphology, proliferation, protein expression, and soluble factor secretion of ASCs following cultivation in these gels without any supplement beyond basic growth media.

2. Materials and methods

2.1. Cell culture

Human ASCs (PT-5006, Lonza) identified as positive for CD29, CD44, CD73, CD90, CD105, CD166 and negative for CD14, CD31, and CD45 were commercially procured. Dulbecco's Modified Eagle Medium (DMEM)-low glucose with 1% Glutamax I (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin–streptomycin was used for the entirety of ASC cultivation, both in the monolayer and 3D hydrogels. The media was exchanged every two days for monolayers and every day for 3D cultivation. ASCs (passage number below 10) were acquired by monolayer expansion at a seeding density of 5000 cells/cm^2 and all cell culture was carried out in an incubator with 5% CO_2 and at 37 °C.

2.2. Fabrication of hydrogel scaffolds and ASC seeding

3D hydrogels were fabricated at 4 mg/ml (final polymer concentration) and at a cell seeding density of 50,000 cells/ml. Gel volumes varied from 0.5 to 3 ml depending on the experiment:

2.2.1. Collagen gel

Rat tail type I collagen solution (5 mg/ml) was purchased commercially (Trevigen). Based on the manufacturer's protocol, the stock solution was combined on ice with cells, ice-cold Dulbecco's phosphate buffered saline (DPBS) (×10), and sodium hydroxide. Solutions were solidified in the 5% CO_2 incubator at 37 °C for 30 min.

2.2.2. Fibrin and P-fibrin gels

Human fibrinogen (Sigma) was dissolved in DPBS (without calcium and magnesium, pH 7.8) at a concentration of 32 mg/ml. For PEGylation, succinimidyl glutarate-modified PEG (SG-PEG-SG; MW 3400, NOF America) was similarly dissolved in DPBS at 3.2 mg/ml and combined with fibrinogen in a 1:1 volume ratio. The fibrinogen-PEG solution was then combined with an equal volume of cell suspension, which was then enzymatically crosslinked by a new equal volume of human thrombin (25 U/mL in 40 mM $CaCl_2$) to form gels. For fibrin gels, DPBS was substituted for the PEG. Gelation was completed by incubating fibrin and P-fibrin solutions in the 5% CO_2 incubator at 37 °C for 10 min.

All gels were formed in a 6 or 12 well plate cell culture insert (membrane pore diameter = 8 μm, BD Biosciences) and the culture media was added both inside and outside the insert.

2.3. ASC morphological analysis

ASC morphology and elongation in the gels were analyzed during cultivation (days 2, 5, and 7) using a phase contrast microscope (EVOS). For clearer analysis of cell-to-cell networks, three-dimensional image-based quantification was also performed. Descriptive network metrics were quantified using our previously described three-dimensional morphometry method [29]. Briefly, the cytoplasm of ASCs in gels was stained with Calcein AM (Life Tech). Image z-stacks were collected with an upright two-photon microscope (Ultima, Prairie Technologies) under a 20× water-immersion objective. The microscope's tunable laser was set to 720 nm for fluorophore excitation and the emitted signal was detected by the green spectrum PMT (455–595 nm). Z-stacks were pre-processed in ImageJ and exported as Visualization Toolkit files (.vtks) for import into 3D Slicer. In 3D Slicer, five individual network structures from each z-stack were segmented in 3D models, which were then exported as coordinate clouds consisting of (x,y,z) coordinates and corresponding model radii. Coordinate cloud data were processed in MATLAB to calculate average metrics per network structure: volume, length, number of branches, and diameter. Metrics were also reported per network branch, which is a sub-segment of a network structure. Volume was calculated using a cylindrical boundary approximation between adjacent coordinate pairs as previously reported. Length was calculated using the three-dimensional distance equation and each branch is a unique linear (non-splitting) path and no two branches within a structure overlap.

2.4. Contiguous dye uptake across multiple interconnected cell bodies

Each gel was fabricated and cultured identically as described in the above method Section 2.2. Gel volume was 500 μl and was cultured in a transwell insert within a 12-well plate. On day 4 of gel culture, 50 μl (including 250 μg) of Texas Red-dextran (Life Tech, 10 kDa) per gel was added to the gel samples. On day 7, gels were destained with PBS for 2 h in a 5% CO_2/37 °C incubator, and PBS solution was exchanged with fresh PBS every 15 min. Then, cells in the gels were fixed with 4% neutral buffered formalin for 15 min at room temperature. The samples were repeatedly rinsed with DPBS 3 times each for 10 min. In addition, DAPI (2 μg/ml, Life Tech) and Alexa Fluor 488-phalloidin (5 units/ml, 0.165 μM, Life Tech) stains were used to visualize the nucleus and F-Actin, respectively. For cell permeabilization, 0.1% saponin in DPBS was used as a washing buffer. Images were captured with 40× magnitude using a confocal microscopy (Zeiss Axio Observer Z1).