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Chapter 1

An Insight into the Abnormal Fibrin Clots — Its Pathophysiological Roles

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Additional information is available at the end of the chapter

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1. Introduction

Blood coagulation and its dissolution (thrombolysis) are integrated and highly regulated process to maintain the homeostasis. The mechanism of blood clotting and declotting and the equilibrium between them exclusively depends on an intricate interplay between series of elements – the coagulation factors (mostly proteolytic enzymes), platelet and endothelium [1]. Under normal conditions, tissue factor (TF) is not expressed by cells that are in direct contact with blood. However, TF is exposed to blood following the damage to the endothelial cell wall, where it is free to bind plasma factor VII and initiate the clotting cascade consisting of two separate pathways – 'intrinsic' and 'extrinsic' that ultimately converge on the 'common' pathway and serve to activate prothrombin, the precursor of the enzyme thrombin (factor IIa) by factor Xa [2]. The intrinsic pathway is initiated by the Hageman factor (factor XII) once it binds to the anionic surfaces *e.g.* polyphosphates from platelets or RNA in inflammatory loci [3,4]. A complex of prekallikrein and High Molecular Weight Kininogen (HMWK) also interacts with the exposed surface in close proximity to the bound factor XII and activates it. During activation, the single chain protein of the native factor XII is cleaved into two chains of 50 and 28 kDa that remain linked by a disulphide bond. The light chain of 28 kDa contains the active site and the molecule is referred to as activated factor XIIa, which in turn activates prekallikrein to form kallikrein. The kallikrein thus produced can then also cleave factor XII and a further amplification mechanism is triggered. The factor XIIa remains in close contact with the activating surface and activate factor XI. This step requires Ca^{2+}. At this stage, HMWK, binds to factor XI and facilitates the activation of factor X to form factor Xa [5,6]. The extrinsic system, in contrast to the intrinsic pathway, involves both blood and vascular elements and provides rapid response to tissue injury by generating activated factor X. TF and factor VII are the unique proteins present in this pathway. Once exposed to blood plasma, TF binds rapidly

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to factor VII which becomes activated to form factor VIIa. Factor VIIa along with Ca^{2+} and a phospholipid rapidly activates factor X forming factor Xa (Figure 1). Factors II, VII, IX, and X are the zymogen forms of vitamin K-dependent serine proteases. Vitamin K is an essential cofactor for post-translational modification of these proteins, in the course of which a carboxyl group is added to the 10 to 12 Glu residues in the amino terminal portion of these proteins. Without this modification, the cell-based coagulation complexes remain unassembled that leads to ineffective clot formation [7].

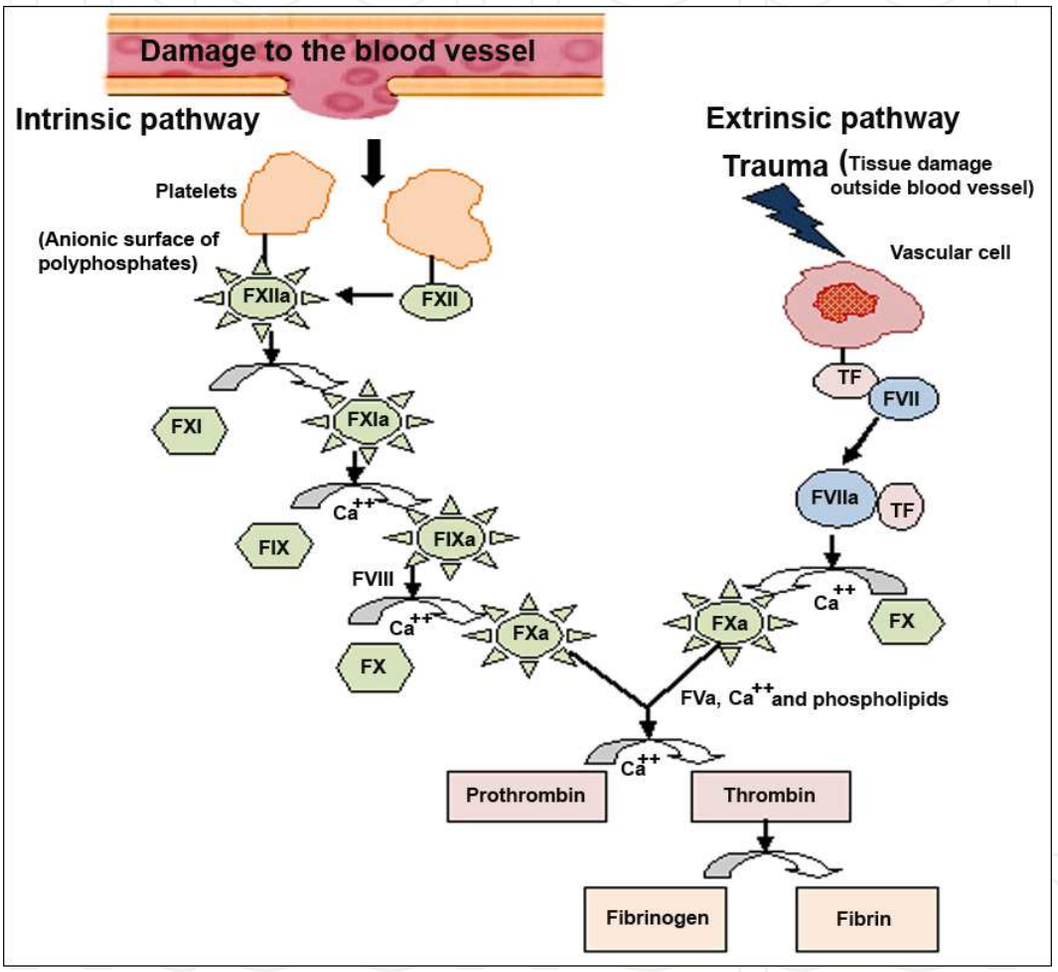
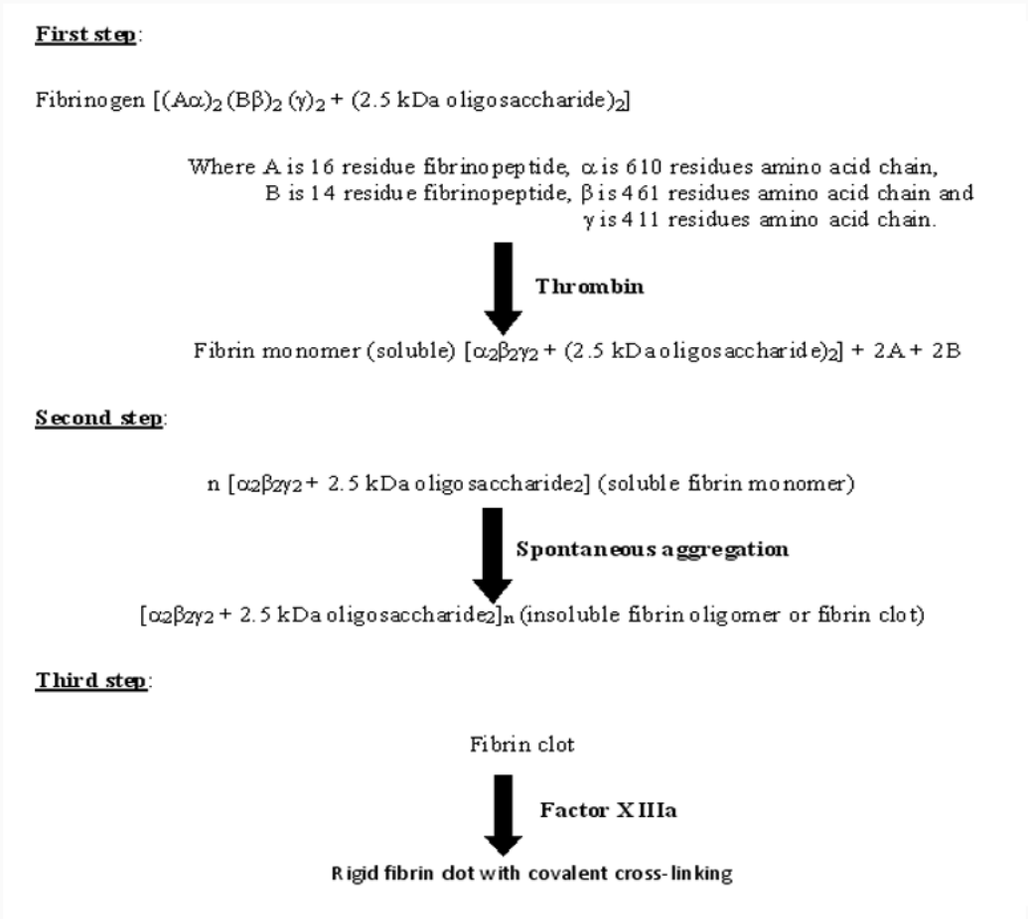


Figure 1. The classical blood coagulation cascade.

2. Formation of fibrin clot

Fibrinogen, a 340 kDa plasma protein, is present at a concentration of 2–4 mg/ml in blood under normal conditions [8]. Composition of fibrinogen, its cleavage by thrombin and subsequent polymerization reactions leading to blood clot are as described in Scheme 1.



Scheme 1. Steps of fibrin clot formation.

It is noteworthy that unlike an organic chemistry reaction of the type A+B →C, where the structures of A, B and C are defined, Scheme I does not imply that the products of the reactions are chemically and physically homogeneous. The extent of cross-linking, elongation and branching of the fibrils, incorporation of other proteins present in blood plasma *etc* affect the molecular weight, conformation, size and shape, stability and rigidity of the fibril structure. Thus the subsequent steps associated with such reactions may be represented as A+B →C →C_1 → C_2 →C_3 … *etc* together with the simultaneous reactions A+B →C →C_1; C → C_2; C→C_3 …. *etc* or a combination thereof. Further, in case of A+B →C, the rate of formation of C is proportional to the concentrations of A and B. However, with variation of fibrinogen and thrombin concentrations in the presence of other interfering blood components, the rate of formation of different conformers of fibrin also varies. Overall, due to so many variable parameters, the process is rather complex and the products formed are still difficult to predict qualitatively and quantitatively.

For a healthy person, when a clot is formed under normal physiological conditions following the above mentioned steps, it may be considered as normal. However, from a chemical point of view, when any one of the reaction conditions is altered, an abnormal clot is likely to be formed. The interferences may originate from alteration of the concentration of the substrate

or corresponding enzyme. In addition, the composition of the reaction medium during the course of the reactions, particularly presence of other biomolecules that interfere, may vary. In either situation, the structure and composition of the products formed may differ significantly. In a diseased condition, the concentration of fibrinogen or thrombin may increase leading to greater accumulation of soluble monomeric form of fibrin [9]. Since the monomeric form of fibrin leads to physically heterogeneous aggregates and the reaction involves bulky macromolecules like proteins, the structure of the product *i.e.* fibrin clot is dependent on the rate of the reaction as well. In other words, unlike the reactions of small organic molecules where the structures of the products are defined and not flexible, structure of fibrin is dependent on the rate of its formation. Further, the process of fibrin aggregation being occurred in a biological environment may recruit other adhering proteins too leading to co-aggregate formation. Thus, whenever the composition of blood differs from normal, the nature and composition of the co-aggregate also differ. The altered structure of fibrin is stabilized in the third step where factor XIIIa enters to form covalent cross links within fibrin clots. If this factor acts favorably, the clot formed will be more stable and hard. The extent of deformation, intramolecular cross linking and incorporation of other proteins in the fibrin structure affect its susceptibility to lysis by plasmin (discussed elaborately later). Usually deformed clots which are resistant to lysis cause medical complications. For a patient, this complication is in addition to those for which normal blood composition is not maintained.

Upon injury, thrombin cleaves off two short peptides from the N-termini of the Aα- and Bβ-chains of fibrinogen, releasing fibrinopeptides A and B, respectively from the center of the fibrinogen molecule, converting it to fibrin monomer, which polymerizes into half-staggered oligomers that lengthen to form ~10 nm wide protofibrils. These protofibrils aggregate laterally to make ~ 100 nm thick fibers. Branching along with lateral and longitudinal growth of fibers leads to the formation of three-dimensional network or gel, which tends to remain localized to the phospholipid-rich sites, *e.g.*, on the surface of activated platelets [10]. Blood clotting factor XIIIa, a plasma transglutaminase specifically cross-links glutamine and lysine residues of adjacent γ- and α-chains of fibrin molecules. These cross-links are formed within and between the protofibrils to stabilize the fibrin gel [11]. The activity of factor XIIIa plays a crucial role at this stage because it determines the stiffness of the clot. In case the clot is too soft, normal pressure of blood flow may break it leading to continuation of bleeding. Alternately, if the clot is too hard, it inhibits the pathway for normal healing of the wound. A serious concern is that the clots may be degraded to clot-lets by the hydrolyzing enzymes and these microemboli[1] enter into the circulation leading to heart attacks and strokes [12]. An optimum degree of cross-linking does not make the fibrin network mechanically very stable rather it can bend where its elasticity is apparent. In a dynamic system, the elasticity offers access of hydrolytic enzymes to the cleavable polypeptide chain of fibrin network [13]. Activation of the clotting cascade subsequently initiates the fibrinolytic cascade that regulates the size of the ultimate clot. Perturbation in this equilibrium due to excess or abnormal thrombus formation may lead to serious pathological problems.

1 When a thrombus detaches from the vessel wall and circulates in the blood.

Based on above discussions, varieties of fibrin clot may be viewed as described in Figure 2. The differences between the clots appear to be the thickness of the fibers and the porosity of the mesh structure that are primary determinants of the action of plasmin. What is hidden is the extent of branching of the chains. All these factors contribute to the elasticity of individual fiber and finally elasticity of the fibrin clot. The elasticity of the clots is an important physical parameter that determines the stability of the clots against the pressure of blood flow. In case the clots are sufficiently elastic, they may get enough time to be degraded by lytic enzymes. Otherwise, clot-lets may form and carried downstream in the blood circulatory system [14].

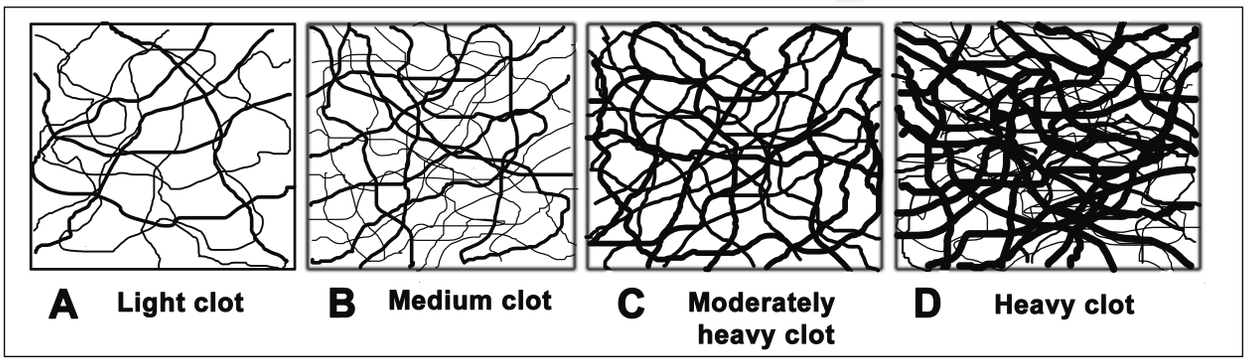


Figure 2. Hypothetical structures of fibrin clot of variable fiber thickness and porosity.

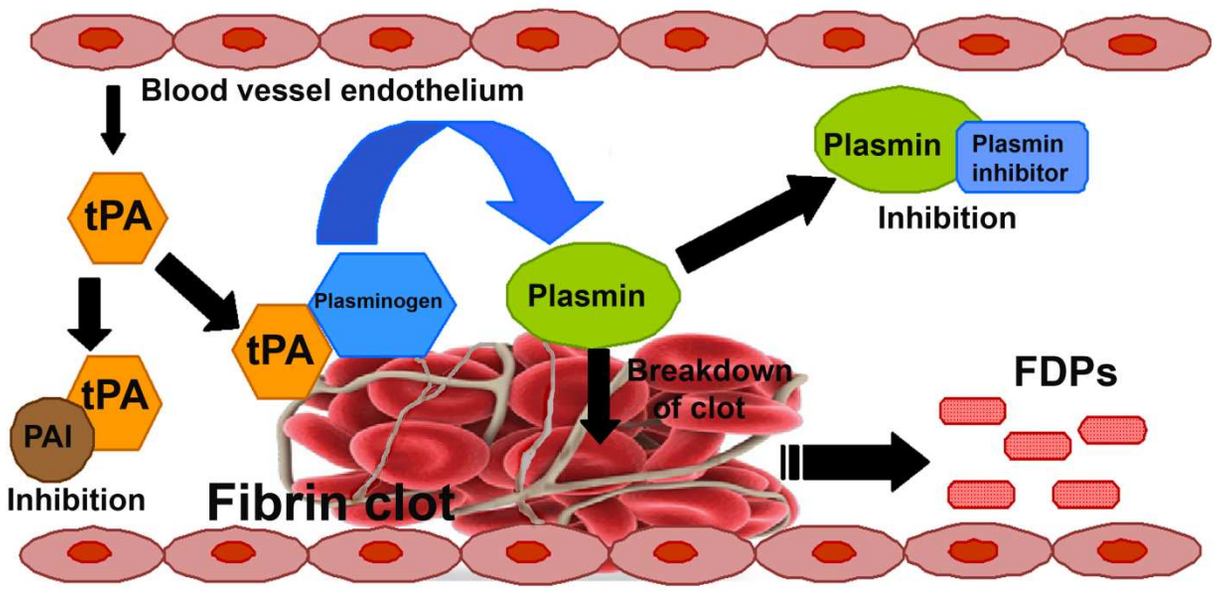


Figure 3. Interplay of enzymes in the process of fibrinolysis. Abbreviations used are FDPs, fibrin degradation products; PAI, plasminogen activator inhibitors; tPA, tissue plasminogen activator.

3. Mechanism of fibrinolysis

When a clot is formed, some mechanism is necessary to limit the clot at the site of injury and ultimately to remove the clot during healing of the injury. Platelet-poor areas of the clot are more prone to fibrinolysis than platelet-rich areas [15]. Fibrin actively regulates its self-dissolution through numerous interactions with fibrinolytic and anti-fibrinolytic components. This pathway consists of plasminogen, a variety of activators and several inhibitors (Figure 3). The 148-160 stretch of residues of the Aα-chain of fibrin becomes exposed and available for plasminogen binding after the conversion of fibrinogen to fibrin. Activation of plasminogen to form plasmin is accomplished either by factor XII-dependent pathway or by plasminogen activators like tissue plasminogen activator (tPA) and urokinase-like plasminogen activator (uPA) [16]. The tPA, which is synthesized primarily by microvascular endothelial cells is most active when attached to fibrin. The affinity for fibrin makes tPA a useful therapeutic agent, since its activity is largely confined to the sites of recent thrombosis [17]. uPA lacks fibrin binding activity, circulates in an inactive single chain form (scu-PA) in plasma and can activate plasmin in the circulation [18]. Plasmin interferes with the fibrin polymerization and initiates cleavage of fibrinogen or soluble fibrin from the C-terminal end of its α-polypeptide chain and gradually forms smaller fragments leading to formation of fibrin degradation products (FDPs) fragments X, Y, D and E in plasma. Cleavage of cross-linked fibrin by plasmin produces degraded products of variable lengths known as X-oligomers, that subsequently degrade into Y, D and E fragments [19]. Elevated levels of FDPs are clinically significant in diagnosing abnormal thrombotic states including Disseminated Intra-Vascular Coagulation (DIC), deep venous thrombosis or pulmonary thromboembolism (described in detail later). The activity of plasmin is tightly regulated to prevent excessive fibrinolysis, which is manifested by a bleeding tendency. Free plasmin rapidly forms a complex with circulating α_2-plasmin inhibitor and is inactivated. Endothelial cells further modulate the coagulation/anticoagulation balance by releasing plasminogen activator inhibitors (PAIs), which block fibrinolysis and confer an overall procoagulation effect. Thrombin also upregulates the expression of uPA and tPA and their inhibitor PAI-1 and regulates fibrinolysis [20].

4. Abnormal fibrin clot

Alteration in fibrin polymerization, heterogeneous fibrillization or unusual structural conformation may lead to the formation of an unstable thrombus. Abnormal fibrin network can make thrombi excessively resistant to degradation or too fragile [21]. Binding of plasminogen to fibrin during fibrinolysis has been reported to be dependent on the fibrin network conformation and fiber diameters. Fibrin fibers are generally intersected laterally rather than by progressive uniform cleavage around the fiber [22]. Clots with a fine fibrin (tight) conformation display a slower lysis than those with a coarse fibrin (loose) conformation, whereas, clots made of thin fibers may be lysed faster than clots having thick fiber. Fibrin network architecture rather than fibrin fiber diameter regulates the distribution or accessibility of fibrinolytic components during the course of fibrinolysis [23]. Longstaff *et al*, (2011) recently showed that

accessibility of the clot to fibrinolytic proteins and alterations in binding of tPA and plasminogen were both regulated by fibrin structure [24].

5. Factors affecting formation of abnormal clots

The structure and functions of fibrin clot are determined by genetic and acquired factors. Other parameters such as microgravity, pH, temperature, reducing agents and concentration of chloride and calcium ions may also affect the conversion of fibrinogen to fibrin; *e.g.* calcium stabilizes the structure of fibrinogen, accelerates fibrin formation and can partially protect fibrinogen from degradation. With advancement of space research substantial rise in quantity and quality of manned space flights has provided opportunity for the eventual long-term inhabitation of space, either on stations or other planets. Within space, a variety of altered circumstances including changes in gravitational status, neuro-immunoendocrine modulations, radiation affect the dynamic equilibrium of human body. Traumatic injuries often occur to the astronaut during space travel for which efficient healing is required. Microgravity plays an important role during wound healing. It has been found that fibrin gels formed in such microgravitational condition are more homogeneous than those formed at normal gravity, although the fibre diameter and matrix porosity remain unaltered. Changes in temperature and concentration of proteins like, fibrinogen and thrombin substantially affect fiber diameters and porosity of fibrin clot [9,25,26].

5.1. Genetic factors

Genetic abnormalities in the fibrinogen genes (4q28.1, 4q28.2, and 4q28.3) may cause low levels of fibrinogen in blood, even fibrinogen production may be stopped. It leads to bleeding problems in patients [27]. On the other hand, other genetic abnormalities may lead to the production of fibrinogen molecules with abnormal structure and function which affect the binding of fibrinogen with thrombin, resulting in the defective polymerization of fibrin molecules or fibrinolytic inactivation by plasmin. This condition, known as 'dysfibrinogenaemia', has an autosomal dominant or recessive mode of inheritance [28]. Changes in chromosomes 5, 6, 9, 16 and 17 in which the quantitative trait loci for fibrin structure are located lead to formation of abnormal architechture of clot [29]. A Common fibrinogen Bβ-chain polymorphism, BβArg448Lys, has been shown to affect fibrin structure in plasma clots. Fibrinogen β-chain plays a crucial role in conformational changes in Cα- region and lateral aggregation of fibrin protofibrils. This determines fiber thickness and final ultrastructure of the clot. The location of the BβArg448Lys polymorphism is relatively close to three important areas, the proposed β-chain polymerization site; a β-chain interaction site for the Cα-region and a β-chain calcium-binding site. Thus this polymorphism affects the fibrin structure and rigidity. Recombinant Lys 448 and wild type fibrinogens also showed differences in fibrin structure, both in purified systems and in plasma [30]. The α-chain Thr312Ala polymorphism is associated with formation of thick fibrin fiber with increased cross-linkage, because, this polymorphism lies close to the factor XIIIa cross-linking site at position Aα 328 [31]. Clots produced with a splice variant of γ fibrinogen (γ′ fibrinogen) have thinner fibers, more branching which

are more resistant to lysis [32]. Fibrinogen Naples I, is an abnormal fibrinogen with a single base substitution (G to A) in the Bβ-chain gene (Ala68Thr). This polymorphism results in inefficient binding between fibrin and thrombin causing decreased release of fibrinopeptide A and B in both homozygous and heterozygous abnormal fibrinogens. Individuals homozygous for this defect had a severe history of both arterial and venous thrombosis [33]. Most of the plasma glycoproteins have N-linked oligosaccharides attached to the appropriate Asn moieties of the peptide core; for example, fibrinogen contains sialic acid, galactose, mannose, and N-Acetylglucosamine which occurs as a biantennary complex, N-linked to Asn364 of each Bβ and to Asn52 of each γ chain [34]. Six hereditary dysfibrinogens have been reported to have an amino acid substitution that generates an Asn-X-Ser/Thr type sequence containing extra oligosaccharide at an Asn residue with the same biantennary structures found in normal fibrinogen. These are, fibrinogen Pontoise at BβAsn333, Asahi at γAsn308, Lima at AαAsn139, Caracas II at AαAsn434, Niigata at BβAsn158 and Kaiserslautern at γAsn380. Carbohydrate moieties in fibrinogen have been proposed to be involved in the regulation of fibrin assembly and form stable fibrin networks. However the extra-glycosylated dysfibrinogens cause altered fibrin assembly at various stages of fibrin network formation [35].

Factor XIII polymorphisms, *i.e*. G to T transition in codon 34, with subsequent replacement of valine with leucine (factor XIII Val34Leu) is associated with altered fibrin structure [36]. Thrombin activates factor XIII Leu34 more efficiently as compared to Val34. Early activation of factor XIII and presence of high concentrations of fibrinogen result in the formation of less permeable clots with smaller pores, thinner fibers and ineffective cross-linking [37]. 'Dusart syndrome' is a congenital dysfibrinogenemia characterized by reduced plasminogen binding, impaired fibrin-dependent plasminogen activation by tPA and abnormal fibrin polymerization and clot structure. 'Dusart' fibrinogen molecules contain disulfide-linked albumin molecules, most of which are bound in the carboxy-terminal region of the Aα554 [38, 39]. The Factor V Leiden is a single point mutation at position 1691 in exon 10 that cause G to A transition resulting in Glu506Arg substitution. G to A transition at position 20210 of the prothrombin gene results in G20210A prothrombin mutations that elevates the plasma concentration of prothrombin and in turn increases thrombin generation. These two mutations affect clot structure resulting in Venous Thrombo-Embolism (VTE) [40].

5.2. Acquired factors

The acquired risk factors include abnormal concentration of thrombin and factor XIII in plasma, blood flow, platelet activation, oxidative stress, hyperglycemia, hyperhomocysteinemia, medications, cigarette smoking, particulate matters in environment and interaction of fibrin with other proteins, the role of which are discussed in details.

Notably prothrombin concentration plays a major role in regulating fibrin structure as the fiber diameter of fibrin decreases with increasing prothrombin levels [41]. In both purified fibrinogen and plasma-based systems, clots produced with high thrombin concentrations (0.25 U/mL) are characterized by thin fibers that form a network with small pores [9]. Whereas in hemophilia B, reduced thrombin generation is associated with the formation of lysis suscep-

tible to loosely packed fibrin with thick fiber. Recombinant factor VIIa increases the rate of thrombin generation and clot stability [42].

There are controversies regarding the effect of blood flow on the structure and physical properties of blood clot. One study found no effect of flow on fiber diameter, whereas another group reported formation of thick and stiff fibers in the direction of flow, with thinner fibers interconnecting these larger fibers perpendicularly [43-45]. Blood flow also causes location-dependent changes in fibrin structure due to mechanical forces (radial, axial and circumferential) acting on the vessel wall. Thinner fibers with smaller pores that are formed on the surface of thrombi are resistant to lysis with plasmin [46].

Fibrinogen is 20 times more susceptible to oxidation than albumin and may therefore scavenge oxidants and protect other proteins from oxidation [47]. Oxidation of fibrinogen following exposure to oxygen, metals and myeloperoxidase-derived oxidants decreases the rate of clot formation, whereas, exposure of fibrinogen to Fe^{3+} ascorbate promotes clot formation and enhances platelet aggregation [47,48]. Fibrin structure and its lysability are also affected by nitration of two β-chain tyrosines in the fibrinogen molecule [49]. F2-isoprostanes, a marker of oxidative stress, shown to be associated with reduced clot permeability and fibrinolysis in cardiovascular patients [50].

Platelets release proteins at the sites of platelet aggregation that alters the properties of fibrin clot. Increased amounts of platelet factor 4 and PAI-1 contributes to the formation of compact clot structures and impaired fibrin degradation [51]. Polyphosphate, a polymer of 60-100 phosphate residues is a platelet-derived proinflammatory and procoagulant mediator that directly bind and activate factor XII. It affects intrinsic pathway by modulating the fibrinolytic system, factor V activation and fibrin structure. Polyphosphates lead to the formation of tight and thick fibrin aggregates having 3-fold higher turbidity [52]. It also impairs binding of plasminogen and tPA to partially lysed fibrin causing prolonged clot lysis. This process is Ca^{2+} dependent and independent of factor XIII activation [4,53].

Altered fibrin structure in hyperglycemia is attributed to fibrinogen glycation, which interferes with fibrin polymerization, cross-linking by factor XIIIa, tPA and plasminogen binding and production of plasmin. Fibrinogen purified from hyperglycemic patients produces denser and lysis-resistant clots. Treatment with insulin makes this fibrin more permeable as it decreases fibrinogen glycation [54].

Homocysteine (Hcy), a product of methionine metabolism, increases the risk for Coronary Artery Disease (CAD) and thrombosis. The ε-amino group of fibrinogen lysines can be modified by a highly reactive thioester, Hcy thiolactone, which is present in small amounts (0.2 nM) in plasma. Elevated level of Hcy thiolactone introduces free sulfhydryl groups to ten lysine residues in the D- and Cα-regions of fibrinogen that increases the size of the modified amino acid. This modification decreases the ability of fibrin to support tPA-induced plasminogen activation. This results in the formation of fibrin with thinner and more tightly packed fibers leading to increased resistance to fibrinolysis [55,56].

The smoking-related fibrin abnormalities appear to be determined largely by elevated fibrinogen and enhanced oxidative stress. It has been reported that following acute exposure to cigarette smoke, fibrin clots have dense and compact fibers compared to nonsmoking

samples [57]. Particulate matter which contained soluble components such as metal ions as well as ultra-fine particles (< 0.22 μm in diameter) has been reported to be capable of causing alterations to fibrin structure and clot permeability in an oxidation-dependent manner [58].

Aspirin (75 mg/day) reversibly increases clot permeability and fiber mass-length ratio up to 65%. *In vitro* model of acetylation of fibrinogen by aspirin showed that acetylation reduced rigidity of clots and enhanced lysis of clot. Aspirin inhibits factor XIII activation and fibrinogen oxidation. Ingestion of 300 mg aspirin increase clot permeability in subjects possessing the Leu34 allele of factor XIII [59,60]. S-nitrosoglutathione (GSNO), a low Mw member of S-nitrosothiols, is an important biological signaling molecule and has been used clinically as an antithrombotic agent. It has been reported to bind to Cα-region of fibrinogen and alters its secondary structure and the kinetics of fibrin polymerization. It also inhibits factor XIIIa activity and fiber cross-linking in a dose-dependent manner. GSNO at higher dose induces abnormal fibrin structures and fibrin agglomerates producing coarse clot networks with decreased fibrin density and increased fiber diameter which are more susceptible to lysis [61]. Fenfluramine (3-trifluoromethyl-*N*-ethylamphetamine), a drug used as a regulator of serotonin has been reported to cause clotting abnormalities [62]. Apart from cholesterol-lowering effects, statins reduce cardiovascular morbidity and mortality by increasing fibrin permeability and shorter lysis time. Quinapril, an angiotensin-converting enzyme inhibitor at 10 mg/day for 1 month can increase clot permeability by decreasing formation of thrombin in CAD patients [63]. Metformin, an oral antidiabetic drug affects the fibrin structure by interfering with fibrin polymerization and reduction of factor XIII-mediated cross-linking that leads to increased lysability [64]. Anticoagulant treatment with vitamin K antagonists, heparins, direct thrombin inhibitors, indirect thrombin inhibitors and direct activated factor X inhibitors affects fibrin structure and physical properties through reduced thrombin generation. This accounts for the formation of less compact and more lysable fibrin [65].

Vascular wall components like proteins (decorin or collagen fragments *etc.*) and glycosaminoglycans (chondroitin sulfate and dermatan sulfate *etc*) entrapped in the fibrin network affect mechanical and chemical stability of fibrin clot. The architecture of the fibrin network is not significantly influenced by the negatively charged glycosmanoglycans, but fully glycosylated decorin, containing the same sugar subunits, modify the fibrin structure. Protein modulators cause faster lysis of the clot, whereas glycosaminoglycans enhance plasmin mediated clot lysis [66].

Several plasma proteins are known to bind to fibrin and change the properties and function of the clot; *e.g.* lipoprotein(a) (Lp(a)) which is structurally similar to plasminogen, can compete with plasminogen for the binding of fibrin and thereby inhibit the formation of plasmin and eventually fibrinolysis. Lp[a] comprises of a heterogenous class of lipoprotein particles having a core of neutral lipids and a protein moiety containing one mole of apoB-100 covalently linked by a di-sulfide bond to one mole of apo[a]. The C-terminal domain of apo[a] containing the catalytic triad, His4350-Asp4393-Ser4481 exhibits a high degree of homology with the serine protease domain of human plasminogen. Apo[a] contains up to 54 kringles, among which, kringles IV and V (KIV and KV) are homologous to plasminogen. Kringles are involved in interactions with small molecules, for example, KIV-5, KIV-8 and KIV-10 have a high binding

affinity for lysine, lysine analogs, and fibrin(ogen) [67]. Elevated Lp(a) levels cause formation of thin fibrin fibers with less permeability and reduced susceptibility to fibrinolysis [68]. The fibrin clot is stabilized against tPA-induced fibrinolysis in the presence of 0.6 to 1.0 μM myosin. In the bound form the tPA-cofactor property of myosin is masked and the fibrin-myosin clot starts disassembling at a slower rate through plasmin degradation than the pure fibrin clot. Myosin weakens the interactions of FDPs leading to its polymerization that increased solubility of the partially degraded fibrin-myosin clot [69].

Binding of fibronectin to a fibrin clot is a two step process; non-covalent and reversible binding of fibronectin to fibrin is preceeded by covalent cross-linking by factor XIIIa. Fibronectin contains three domains with fibrin binding affinity. Low-affinity fibrin-binding sites are contained within the C-terminal region and the high-affinity fibrin binding site resides within the NH_2- terminus of the fibronectin molecule. Covalent cross linking between glutamine in fibronectin and the ε-amino group of a lysine residue in the α-chain of fibrin is mediated by factor XIIIa [70] Binding of fibronectin to fibrin upon injury is important for wound healing and tissue repair [71]. Increased concentration of fibronectin in blood causes the fibrin network to have thicker fibers and larger pores and is associated with arterial thrombosis [72]. Other proteins that bind to fibrin includes α_2-plasmin inhibitor, plasminogen activator inhibitor-2 (PAI-2), hepatocyte-derived fibrinogen-related protein-1 (HFREP-1), albumin, fibroblast growth factor-2, vascular endothelial growth factor, interleukin-1b, factor Xa, tissue factor pathway inhibitor, thrombin-activatable fibrinolysis inhibitor (TAFI), von Willebrand factor, thrombospondin, actin, factor V and factor XIII. α_2-plasmin inhibitor, PAI-2, TAFI, von Willebrand factor, thrombospondin, actin and factor V have been reported to cross-link with fibrin via factor XIIIa. Howes *et al*, described the total protein composition of the whole clot and identified proteins that are cross-linked via factor XIIIa [73]; whereas Talens et al, identified eighteen different fibrin clot-bound proteins, which are not cross-linked to fibrin via factor XIIIa [74]. These authors classified eleven out of the eighteen fibrin clot-bound proteins has been classified into three groups related to their function: blood coagulation, protease inhibition and high density lipoprotein (HDL) metabolism. Plasminogen, factor XIII and thrombin are involved in blood coagulation while α_2-macroglobulin and α_1-antitrypsin are protease inhibitors and haptoglobin, serum amyloid P and apolipoproteins A-I, A-IV, J and E are involved in HDL metabolism [74]. β-amyloid 1-42 (Aβ42) peptide may bind to each identical ends of fibrinogen and specifically interacts near the C-terminus of the fibrinogen β-chain and induces oligomerization. Fibrin clots formed in the presence of Aβ42 have abnormal structure and are resistant to degradation by plasmin. This abnormal fibrin co-deposit with Aβ42 and increase neurovascular damage in blood vessels [75]. Figure 4 shows co-aggregation of different plasma proteins with fibrin. When fibrinogen (1.5 μM) was incubated with thrombin (100 μU/ml), it forms a thread like fibrin fibers. Upon incubation with 1 μM of the plasma proteins, the fibrin forms unusually dense network with less porous structure. Fibrin and α-synuclein co-aggregate forms thread-like structure interspersed with dense network. These SEM images of co-aggregates support the previous finding that binding of plasma proteins with fibrin alters its fiber diameter, porosity. The dense clumps of aggregates might show poor lysability.

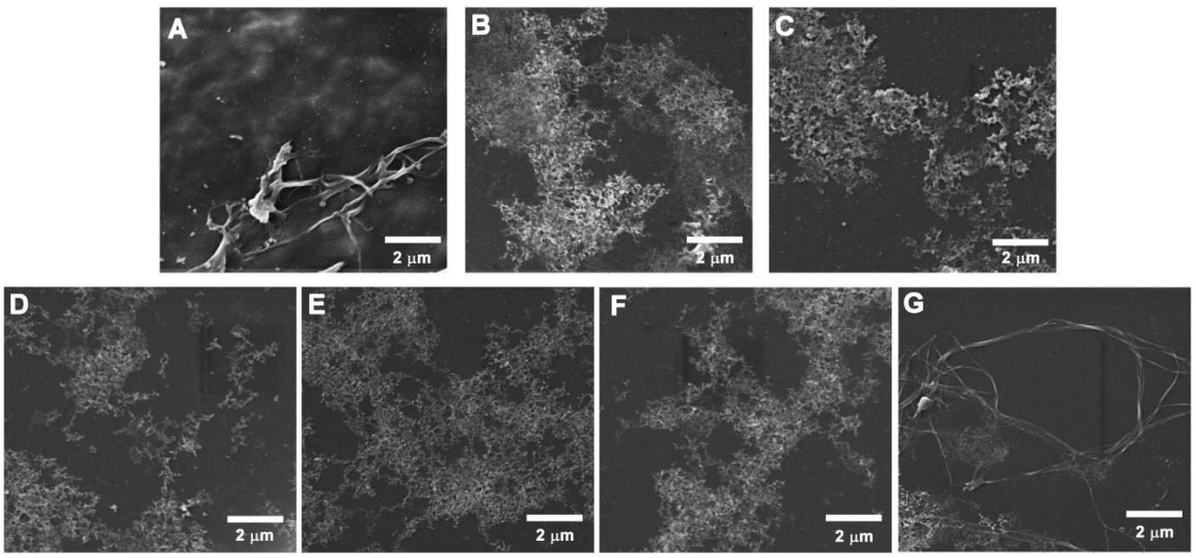


Figure 4. Morphology of abnormal fibrin co-aggregate. A. Normal fibrin network; B. Fibrinogen - fibronectin co-aggregate; C. Fibrinogen- Aβ42 co-aggregate; D. Fibrinogen - transthyretin co-aggregate; E. Fibrinogen - human serum albumin co-aggregate; F. Fibrinogen - lysozyme co-aggregate and G. Fibrinogen-A53T α-synuclein co-aggregate. In these experiments, 1.5 μM fibrinogen was incubated with 100 μU/ml of thrombin (after dilution from bovine thrombin, 1000 NIH Units, Sigma Aldrich, USA) at 37°C for 24 hr to form fibrin clot. The co-aggregate was prepared under identical conditions with 1 μM of the plasma proteins as stated above. Morphological analysis of protein aggregates was done using scanning electron microscope (Model: Vega II LSU, Tescan Digital Microscopy Imaging, Czechoslovakia). The sample (10 μl) was placed on a carbon coated 300-mesh grid for 5 min at 25ºC and the unbound substrate was removed by blotting paper. To stain the adhered particles, the grid was treated with 2% uranyl acetate for 20 sec and the excess reagent was removed as stated. The grid was dried under vacuum, sputter coated with gold-palladium alloy and viewed under SEM at 10.0 kV voltages and 20,000 x magnification.

6. Pathophysiological role of abnormal clots

Abnormally structured clots may also generate emboli that can lodge in critical organs, disrupting the blood flow with potentially fatal consequences like liver diseases, ischemic stroke, myocardial infarction, venous thromboembolism, atherothrombotic vascular disease, peripheral artery disease, coronary artery disease, adult respiratory distress syndrome, retinal vein occlusion, end-stage renal disease, acute pancreatitis, rheumatoid arthritis, type 1 and type 2 diabetes and Alzheimer disease.

6.1. Liver diseases

The increased sialic acid content in the oligosaccharide of the abnormal fibrinogen impairs polymerization of fibrin monomers leading to severe dysfibrinogenemia in patients with End Stage Liver Disease (ESLD) [76,77]. Accelerated fibrinolysis is attributed by the impaired clearance of tPA and other fibrinolytic enzymes by the diseased liver, without an appropriate increase in plasminogen activator inhibitors [78]. Impaired hepatic synthesis of fibrinolytic inhibitors like, α_2-plasmin inhibitor and TAFI contributes to increased levels of plasmin. The

hemostatic imbalance in ESLD occasionally favors hypercoagulability, predisposing to thrombosis [79].

6.2. Ischemic stroke

Four types of inherited abnormalities of fibrinolysis (plasminogen deficiency, plasminogen activator deficiency, dysfibrinogenemia and factor XII/prekallikrein deficiencies) are related to thrombosis. Fibrin clots were formed more rapidly and had a compact structure composed of thicker fibers and reduced permeability compared to those made from plasma obtained from healthy controls [80].

6.3. Myocardial infarction (MI)

The term myocardial infarction pathologically denotes the death of cardiac myocytes due to extended ischemia. Rupture of the atherosclerotic plaque[2] in an epicardial coronary artery contributes to the activation, adhesion and aggregation of platelets and the production of thrombin, causing subsequent thrombus formation which occludes the vessels and impedes blood flow [81]. Acute MI patients show the tendency to form less permeable and lysable fibrin clots that are composed of thicker fibers [82].

6.4. Venous thromboembolism (VTE)

Forming thrombus inside the vessel (intravascular thrombosis) of the lower extremities and to a lesser extent in the upper extremities may lead to partial or complete blockage of blood flow through this vessel causing a serious pathological problem known as deep vein thrombosis (DVT). When an embolus goes up through the circulation settling in an arterial branch in the lungs, it cause pulmonary embolism (PE). DVT and PE together are called venous thromboembolic disorders (VTE). In addition to abnormalities in the blood coagulation system due to increased thrombin generation, it can be caused by defective plasminogen, tPA deficiency and higher level of TAFI. These indicate enhanced fibrin formation and degradation [83]. Curnow *et al*, 2007 showed that patients with arterial thrombosis, VTE, pregnancy complications or autoimmune diseases have increased fibrin generation and reduced fibrinolysis [84].

6.5. Peripheral arterial disease (PAD)

It results from progressive narrowing of the peripheral arteries, most commonly in the pelvis and legs. In middle-aged and elderly PAD patients, it has been shown that plasma fibrin clots contain thicker fibers and smaller pores, which form more rapidly, but are lysed at a reduced rate, compared with those made from plasma obtained from healthy individuals [16].

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2 Deposition of a solid substance in the lining of the artery wall leading to hardening of the arteries. The core of the plaque is made of fatty substances, cholesterol, waste products from the cells, calcium, and fibrin, which is separated from arterial bloodstream only by a slender and fragile layer of tissue, the fibrous cap.

6.6. Coronary artery disease (CAD)

It is caused by the narrowing and hardening (atherosclerosis) of arteries which supply blood to the cardiac muscles. In CAD patients, plasma fibrin clots are denser and less permeable than healthy individuals. The fibrin clots with tightly packed, thin fibers and small pores are associated with the number and severity of coronary artery stenoses (diseased arterial tissue) documented by angiography. C reactive protein (CRP) binds to fibrin(ogen) and may alter fibrin network formation, clot permeability and susceptibility to lysis both in healthy and CAD patients [85].

6.7. Adult respiratory distress syndrome (ARDS)

Alveolar fibrin deposition is one of the hallmarks of this syndrome. In ARDS the increased PAI and α_2-plasmin inhibitor levels lead to decreased fibrinolytic activity and increased alveolar fibrin deposition [86]. It appears to contribute to the magnitude of the inflammatory response by virtue of their ability to cleave and degrade products to promote chemotaxis, increase vascular permeability and exert modulatory effects on various immune cells. It also causes lung fibrosis by providing a matrix for macrophage migration and by promoting angiogenesis and collagen deposition [87].

6.8. Rheumatoid arthritis (RA)

During inflammation, the exudation of plasma into joints results in accumulation of high concentration of coagulation factors at the synovial fluid and often accompanied by fibrin deposits. Patients with RA have faster clot formation, higher clot absorbance at 405 nm, indicating presence of thicker fibrin fibers than healthy individuals. Moreover, the clot is less permeable and lysis time is longer. Fibrin deposition is correlated with fibrinogen, tPA, PAI-1, PAI-2, CRP, platelet count and 8-iso-prostaglandin F_2 alpha, an inducer of oxidative stress [88]. Local activation of complement system helps to stabilize fibrin clots thereby decreasing the fibrinolytic potential at the joint. Local production of the regulatory factor C4B-binding protein (C4BP) by rheumatoid synovial fibroblasts as well as its co-localization with fibrin-rich areas at the synovial tissue contributes to fibrin deposition at synovial joints. Fibrin is one of the major substrate for peptidyl deiminases that transform Arg residues into Cit (citrulline)of fibrin and subsequently change its physical properties inside inflamed joints. This modification makes the clot resistant to proteolytic degradation by altering the binding sites for plasmin. Further, the deformity turns the molecule antigenic [89].

6.9. Type 1 and type 2 diabetes

Diabetic patients suffer from persistent hyperglycaemia, which cause protein glycation. Protein glycation generates glycoaldehyde that induces post-translational modification in fibrinogen, which impairs the fibrinolytic process. Decreased binding of tPA and plasminogen to fibrin, reduced plasmin generation on the clot surface and increased cross-linking cause formation of dense, less porous fiber with reduced lysability in diabetic patients as compared to healthy non-diabetic persons [90].

6.10. Retinal vein occlusion (RVO)

Abnormal fibrin clot with poor lysability contribute to the hyperviscosity reported in this disease. Elevated level of CRP, which binds to fibrinogen might be responsible for this abnormality [91].

6.11. End-stage renal disease (ESRD)

In ESRD patients, plasma fibrin clots have reduced permeability, faster protofibril formation, increased fiber size and mass, decreased susceptibility to fibrinolysis, compared with healthy individuals. The plasma concentration of the acute phase protein fibrinogen plays major role in regulating fibrin structure properties. Besides the levels of other acute phase proteins such as orosomucoid, CRP and interleukin 6 (IL-6) have also been reported to affect the tightness and density of plasma clots [50,92,93].

6.12. Alzheimer disease (AD)

Fibrinogen circulates through the brain and spinal cord vasculature without entering the central nervous system (CNS) due to blood brain barrier (BBB) [94,95]. However, in pathological conditions like injury or diseases associated with vascular disruption, infection or inflammation, the concentration of fibrinogen increases beyond its normal limit (2-4 mg/ml) and enters into CNS through disrupted BBB [96]. The synergistic effect of higher fibrinogen level and presence of Aβ peptide produce lysis resistant clots in neurovascular diseases, which contributes to vascular deficiencies, decreased blood flow, increased inflammation and neuronal death leading to higher severity of AD [75]. Aβ can alter fibrinolysis by three independent mechanisms; Aβ intercalates into fibers during formation of fibrin network promoting generation of clots with an abnormally dense fiber network, blocks binding of plasminogen to fibrin and therefore blocks generation of plasmin and finally as a result, alters the rate of plasmin-mediated fibrinolysis [97,98].

7. Role of plasmin

The suitability of a protein as a substrate of a proteolytic enzyme primarily depends on two factors; specificity of the enzyme *i.e.* the peptide bond of the amino acids that the enzyme targets to hydrolyze and accessibility of the hydrolysable bond to the catalytic site of the protease. Thus, mere existence of a proteolytically cleavable bond in the primary amino acid sequence of a protein does not ensure it to be hydrolyzed by a protease until the bond is physically accessed by the catalytic site of the enzyme. Because of this stringency, many proteins can survive proteolysis while maintaining compact configuration of the native state in an environment of proteases whereas the partially or fully denatured state of the same protein is easily degraded by the proteases. Sometimes it also happens that a proteolytically sensitive region of a protein is first cleaved off by a protease followed by complete unraveling

of the molecule in a cooperative manner leading to its fragmentation. For large multi-domain proteins, usually the domains are connected by proteolytically sensitive hinge regions. Once the domains are cleaved off, structural integrity of each domain is lost facilitating digestion by the proteases. Therefore, if the peptide bonds of fibrin polymer (clot) that are otherwise hydrolysable by plasmin are no more accessible to the enzyme due to alteration of the structure of the clots, fibrin in its modified form may be partly or completely resistant to plasmin. For example, tighter fibrin networks composed of thin fibers are degraded less efficiently by plasmin than those composed of thick fibers due to two reasons; first, an increased number of fibers to be cleaved and second, decreased porosity of tighter fibrin networks make the fibrinolytic enzymes inaccessible to the hydrolysable bonds [99]. In case the fibrin clot is not constituted of pure fibrinogen rather a copolymer with other protein/s or ligand, the situation becomes even more complicated. The added molecules may sterically protect the hydrolysable bonds of fibrin from the action of plasmin. Taken together, the fibrin clot may be completely resistant to plasmin, *e.g*. Aβ binds to the fibrinogen β-chain near the β-hole, which is in close proximity to residues 148-160 of the Aα-chain and modifies the structure in such a way that it inhibits plasmin to bind the copolymer [98].

In the dissolution of the clots, the substrate (fibrin) is virtually static. It is only the enzyme plasmin that is free in the solution and is capable of searching and recognizing the hydrolysable bonds. From an enzymologist's point of view, these reactions are difficult to take place, slow and are not supposed to follow normal Michaelis-Menten relation of enzyme kinetics. From the above discussion, it is apparent that when dealing with abnormal clots, presence of plasmin may not be limiting. It is the deformation of the structure of the fibrin clot that prevents it from being degraded by plasmin.

8. Prevention and treatment

At present, there are three major classes of medicines to treat patients with a thrombophilic disorders: antiplatelet, anticoagulant and thrombolytic agents. There are several medications that are used to inhibit platelet aggregation through the process by which platelets clump together to plug the injured surface. Among them aspirin, dypyridamole, ticlopidine and clopidogrel are orally administered, glycoprotein IIb/IIIa (GP IIB/IIIA) inhibitors are intravenous (IV) forms, whereas, non-steroidal anti-inflammatory drugs (NSAIDs) are available in either of the forms stated. The anticoagulants, which act through inhibiting or altering steps in the coagulation cascade, include warfarin (Coumadin), heparin *etc*. Thrombolytic medications serve to break up the fibrin clot. This includes streptokinase, uPA, tPA and their recombinant variants. Streptokinase binds with plasminogen and ultimately forms streptokinase and plasmin complexes. These complexes are more efficient than plasmin alone at breaking down a clot. Excessive bleeding is a serious consequence of using these medications [100]. The specific impact of the abnormal fibrin structure on the efficiency of each of these therapeutic agents has not been comprehensively characterized to date.

The activity of fibrinolytic enzymes isolated from natural sources often resembles the activity of plasmin and plasminogen activators [101]. Earthworms have been used in East-Asian traditional folk medicine for thousand years for the antithrombotic effect. Later, Mihara *et al*, 1983 first isolated lumbrokinase (LK), a fibrinolytic enzyme from the *Lumbricus rubellus* (earthworm) [102]. Few fibrinolytic enzymes have been isolated from earthworm *Perionyx excavates*, which show rapid hydrolysis on both coagulated fibrous fibrin and soluble fibrinogen monomers in absence of activators such as tPA or urokinase [103]. Snake venom proteases possess coagulatory and fibrinolytic activities. Fibrino(geno)lytic enzymes have been isolated from the venoms of *Agkistrodon acutus*, *A. contortrix*, *A. rhodostoma*, *A. halys brevicaudus*, *A. piscivorus piscivorus*, *A. piscivorus conami* and *Crotalus atrox etc* [104]. Russell's viper (*Daboia russelli russelli*) venom contains a fibrinolytic enzyme that also shows hemorrhagic activity. Exposure to 90°C irreversibly destroys the hemorrhagic activity of this enzyme while its fibrinolytic activity could be restored on cooling [105]. Caffeic acid phenethyl ester (CAPE), a phenolic compound found in honey bee product has been reported to have fibrinolytic activity [106].

Microbial fibrinolytic enzymes have been isolated from bacteria (*e.g. Streptomyces sp*, *Actinomyce sp etc*), fungi, and algae. Streptokinase and staphylokinase are two well-known plasminogen activators from *Streptococcus hemolyticus* and *Streptococcus aureus*, which have been found to be effective in thrombolytic therapy. Fibrinolytic enzymes have also been purified from fermented products like Japanese natto, Korean Chungkook-Jang soy sauce, dochi, fermented shrimp paste, salt-fermented fish, fermented vegetables, *e.g.* Kimchi and Indonesia soy products, *e.g.* Tempeh [107]. The first commercial fibrinolytic enzyme, nattokinase was purified and characterized from natto, a popular soybean food in Japan, which is fermented by *Bacillus subtilis natto* [108]. Fibrin(ogen)olytic enzymes have been identified from mushrooms like *Pleurotus ostreatus*, *Armillaria mellea*, *Tricholoma saponaceum*, *Cordyceps militaris*, *Ganoderma lucidum*, *Fomitella fraxinea*, *Cordyceps sinensis*, *Flammulina velutipes*, *Fusarium sp.* and *Schizophyllum commune* [107].

Antioxidant therapy using vitamin C showed satisfactory result in patients having type 2 diabetes with CAD by regulating the fibrinolytic system [109]. High dosages of vitamin C and vitamin E in combination have been reported to improve endothelial function and decrease plasma levels of PAI-1, von Willebrand factor and PAI-1/tPA ratio in chronic smokers, thereby keeping under control the excessive thrombotic trend in these patients [110]. Astaxanthin, a red pigment carotenoid found in salmons and crustacean species, protects experimental animal models from vascular oxidative damage, hypertension and cerebral thrombosis [111]. It has been found that folic acid, vitamin B6 and vitamin B12 are very effective at lowering homocysteine and thereby prevent thrombosis [112]. Therapeutic approaches using these natural product-derived fibrin(ogen)olytic enzymes and antioxidants showed promising results in both experimental and clinical settings. However, there are no reports regarding the application of these molecules for prevention of abnormal fibrin clot formation.

9. Conclusion

Abnormal blood clots are formed by a variety of reasons leading to variable structures of the clots. Therefore, it is difficult to conceive that they could be treated by a general protocol. Information on this part is scanty. Our ongoing investigations indicate that there are fibrinolytic enzymes from plant and mammalian sources that are capable of efficient degradation of the fibrin-plasma protein co-aggregates (P. Bhattacharjee and D. Bhattacharyya, manuscript to be communicated elsewhere). Whether these enzymes may be upgraded to drugs remains speculative at this stage. We have a feeling that remedies for combating abnormal clots will be available from natural sources in due course.

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