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Fibrin-based 3D Matrices Induce Angiogenic Behavior of Adipose-derived Stem Cells

Eunna Chung¹, Julie A Rytlewski¹, Arjun G Merchant¹, Kabir S Dhada¹, Evan W Lewis¹, and Laura J. Suggs¹

Eunna Chung: enchung@utexas.edu

¹Department of Biomedical Engineering, The University of Texas at Austin, TX, U.S.A, 78712-0238

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.

Keywords

adipose-derived stem cells; collagen; fibrin; hydrogel; angiogenesis; tissue engineering

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

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Corresponding Author: Laura J. Suggs, Phone: +1 (512) 232-1671, suggs@utexas.edu.

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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]. ASC 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 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 glycols (PEGs). 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.

Materials and Methods

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 5,000 cells per cm² and all cell culture was carried out in an incubator with 5% CO₂ and at 37°C.

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 0.5–3 ml depending on the experiment:

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% CO₂ incubator at 37°C for 30 minutes.

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 CaCl₂) 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% CO₂ incubator at 37°C for 10 minutes.

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.

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. 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 720nm for fluorophore excitation and the emitted signal was detected by the green spectrum PMT (455–595nm). 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.

Contiguous dye uptake across multiple interconnected cell bodies

Each gel was fabricated and cultured identically as described in the above method section (*Fabrication of hydrogel scaffolds and ASC seeding*). 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 hours in a 5% CO₂/37°C incubator, and PBS solution was exchanged with fresh PBS every 15 minutes. Then, cells in the gels were fixed with 4% neutral buffered formalin for 15 minutes at room temperature. The samples were repeatedly rinsed with DPBS 3 times each for 10 minutes. 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).

Cell proliferation assay

To compare the effects of different hydrogels on ASC growth, ASC metabolic activities following cultivation at the certain time-points (days 1, 4, 7, and 10) were measured using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega). In brief, MTS CellTiter 96 solution was added as 20% (v/v) of the gel culture media and incubated in

the 5% CO₂ incubator at 37°C for 4 hours. After incubation, the supernatant was collected from each gel sample and absorbance measured at 490 nm using a microplate reader (BioTek).

Real time qRT-PCR

To compare the gene expression of ASCs, ASCs were cultured in the three different hydrogels for 7 days as described above. 3D hydrogels (1 ml) including ASCs were homogenized and RNA samples were collected in Trizol® reagent (Life Tech). After precipitating with isopropanol and ethanol solutions, reverse transcription of isolated RNA was carried out using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems) by incubating for 120 minutes. CDNA was mixed with 2× TaqMan® Fast Universal PCR Master Mix (no AmpErase® UNG, Applied Biosystems) and specific Gene expression assays (Applied Biosystems) using ViiA™ 7 (Applied Biosystems). The gene expression assay ID was Hs00426835_g1, Hs00169795_m1, and Hs00900054_m1 for SMA, vWF, and VEGF-A, respectively. We normalized the Ct value by a house keeping gene, (gene encoding beta actin). Also, the presented gene data was normalized by the relative fold induction level compared to collagen.

Protein secretion analysis

Enzyme-linked immunosorbent analysis (ELISA) was applied to analyze changes in VEGF secretion of ASCs after they were cultured in different gel substrates. A Quantikine ELISA Kit (R&D Systems) against human VEGF was used according to the manufacturer's direction for cell culture supernatants. The culture media was collected after 24 hour time periods: day 0–1, 3–4, and 6–7. To compare VEGF secretion levels per cell, total VEGF secretion was normalized to cell number, as determined by the CellTiter 96 assay. In addition, culture media from day 6–7 was analyzed by a RayBio® Human Angiogenesis Array C1000 (Raybiotech) as a broad-spectrum screen for secreted angiogenic factors. Briefly, according to the manufacturer's protocol, these membranes, printed with antibody spots against multiple antigens, were incubated with media samples, and biotinylated antibodies and HRP-conjugated streptavidin that can specifically detect biotin were continuously added. The chemiluminescent signal was acquired using a FluorChem Q (ProteinSimple) device. The signal was then quantified in AlphaView SA (ProteinSimple).

Analysis of intracellular protein expression

Western blot—Smooth muscle alpha actin (α -SMA) and von Willebrand factor (vWF) were chosen to demonstrate whether hydrogels modify the intracellular protein expression of ASCs. Cell lysates were collected using ice-cold RIPA buffer (Santa Cruz Biotechnology). The concentration of acquired protein was measured by BCA Protein Assay Kit (Pierce). Protein (15 ug) was combined with an equal volume of loading buffer, consisting of Laemmli sample buffer (Bio-Rad Laboratories) with 5% (v/v) β -mercaptoethanol. Protein was denatured by heating to 95–100°C for 5 minutes. Samples were loaded in the wells of 10% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories). After gel electrophoresis for 60 minutes at 100V, the proteins were blotted onto PVDF membranes in a wet tank setup for 1h at 100V. Immunoblotting was performed by incubating the membrane

in 5% non-fat dry milk solution in 1% (v/v) Tween-20 in Tris buffered saline. The following primary antibodies against each target protein were added to the membranes for 1h: mouse anti- α -SMA (ab7817, Abcam), rabbit anti-vWF (ab6994, Abcam), and (loading control) rabbit anti-beta actin (ab75186, abcam). After washing, HRP-conjugated secondary anti-IgG antibodies were added for another hour according to the species of the primary antibody: goat anti-mouse (sc-2005, Santa Cruz) and goat anti-rabbit (ab6721, Abcam). Immunoblotted protein bands were visualized with a chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate, Pierce) and imaged using FluorChem Q (ProteinSimple). AlphaView software was again used for signal quantification.

Immunofluorescence stain—Gels cultured with encapsulated ASCs were fixed with 4% neutral buffered formalin for 15 minutes at specific time points (day 1, 4, and 7). After fixation and washing, ASCs in gels were permeabilized with 0.2% Triton X-100 for 15 minutes. After washing with DPBS, samples were blocked with 10% normal goat serum/0.1% saponin. A 1:100 dilution with anti- α -SMA (ab7817, Abcam) was incubated with the samples overnight at 4°C; normal mouse IgG (sc-2025, Santa Cruz, 400 ug/ml) was used as an isotype control. We diluted the primary antibody, anti-SMA (ab7817, abcam) at the concentration of 1:100 (v/v). The final concentration of both anti-SMA and the relevant isotype antibody are 2 μ g/ml. The next day, samples were washed and incubated with Alexa 488-conjugated goat anti-mouse IgG (H+L) (Life Tech) with 1% normal goat serum/0.1% saponin for 1 hour at room temperature. We counterstained cell nuclei with 1 μ g/ml DAPI (Life Tech) for 15 minutes and mounted gels in fluorescence-antifading media (Life Tech). Image z-stacks were collected with an upright two-photon microscope (Ultima, Prairie Technologies) under a 20 $\times$ water-immersion objective. The microscope's tunable laser was set to 720nm for simultaneous excitation of all fluorophores. Emitted signal for α -SMA was detected by the green spectrum PMT (455–595 nm) and nuclei counterstain was detected by the blue spectrum PMT (410–510nm). Z-stacks were flattened into z-projections prior to fluorescence signal quantification. Average pixel intensity was measured in ImageJ by sampling 20 intracellular image areas per z-projection. Fluorescence intensity was corrected for sampled pixel area and background signal.

Effects of conditioned media on endothelial metabolic activity

Conditioned media was prepared by collecting the culture media daily from each ASCs gel culture and concentrated 10-fold using Amicon Ultra-15 Centrifugal Filter Units (MW 3K cutoff, Milipore). Human Dermal Microvascular ECs (HDMECs) were acquired commercially from Promocell. Passage 4 HDMECs were seeded onto a 48-well plate at a density of 20,000 cells/cm². HDMECs adhered overnight with endothelial growth media consisting of MCDB131 basal media supplemented by an EGM-2MV bullet kit (Promocell), 10% FBS, 1% penicillin-streptomycin, and 1% Glutamax™. The next day, the endothelial growth media was removed and cells were washed with DPBS (without calcium or magnesium). Washed HDMECs were treated with 500 μ l of various media conditions and cultured further for 48 hours. The media conditions were as follows: 1) 500 μ l serum-free MCDB131, 2) 200 μ l 10% FBS-MCDB131 media mixed with 300 μ l serum-free MCDB131, 3) 500 μ l 10% FBS media, 4) 500 μ l endothelial growth media, and 5–7) 200 μ l 10% FBS-MCDB131 media mixed with 300 μ l conditioned media from either collagen,

fibrin, P-fibrin gel cultures. After 48 hours, the CellTiter 96 assay was again performed to analyze the metabolic activities of HDMECs.

Statistical analysis

For quantitative analysis, test groups were prepared and tested at a minimum of three individual samples per experimental group ($n=3-5$). Plotted data were presented as mean values with an error bar of one standard deviation. Statistical analysis was performed by ANOVA corrected by Tukey's method for multiple comparisons. The data was considered significantly different when the p value was below 0.05.

Results

Material analysis

Digital camera imaging and rheological measurements were performed to compare the properties of three different gels (collagen, fibrin, and P-fibrin) prior to cell experiments. All materials maintained their original shape and formed stable gels in the transwell insert (Fig. 1A–C). Without cells, gels did not visibly degrade or collapse in the presence of culture media. However, the gel opacity and media perfusion rate were different between the three materials. Collagen and fibrin gels were opaque while P-fibrin gels were optically clear. The media perfusion was most rapid in collagen gels, followed by fibrin, and lastly P-fibrin. The rheological properties of each gel type were characterized (Fig. 1D). Both storage and loss moduli of collagen gels were significantly higher than those of fibrin-based gels. The loss factor ($\tan \delta$) of P-fibrin was significantly lower than that of collagen or fibrin gels (data not shown here).

ASC morphology

As shown in Fig. 2, ASCs in the P-fibrin gel developed highly elongated morphologies and interconnected networks as we have previously demonstrated using ASCs and BMSCs [7, 29]. On day 2, no notable changes in the residing cells were observed in any gel culture; at later time points (day 5 and 7), a spindle-like cell shape was more apparent in the P-fibrin gels, and the cells went on to develop uniform interconnected networks. On day 7, the networks in P-fibrin became more complex than those on day 5 and the gel shape was stably maintained, indicating minimal matrix degradation. However, the degradation and collapse of fibrin gels was observed during the cultivation period. Collagen gels did not induce elongated cellular networks, but the gel shape was maintained.

To compare the gel matrix-mediated modification in cell morphology and cell-to-cell interaction more clearly, we applied image stack segmentation for three-dimensional quantitative analysis. Quantification of ASC networks showed that P-fibrin promotes significantly more developed network morphology than either collagen or fibrin matrices. Morphological differences between ASCs in collagen and fibrin were not observed. ASC networks in P-fibrin had the largest net volumes, longest net lengths, and most branches of the three experimental groups (Fig. 3A–C; filled bars). However, normalizing network volume and length to the number of branches (per network) eliminated any statistically significant variation (Fig. 3A, B; white bars). Since all networks were composed of similarly

sized branches, any increase in ASC network size arose solely from an increase in branch number—not from an increase in individual branch dimensions. Hence, networks in P-fibrin were the largest because the ASCs developed significantly more branches per network (Fig. 3C). We also derived diameter by dividing the volume network with length, as shown in Fig. 3D, and the networks in the fibrin gel had significantly greater diameter than any other gels. Furthermore, to access the connectivity of cell-to-cell networks in each gel, as shown in Fig. 4, 10 kDa dextran dyes were used with the cytoskeletal and nuclear counterstains. Even though dye uptake by ASCs showed cell-to-cell variation, more continuously-filled dextran dye was detected in the elongated cellular networks of PEGylated fibrin gels. In contrast, there were signals from dye that was not taken up by cells in the collagen and fibrin gels.

ASC proliferation

All gel materials showed similar ASC growth rates, as measured over a 10 day period (Fig. 5). However, ASCs in fibrin initially proliferated more rapidly than in collagen and P-fibrin; subsequently, significantly more ASCs were present in fibrin gels at day 4 than in collagen. ASC metabolic activities on day 10 were comparable across all gel systems.

ASC gene and protein expression

Intracellular gene and protein analysis showed inverse trends between SMA and vWF expression, as shown by qRT-PCR and western blot analysis in Fig. 6. ASCs showed decreased levels of transcripts coding for SMA expression in fibrin and P-fibrin gels compared to collagen on day 7. Conversely, vWF gene expression was slightly upregulated in fibrin and P-fibrin gels although this did not achieve statistical significance (Fig. 6A). ASCs expressed the greatest levels of SMA protein in collagen gels followed by fibrin and then P-fibrin gels, which were both slightly lower (Fig. 6B). This trend was confirmed more clearly via immunofluorescence staining (Fig. 7). The SMA-positive signal from ASCs in P-fibrin gels was lower than ASCs in either collagen or fibrin. In contrast, vWF protein expression in P-fibrin on day 7 was significantly greater than any other gel group (Fig. 6C).

ASC protein secretion and paracrine effects

The angiogenic paracrine potential of ASCs was investigated by VEGF qRT-PCR and ELISA analysis (Fig. 8). VEGF mRNA was upregulated in fibrin and P-fibrin gels compared to collagen, with the fibrin gel achieving significance. VEGF secretion was similar at days 1 and 4 in all gels. However, we observed significantly greater levels of VEGF in fibrin than collagen and P-fibrin, and P-fibrin showed a higher level than collagen at day 7. VEGF secretion per cell decreased dramatically in the time period from day 1 to 4 in all gels. Furthermore, the normalized VEGF level per cell in fibrin at day 1 was lower than collagen and P-fibrin.

HDMECs were able to maintain a higher level of metabolic activity following treatment with conditioned media from both fibrin-based gels than that of collagen gels (Fig. 9A). HDMECs did not survive in the serum-free media and showed maximum metabolic activity in complete endothelial growth media. Full FBS-containing media and partially serum-added groups (200 μ l of 10% FBS containing media mixed with 300 μ l serum free) were not demonstrably different. We therefore attributed the diminished cell proliferation in collagen

to factors from the collagen conditioned media interfering with EC growth. The fibrin-based gels, however, promoted cell growth to the level that full FBS-containing media would demonstrate.

In addition, ELISA-based arrays revealed that ASCs in fibrin-based gels secreted more soluble angiogenic factors than ASCs in collagen, both in quantity and diversity (Fig. 10). Several angiogenic/proinflammatory proteins such as GRO, MCP-1, IL-6, and IL-8 were dramatically up-regulated in fibrin and P-fibrin gels compared to collagen.

Discussion

Our current study expanded upon the present understanding of how 3D matrices—collagen, fibrin, and P-fibrin—can influence the angiogenic programming of adipose tissue-derived stem cells. ASCs were chosen for the current study. They are a mesenchymal-like stem cells isolated from the vascular fraction of adipose tissue and sorted based on several mesenchymal indicating surface markers (*e.g.* CD29⁺CD44⁺CD105⁺CD106⁺CD14⁻CD45⁻). Adipose tissue alone has previously shown enhanced skin regeneration and angiogenesis, more than localized delivery of growth factors such as bFGF and EGF [30]. While adult stem cells isolated from bone marrow tissue have been widely used in tissue engineering, more recent studies have highlighted the comparable potential of ASC populations. Unfortunately, their behavior in 3D matrices is less well understood. ASCs from the vascular fraction of adipose tissue can be categorized as mesenchymal stromal cells or (more specifically) vascular progenitor cells (CD34⁺/CD31⁻) due to their mitogenic abilities, surface markers, and unstable expression profiles over time [13, 31]. In spite of these concerns, utilization of ASCs has advanced: strategies for isolation and clinical use have become more refined. Chan *et al* recently reported that ASCs isolated from the debrided skin tissue of burn injuries possessed comparable regeneration potential to other fat sources [32]. Our study used a commercial mesenchymal-like stem cell line, which is similarly immunophenotyped to BMSCs, as an ASC source.

BMSCs have been reported to adopt a pericytic role and support EC stabilization in the microvascular niche [33–35]. Similarly, ASCs can also assemble and mechanically support recruited ECs, leading to superior pre-vascularization [36, 37]. Prior studies by our laboratory and collaborating teams have demonstrated that P-fibrin gels have unique angiogenic/vasculogenic properties over unmodified fibrin gels [6, 29]. These characteristics suggest that P-fibrin has potential for cardiovascular and skin tissue engineering applications, since giving additional chemical cues to damaged tissue through the use of a cell-gel construct may be able to accelerate angiogenesis. In current study, we found that the morphological changes of ASCs in P-fibrin followed similar trends in cellular morphology as previously-reported BMSCs, however, it was unknown how these cells compared to those cultured in fibrin or collagen [29]. In addition, ASCs in collagen gels did not make interconnected elongated networks, but they readily developed network formations in the P-fibrin gel (Fig. 2–4) and the quantitative levels (segment numbers, volume and length) of elongated network systems increased in the order of collagen, fibrin and P-fibrin gels. Interestingly, our data demonstrated that ASCs were significantly elongated in P-fibrin gels, which possessed a mid-range storage modulus between collagen and fibrin gels.

Furthermore, no difference was quantifiably detected in ASC morphology between collagen and fibrin gels, even though the modulus of collagen gels was significantly greater than fibrin or P-fibrin gels.

Cellular effects on ASCs were observed in the current study and may result from the differences in the viscoelastic properties of collagen and fibrin gels and/or different interactions between cellular receptors and material ligands. The mechanical modulus of collagen gels was found to be comparable to that of approximately two-fold higher concentration of fibrin gels [38]. Collagen is known for greater mechanical strength, facilitating its use for mechanically loaded tissues [21]. The higher modulus of collagen is known to result in low endothelial cell sprouting compared to fibrin [19, 39]. In studies, fibrin showed a 32.4% increase in microvessel formation from ECs and a 73.5% wider vascular area than collagen [40]. However, the unique mechanism whereby P-fibrin induces ASC elongation and network formation is not yet understood. While collagen is a stiffer material than fibrin and P-fibrin (Fig. 1B), network formation did not correlate directly with rheological characteristics. Even though we have chosen to use one uniform concentration, the difference in cell behaviors and morphologies in each gel can be a result of combined characteristics such as biochemical and biomechanical cues. According to a literature, in cell-matrix interactions, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins dominate in fibrin-based environments and $\alpha_2\beta_1$ integrins dominate in collagen [19, 41]. According to Feng *et al*'s study, the sprouting action of ECs was enhanced in fibrin over collagen, as determined by microbead outgrowth assay [19]. ECs in that study did not form tubular networks, however, without soluble growth factors such as VEGF and bFGF, suggesting biochemical cues are required for cells to form capillary-like networks in unmodified fibrin matrices [19].

Increasing network development of ASCs did correlate with elevated angiogenic paracrine secretion. ASCs in P-fibrin showed significant up-regulation of vWF. Conversely, increasing network development seems to be related to the down-regulation of α -SMA expression. ASCs preferentially express high levels of SMA but low levels of vWF [13, 42]. However, our data indicate that hydrogel matrices can modulate this expression. Moreover, the inverse trend we observed between smooth muscle and EC markers in 3D hydrogels are similar to those of Wingate *et al*'s study, which analyzed MSC differentiation in photopolymerized PEG dimethacrylate gels [43]. They reported that stiffer PEG-DMA gels (9 kPa) led to more SMA expression, whereas softer gels (3 kPa) led to more Flk-1 expression [43].

For the first time, our studies indicate that angiogenic protein secretion by ASCs in a direct comparison can be modified via biochemical cues of hydrogel matrices. While ASCs in collagen and P-fibrin gels proliferated very similarly, their secretion profiles of angiogenic factors were significantly different (Fig. 5, 8, and 10). In addition, ASCs in P-fibrin did not express CD31 strongly or uptake LDL, both of which are standard benchmarks for identifying mature ECs (data not shown); however, these cells still maintained their ability to secrete angiogenic factors such as VEGF, and in our study this secretion was enhanced in fibrin and P-fibrin relative to collagen. Park *et al* demonstrated that conditioned media from ASCs (4×10^5) cultured over 72 hours *in vitro* included fibronectin (1466 ng/ml), type I collagen (921 ng/ml), VEGF (809 ng/ml), and HGF (670 ng/ml) [8]. In addition, Nie *et al*

demonstrated that ASCs (with an immunophenotype of CD29⁺, CD44⁺, CD90⁺, CD31⁻, CD45⁻) showed significantly greater paracrine contribution of VEGF and HGF into the wound tissue environment compared to fibroblasts [44]. Our CAM assay showed that newly-generated blood vessels were guided by implanted ASCs in fibrin-based gels compared to collagen (supplementary data). In addition, the increased secretion of inflammatory cytokines by ASCs in P-fibrin suggests that these cells could also modulate inflammatory cells in the immunocompetent organism, which may play a role in wound healing.

The clinical benefits of stem cell-based therapies are thought to derive from one (or both) of two key stem cell roles: a paracrine chemical stimulator of neighboring cells and a differentiation-mediated cell replacement [44–46]. However, the question of which mechanism is more critical for achieving therapeutic benefit remains unanswered. A recent report demonstrated that ASCs transplanted to the myocardium, post infarct, decreased infarct-associated cell death and stimulated reperfusion of damaged heart tissue [47]. These benefits were linked to angiogenic paracrine secretion of ASCs, rather than trans-differentiation into cardiomyocytes [47]. Similarly, Nakagami *et al*'s study described the interactions of adipose-derived mesenchymal stem cells with ECs via soluble communicators such as VEGF and HGF; here, too, GFP-tagged ASCs delivered to the ischemic tissue did not express vascular phenotype markers such as CD31, vWF, and SMA [48]. Moreover, adipose-derived stromal cells cultured with ECs had diminished differentiation capacities towards the adipocyte lineage but still actively communicated with ECs [49].

ASCs are known to differentiate into ECs in response to certain chemical and physical cues such as growth factors, hypoxia, and mechanical shear stress [3, 12, 50]. A majority of relevant literature regarding stem cells considers several growth factors essential to induce endothelialization or improve endothelial cell activity, including HGF, bFGF, and VEGF [12, 48, 51]. According to Zhao *et al*'s study, human ASCs (CD31⁻, CD34⁻, CD106⁻, and fetal liver kinase⁺) stimulated with VEGF *in vitro* or cultured under ischemia *in vivo* can differentiate into endothelial-like cells expressing CD31, VE-cadherin, and eNOS [12]. Smith *et al* also demonstrated that hypoxic culture conditions induce greater EC elongation and sprouting and increased expression of Factor VIII, to which vWF binds [50]. However, our previous study suggested that vWF expression of ASCs in P-fibrin is a more sensitive marker than CD31 [6, 23]. Several papers have previously described vascular-like morphogenesis of stem cells without a high level expression of CD31 [52]. Since we do not add any growth factors to culture media, complete endothelial differentiation of our cell populations may be limited. Furthermore, the up-regulation of vWF in ASCs cultured in fibrin-based gels may not be directly indicative of differentiation towards an endothelial lineage. Rather, vWF can be viewed as a critical regulator of angiogenesis and wound healing [30, 53].

Conclusions

The current comparative study evaluates how 3D hydrogel cues can modify the behavior of adipose-derived stem cells, using an *in vitro* culture model without added soluble factors. In

particular, we investigate how P-fibrin gels provide unique advantages over collagen or fibrin matrices for promoting therapeutic angiogenesis. Based on the observed angiogenic properties of ASCs in P-fibrin, PEGylated fibrin may stably localize and mechanically support ASCs, and biochemically support EC recruitment and invasion into the gel matrix and nearby tissues.; collectively, these mechanisms were superior in P-fibrin gels over both collagen and fibrin gel materials. In P-fibrin gels, ASCs not only formed more elongated and interconnected networks but also up-regulated angiogenic and endothelial phenotype proteins. In addition, this P-fibrin-based modulation of ASC behavior led to improved metabolic activity of ECs, suggesting better communication between stem cells and surrounding ECs. Through this study, the understanding of ASC regenerative potential in a hydrogel-based carrier was expanded for tissue engineering applications. In the future, our studies will focus on investigating how ASC morphological changes in the PEGylated hydrogel can contribute to their interaction with macrophages and ECs, which are pivotal cell types involved in wound healing and angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Hydrogel characterization. Gel morphologies of Collagen (A), Fibrin (B), and P-Fibrin (C) were acquired by a digital camera. (D) Rheological modulus. Asterisks and hashtags denote the significance between collagen and other gels in storage and loss modulus, respectively ($p < 0.05$).

Figure 2.

ASC morphologies in 3D matrices following *in vitro* cultivation. Phase-contrast microscopy demonstrated cell elongation in 3D gel systems including collagen (A, D, and G), fibrin (B, E, and H), and P-fibrin (C, F, and I). Image data was collected on days 2 (A–C), 5 (D–F), and 7 (G–I). Scale bar denotes 300 μm.

Figure 3.

Quantitative network metrics of ASCs in 3D matrices on day 7. (A) Average network volume in nanoliter (nL), per structure (shaded) and per structure branch (white). (B) Average network length in μm , per structure (shaded) and per structure branch (white). (C) Average number of branches per network structure. (D) Average branch diameter. P-values < 0.05 were considered significant ($n = 5$): * ($p < 0.05$), # ($p < 0.001$), and ** ($p < 0.01$).

Figure 4. Dextran dye uptake of ASCs cultured in each gel for 7 days. (A) Collagen, (B) Fibrin, (C) P-fibrin. F-actin, the nucleus, and dextran dye are visualized as green, blue, and red fluorescence. The scale bar is 50 μm.

Figure 5.

ASC proliferation in 3D hydrogels. MTS assay was performed to measure cell growth over time (days 1, 4, 7, and 10). Absorbance at 490 nm were measured to represent ASC metabolic activity. P-values (< 0.05 , *) were considered as significant.

Figure 6.

qRT-PCR and Western blot analysis on SMA and vWF gene and protein expressions following cultured in different gel matrices for 7 days. mRNA levels (A) of SMA and vWF in fibrin and P-fibrin gels was quantitatively compared to the collagen level. SMA (B) and vWF (C) specific bands in the blotting membrane, and the relevant quantified graphs. Beta actin protein was used to normalize protein level per identical amount of the cellular protein. Asterisks represents the significance between test groups. P-values < 0.05 were considered as significant.

Figure 7. Smooth muscle actin expression in ASCs as detected by immunofluorescence. (A–F) Fluorescent z-projections from two-photon microscopy z-stacks. Samples shown on days 1 (A, C, E) and 7 (B, D, F) in collagen, fibrin, and P-fibrin matrices. Scale bar represents 100 μm . (G) Quantification of immunofluorescence signal, normalized to sampled pixel area ($n = 20$ sampled areas) and background fluorescence. P-values < 0.05 were considered as significant.

Figure 8.

VEGF mRNA expression and protein secretion of ASCs cultured in different 3D matrices. mRNA levels (A) VEGF-A in fibrin and P-fibrin gels were analyzed relative to the collagen gels. (B) VEGF concentration in the culture media collected from ASC-gel culture system. (C) Normalized VEGF production per cell. Cell numbers were derived from MTS assay. P-values < 0.05 were considered as significant.

Figure 9. HDMEC metabolic activity measured 48 hours following treatment with conditioned media collected from different gel matrices. MTS assay was used to compare HDMEC proliferation.

Figure 10.

Modified secretion of angiogenic proteins by ASCs analyzed by human angiogenesis membrane array following culture in 3D gels. Blotted membranes (A) and quantification (B). Media samples were collected for 24 hours from day 6 to day 7.