

Concentration of Fibrin and Presence of Plasminogen Affect Proliferation, Fibrinolytic Activity, and Morphology of Human Fibroblasts and Keratinocytes in 3D Fibrin Constructs

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Fibrin is a hemostatic protein found in the clotting cascade. It is used in the operating room to stop bleeding and deliver cells and growth factors to heal wounds. However, formulations of clinically approved fibrin are optimized for hemostasis, and the extent to which biochemical and physical cues in fibrin mediate skin cell behavior is not fully understood nor utilized in the design of biomaterials. To determine if the concentration of fibrinogen and the presence of plasminogen affect cell behavior relevant to wound healing, we fabricated three-dimensional fibrin constructs made from 5, 10, or 20 mg/mL of clinical fibrin or plasminogen-depleted (PD) fibrin. We cultured dermal fibroblasts or epidermal keratinocytes in these constructs. Fibroblasts proliferated similarly in both types of fibrin, but keratinocytes proliferated more in low concentrations of clinical fibrin and less in PD fibrin. Clinical fibrin constructs with fibroblasts were less stiff and degraded faster than PD fibrin constructs with fibroblasts. Similarly, keratinocytes degraded clinical fibrin, but not PD fibrin. Fibroblast spreading varied with fibrin concentration in both types of fibrin. In conclusion, the concentration of fibrinogen and the presence of plasminogen affect fibroblast and keratinocyte proliferation, morphology, and fibrin degradation. Creating materials with heterogeneous regions of fibrin formulations and concentrations could be a novel strategy for controlling the phenotype of encapsulated fibroblasts and keratinocytes, and the subsequent biomechanical properties of the construct. However, other well-investigated aspects of wound healing remain to be utilized in the design of fibrin biomaterials, such as autocrine and paracrine signaling between fibroblasts, keratinocytes, and immune cells.

Introduction

CHRONIC WOUNDS, burns, and blistering skin diseases have prompted the development of engineered skin equivalents.^{1–3} These include sheets of autologous or allografted skin cells, synthetic polymer scaffolds, and scaffolds derived from extracellular matrix (ECM) proteins. One such ECM protein is fibrin, which forms from fibrinogen in the final step of the coagulation cascade triggered by tissue damage.⁴ Because fibrin is a biocompatible and biodegradable hemostatic protein critical for wound healing, it is currently used as a surgical adhesive and skin graft matrix in the clinic.^{5–10} In addition to reducing blood loss, fibrin sequesters growth factors and forms a scaffold for immune cells, fibroblasts, and keratinocytes.¹¹ A deeper understanding of cell–fibrin interactions could translate to novel regenerative therapeutics, and is thus an active area of tissue engineering research.¹²

Fibrin properties and subsequent cellular responses can be controlled by manipulating polymerization conditions such as fibrinogen and thrombin concentrations, solution pH, and ionic strength.^{13–16} Altering fibrin polymerization parameters also affects biochemical cues in fibrin, such as the concentration and distribution of ECM ligands, cytokines, and growth factors.¹⁷ These biochemical cues affect cell proliferation, differentiation, and expression of growth factors. In conjunction with biochemical stimuli, physical cues such as matrix stiffness, fibril geometry, and transduced force also affect cell behavior.^{18–23} Mechanical load alone causes scarring by inhibiting fibroblast apoptosis through an Akt-dependent mechanism, which results in increased collagen deposition due to higher cellularity.²⁴ We have previously shown that fibrin concentration affects the behavior of encapsulated fibroblasts,²⁵ keratinocytes,²⁶ and mesenchymal stem cells.²⁷ Recently, we also demonstrated that

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mesenchymal stem cell differentiation is affected by fibrinogen, collagen type I, and fibronectin.²⁸ Unfortunately, fibrin composition and concentration varies from study to study, invalidating the direct comparison of data.

Fibrin composition varies because fibrinogen is obtained from human plasma, which contains a diverse array of proteins such as transforming growth factor beta-1, basic fibroblast growth factor, fibronectin, coagulation cascade factors, plasminogen activator inhibitor, albumin, von Willebrand factor, and immunoglobulins.^{29,30} Laboratory-grade fibrinogen is obtained by purifying fibrinogen through immunoprecipitation, which removes specific plasma proteins such as plasminogen—a protease zymogen that becomes plasmin, cleaves fibrin polymer, and is critical for the terminal phases of wound healing after the fibrin scaffold has served its purpose.^{31,32} Purifying fibrin also changes the mechanical properties of polymerized constructs. Dickneite *et al.* found significant differences in adhesive clot strength and hemostatic efficiency of 12 commercially available fibrin sealants,³³ but cellular responses to varying fibrin formulations have not been reported. To investigate how fibrin formulation and concentration affect cell behavior relevant to wound healing, we fabricated three-dimensional (3D) fibrin constructs made from 5, 10, or 20 mg/mL of clinical fibrinogen or laboratory-grade plasminogen-depleted (PD) fibrinogen. This article reports the effects of fibrin parameters on the *in vitro* proliferation, morphology, and fibrinolytic activity of fibroblasts and keratinocytes.

Materials and Methods

Cell culture

Human foreskin fibroblasts (BJ HFF; American Type Culture Collection [ATCC] CRL-2522) were cultured in Dulbecco's modified Eagle's medium (DMEM) (CellGro) with 10% fetal bovine serum and 1% antibiotic solution in 5% CO₂ at 37°C. Media were changed twice a week, and cells were passaged to new flasks upon reaching confluence. Passage 7–12 fibroblasts were used. Human epithelial keratinocytes (HEK001; ATCC CRL-2404) were cultured in keratinocyte serum-free media (Gibco) in 5% CO₂ at 37°C. Media were changed every 3 days, and cells were passaged to new flasks upon reaching confluence. Passage 6–10 keratinocytes were used.

Fabrication of 3D fibrin constructs with encapsulated fibroblasts or keratinocytes

Three-dimensional fibrin constructs with encapsulated fibroblasts were fabricated with a protocol similar to Duong *et al.*¹⁶ Constructs were made from two fibrin formulations. The first formulation was clinical fibrin (Tisseel™; Baxter Healthcare Corp., BioScience). Tisseel contains fibrinogen (70–110 mg/mL), albumin (15 mg/mL), fibronectin (6 mg/mL), plasminogen (40–120 µg/mL), factor XIII (10 U/mL), and trace amounts of plasmin inhibitor, von Willebrand factor, growth factors, immunoglobulins, histidine, and niacinamide.²⁹ The second formulation was PD fibrin (Enzyme Research Laboratories) in a buffer of 20 mM sodium citrate-HCl, with a pH of 7.4. The main difference between the two types of fibrin was the depletion of plasminogen in the PD fibrin. Fibroblasts were seeded at 200,000 cells/mL. Lyo-

philized fibrinogen was suspended in 40 mM tris-CaCl₂. We omitted aprotinin, a serine protease inhibitor, to allow constructs to degrade. Fibroblasts were trypsinized, centrifuged, counted, diluted in media, and added to fibrinogen. Three hundred fifty microliters of fibrinogen–cell solution was pipetted into a 48-well tissue culture plate. Fifty microliters of thrombin at 40 IU/mL was added to each well and mixed to a final concentration of 5 IU/mL. Keratinocyte constructs were fabricated with an identical protocol. After polymerization, 400 µL of DMEM for fibroblasts or 400 µL of HEK media for keratinocytes was added to each well. Plates were incubated in 5% CO₂ at 37°C. Final constructs were 400 µL in volume, 9 mm tall, 3 mm in diameter, with fibrin concentrations of 5, 10, and 20 mg/mL. To image cells, thinner 3D fibrin constructs were fabricated in 24-well plates. Although the dimensions of the 24-well constructs differed from those of the 48-well constructs, cells proliferated to the same extent in the 24-well versus the 48-well constructs. Cell proliferation was assessed through the alamarBlue® assay (Invitrogen Corp.) (data not shown). Constructs were fabricated in triplicate for each time point, during which cell proliferation, morphology, and 3D fibrin construct stiffness were assessed. Time points for clinical fibrin constructs were taken on days 1, 3, and 5. Time points for PD fibrin constructs were taken on days 1, 4, and 7 due to slower degradation. Because preliminary data showed that keratinocytes do not degrade PD fibrin over 1 week, time points for keratinocytes were taken on days 1, 3, and 5. We prepared gels from separate batches of the same lot (lot number 08P1498C). One batch of fibrin was used to create each triplicate set of gels.

Fibroblast and keratinocyte proliferation in 3D fibrin constructs

At each time point, the supernatant was removed, constructs were rinsed with PBS, and incubated with 10% alamarBlue in media at 37°C for 2 h. One hundred microliters of assay media was aliquoted to a 96-well plate. Fluorescent intensity was read at 535 nm excitation and 595 nm emission with a multi-well plate reader (Infinite® F200; Tecan Group Ltd.).

Fluorescent imaging of cells in 3D fibrin constructs

Fibroblast and keratinocyte constructs in 24-well plates were stained with the Live/Dead Kit (Invitrogen). The supernatant was aspirated, and constructs were incubated for 1 h with 500 µL of DMEM with CalceinAM diluted at a 1:2000 volumetric ratio and ethidium homodimer (EthD-1) diluted 1:500 (volumetric). Labeled cells were imaged at a focal depth in the middle of the construct (Nikon). Red fluorescence was negligible, suggesting minimal cell death, so only green fluorescent images were analyzed further. Because cell spreading affects proliferation and apoptosis, we quantified fibroblast spreading morphology by calculating cell circularity with ImageJ software.^{34–36} Circularity is defined as 4π (area/perimeter²), with a value of 1.0 indicating a perfect circle. At least three images per construct were taken (representative images shown) and > 15 cells per image were analyzed.

Measuring stiffness of 3D fibrin constructs

We measured the Young's modulus at the surface of the construct to quantify fibrin degradation. The Young's modulus E is the ratio of elastically deformable stress to

strain of 3D fibrin constructs and is a measure of stiffness (see equation below). Stiff materials such as concrete, metal, and glass have high E values, while soft materials such as gelatin, fibrin, and cartilage have low E values.

$$E \approx \frac{\text{tensile stress}}{\text{tensile strain}} = \frac{\sigma}{\varepsilon} = \frac{\frac{F}{A_0}}{\frac{\Delta L}{L_0}} = \frac{FL_0}{A_0 \Delta L}$$

where E is the Young's modulus; F is the force exerted on the fibrin construct; A_0 is the original cross-sectional area through which the force is applied; ΔL is the change in length of the fibrin construct; L_0 is the original length of the fibrin construct.

Indentation testing was performed with an Instron 5564 Universal Material Testing Machine and a 1.5-mm-diameter cylindrical aluminum punch. One indentation per construct was performed and three constructs were tested per condition.

Statistical analysis

Data are presented as the mean \pm standard error, with $n=3$ (triplicate constructs per condition, repeated three times). To assess if cell proliferation or construct stiffness significantly differed from one time point to the next, data were assessed using Student's t -test, with $p < 0.05$ considered significant. To determine if cell proliferation, construct stiffness, or fibroblast spreading differed among the three fibrin concentration groups at a given time point, single-factor analysis of variance (ANOVA) without replication was performed, with $p < 0.05$ considered significant. Excel Analysis ToolPak was used for statistical analysis, and figures were generated in MATLAB.

Results

Fibroblasts proliferate similarly in clinical and PD fibrin

Fibroblasts in clinical fibrin proliferated 20-fold over 5 days (Fig. 1A). Day 3 proliferation differed among clinical fibrin concentrations (ANOVA, $p < 0.01$) with the most proliferation in 10 mg/mL clinical fibrin. Day 5 proliferation

did not differ among clinical fibrin concentrations (ANOVA, $p = 0.21$). For each concentration group of clinical fibrin, proliferation increased from each time point to the next (Student's t -test, $p < 0.05$). Fibroblasts proliferated 15- to 20-fold in PD fibrin over 7 days (Fig. 1B). For each concentration of PD fibrin constructs, proliferation increased from each time point to the next (Student's t -test, $p < 0.05$). Day 4 proliferation did not differ among PD fibrin concentrations (ANOVA, $p = 0.10$). Day 7 proliferation differed among PD fibrin concentrations (ANOVA, $p < 0.01$) with the greatest proliferation in 20 mg/mL. Fibroblast proliferation differed in varying concentrations of fibrin on day 3 for clinical fibrin and on day 7 for PD fibrin, although the overall growth of fibroblasts in both types of fibrin was similar. Depletion of plasminogen did not affect fibroblast proliferation in the tested concentration range.

Keratinocytes proliferate better in low concentrations of clinical fibrin and do not proliferate well in PD fibrin

Keratinocytes in clinical fibrin proliferated two- to four-fold over 5 days (Fig. 2A), with the highest proliferation in the lowest concentration of 5 mg/mL. Keratinocytes in PD fibrin proliferated slightly at day 3, but on day 5, proliferated only between 50% and 150% of day 1.

Clinical fibrin constructs with fibroblasts are less stiff and degrade faster than PD fibrin constructs with fibroblasts

Day 1 clinical fibrin construct stiffness varied with fibrinogen concentrations, ranging from 0.3 kPa for 5 mg/mL to 4.5 kPa for 20 mg/mL (Fig. 3A). For each concentration group of clinical fibrin, stiffness decreased from day 1 to 3 and from day 3 to 5 (Table 1). Fibroblasts completely degraded clinical fibrin constructs between days 3 and 5. Acellular control constructs made from clinical fibrin did not degrade over 5 days, suggesting that stiffness decrease was due to fibroblast-mediated fibrinolysis (data not shown).

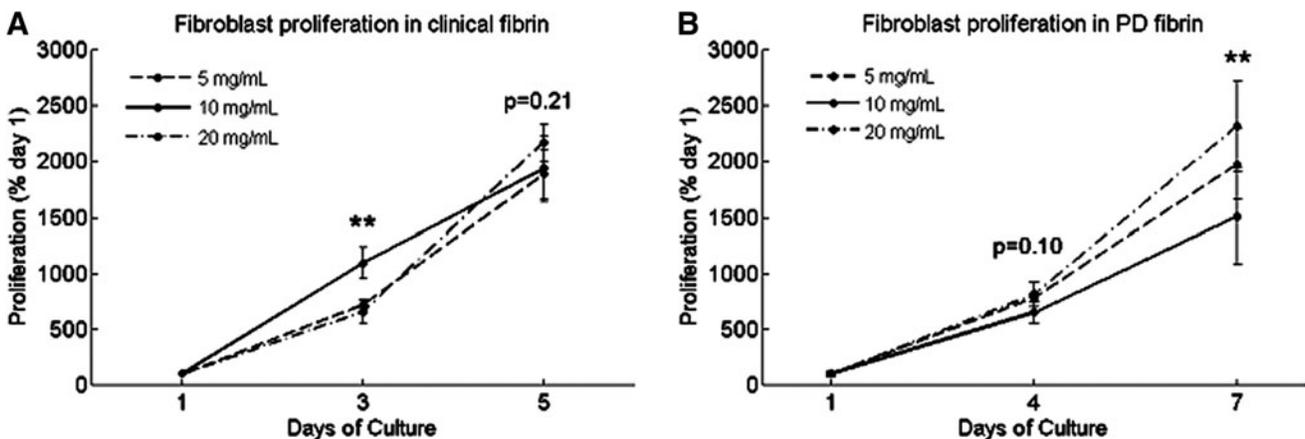


FIG. 1. Proliferation of human foreskin fibroblasts cultured in three-dimensional (3D) fibrin constructs at (A) days 1, 3, and 5 in clinical fibrin and (B) days 1, 4, and 7 in plasminogen-depleted (PD) fibrin, measured by alamarBlue[®]. Cell growth is presented as a percentage of day 1. Data are mean \pm SEM, $n=3$. At each time point after day 1, a single-factor analysis of variance (ANOVA) without replication was performed to compare fibroblast proliferation in different fibrin concentrations. “**” Indicates significance at $p < 0.05$, and “***” indicates significance at $p < 0.01$. p -Values are reported on the figure for nonsignificant results. From each time point to the next, fibroblast proliferation significantly increased in each concentration group ($p < 0.05$, * not shown).

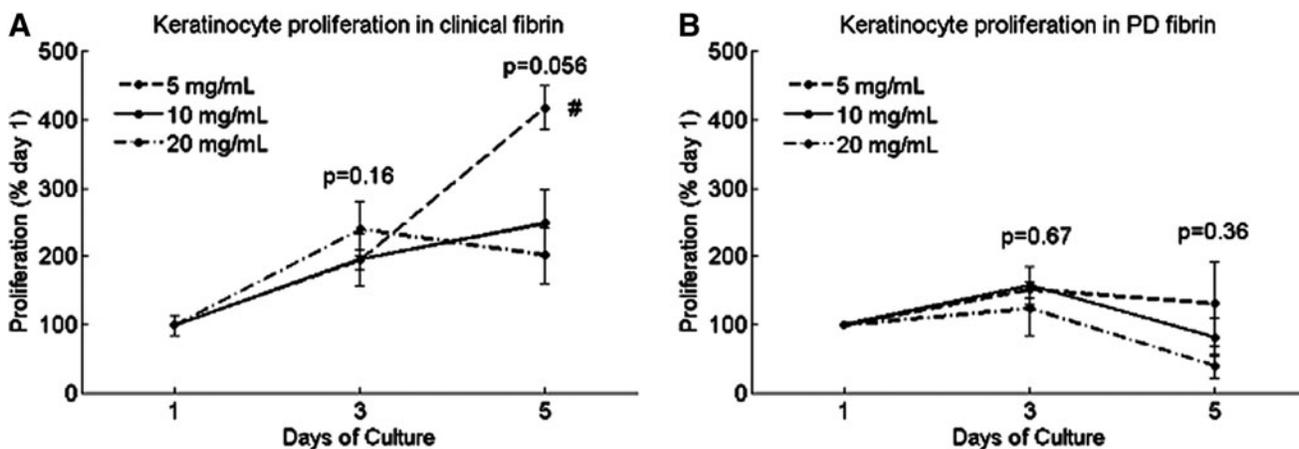


FIG. 2. Proliferation of human epidermal keratinocytes cultured in 3D fibrin constructs at (A) days 1, 3, and 5 in clinical fibrin and (B) days 1, 3, and 5 in PD fibrin, measured by alamarBlue. Cell growth is presented as a percentage of day 1. Data are mean \pm SEM, $n=3$. At each time point after day 1, a single-factor ANOVA without replication was performed to compare keratinocyte proliferation in different fibrin concentrations. “*” indicates significance at $p < 0.05$. From each time point to the next, fibroblast proliferation significantly increased in each concentration group ($p < 0.05$, * not shown). “#” indicates a significant difference on day 5 between 5 and 10 mg/mL clinical fibrin ($p = 0.046$), as well as between 5 and 20 mg/mL clinical fibrin ($p = 0.015$).

PD fibrin constructs were stiffer than those made of equivalent concentrations of clinical fibrin (Fig. 3B). Initial stiffness varied with fibrinogen concentration, ranging from 2.0 kPa for 5 mg/mL to 7.2 kPa for 20 mg/mL PD fibrin. On day 7, the stiffness of PD fibrin constructs decreased to 50–80% of initial values, but the constructs did not completely degrade. Stiffness decreased from each time point to the next for 5 mg/mL PD fibrin (Table 1). However, from day 1 to 4, the 10 and 20 mg/mL PD fibrin constructs did not degrade. Additionally, from day 3 to 7, the 10 mg/mL PD fibrin constructs did not degrade. Acellular control constructs made from PD fibrin did not degrade over 7 days, suggesting that

stiffness decrease was due to fibroblast-mediated fibrinolysis (data not shown). PD fibrin constructs lacking plasminogen and other plasma proteins were stiffer and more resistant to fibroblast-mediated degradation than clinical fibrin constructs.

Keratinocytes degrade clinical fibrin constructs, but do not degrade PD fibrin constructs

On day 1, the stiffness of clinical fibrin constructs with keratinocytes varied with fibrinogen concentrations, ranging from 0.3 kPa for 5 mg/mL to 3.8 kPa for 20 mg/mL (Fig. 4A). These values were similar to stiffness values of

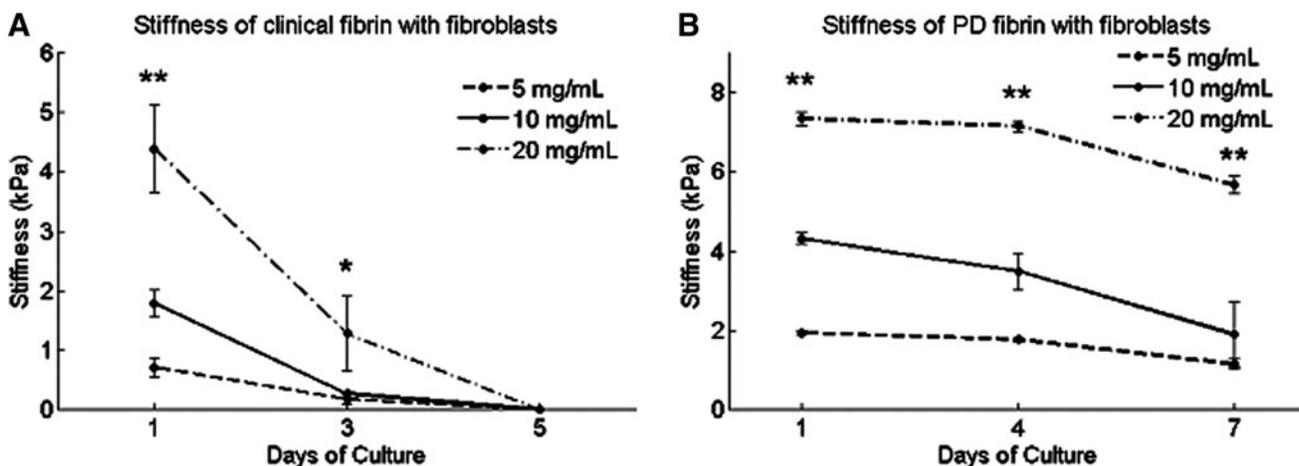


FIG. 3. Stiffness of fibroblast-encapsulating 3D fibrin constructs fabricated from clinical or PD fibrin. Stiffness was measured by means of mechanical indentation testing. (A) Clinical fibrin construct stiffness varies with fibrinogen concentration, ranging from 0.7 kPa for 5 mg/mL to 4.4 kPa for 20 mg/mL clinical fibrin. Clinical fibrin constructs with fibroblasts degrade over 5 days of culture. (B) PD fibrin construct stiffness also varies with fibrinogen concentration, ranging from 2.0 kPa for 5 mg/mL to 7.2 kPa for 20 mg/mL PD fibrin. Fibroblasts degrade PD fibrin constructs less than clinical fibrin constructs. Data are mean \pm SEM, $n=3$. At each time point after day 1, a single-factor ANOVA without replication was performed to compare if construct stiffness differed among different concentrations. “**” indicates significance at $p < 0.05$, and “***” indicates significance at $p < 0.01$.

TABLE 1. *P*-VALUES FROM STUDENT'S *T*-TEST COMPARING STIFFNESS OF CLINICAL AND PLASMINOGEN-DEPLETED FIBRIN CONSTRUCTS WITH FIBROBLASTS

	5 mg/mL	10 mg/mL	20 mg/mL
Clinical fibrin			
Day 1 vs. 3	<0.01	<0.01	<0.01
Day 3 vs. 5	<0.01	0.02	0.03
PD fibrin			
Day 1 vs. 4	<0.01	0.07	0.32
Day 3 vs. 7	<0.01	0.06	<0.01

PD, plasminogen depleted.

the same constructs with fibroblasts. For each concentration group of clinical fibrin, stiffness decreased from day 1 to 3 and from day 3 to 5, suggesting keratinocyte-mediated degradation (Table 2). One exception was 20 mg/mL clinical fibrin, which interestingly increased in stiffness to 6 kPa on day 3, but fell to 2.5 kPa on day 5, enduring 5 days of culture. Lower concentration clinical fibrin constructs (5 and 10 mg/mL) completely degraded between days 3 and 5. Acellular control constructs made from clinical fibrin did not degrade over 5 days, suggesting that the decrease in stiffness was due to keratinocyte-mediated fibrinolysis and not due to hydrolysis or proteolytic factors in culture media (data not shown). Each line in both Figure 4A and B represents one fibrinogen concentration.

PD fibrin constructs were stiffer than those made of equivalent concentrations of clinical fibrin (Fig. 4B). At each time point, the stiffness of PD fibrin constructs with keratinocytes varied with fibrin concentrations (ANOVA, $p < 0.01$). These values were similar to stiffness values reported for the same constructs with fibroblasts. PD fibrin constructs with keratinocytes did not degrade over 7 days of culture. Keratinocyte-mediated fibrinolysis did not require plasminogen or other plasma proteins absent in PD fibrin.

Fibroblasts spread in both clinical and PD fibrin, although cell spreading is decreased in high concentrations of PD fibrin

In two-dimensional (2D) and 3D culture, fibroblasts adhere to the substrate and spread out. We performed fluorescent microscopy of CalceinAM-stained fibroblasts cultured in clinical fibrin and observed spread fibroblast morphology by day 3 in lower fibrinogen concentrations of 5 and 10 mg/mL (Fig. 5A). Qualitatively, fibroblasts appeared less elongated and more circular in 20 mg/mL. By day 5, clinical fibrin constructs were mostly degraded and fibroblasts spread in a confluent monolayer over the entire area of the well. Fibroblasts in the remaining clinical fibrin networked together while spreading. Imaging was also performed on fibroblasts cultured in PD fibrin constructs, in which fibroblasts elongated and spread by day 4 in 5 and 10 mg/mL, but maintained ball-like morphology with low spreading in 20 mg/mL (Fig. 5B). This trend continued to day 7, where fibroblasts in 20 mg/mL PD fibrin constructs spread less than in 5 and 10 mg/mL.

We quantified fibroblast cell spreading by calculating 1 - circularity, where a higher value indicated more spreading. At each time point after day 1, fibroblast spreading in clinical fibrin constructs differed among concentration groups (Table 3). On day 5, the spreading ranged from 0.5 to 0.7, with fibroblasts in the lowest concentration of clinical fibrin spreading the most. A more noticeable difference was observed in PD fibrin constructs. Similar to the fibroblasts in clinical fibrin, spreading in PD fibrin differed among concentration groups (Table 3). However, on day 7, fibroblasts cultured in the lower PD fibrin concentrations of 5 and 10 mg/mL both had a spread of 0.9, while fibroblasts cultured in the highest PD fibrin concentration of 20 mg/mL had a lower spread of 0.25. Plasminogen and other plasma proteins absent in PD fibrin, and/or the stiffer mechanical environment may have opposed cell spreading in lower concentrations of fibrin.

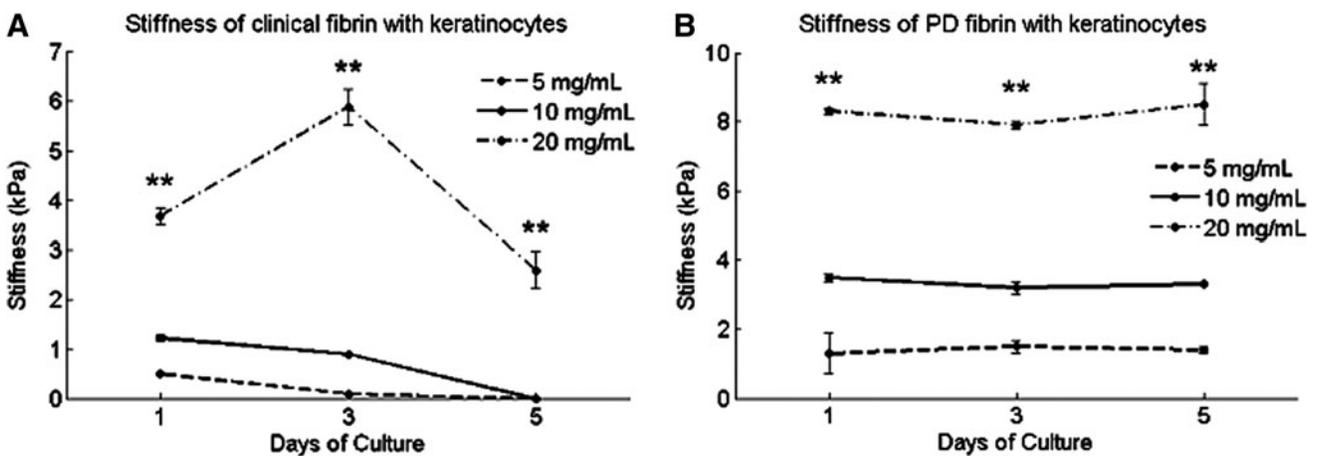


FIG. 4. Stiffness of keratinocyte-encapsulating fibrin constructs fabricated from clinical or PD fibrin. Constructs contained 200,000 keratinocytes/mL. Stiffness was measured by means of mechanical indentation testing. (A) Keratinocytes degrade clinical fibrin constructs over 5 days. (B) Keratinocytes do not degrade PD fibrin. Data are mean \pm SEM, $n = 3$. At each time point after day 1, a single-factor ANOVA without replication was performed to compare if construct stiffness differed among different concentrations. “***” indicates significance at $p < 0.01$. Each line represents one fibrinogen concentration.

TABLE 2. *P*-VALUES FROM STUDENT'S *T*-TEST COMPARING STIFFNESS OF CLINICAL AND PLASMINOGEN-DEPLETED FIBRIN CONSTRUCTS WITH KERATINOCYTES

	5 mg/mL	10 mg/mL	20 mg/mL
Clinical fibrin			
Day 1 vs. 3	<0.01	0.012	<0.01
Day 3 vs. 5	<0.01	<0.01	<0.01
PD fibrin			
Day 1 vs. 3	0.67	0.21	0.12
Day 3 vs. 5	0.46	0.50	0.45

Keratinocyte morphology is qualitatively identical in clinical and PD fibrin

Keratinocytes assume a cobblestone morphology *in vitro* and do not extend and spread like fibroblasts. We performed fluorescent microscopy of CalceinAM-stained keratinocytes cultured in clinical fibrin and observed normal cobblestone morphology (Fig. 6A). On day 5, keratinocytes clustered together more in 10 and 20 mg/mL clinical fibrin than in 5 mg/mL, or in any PD fibrin construct. Imaging was also performed on keratinocytes cultured in PD fibrin constructs, in which identical morphologies were qualitatively observed, although with less keratinocyte clustering on day 5 (Fig. 6B).

Discussion

In this study, we investigated if (1) the presence of plasminogen and other plasma proteins and (2) fibrinogen concentrations affect the behavior of skin cells. We fabricated cellularized 3D fibrin constructs made from 5, 10, or 20 mg/mL of clinical or PD fibrin, and examined the proliferation and morphology of fibroblasts and keratinocytes. We also assessed cell-mediated fibrinolysis by measuring construct stiffness.

Fibroblasts proliferated at least 15-fold regardless of fibrin composition and concentration (Fig. 1). This may be

due to competing biochemical and physical cues that vary with fibrinogen concentration—when one increases, the other decreases. The ECM structure mediates cell behavior by presenting ligands to bind to integrin receptors on the cell surface.³⁷ Fibrinogen and fibronectin, two such ligands, bind fibroblasts through the $\alpha\text{v}\beta\text{3}$ integrin, which stimulates cell adhesion and proliferation.³⁸ A higher concentration of fibrinogen provides a higher ligand density for integrin binding, which regulates actin filament organization, cell adhesion, and motility.³⁹ The concentration of fibrinogen also determines the fibril geometry and size, factors also mediating cell behavior. Fibroblasts cultured on collagen grooves demonstrate different gene expression patterns than when cultured on flat surfaces,⁴⁰ potentially due to physical interactions between the microenvironment, cytoskeleton, and nuclear matrix.³⁶ However, too high concentrations of fibrin prevent fibroblast spreading and mechanically inhibit proliferation in 3D culture.^{26,41} On the other hand, fibrin at a low concentration features a porous microstructure that encourages fibroblast spreading and proliferation, but presents fewer ECM ligands and growth factors. Although PD fibrin is stiffer than clinical fibrin at identical concentrations (Figs. 3 and 4), fibroblasts may receive more biochemical input from clinical fibrin and more biophysical input from PD fibrin, resulting in similar proliferation profiles.

Fibrin, fibronectin, and plasminogen represent some components of the natural wound milieu, which support keratinocyte growth. Keratinocytes actually do not express integrin $\alpha\text{v}\beta\text{3}$ —the receptor for fibrin and fibrinogen. Fibrin indirectly mediates keratinocyte behavior by presenting fibronectin and releasing plasminogen.^{42,43} In response to wounding, keratinocytes upregulate integrin subunit α5 , the receptor for fibronectin.^{44,45} This subunit promotes keratinocyte migration and survival in a Bcl-2 pathway-dependent fashion.^{46–48} Keratinocytes proliferating in clinical fibrin therefore adhered to fibronectin present in the construct. However, keratinocytes did not proliferate in PD fibrin (Fig. 2). Plasminogen activates matrix metalloproteinases crucial

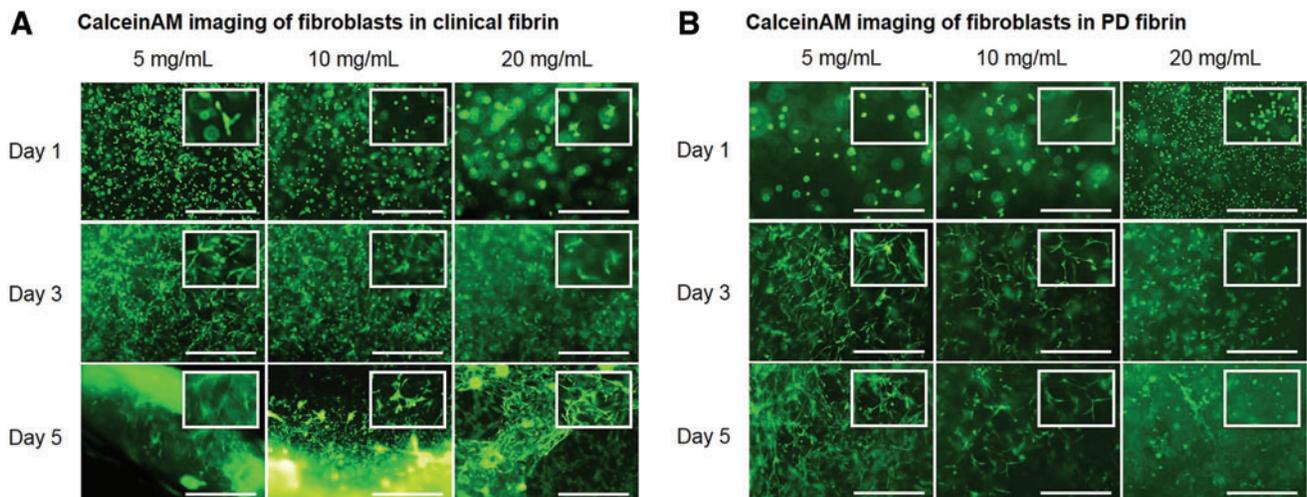


FIG. 5. Fluorescent imaging and spreading quantification of CalceinAM-stained fibroblasts (scale bar = 250 μm). (A). Fibroblasts in clinical fibrin constructs spread by day 3 in all concentrations and degraded most of the constructs by day 5. (B) Fibroblasts in PD fibrin constructs spread more in 5 and 10 mg/mL and less in 20 mg/mL. Insets feature a magnified region of the image to highlight cellular morphology. Color images available online at www.liebertpub.com/tea

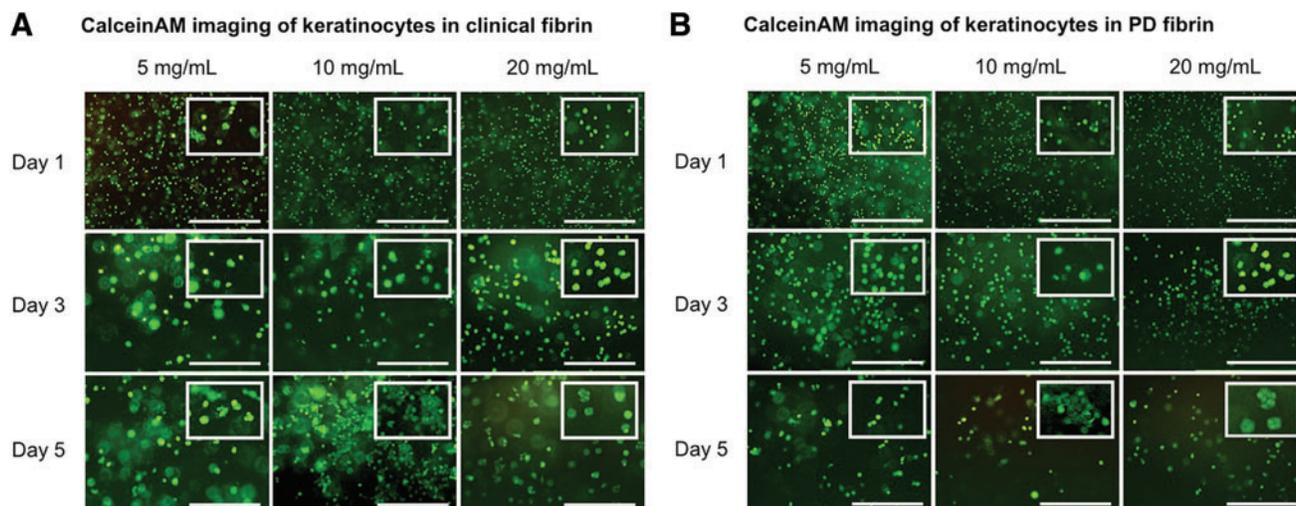


FIG. 6. Fluorescent imaging of CalceinAM-stained keratinocytes in (A) clinical fibrin and (B) PD fibrin (scale bar = 250 μ m). On day 5, keratinocytes clustered together more in 10 and 20 mg/mL clinical fibrin than in 5 mg/mL clinical fibrin, or in any PD fibrin construct. Insets feature a magnified region of the image to highlight cellular morphology. Color images available online at www.liebertpub.com/tea

for epithelial cell migration,⁴⁹ converts latent transforming growth factor beta and hepatocyte growth factor into active forms,^{50,51} and induces keratinocyte migration, while reducing proliferation.⁵² Our data suggest that plasminogen may be a vital component of a fibrin construct containing keratinocytes.

Whereas fibroblast proliferation was nearly identical in clinical fibrin compared with PD fibrin, the stiffness and degradation of fibrin constructs varied with composition and concentration (Table 4). Fibroblasts rapidly degraded clinical fibrin within 3–5 days, with higher concentrations of fibrin corresponding to stiffer constructs, which degraded more slowly than constructs fabricated from lower concentrations (Fig. 3A). In agreement with our results, several studies have shown that a lower fibrinogen concentration results in thicker fibers, which Gabriel *et al.* demonstrated to be more susceptible to plasmin digestion than thin fibers found in higher fibrin concentrations.⁵³

PD fibrin constructs with fibroblasts were twice as stiff and degraded slower than clinical fibrin constructs (Fig. 3B), likely due to the lack of plasminogen. Compared with clinical fibrin, PD fibrin may contain different amounts of structurally important proteins such as collagen, factor XIIIa, fibronectin, and albumin. Even without

plasminogen, fibroblasts degraded PD fibrin over 1 week (Fig. 3B), suggesting activity of other proteases, such as matrix metalloproteinases.⁵⁴ We omitted aprotinin to allow fibroblasts to degrade fibrin and observed 15- to 20-fold proliferation in both types of fibrin. Fibroblasts have been shown to proliferate significantly less (only three-fold) in fibrin constructs fabricated with aprotinin.²⁵ Aprotinin prevents the degradation of the ECM, which may prevent subsequent liberation of degradation products and growth factors, thus reducing the survival, proliferation, and differentiation of both encapsulated and endogenous cells. The initial composition of fibrin may play a large role in mediating the dynamic reciprocity of the wound microenvironment.⁵⁵

TABLE 3. *P*-VALUES FROM STUDENT'S *T*-TEST COMPARING FIBROBLAST SPREADING IN CLINICAL AND PLASMINOGEN-DEPLETED FIBRIN CONSTRUCTS

	5 mg/mL	10 mg/mL	20 mg/mL
Clinical fibrin			
Day 1 vs. 3	<0.01	<0.01	0.46
Day 3 vs. 5	<0.01	0.65	<0.01
PD fibrin			
Day 1 vs. 4	<0.01	<0.01	<0.01
Day 3 vs. 7	<0.01	<0.01	<0.01

TABLE 4. SUMMARY OF FIBROBLAST AND KERATINOCYTE PROLIFERATION, AND FIBRIN CONSTRUCT STIFFNESS

	Final cell proliferation (% of day 1)		
	5 mg/mL	10 mg/mL	20 mg/mL
Fibroblasts			
Clinical fibrin	1890 \pm 220	1940 \pm 300	2170 \pm 160
PD fibrin	1980 \pm 310	1510 \pm 430	2320 \pm 410
Keratinocytes			
Clinical fibrin	420 \pm 30	250 \pm 50	200 \pm 40
PD fibrin	130 \pm 60	82 \pm 30	40 \pm 20
	Final construct stiffness (kPa)		
	5 mg/mL	10 mg/mL	20 mg/mL
Fibroblasts			
Clinical fibrin	0	0	0
PD fibrin	1.2 \pm 0.1	1.9 \pm 0.8	5.7 \pm 0.2
Keratinocytes			
Clinical fibrin	0	0	2.6 \pm 0.4
PD fibrin	1.4 \pm 0.1	3.3 \pm 0.1	8.5 \pm 0.6

Mean values \pm standard error.

Keratinocytes slightly degraded clinical fibrin over 5 days (Fig. 4A). The stiffness of 20 mg/mL clinical fibrin constructs with keratinocytes rose at day 3 but fell at day 5. Batch variation of clinical fibrin may explain the increased day 3 stiffness. However, the overall trend of keratinocyte-mediated fibrin degradation in clinical fibrin was similar to that from fibroblast constructs. As expected, higher concentrations of fibrin resulted in stiffer constructs, both for clinical and PD fibrin. Unlike fibroblasts, keratinocytes did not degrade PD fibrin (Fig. 4B). Keratinocytes may express plasminogen activators ineffective without plasminogen or may not express sufficient amounts of nonplasmin proteases to degrade PD fibrin.

Fibroblasts exhibited spreading morphology in both clinical fibrin (Fig. 5A) and PD fibrin (Fig. 5B), except in the highest concentration of PD fibrin. Surprisingly, the proliferation in 20 mg/mL PD fibrin constructs was similar to the proliferation in lower concentration constructs. Several studies have shown that substrate stiffness plays a critical role on cell morphology, survival, and differentiation. Yeung *et al.* found that the stiffness of a 2D substrate must be at least 2–3 kPa for fibroblast spreading, a range similar to that of our data.⁵⁶ At each time point, fibroblast spreading differed significantly depending on the fibrin concentration (Fig. 5C, D). Interestingly, fibroblasts in 10 mg/mL clinical fibrin appear to spread the least, although significant differences were observed between all three concentration groups. Fibroblasts in 5 and 10 mg/mL PD fibrin spread similarly, while fibroblasts in 20 mg/mL PD fibrin did not, suggesting a threshold of mechanical resistance beyond which fibroblasts cannot spread. Our data agree with previous work showing that mechanical stiffness regulates cell spreading. Plasminogen may also mediate cell morphology and spreading in 3D culture, although we are yet to discern the relative contributions of mechanical stiffness and biochemical signaling toward cell spreading. If ECM ligands such as fibronectin and fibrin enable cell spreading and mechanical resistance opposes cell spreading, fibroblast spreading should be greater in clinical fibrin than in PD fibrin. However, clinical fibrin constructs may have degraded before fibroblasts could maximally spread, while stiffer and more degradation-resistant PD fibrin constructs could allow more time for fibroblasts to spread.

We also performed fluorescent microscopy on CalceinAM-stained keratinocytes in clinical and PD fibrin. Keratinocytes demonstrated normal cobblestone morphology (Fig. 6A) and were distributed homogeneously throughout the construct. On day 5, keratinocytes clustered more in high-stiffness clinical fibrin. Keratinocytes are known to form focal adhesions to the ECM in a high-stiffness environment.^{57,58} However, keratinocytes did not cluster in PD fibrin constructs that were stiffer than clinical fibrin constructs of the same fibrinogen concentration (Fig. 6B). These data, consistent with other studies previously discussed, demonstrate that plasminogen and other plasma proteins, which may be absent in PD fibrin, mediate keratinocyte adhesion.

In summary, our data show that fibrin stiffness and the presence of plasminogen affect the behavior of fibroblasts and keratinocytes. These results could be translated into a novel strategy of delivering cells to a dermal wound site using a heterogeneous fibrin construct, containing subregions of biomaterial optimized for controlling specific cell phenotypes. However, the interplay between various cells

and the matrix is complex, and different wound pathologies present unique spatiotemporal profiles of inflammatory molecules, ECM ligands, and proteases.

Fibroblasts and keratinocytes signal to each other through autocrine and paracrine mechanisms to regulate tissue homeostasis, as well as wound healing. Further complicating matters, cells bind to the ECM that changes cell behavior, which in turn leads to changes in the ECM—a phenomenon coined “dynamic reciprocity.”⁵⁵ To better understand the complexity of the wound-healing milieu, we are currently investigating constructs with multiple cell types and ECM proteins. Adding more components to our *in vitro* models will improve our understanding of (1) the relative contribution of fibrinogen, fibronectin, plasminogen, and type I collagen toward the mechanical and biological properties of the microenvironment and (2) how communication between dermal, epidermal, immune, and mesenchymal stem cells regulates cell proliferation, migration, differentiation, and fibrinolysis.^{59–64} Once we identify *in vitro* fibrin construct formulations and cell profiles that optimally form skin-like phenotypes, we will test the ability of these constructs to heal wounds in animal models. Studying simplified *in vitro* models of wound healing using individual cell populations, specific growth factors, or biomechanical forces is important, but findings must be scaled to more complex *in vivo* models that translate into novel therapeutic strategies.

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Disclosure Statement

No competing financial interests exist.

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