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Nanomechanics of Blood Clot and Thrombus Formation

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Abstract

Mechanical properties have been extensively studied in pure elastic or viscous materials; however, most biomaterials possess both physical properties in a viscoelastic component. How the biomechanics of a fibrin clot is related to its composition and the microenvironment where it is formed is not yet fully understood. This review gives an outline of the building mechanisms for blood clot mechanical properties and how they relate to clot function. The formation of a blood clot in health conditions or the formation of a dangerous thrombus go beyond the mere polymerization of fibrinogen into a fibrin network. The complex composition and localization of in vivo fibrin clots demonstrate the interplay between fibrin and/or fibrinogen and blood cells. The study of these protein–cell interactions and clot mechanical properties may represent new methods for the evaluation of cardiovascular diseases (the leading cause of death worldwide), creating new possibilities for clinical diagnosis, prognosis, and therapy.

Contents

1. BLOOD COAGULATION

The formation of fibrin clots is the ultimate step of a series of enzymatically catalyzed reactions belonging to the blood coagulation cascade (103). These clots are made up of a three-dimensional biomechanical material that forms a temporary platform for tissue repair, aiming to stop the bleeding when a blood vessel ruptures. However, blood clotting may also have a dark side. When a pathological fibrin clot is formed in the blood vessel lumen, it may limit or impair blood flow, leading to thrombosis (4, 15, 34, 135). The formation of this network follows an ordered series of events that goes from activation of the coagulation cascade to formation of insoluble fibrin fibers due to the polymerization of the soluble fibrinogen protein that circulates in the blood plasma.

Fibrinogen is a soluble, 45-nm-long, 340-kDa glycoprotein composed of six (three pairs) polypeptide chains: two Aα, two Bβ, and two γ chains. This complex protein is one of the most abundant in the blood plasma, at a concentration of 2–4 g/L, and is converted to insoluble fibrin by thrombin-mediated proteolysis of the N-terminal domains of the Aα and Bβ chains. During thrombin proteolytic activity, fibrinopeptides A and B are released, exposing new amino acid residue sequences on fibrin monomers, so-called knobs A and B (148). The newly exposed knobs from one fibrin monomer will then interact with other fibrin molecules on the corresponding binding sites, termed holes a and b, located in the Aβ and γ nodules, forming the protofibrils (148). For the fibers to be formed, protofibrils must bundle together laterally through interactions between the αC regions in adjacent protofibrils (89). The entangling of fibrin fibers leads to the formation of the 3D mesh-like clot structure. Last, the fibrin clot is stabilized by intermolecular covalent γ-glutamyl-ε-lysyl cross-links between residues in the Aα and γ chains of fibrin monomers by factor XIIIa (FXIIIa) (148), culminating in a clot that is more resistant to enzymatic cleavage and shear forces (**Figure 1**).

Thrombosis: mechanism of formation and development of a thrombus; can occur whenever the blood flow in arteries or veins is hampered

The mechanical stability of a clot is determined mainly by its fibrin content. Because a blood clot exists in an environment with hemodynamic shear force (blood flow) and contractile tension (muscle contraction), it is important for it to retain some stiffness to keep it from rupturing and releasing part of its scaffold, forming an embolism. However, the fibrin clot must also have some plasticity (the ability to permanently deform in response to blood flow shear forces) to keep from imposing a rigid barrier to blood flow upon tissue repair. In this way, the normal blood flow is not perturbed. The biomechanical response of fibrin clots is remarkably like those

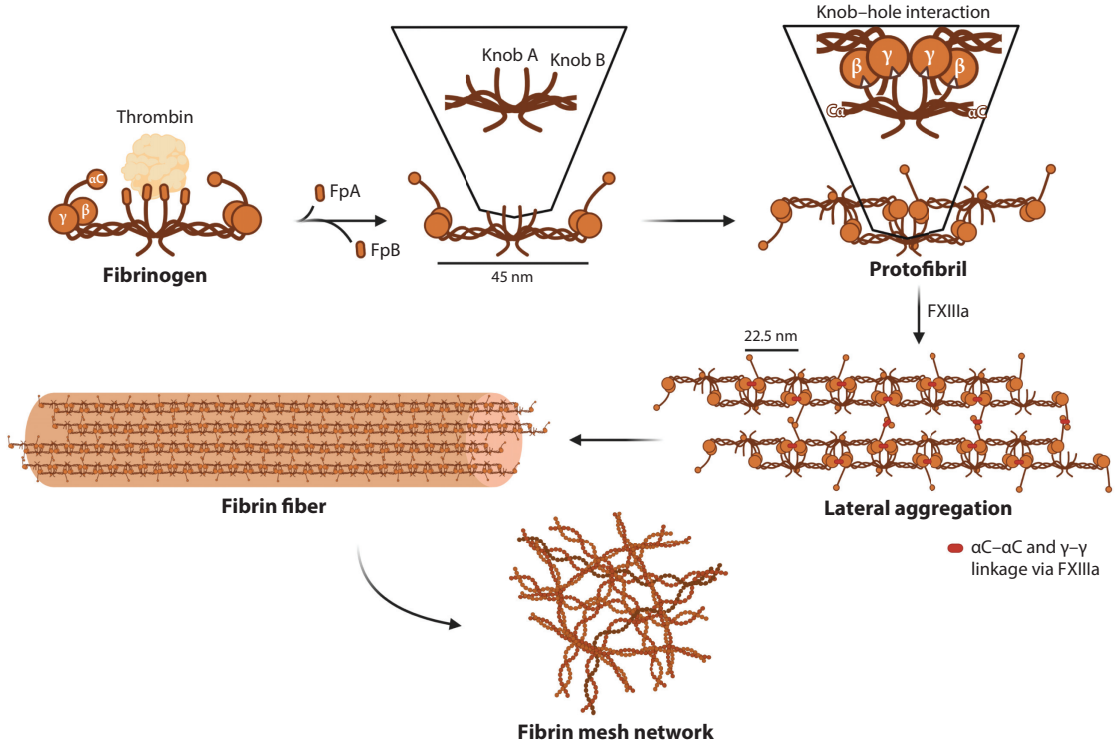

Figure 1

Fibrin polymerization. Thrombin cleaves fibrinopeptides from the 45-nm-long fibrinogen molecule to create fibrin monomers. The cleavage of fibrinopeptides exposes the fibrin knobs A and B, enabling knob–hole interactions between fibrin monomers and causing the fibrin monomers to polymerize into 22.5-nm half-staggered oligomers, which enlarge into protofibrils. Protofibrils then aggregate laterally, forming fibrin fibers. Last, fibrin fibers entangle, forming branching points that culminate in the fibrin mesh network. The fibrin clot is then stabilized by factor XIIIa (FXIIIa), a transglutaminase that cross-links a glutamine residue on one fibrin molecule to a lysine residue on another fibrin molecule. This cross-linking occurs between the C-terminal domains of the Aα chains, as well as between the C-terminal domains of the γ chains. These cross-links help strengthen the fibrin clot, making it more resistant to physical and chemical damage. Figure adapted from images created with BioRender.com.

of other biopolymers, such as actin, collagen, and vimentin, showing a nonlinear behavior of the deformation with stress, called strain-hardening (18). The network structure is responsible for adaptability to the external exerted forces, from macroscopic deformation of fibrin fibers to molecular unfolding of fibrin monomers.

2. BLOOD CLOT MECHANICS

2.1. Large-Scale Clot Mechanics

Several studies have been performed to investigate the structural changes in the clot in response to shear and tensile forces using rheology-based techniques (see the sidebar titled Rheology Measurements). Early works from John D. Ferry's group, reported in the 1970s, shed some light on clot viscoelasticity. In their studies, it was demonstrated that clots, with fibers cross-linked by FXIIIa, have less deformation and are more likely to recover their original structure upon release of the stress compared to clots formed in the absence of FXIIIa (98). The result of the study showed that fiber cross-linking by FXIIIa is related to increased clot stiffness and

Stress: a force imposed on the system to cause material creep (deformation)

Strain-hardening: elastic property where a material shows increased strain stiffening with increasing shear and tensile forces

RHEOLOGY MEASUREMENTS

Rheology apparatuses, such as rheometers, thromboelastography, magnetic tweezers and optical tweezers, have been employed to study fibrin clot mechanical responses (13, 15, 17, 27). Rheology measures the stiffness or storage modulus (G') and the viscous or loss modulus (G'') components of the complex modulus (G^*), where $G^* = G' + iG''$. The loss tangent ($\tan\delta$) is the ratio between viscous and elastic properties. For a viscous material, $\tan\delta \gg 0$, i.e., $G'' \gg G'$, whereas for an elastic solid, $\tan\delta \ll 0$, i.e., $G' \gg G''$. Common rheometers apply a shear stress or a shear rate to the materials and measure the clot response as a displacement material deformation (shear strain) or the shear stress, respectively. The shear strain (γ) equals s/h, where s is the deformation, and h is the thickness of the material, while the shear stress (τ) equals F/A, where F is the shear force, and A is the shear area. Rheometers determine G' and G'' from the in-phase sinusoidal response and the out-of-phase response stress, respectively. In microrheology studies using magnetic tweezers, the frequency-dependent modulus (18) is calculated from the time-dependent compliance, $J(t) = \gamma(t)/\sigma_0$, i.e., the ratio of the time-dependent shear strain, $\gamma(t)$, to the magnitude σ_0 of the constant stress that is switched on at time $t = 0$.

clot elastic moduli. Similarly, other factors also play a role in clot stiffness modulation. Ryan et al. (115) studied the effects of fibrinogen, calcium ion, and thrombin concentrations on clot rheology. Their data pointed out that, at constant fibrinogen but varying thrombin and calcium concentrations, the stiffness of the clot was controlled by a balance between large fiber sizes and increased branching points. Increasing fiber and branching point densities at higher fibrinogen concentrations also enhanced clot rigidity. In agreement with what was previously described in Ferry's work, the study demonstrated that FXIIIa-catalyzed fiber cross-linking increases clot stiffness. Altogether, rheological studies suggest that clots composed of thin, densely packed fibers are stiffer than those composed of thick fibers with larger pore sizes (67, 113, 115).

When it comes to clot resistance, Brown et al. (16) showed that fibrin clots could be stretched to over three times their relaxed state length before rupture. In addition to the remarkable compliance of the clot, a decrease in clot volume upon stress loading was also detected. The decrease in volume was associated with water being expelled from the clot due to the increase in protein density during the threefold clot stretching, while the high extensibility can be explained by protein secondary structure transitions (50, 82, 147). Confocal and electron microscopy images of clots show that they have network compactness, with fibrin fibers being thinner and aligned in the direction of the stress. Furthermore, the compressive forces lead to the transition to a more compact clot, with increased fibrin fiber crisscrossing (16, 82, 83). Another study observed that the clot network undergoes a strong and irreversible increase in their mechanical rigidity in response to compression (143). Because clots form under different environment shear forces, this may have an impact on the final structure and the mechanical behavior of the scaffold. One study showed that fibrin polymerized under continuous oscillatory shear perturbations forms rigid clots that exhibit later onset of strain-stiffening and a postponed rupture (96). These changes in mechanical properties arise from changes in the clot network, enabling the clots to serve their function over a wide range of mechanical challenges.

Compliance: ability of the biomaterial to respond to a mechanical load

Toughness: distinct from deformation and viscoelasticity and related to the ability to deform and absorb energy without fracture

An important aspect of studying the mechanical properties of a clot is the assessment of its toughness, with the aim of understanding the embolization of thrombi. Tutwiler et al. (134) measured the toughness of plasma-derived fibrin clots and showed that clots could hold higher forces when they did not have any crack in their structure. Collet et al. (35) demonstrated a correlation between clot stiffness and premature coronary artery disease using patient blood samples. Although, characteristically, the clots exhibit strain-hardening (essentially at low frequencies),

Domínguez-García et al. (42) demonstrated, using Brownian optical tweezers in non-cross-linked fibrin fibers, that, at high frequencies, the clot network undergoes significant fluidification, where the loss modulus is higher than the storage elastic modulus. This means that, instead of hardening, the clot becomes softer.

Several studies have been conducted to study clot composition and the contribution of each component to the biomechanical behavior. Chernysh et al. (32) measured the mechanical response of an embolus clot, concluding that an embolus clot enriched in fibrin fibers has a higher stiffness than a clot enriched in red blood cells (RBCs). Fereidoonnezhad et al. (49) obtained a similar result, demonstrating that fibrin-enriched clots have higher fracture resistance than RBC-enriched clots. This may have an impact on the propensity of a clot to embolize, eventually creating a possible avenue for developing new treatments and assessing a series of risk factors for cardiovascular complications.

The architectural adjustment to the specific loading conditions of the environment may enable blood clots to maintain their nonlinear properties and prevent early rupture, thus allowing them to fulfill their function despite a wide range of mechanical challenges.

Loss modulus: related to the energy dissipated upon stress application

Young modulus: a mechanical property that quantifies the stiffness of a material

Fibrinolysis: process in which the fibrin clot is degraded by plasmin activity, releasing the fibrin degradation products

2.2. Fibrin Fiber Clot Mechanics

As fibrin fibers are the mechanical backbone of the clots, several studies have been carried out to clarify the viscoelastic properties of these fibers. According to research on clot networks, fibrin fibers possess a high extensibility, with Young moduli of the order of 1 MPa and 10 MPa for non-cross-linked and cross-linked fibrin fibers, respectively (83, 86). Compressive studies on clots show that the material responds by lowering resistance to the movement and softening the material. This effect under low compressive forces results from the buckling and bending of the fibrin fibers, which, at larger compressive strains, have less impact than the fibrin fiber compactness (71). Based on the structure of fibrin fibers, several studies have been conducted to determine specific patterns that influence the mechanical properties of the clot scaffold. Fibers with truncated αC regions (Aα251) (**Figure 1**) are thinner, with lower stiffness and enhanced fibrinolysis, emphasizing the importance of this region of the fibrin molecule to stress-strain behavior (5, 36). The study of a recombinant fibrin with mutated γ chain cross-linking sites (Q398N, Q399N, and K406R) revealed that the loss of fiber elasticity and extensibility is primarily due to Aα chain cross-linking (63). Loss of fiber extensibility may explain the recent observation that, during clot retraction, erythrocytes are trapped in FXIIIa cross-linked fibrin networks without being covalently bound to the fibers, while erythrocytes in non-cross-linked fibers are extruded (2, 19).

Thrombin concentration, as a key component in the clotting process, plays a role in regulating fiber formation, leading to different clot network structures and mechanical responses. Higher thrombin concentrations are associated with denser clots with small porosity, composed of thinner fibers (41). In contrast, lower concentrations of thrombin lead to the opposite: clots of large porosity, made of thicker fibers. A study showed that, in fully hydrated conditions, thrombin and γ′ fibrinogen (see below) alter the protofibril content and that protein density within fibers correlates with the strength of the fibrin network (41). As mentioned above, FXIIIa is another regulator of fibrin clot strength and fibrinolysis. FXIIIa cross-linking has a dramatic effect on fibrin mechanical properties. Fully cross-linked fibers are 2- to 10-fold stiffer, are 50% less elastic, and have 40–50% lower extensibility than partially cross-linked fibers (63, 86). Although cross-linking by FXIIIa does not significantly affect the structure at the network level, it leads to fiber compaction by tightening the coupling between protofibrils (76). This structural feature explains the stiffening of fibrin fibers, which eventually leads to a rigid clot, as discussed above. In rheological studies, where network mechanical properties typically depend more on fiber structural rigidity and

network rearrangement than on fiber stretching, networks composed of cross-linked fibers also exhibited a two- to fivefold higher elastic modulus (stiffness) and a twofold higher loss modulus (36, 76, 126).

Fibrin fiber mechanics has a higher impact on fibrin clot mechanical behavior under stress, such as in blood flow. Nevertheless, the mechanical properties of fibrin fibers contribute to fibrinolysis susceptibility. Varjú et al. (140) and Liu et al. (86), in two independent studies, showed that fiber stretching decreases plasminogen activation and lysis, suggesting that stretching is a mechanosensitive factor that regulates blood clot dissolution. Under certain conditions, thick fibers elongate during lysis, reaching a lysis-resistant state (17). One factor that may explain this phenomenon is that thicker fibers are likely under higher tension than are thinner fibers due to protofibril packing. As fibers are lysed, they lose their tension, leading to elongation, which hinders fiber lysis. Elongation is more prominent in thicker fibers, providing an additional explanation for why thinner fibers lyse more rapidly. Another study by Adhikari et al. (1) found a 10-fold reduction in plasmin degradation of strained clots, correlating this with a reduction in diffusive transport into the network. Taken together, these results indicate that fiber tension and stretching play an important role in the regulation of fibrinolysis, influencing the binding of plasminogen activators, the availability of fibrinolytic enzymes, and the activity of plasmin.

As described above, clot extensibility arises from fibrin fiber elasticity. The basis of fibrin fiber extensibility has been the focus of several studies and is likely due to structural rearrangements of fibrin monomers. Unfolding of the α-helical coiled-coil or γ nodule and stretching of αC domains have been proposed as possible foundations of fibrin fiber stretching capacity (16, 65, 154).

Small-angle X-ray scattering revealed that protofibril aggregation into a fibrin fiber leads to a periodic pattern, occurring approximately every 22.5 nm, due to the half-staggering of 45-nm molecules (**Figure 1**). Vos et al. (144) showed that, under strain, fiber stretching induces a negligible change in the periodicity pattern when compared to the 80% length increase of the fibers. They concluded that the unfolding of the fibrin monomers that lead to high extensibility come from αC regions that are interconnected within protofibril packing, without unfolding of other domains (144). This means that there is a sliding effect coming from the extensibility and unfolding of the αC-terminal regions that connect protofibrils. The molecular involvement of the αC-terminal regions is not surprising, as the presence of FXIIIa increases fiber stiffness (36). This may lead to a lower extensibility of the αC-terminal regions. Other studies revealed that, under higher strains, following αC domain involvement in the stretching capacity, unfolding of the coiled-coil domains occurs, with an increase in the β-sheet content of the α-helix domains; refolding occurs after strain release (82, 147). Due to the difficulty in distinguishing several unfolding regions in the fibrinogen molecule by atomic force microscopy, molecular dynamics (MD) approaches were used. Through MD simulations, it was possible to understand that not only was the coiled-coil region unfolded, but also the γC domain was unstructured (153).

As mentioned above, fiber stretching leads to higher resistance to fibrinolysis. As the mechanical stresses induce molecular changes in fibrin monomers, it was proposed that the partial unfolding may alter or partially block enzyme binding to the cleavage sites (66). Further work on the mechanical properties of the clot network and its correlation to fibrinolysis would enable better prediction of pathological consequences.

3. FIBRIN(OGEN) INTERACTION WITH BLOOD CELLS

Clot components, including fibrinogen, platelets, and RBCs (erythrocytes), contribute to the mechanical properties of the clot. The fibrin mesh acts as a structural scaffold to entrap and capture blood cells and the components of blood plasma. The presence of these cells in the clot has been

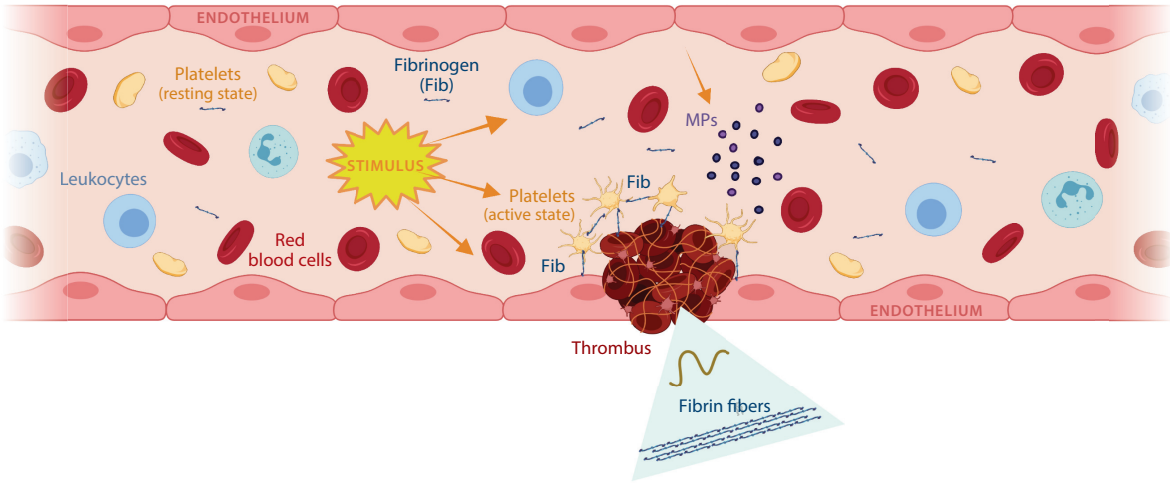

Figure 2

In healthy conditions, the blood flow transports red blood cells (RBCs) (erythrocytes), white blood cells (leukocytes), and platelets. RBCs flow predominantly in the center of the lumen of the blood vessel, while platelets travel closer to the endothelial cells on the vessel wall. In low-hematocrit conditions, platelets travel closer to the center of the vascular lumen, being less likely to interact with the subendothelium. RBCs and platelet-derived microparticles (MPs) can influence thrombin production (119). After formation of the fibrin plaque, RBCs become entrapped within the thrombus to stabilize and support its structure. Figure adapted with permission from Reference 133, and from images created with BioRender.com.

shown to have an impact on fiber thickness, density, and elasticity, as well as on some properties of the clot itself, including its network stiffness and resistance to lysis.

Differences in the mechanical properties of blood clots between healthy individuals and those with pathological conditions have already been reported (3, 95). Venous thromboembolism (VTE) is a medical disorder that involves the formation of a fibrin-rich thrombus in a deep vein. Scanning electron microscopy images of venous thrombi demonstrated that they contain a fibrin meshwork with entrapped erythrocytes, few platelet aggregates, and some white blood cells (leukocytes) (52, 142). The main thrombus component is fibrin fibers (approximately 60%), with RBCs, platelets, and leukocytes making up the remaining approximately 40% (72).

The biochemical and biophysical interactions that occur between fibrin(ogen) and blood cells are extremely important for in vivo hemostasis and thrombosis (**Figure 2**). Glycoproteins belonging to the integrin family mediate cell adhesion and migration through their integration into the extracellular matrix (ECM) with the cytoskeleton (105).

3.1. Fibrin(ogen) Interaction with Platelets

Platelet membrane glycoproteins include adhesion molecules identified in other cell types in the late 1980s (102, 104). Platelet membrane glycoproteins are important in the formation of a response when an injury to the vascular wall occurs. They are present on the platelet surface or inserted into the α-granule membrane and play a key role in normal platelet adhesion or aggregation.

The recognition of the fibrinogen molecule by the activated platelet glycoprotein $\alpha_{IIb}\beta_3$ receptor (an integrin) occurs through four RGD (Arg-Gly-Asp) amino acid residue sequences at the fibrinogen Aα chains (3, 64, 81). Amino acid residues 118–131 of the receptor β_3 subunit were identified as the sequences of recognition of the RGD sequences (44). Fibrinogen-platelet binding can also occur through a non-RGD sequence, the dodecapeptide (HHLGGAKQAGDV)

Venous thromboembolism (VTE): a medical disorder that involves the formation of a fibrin-rich thrombus in a deep vein

Hemostasis: maintenance of blood vessel flow, avoiding bleeding either by natural (clot formation, vessel spasm) or artificial (compression, ligation) mechanisms

Integrin: cell surface glycoprotein that facilitates cell–cell adhesion and cell–ECM adhesion, acts as an adhesion receptor for extracellular ligands, and transduces biochemical signals into the cell

von Willebrand factor (VWF): adhesive and multimeric glycoprotein; participates in coagulation by binding to proteins (e.g., factor VIII) and is important in platelet adhesion to wound

sequence in the γ chain C-terminal region (residues 400–411), which binds amino acid residues 294–314 on the α_{IIb} subunit (11, 43, 81). The $\alpha_{IIb}\beta_3$ integrin is required for platelet aggregation, being the receptor for fibrinogen, von Willebrand factor (VWF), fibronectin, and vitronectin (51). There are approximately 80,000 $\alpha_{IIb}\beta_3$ receptors on the surface of each platelet (145). Further details on the regulation of $\alpha_{IIb}\beta_3$–ligand binding and the distinct functions of the α_{IIb} and β_3 subunits can be found elsewhere (11).

In certain individuals, the blood coagulation mechanism does not occur under physiological conditions. One of the many possible causes for this problem is associated with abnormalities on the platelet $\alpha_{IIb}\beta_3$ receptor or to its absence, Glanzmann's thrombasthenia disease (51).

Platelets initiate and propagate the formation of fibrin networks, also changing the clot stiffness by platelet retraction (30, 100, 132). Pathare et al. (100) suggested that platelet retraction induces a prestress in fibrin fibers, increasing the effective stiffness in both cross-linked and non-cross-linked clots. Their results provide evidence for fibrin compaction at discrete nodes as a major determinant of the mechanical response to applied loads.

Thrombi in arteries are rich in fibrin fibers and platelets, whereas thrombi formed in veins have a fibrin network with platelet clusters and RBCs inside the clot (12). Microfluidics studies provided new observations into these processes (128, 129). Both platelet and coagulant activity depend on the local blood flow and shear flow conditions. In arteries, platelet deposition and thrombus formation increase with the shear flow rate via interaction between the glycoprotein Ib-V-IX complex and VWF. Thus, depending on the flow conditions, either platelet adhesion (high shear) or thrombin and fibrin generation (low flow) can act as driving factors for thrombus formation (128).

3.2. Fibrin(ogen) Interaction with Red Blood Cells

RBCs are the most abundant cells in the blood, corresponding to approximately 35–45% of the total blood volume. The major function of RBCs is to transport oxygen bound to hemoglobin through the whole body. Their discoid shape gives RBCs specific biological and mechanical properties required to accomplish their functions (150). RBCs are very elastic and deformable as a consequence of their membrane structure, in which the lipid bilayer is anchored to an elastic net of skeletal proteins through binding sites on cytoplasmic domains of transmembrane proteins (94). However, it is important to bear in mind that the uncommon elastic properties of RBCs are due not only to the proteins of their cytoskeleton, but also to the unique lipid composition of their membrane. Importantly, the RBC lipid bilayer is composed of an abnormally high percentage of cholesterol (values between 40 and 55 mol% have been reported, depending on the sample and technique used). This high percentage of cholesterol renders the membrane less fluid than that of other cells with lower cell membrane cholesterol levels. This seems counterintuitive for a cell with such a need to change shape during its path in the bloodstream, namely, upon the crossing of blood vessels with a lower caliber. However, a lower cholesterol level on the RBC lipid bilayer would make the cell both more fluid and more susceptible to wear under the elastic stresses to which erythrocytes are exposed (54).

Carvalho et al. (22, 23) and Guedes et al. (60) identified the receptor for fibrinogen on the erythrocyte membrane, the $\alpha_v\beta_3$ integrin, mostly using atomic force microscopy (AFM)-based force spectroscopy measurements (see the sidebar titled Atomic Force Microscopy). The simultaneous binding of fibrinogen to such receptors on two different erythrocytes may transiently bridge these cells, increasing blood viscosity at the microcirculatory level, impairing proper blood flow, and increasing vascular risk. Lominadze et al. (87) previously suggested that fibrinogen can specifically interact with erythrocyte membranes. On the fibrinogen molecule, the interaction with erythrocytes involves the Aα chain (109). In a previous study by Carvalho et al. (25), it was possible

ATOMIC FORCE MICROSCOPY

AFM is a scanning probe microscopy technique that enables the construction of images of surfaces, in solution or in air, but also the study of molecular interactions and their mechanical forces (6). AFM lateral resolution can go down to approximately 1 nm, making it able to image molecules, supramolecular assemblies, cells, and tissues. The vertical resolution limit goes down to subatomic resolution (<1 Å) (112).

The basic quantitative concept of AFM lies in Hooke's law of elasticity—a simple equation expressing the force (F) generated within a spring (like an AFM cantilever) when it is compressed in terms of a known spring constant (k) and the length of the deflection (Δx) of the spring ($F = -k\Delta x$).

AFM-based force spectroscopy allows the measurement of inter- and intramolecular interaction forces required to separate the tip from the sample with piconewton resolution. It is possible to modify the surface and manipulate individual molecules at physiological conditions using AFM (24, 26). In this mode, the cantilever moves in the vertical direction (z axis) toward the surface and then in the opposite direction. The result is a force–distance curve. Force spectroscopy has been used to study cell stiffness, protein unfolding, polymer and fiber elasticity, single-molecule interactions, and cell–cell adhesion, among other biological and biomedical applications (22–24, 26, 60).

to identify that the RGD sequence of fibrinogen Aα chain residues 95–97 is involved in its interaction with RBCs. The fibrinopeptide-containing region in the central domain (including Aα and Bβ chains) was shown to be repulsive to the negatively charged erythrocyte membrane. A contribution of the C-terminal region of the fibrinogen γ chain for erythrocyte binding must also be taken into consideration, as it is in fibrinogen–platelet binding, but this contribution is not yet clear (91).

The inclusion of RBCs in clots leads to heterogeneity in the fibrin network structure and changes in clot stiffness, with the specific effects depending on the RBC concentration (57). The incorporation of RBCs affects clot microstructure by increasing fibrin fiber diameter, pore size, and network heterogeneity. RBCs inhibit branch protrusion of growing fibers, forcing fibrin monomers to align with previously settled fibers and leading to the formation of thicker fibers (57).

A reduction in bleeding and an increase in susceptibility to thrombosis associated with an increase in RBC count (or high hematocrit), inherited erythroid diseases, and several acquired pathological disorders that change RBC properties have been reported (149). RBC transfusions are associated with a rather high incidence of thrombotic complications (84). This observation also evidences the contribution of RBCs to blood clotting disorders, while the thrombosis risk could also be associated with the underlying disease. This fact may weaken the connection between blood transfusions and thrombosis (146).

RBCs may contribute to both arterial and venous thrombosis. In arterial thrombosis, RBCs help with the transport and accumulation of platelets near the vessel wall (platelet margination), promoting the interactions between platelets and the thrombus, platelet activation, and adhesion (133). In veins, blood viscosity is increased by erythrocyte hyperaggregation into rouleau structures. RBCs also adhere to the vessel wall and contribute to thrombin generation inside the thrombus (20). Then, as the size of the thrombus increases, its permeability and vulnerability to lysis decrease. During thrombosis, RBCs may also adhere to the endothelium or ECM, activate platelets and other cell types, and increase local thrombin concentration (20).

Hematocrit: the ratio (usually expressed as a percentage) of the volume of RBCs to the total volume of blood

Rouleau: a stack of RBCs resembling a roll of coins; occurs in vivo as concentrations of proteins (such as fibrinogen, immunoglobulins) increase

3.3. Fibrin(ogen) Interaction with White Blood Cells

Under physiological conditions, inactive white blood cells (leukocytes) promote the maintenance of blood fluidity. White blood cells express and release coagulation and fibrinolytic factors and interact with the hemostatic system through innate immune functions (130). Furthermore, they

Ischemic stroke: stroke caused by reduced blood flow to a specific brain artery; consequence of a clot in the artery

produce cytokines that modulate the expression of procoagulant and adhesive molecules on vascular endothelial cells.

White blood cells may also induce venous, arterial, and microvascular thrombus formation. Two different types of white blood cells, monocytes and neutrophils, once activated, play a role in the formation of a blood clot (28). Neutrophil extracellular traps (NETs), which are DNA complexes with nuclear fibers, influence thrombus organization and stability (52, 142). NETs increase fibrin fiber stability and rigidity, making the fibers less prone to fibrinolysis (88). Additionally, NETs have different binding sites for monocyte-derived tissue factor, fibrinogen, fibronectin, and VWF, all of which contribute to platelet activation and fibrin clot development (52, 142).

In deep vein thrombosis patients, an increase in circulating markers of NETs and neutrophil activation has been observed (40), as well as increased monocyte tissue factor (68). White blood cells also accumulate at a site of vascular injury, being incorporated into the thrombus. The influence of white blood cells on blood coagulation and platelet activation, the contribution of white blood cells to thrombosis, and the possible role of white blood cells in the development of new antithrombotic strategies are reviewed in detail elsewhere (130).

3.4. Fibrin(ogen) Interaction with Endothelium and Blood Vessel Walls

The endothelial ECM contains protein fibrils 30–70 nm in diameter, which can be packed into larger fibers (up to 1 μm). These cell fibers facilitate the packing of a huge number of cells (135). In the clot, fibrin fibers are aligned in the direction of the blood flow, increasing clot stiffness and resistance to fibrinolysis (21, 56, 97). Some authors reported that blood flow has no effect on fiber diameter (56), whereas others described formation of thicker fibers in the direction of flow, with thinner fibers intersecting these larger fibers perpendicularly (21, 135).

The molecular mechanisms that regulate clot structure close to the endothelium are still unknown but could involve local tissue factor activity, changes in thrombomodulin concentration, or the endothelial fibrin(ogen) receptor $\alpha_V\beta_3$. As pointed out above, fibrinogen contains two RGD integrin-binding sites, which may bind endothelial cells and fibroblasts (53, 108, 118).

Fibrin promotes wound healing by acting as a temporary ECM for fibroblasts that realign and degrade fibrin fibers. In 2018, Van Esterik et al. (46) reported that the incorporation of fibroblasts into fibrin networks increases the elastic moduli and decreases the viscous moduli of fibrin networks, which might drive the fibrin networks into a nonlinear stress-stiffened state. The luminal surface of venous thrombi consists of a barrier of platelet endothelial cell adhesion molecule 1 (PECAM-1)-positive endothelial cells. This endothelial barrier prevents the fibrinolysis agents present within the blood plasma from getting inside the thrombus (28, 127).

Biophysical studies describe thrombus growth under flow based on local shear (10). Experimental studies and mathematical models suggest that the platelet aggregation rate under flow is nonlinearly dependent on the shear rate (77, 121).

4. PATHOLOGY AND CLINICAL PROGNOSIS

Fibrinogen and fibrin are known to be key players in different pathological settings. Fibrinogen's high plasma concentrations, its altered structural properties, and the impact of its polymorphisms may influence clot permeability, stiffness, and resistance to lysis. Whether the abundance of fibrin(ogen) has a causal role or is a consequence of the underlying pathology should be of extreme relevance.

In response to a vascular injury, blood clots are formed to help stop bleeding and maintain homeostasis. When a blood clot arises in undesired sites or is shed from the vascular wall to the bloodstream, conditions such as deep vein thrombosis, heart attacks, and ischemic strokes may

occur (5). Fibrinogen is present within atherosclerotic plaques, indicating a potential role in their development and stability (125). An inverse relationship between plasma levels of fibrinogen and thickness of the fibrous cap of atheroma has been observed that results in higher incidence of plaque rupture and thrombosis in subjects with increased fibrinogen levels (116).

γ′ fibrinogen is an alternative splicing variant of fibrinogen with an additional sequence of amino acid residues on one of its γ chains (heterodimer) or on both of them (homodimer), commonly found in vivo at low concentrations. Clinical studies revealed its importance as a cardiovascular disease biomarker. Increased γ′ fibrinogen was associated with a higher risk of venous thrombosis (48). Clots produced with γ′ fibrinogen have thinner fibers, more branching points, and increased resistance to fibrinolysis (37, 135). In 2019, van Dijk et al. (139) showed that total fibrinogen and γ' fibrinogen are inversely associated with intraplaque hemorrhage volume and lipid-rich necrotic core volume, independently of inflammation. Guedes et al. (59) also reported that the interaction between γAγ′ (γ′ heterodimer) fibrinogen and RBCs leads to heterogeneous clots, with areas of denser and highly branched fibrin fibers. The presence of RBCs also increased the stiffness of γAγ′ fibrin clots, which are less permeable and more resistant to lysis than γAγA (the most common form of fibrinogen) clots. γ′γ′ (γ′ homodimer) fibrinogen also increases the binding force and the frequency of the binding to RBCs compared to γAγA fibrinogen, promoting cell aggregation (58).

Homocysteine, a product derived from the methionine metabolism, is associated with an increased risk for coronary artery disease and thrombosis (135). Hyperhomocysteinemia was associated with thinner and more tightly packed fibrin fibers and increased resistance to fibrinolysis (122). Homocysteine addition in vitro leads to the formation of clots with shorter fibers and a more compact structure (79).

Fourier-transform infrared spectroscopy (FTIR), Raman spectroscopy, and AFM were used to examine two types of thrombus derived from different ischemic stroke etiologies: cardioembolic origin and large vessel origin (14). FTIR and Raman analysis of the fibrin-predominant thrombus obtained from a patient after aortic dissection enabled the discrimination of three molecular regions: the fibrotic, heme, and lipid regions (14). In 2011, Pretorius et al. (106) investigated fibrin fiber diameters from stroke patients and compared them with diameters from healthy individuals. They concluded that there is a statistical difference between fibrin fiber thickness during thromboembolic ischemic stroke and normal thickness. Stroke patients have thicker fibers compared to healthy donors. Patients within the acute phase of ischemic stroke demonstrated reduced clot permeability and decreased fibrinolysis, contributing to the thrombosis mechanism (93, 137, 138). Rooth et al. (114) showed that the changes observed during the acute phase of ischemic stroke persist for more than 60 days after the stroke event. Patients with acute ischemic stroke associated with carotid artery disease (CAD) exhibited slower clot lysis compared to controls without CAD history (93). Fibrin clot density is correlated with neurological deficit upon both admission and discharge of acute ischemic stroke patients (137). Highly dense plasma clots (that are thus more resistant to fibrinolysis) were produced from the plasma of patients who suffered from strokes with an unknown cause 3–9 months earlier (136).

In 2016, mostly using AFM, Guedes et al. (60) were able to show that ischemic heart failure patients displayed increased fibrinogen–erythrocyte binding forces compared to nonischemic patients and healthy controls. Importantly, patients with higher fibrinogen–erythrocyte binding forces, as measured by AFM at the beginning of the study, had a higher probability of being hospitalized due to cardiovascular complications during a subsequent 12-month clinical follow-up, indicating the potential of this experimental approach at the level of the clinical prognosis to pinpoint the patients at higher cardiovascular risk for personalized medical interventions (60).

Guedes et al. (61) also reported that the work (energy) and force necessary for erythrocyte–erythrocyte detachment are higher for essential arterial hypertension (EAH) patients than for healthy blood donors and increase with increasing fibrinogen concentration. In another study, Guedes et al. (62) reported stronger fibrinogen binding to RBCs in EAH patients than in controls, together with an increase in RBC stiffness.

Patients with chronic kidney disease also have increased risk of thrombotic events (70). Fibrinogen purified from patients with chronic kidney disease on hemodialysis showed evidence of glycosylation and guanidinylation (70, 123). Clots made with guanidinylated fibrinogen have significantly thinner and denser fibrin fibers. In hemodialysis patients, denser fibrin complexes were independently associated with the risk of mortality (123). In 2016, Lau et al. (78) also reported major changes in fibrin clot structure across different degrees of renal function, as classified by chronic kidney disease stages.

Vascular complications are common in patients with long-standing type 2 diabetes mellitus (DM) (92). The presence of metabolic syndrome and hyperfibrinogenemia may contribute to initial progress of macro- (ischemic heart disease) and micro- (retinopathy) vascular complications. Increased plasma fibrinogen was independently associated with major adverse cardiovascular events in CAD patients, especially among those with pre-DM and DM (85). Fibrinogen may be useful in the cardiovascular risk stratification of pre-DM and DM patients.

Fibrin(ogen) also likely plays an important role in fibrotic and arthritic diseases (141). High levels of fibrinogen in plasma could inhibit the production of matrix metalloproteinase 2 (MMP-2), which is vital for healthy organ development and repair. Lower levels of MMP-2 could lead to arthritic and cardiac disorders (120).

Inherited fibrinogen disorders are classified into two different types (93). Type I (referring to reduced quantity of fibrinogen) includes afibrinogenemia (plasma fibrinogen levels below 0.1 g/L) and hypofibrinogenemia (plasma fibrinogen levels of 0.1–1.5 g/L). Dysfibrinogenemia is a type II inherited fibrinogen disorder characterized by an anomaly in the quality of fibrinogen: normal fibrinogen levels but with low functional activity (74, 93). Interestingly, inherited fibrinogen disorders confer both an increased bleeding risk and an increased risk of thromboembolic complications. Dysfibrinogenemias resulting from mutations in the fibrinogen genes are linked with arterial or venous thrombosis in approximately 25% of cases (38, 135). Congenital hypofibrinogenemia is much more common than afibrinogenemia and is often caused by heterozygous fibrinogen gene mutations (141). Recently, a systematic analysis of exome and genome data from approximately 140,000 individuals belonging to the Genome Aggregation Database showed that the worldwide prevalence of recessive fibrinogen disorders varies from 1 in 106 persons among East Asians to approximately 25-fold more among non-Finnish Europeans (99). In some cases, due to mutations in *FGG*, the mutant fibrinogen forms aggregates in the endoplasmic reticulum of hepatocytes, potentially causing liver disease (39). Patients with dysfibrinogenemia are usually asymptomatic but can suffer from bleeding and/or thromboembolic complications (27). Low fibrinogen levels due to disseminated intravascular coagulation are frequently detected in patients with acute promyelocytic leukemia (29). Patients with liver disease can also have low plasma fibrinogen levels due to impaired production (13).

Cancer development and progression have been associated with high plasma fibrinogen levels (141). Fibrinogen can be produced by some non-hepatocyte-derived cancer cells and present in the surroundings of tumors, such as in breast cancer (124). Decreasing plasma fibrinogen concentrations, either via drug therapy or lifestyle changes, could help to increase survival in cancer patients. Targeting fibrinogen-specific receptor interactions (e.g., inhibitors of fibrinogen–$\alpha M\beta 2$ interactions) may be beneficial in cancer treatment and/or prevention (101).

Smoking habits also increase thrombotic risk by increasing fibrinogen levels. Following acute exposure to cigarette smoke, fibrin clots become denser and composed of thinner fibers compared with nonsmoking and presmoking samples (8, 135). Thromboelastography performed in whole blood of individuals before and after smoking two cigarettes showed lower lysis efficiency after smoking (7).

Thromboelastography: evaluates the ability of whole blood to coagulate and measures the time for the blood to clot and clot shear strength

Vitamin K: molecules obtained in the human diet (vitamin K_1, vitamin K_2); cofactor in protein carboxylation by the γ-glutamyl carboxylase, required for coagulation

5. CLOT MECHANICAL PROPERTIES UNDER THERAPEUTIC APPROACHES

Typically, monitoring of anticoagulation effects during treatment is performed in plasma by the international normalized ratio (INR), which measures the time necessary for the blood to clot (117). However, the INR test does not consider differences among the factors that influence clotting, such as fibrinogen concentration. Due to this, it is hard to get a clear idea of the effect of the anticoagulation treatment. An alternative to the INR test is the evaluation of plasma thrombin generation by thrombin generation assays, which provide information on the initiation, amplification, and propagation of the coagulation cascade (33, 45, 55). Other alternatives are the whole-blood viscoelastic hemostasis assays, namely, thromboelastometry and thromboelastography (TEG), which provide information on coagulation kinetics, clot strength, hypercoagulability, and fibrinolysis. Both use whole blood, without a need for further sample processing, and rapidly provide test results (90, 151). Viscoelastic methods can be used for real-time measurement of the clot formation rate and stability. Further details on viscoelastic tests on clinical approaches can be found elsewhere (117).

Current evidence indicates that both the approach of using fractal dimension (Df) based on a measurement of the gel point (GP) of a whole blood clot and the approach of using typical markers describing clot mechanical properties (such as G′ and G″) are able to detect changes in the clot microstructure related to anticoagulant therapy and deserve to be considered as global markers of hemostasis (47, 80). The GP measurement and Df determination through rheological technical approaches can provide information about the clot microstructure. Lower Df values would be characterized by reduced strength (elasticity) and a less dense, porous network structure (features typically associated with hypocoagulable states). Conversely, higher Df values would indicate a clot that is mechanically far stronger, with a more compact microstructure, corresponding to a hypercoagulable state.

To date, little is known about the impact of therapeutic approaches on viscoelastic properties of plasma clots, reflected by G′ and G″, and how these therapeutic approaches could be useful tools for evaluating anticoagulation effectiveness or safety. Clinicians often use anticoagulants and vitamin K antagonists (VKAs) as resources to tackle thromboembolism-related diseases (9). The pathophysiological role of increased clot stiffness and its on-treatment values may have potential clinical implications. Kopytek et al. (73) studied the effects of rivaroxaban (an inhibitor of factor Xa) and the VKAs warfarin and acenocoumarol on clot viscoelastic properties from VTE patients' plasma samples. A main difference between this study and conventional clinical setups is that the study was not performed in whole-blood samples, which may impair its clinical significance, as platelets and erythrocytes modulate clot mechanics (31, 71, 84). Results indicate that VTE patients taking rivaroxaban or VKAs have similarly improved clot viscoelastic properties to those of VTE patients after oral anticoagulant treatment cessation. Another study on the mechanical properties of fibrin clots prepared from whole blood of VTE and non-VTE patients taking warfarin (mean INR 2.7 for both groups) has shown that VTE subjects are still characterized by abnormal clot microstructure through increased Df and probably require more efficient anticoagulant therapy (80).

Young et al. (152), through TEG measurements, evaluated the effect of direct thrombin inhibitors (DTIs) on clot strength. They showed that the DTIs argatroban and bivalirudin do not alter clot rigidity or elasticity. Furthermore, the reduced bleeding reported with DTIs versus heparin-based molecules may relate to the fact that clots are formed with normal rigidity and elasticity.

Recently, a therapeutic approach for coagulopathy-related phenomena occurring after high-dose, low-molecular-weight heparin, was tested in an intensive care unit on COVID-19 patients with antifactor Xa (117). The authors of this study observed that antifactor Xa activity was within the range of the pharmacodynamic endpoint, but viscoelastic tests demonstrated a procoagulant pattern, with maximum clot strength and fibrin clot strength above the reference values. There is still open debate about how to achieve the optimal doses of the treatment. However, apixaban, another factor Xa inhibitor, was shown to decrease clot stiffness in a concentration-dependent manner, in blood samples from healthy donors, by slowing down thrombin generation (107).

Acquired fibrinogen deficiency is a major determinant of severe bleeding in different clinical conditions, including cardiac surgery, trauma, postpartum hemorrhage, liver surgery, and transplantation (75, 111). Existing guidelines recommend supplementing fibrinogen to patients with severe bleeding when fibrinogen concentration is below 1.5 g/L (75, 111, 131). Viscoelastic tests provide a fast determination of the fibrinogen contribution to clot stiffness and allow prompt treatment of acquired fibrinogen deficiency (110).

As mentioned above, several chemical treatments for clotting disturbances use anticoagulants and VKAs. Although these treatments are efficient in preventing blood clotting, when it occurs a more invasive mechanical approach is needed to remove the clot, such as endovascular thrombectomy. A recently published work showed that clot stiffness affects the efficacy of different thrombectomy devices on recanalization rates (69).

DISCLOSURE STATEMENT

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