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CHAPTER 3

FIBRIN NETWORK ADAPTATION TO CELL-GENERATED CONTRACTILE STRESS

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ABSTRACT
Fibrin is the main structural protein in blood clots, which stops bleeding, and promotes wound healing by serving as a provisional extracellular matrix for cells such as fibroblasts. Fibroblasts can sense and respond to their surrounding substrate in a mechanical-feedback loop. For tissue engineering it is essential to elucidate this mechanical-feedback loop at the micron scale. Therefore we aimed to study the mechanical adaptation of fibrin networks due to cell-generated contractile stress at the micron scale. Viscoelastic properties of fibrin networks and fibroblast-seeded fibrin networks were measured by one-particle passive microrheology 1 h after polymerization. Fibrin networks and fibroblasts-seeded fibrin networks were prepared with 1, 2, or 3 mg/ml fibrinogen polymerized with 0.5 IU/ml thrombin, and 1.0 μm polystyrene beads were embedded. Fibroblasts were round in all fibrin networks within 2 h after cell seeding, while after 24 h the fibroblasts were elongated-shaped in fibrin networks with 1 and 2 mg/ml fibrinogen, and cobblestone-shaped in fibrin networks with 3 mg/ml fibrinogen. At low frequencies $f < 10^2$ Hz, $G'$ of fibroblast-seeded fibrin networks with fibrinogen concentrations $\geq 2$ mg/ml increased by 3-fold compared to fibrin networks. At high frequencies $f > 10^3$ Hz, $G''$ of fibrin networks decreased as a power-law in frequency with exponents ranging from 0.76±0.05 to 0.41±0.01 Pa.s, while $G''$ of fibroblasts-seeded fibrin networks increased as a power-law in frequency with exponents ranging from 0.57±0.02 to 0.68±0.02 Pa.s. In conclusion, the presence of fibroblasts in fibrin networks contributed actively to a change in the viscoelastic properties of fibrin at the micron scale, suggesting that fibrin networks adapt to cell-generated contractile stresses. These data have wide ranging implications for understanding processes for improving wound healing, treating pathological tissue development, and tissue engineering applications.

Key words: One-particle microrheology, Microenvironment, Fibroblasts, Fibrin, Mechanosensing
INTRODUCTION
Fibrin is the main structural protein in blood clots, which stops bleeding, and promotes wound healing by serving as a provisional extracellular matrix for cells such as fibroblasts. Upon vascular injury, the enzyme thrombin converts the plasma protein fibrinogen in fibrin that can withstand forces exerted by flowing blood and by embedded cells. Fibrin displays non-linear mechanical properties such as strain-stiffening, whereby the material is compliant at normal strain levels and stiffens strongly at larger deformations, and thereby becoming increasingly resistant to further deformation. Strain stiffening protects tissues from tearing under large stresses and can also play a role in tissue development, homeostasis and repair.

A variety of tissue-forming cells such as fibroblasts can sense and respond to mechanical properties of their surrounding substrate by locally pulling on the matrix, and at the same time actively change the stiffness and tension of the surrounding substrate by applying localized forces, so-called traction forces, in a mechanical-feedback loop. Fibroblasts affected the macroscopic mechanical properties of fibrin by actively stiffening of fibrin networks by applying traction forces. Macroscopic measurements are often insensitive to local micron scale heterogeneities, but how the contractile activity of cells affects the mechanical properties of their microenvironment and vice versa is important for tissue engineering.

Since it is essential to elucidate this mechanical-feedback loop at the micron scale, local matrix stiffness and cell-generated contractile stress are important parameters. Therefore, we aimed to study the mechanical adaptation of fibrin networks due to cell-generated contractile stress at the micron scale. We hypothesized that fibrin networks adapt to cell-generated contractile stresses. We expected that cell-generated contractile stresses will increase the micromechanical properties of fibrin network. Fibrin networks and fibroblasts-seeded fibrin networks were prepared with 1, 2, or 3 mg/ml fibrinogen polymerized with 0.5 IU/ml thrombin, and 1.0 μm polystyrene beads were embedded. Viscoelastic properties of fibrin networks and fibroblast-seeded fibrin networks were assessed by one-particle passive microrheology 1 h after polymerization.

MATERIALS AND METHODS
Cell culture
Human CCL-224 fibroblasts were cultured in T-75 culture flasks (Nunc, Roskilde, Denmark) in Dulbecco’s Modified Eagle Medium (D-MEM; Gibco, Paisley, UK) with 20% fetal bovine serum (FBS; Gibco, Paisley, UK), and 1% penicillin streptomycin (Gibco, Paisley, UK), and incubated at 37°C in a humidified atmosphere with 5% CO₂. After 24 h, cells were cultured in D-MEM with 10% FBS, and 1% penicillin streptomycin. The medium was refreshed every 3-4 days. After reaching confluency, cells were harvested by incubation with 0.25% trypsin-
ethylenediaminetetraacetic acid (Gibco, Paisley, UK), replated, cultured until passage 11, and used for preparation of fibroblasts-seeded fibrin networks (see below).

**Fibrin networks and fibroblasts-seeded fibrin networks**

For fibrin networks preparation, human fibrinogen (17.86 mg/ml; Enzyme Research, South Bend, IN, USA) in a buffer of 20 mM sodium citrate–HCl, pH 7.4, was thawed. Then fibrinogen solutions were mixed in fibrin assembly buffer containing 20 mM Heps (Sigma-Aldrich, St. Louis, MO, USA), 150 mM NaCl, and 5 mM CaCl$_2$ (pH 7.4), for 10 min at 37°C in a dry bath (Benchmark Scientific, Inc., Edison, NJ, USA). Fibrin networks were prepared with 1, 2, or 3 mg/ml fibrinogen with 1.0 µm diameter polystyrene beads (Phosphorex, Hopkinton, MA, USA) embedded, and polymerized with 0.5 IU/ml human α-thrombin (Enzyme Research, South Bend, IN, USA) in a custom built chamber at room temperature for 1 hour.

Fibroblasts-seeded fibrin networks for experiments were prepared by dissolving 1, 2, or 3 mg/ml human fibrinogen in fibrin assembly buffer (see above). Fibroblasts were resuspended in fibrinogen solutions with 1.0 µm diameter polystyrene beads and seeded at 600–800 cells/µl fibrinogen. Fibroblasts-seeded fibrinogen was polymerized with 0.5 IU/ml human α-thrombin in customized petri dishes (Greiner Bio-One GmbH, Frickenhausen, Germany). Fibroblasts-seeded fibrin networks were cultured in D-MEM/CO$_2$-independent medium (Gibco) with 10% FBS, and 1% penicillin streptomycin, and incubated at 37°C in a humidified atmosphere with 5% CO$_2$ for 1 hour.

For visualizing the dynamics of cell spreading in fibrin networks, fibroblasts-seeded fibrin networks were imaged using a TCM400 Inverted Bright Field microscope (LaboMed America, Inc., Fremont, CA, USA) immediately after seeding, and at 1, 2, and 24 h after cell seeding.

**Passive micro rheology**

One-particle passive micro rheology was performed with optical tweezers 1 h after polymerization. Polystyrene beads in the fibrin networks and fibroblasts-seeded fibrin networks were weakly trapped using a laser with wavelength of 1064 nm. The motions of the beads were tracked by back-focal-plane interferometry. Fluctuations of the beads were detected by a quadrant photodiode at a sampling rate of 195 kHz and converted to shear moduli using linear response theory.

**RESULTS**

**Fibroblasts spread in fibrin networks**

For visualizing the dynamics of cell spreading in fibrin networks, fibroblasts-seeded fibrin networks were imaged with bright field microscopy immediately after seeding, and at 1, 2,
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and 24 h after cell seeding. Fibroblasts were rounded in all fibrin networks within 2 h after cell seeding, and spread in all fibrin networks after 24 h after cell seeding (Fig. 1). Twenty-four hours after cell seeding, cells were elongated in fibrin networks with 1 and 2 mg/ml fibrinogen and had a cobblestone-shape in fibrin networks with 3 mg/ml fibrinogen (Fig. 1).

Figure 1. Morphology of fibroblasts in fibrin networks with 1, 2, or 3 mg/ml fibrinogen polymerized with 0.5 IU/ml thrombin immediately after seeding, 1, 2, and 24 h after cell seeding. Cells were visualized by bright field microscopy. Magnification: 20x.

Mechanics of fibrin networks and fibroblasts-seeded fibrin networks
The elastic modulus, $G'$, and viscous modulus, $G''$ of fibrin networks and fibroblast-seeded fibrin networks with different concentrations of fibrinogen were measured 1 h after polymerization by one-particle passive microrheology. In fibrin networks, the elastic modulus
G’ (Fig. 2A), and the viscous modulus G” (Fig. 2B) increased by increasing fibrinogen concentration from 1 mg/ml to 3 mg/ml. In fibroblasts-seeded fibrin networks, the elastic modulus G’, and the viscous modulus G” increased by increasing fibrinogen concentration from 1 mg/ml to 2 mg/ml, while G’ and G” of fibroblasts-seeded fibrin networks with 2 mg/ml were similar to fibroblasts-seeded fibrin networks with 3 mg/ml (Fig. 4). The elastic modulus of fibrin networks and fibroblast-seeded fibrin networks was higher in magnitude than the viscous modulus at lower frequencies f < 10^2 Hz (Fig. 2,4).

Figure 2. Viscoelastic properties of fibrin networks with 1, 2, or 3 mg/ml fibrinogen polymerized with 0.5 IU/ml thrombin measured by one-particle passive microrheology 1 h after polymerization. (A) Elastic moduli G’ of fibrin networks. (B) Viscous moduli G” of fibrin networks.

Fibrin networks and fibroblasts-seeded fibrin networks showed frequency-independent (Fig. 3A), and fibrinogen concentration-dependent (Fig. 3C) elastic moduli at low frequencies f < 10^2 Hz. At low frequencies f < 10^2 Hz, the elastic plateau G_0 increased by increasing fibrinogen concentration c following the power-law dependence G_0 \approx c^{1.8 \pm 0.5} (Fig. 3C) for fibrin networks, and G_0 \approx c^{3.0 \pm 1.0} for fibroblasts-seeded fibrin networks. The elastic modulus G’ of fibroblasts-seeded fibrin networks with fibrinogen concentrations c \geq 2 mg/ml increased by 3.0-fold compared to fibrin networks (Fig. 3A,B). The elastic modulus of fibrin networks and fibroblasts-seeded fibrin networks was lower in magnitude than the viscous modulus at high frequencies f > 10^3 Hz (Fig. 4). Fibrin networks and fibroblasts-seeded fibrin networks showed frequency-dependent (Fig. 3B), and fibrinogen-dependent (Fig. 3D) viscous moduli at high frequencies f > 10^3 Hz. At high frequencies f > 10^3 Hz, G” of fibrin networks decreased with increasing concentration following the power-law dependence in frequency with G” \approx \omega^{0.76 \pm 0.05} for 1 mg/ml fibrinogen, G” \approx \omega^{0.66 \pm 0.04} for 2 mg/ml fibrinogen, and G” \approx \omega^{0.41 \pm 0.01} for 3 mg/ml fibrinogen (Fig. 3D). At frequencies f > 10^3 Hz, G’ of fibroblasts-seeded fibrin networks increased with increasing concentration following the power-law in frequency with
G'' ≈ ω^{0.57±0.02} for 1 mg/ml fibrinogen, G'' ≈ ω^{0.66±0.02} for 2 mg/ml fibrinogen, and G'' ≈ ω^{0.68±0.02} for 3 mg/ml fibrinogen (Fig. 3D).

**Figure 3.** Viscoelastic properties of fibrin networks and fibroblasts-seeded fibrin networks with 1, 2, and 3 mg/ml fibrinogen polymerized with 0.5 IU/ml thrombin measured by one-particle passive microrheology 1 h after polymerization. (A) Elastic moduli G' of fibrin networks and fibroblasts-seeded fibrin networks at low frequencies f < 10^2 Hz. (B) Viscous moduli G'' of fibrin networks and fibroblasts-seeded fibrin networks at high frequencies f > 10^3 Hz. (C) Elastic plateau G_0 of fibrin networks and fibroblasts-seeded fibrin networks at low frequencies f < 10^2 Hz. (D) Slope of viscous moduli G'' of fibrin networks and fibroblasts-seeded fibrin networks at high frequencies f > 10^3 Hz.
**Figure 4.** Viscoelastic properties of fibrin networks and fibroblasts-seeded fibrin networks with 1, 2, and 3 mg/ml fibrinogen polymerized with 0.5 IU/ml thrombin measured by one-particle passive microrheology 1 h after polymerization. (A,B) Fibrin networks and fibroblasts-seeded fibrin networks with 1 mg/ml fibrinogen. (C,D) Fibrin networks and fibroblasts-seeded fibrin networks with 2 mg/ml fibrinogen. (E,F) Fibrin networks and fibroblasts-seeded fibrin networks with 3 mg/ml fibrinogen. (A, C, E) Elastic moduli $G'$ of fibrin networks and fibroblasts-seeded fibrin networks. (B, D, F) Viscous moduli $G''$ of fibrin networks and fibroblasts-seeded fibrin networks.

**DISCUSSION**

We studied the mechanical adaptation of fibrin networks due to cell-generated contractile stress at the micron scale. We found that (i) Fibroblasts were round in fibrin networks with 1, 2, and 3 mg/ml fibrinogen within 2 h after cell seeding, while 24 h after cell seeding,
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Fibroblasts were elongated-shaped in fibrin networks with 1 and 2 mg/ml fibrinogen, and cobblestone-shaped in fibrin networks with 3 mg/ml fibrinogen; (ii) At low frequencies $f < 10^2$ Hz, the elastic modulus $G'$ of fibroblasts-seeded fibrin networks increased by 3-fold compared to fibrin networks; (iii) At high frequencies $f > 10^3$ Hz, the viscous modulus $G''$ of fibrin networks decreased as a power-law in frequency with exponents ranging from 0.76±0.05 to 0.41±0.01 Pa.s, while $G''$ of fibroblasts-seeded fibrin networks increased as a power-law in frequency with exponents ranging from 0.57±0.02 to 0.68±0.02 Pa.s. Therefore, our results showed that fibroblasts generated contractile stress causing microscopic matrix stiffening, suggesting that fibrin networks adapt to cell-generated contractile stresses.

Fibroblasts were initially round in morphology, and began to extend and adhere to the fibrin fibers 2 h after cell seeding. Cells embedded in fibrin networks attach and exert traction forces on the fibers of the network. These forces can lead to local reorganization and realignment of the network microstructure. Reorganization is the first stage of the matrix remodeling process and takes place over a few hours to a few days. The differences found in cellular shape between the dilute fibrin networks with 1 or 2 mg/ml fibrinogen and the denser fibrin network with 3 mg/ml fibrinogen one day after cell seeding, indicates differences in reorganization. Fibroblasts change cellular shape most pronouncedly in dilute fibrin networks with fibrinogen concentrations $c \leq 2$ mg/ml compared to denser fibrin networks with fibrinogen concentrations $c$ between 3 and 6 mg/ml. Cells in dilute fibrin networks are elongated and extend several thin protrusions into the network, while cells in denser fibrin networks remain round and extend short and thin protrusions into the network. Since mesh size decreases with the square-root of fibrinogen concentration, the smaller mesh size in dense fibrin networks can constrain the cell body, and can impede the extensions of protrusions. Thus the structure of the microenvironment around cells in dilute fibrin networks differs from dense fibrin networks which might lead to altered mechanical properties of the microenvironment. Since we studied the micromechanical properties of fibrin networks and fibroblasts-seeded fibrin networks 1 h after polymerization, further studies are needed to correlate the onset of cell spreading and the onset of gel-stiffening.

The elastic modulus in the elastic plateau region $G_0$ of semiflexible polymers follows a power-law in concentration with an exponent of 11/5. In our study, $G_0$ of fibrin networks followed a power-law in concentration with an exponent of 1.8. This exponent is in good agreement with prior measurements on fibrin networks where $G_0$ followed a power-law in concentration with an exponent of nearly 2.0 measured by macrorheology, and 2.3 measured by microrheology. The magnitude of the moduli measured by both macrorheology and microrheology is greater than our measurement. Fibrin networks were prepared under similar fibrin conditions, but in our study the fibrinogen concentration range was small, i.e. 1 to 3 mg/ml compared to other studies where the range was 0.1 to 6 mg/ml.
and 0.1 to 8 mg/ml. The difference in magnitude of the moduli can be explained by the smaller range of fibrinogen concentration used in our study.

The elastic modulus of fibroblasts-seeded fibrin networks was threefold higher than fibrin networks when the fibrinogen concentration was ≥ 2 mg/ml. This is in good agreement with prior macrorheology measurements on fibrin networks with and without fibroblasts in which also a threefold higher $G'$ for fibroblast-seeded fibrin networks was found, but for fibrinogen concentrations ≤ 2 mg/ml. Both microrheology and macrorheology measurements showed that cells stiffened the fibrin networks starting 1 h after cell seeding.

The viscous modulus $G''$ of semiflexible networks follows a power-law in frequency with exponent $\frac{3}{4}$. In our study, the exponents most probably decreased exponentially which could be related to the stiffening of fibrin as well as decrease in mesh size with increasing fibrinogen concentration. The opposite result was found for fibroblasts-seeded fibrin networks where the exponents increased with increasing concentration. This might indicate that cells constrain the formation of an uniform fibrin network which results in a highly viscous network.

To unravel the mechanical-feedback loop, it is important to also take the nucleus as well as the ability of cells to push against the matrix into account. The nucleus also exhibits viscoelastic properties. The nucleus is tightly coupled to the surrounding cytoskeleton, which in turn is physically connected to the extracellular matrix. Deformations of the nucleus can have important consequences on cellular functions such as gene expression in cells, resulting in defective nuclear cytoskeletal coupling and defects in cytoskeletal organization and stiffness, thereby affecting cellular and tissue function. Cells have shown besides being able to pull at the surrounding matrix, also to be able to push against the matrix. These pushing forces indicate that compressive forces are present within the cell which might be the result of nuclear deformation. Future studies are needed to investigate the mechanism underlying how cells push against the matrix.

In summary, cellular shape is both fibrinogen concentration and time-dependent. The incorporation of fibroblasts into fibrin networks increased the elastic moduli and decreased the viscous moduli of fibrin networks driving the fibrin networks into a nonlinear stress-stiffened state. Therefore, our results showed that the presence of fibroblasts in fibrin networks contributed actively to a change in the viscoelastic properties of fibrin at the micron scale, suggesting that fibrin networks adapt to cell-generated contractile stresses. These data have wide ranging implications for understanding processes for improving wound healing, treating pathological tissue development, and tissue engineering applications.
REFERENCES