FIBRINOGEN HETEROGENEITY AND CONTRIBUTION TO WOUND HEALING

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ABSTRACT

Fibrinogen is an essential matrix to prevent local hemorrhage, to interact with proteins, proteases, protease inhibitors, growth factors and cells, to induce and modulate cell responses, and to facilitate the wound healing process. Since fibrin matrices are a great potential for wound repair and tissue regeneration, they have been utilized as fibrin sealants for multiple tissue engineering applications, including peripheral nerves repair, bone regeneration, skin grafting after burn, and induction of angiogenesis.

HAEMOSTASIS

Maintaining the integrity and patency of the vascular system is essential for the viability of humans. When vascular injury has occurred, fast formation of a thrombus at the site of injury is essential to seal the wound, resulting in haemostasis. Haemostasis is a tightly regulated process, which involves the activation of endothelial cells, platelets, procoagulants and the inhibition of fibrinolytic factors. Haemostasis can be separated in two phases called primary and secondary haemostasis, which occur simultaneously. In primary haemostasis, a platelets plug is rapidly formed at the site of injury, whereas in secondary haemostasis, blood coagulation is initiated, either with negatively charged surfaces (intrinsic pathway) or with tissue factor (extrinsic pathway). The cascade leads to the generation of thrombin and the formation of a fibrin network.[1] The thrombus provides an effective restriction for bleeding. Hence, an imbalance of normal haemostasis caused by pathologic disorders may lead to thrombosis or hemorrhage, which may account for morbidity and mortality.

FIBRINOGEN

Fibrinogen is a central protein in the hemostatic system. At the final stage of the blood coagulation system, thrombin converts the soluble fibrinogen into fibrin monomers, which then polymerize to an insoluble fibrin clot. Fibrinogen is a plasma glycoprotein that is mainly synthe-

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sized by hepatocytes in the liver. In plasma, the circulating concentration of fibrinogen is maintained between 2 and 4 mg/mL (6 -12 μM), and its half-life is about 3-4 days. Fibrinogen is an acute phase protein, its levels are up-regulated 2 to10-fold through the action of interleukin 6 (IL-6) and glucocorticoids during the acute-phase response to inflammation, infection and tissue injury.[2-3]

Plasma fibrinogen levels are associated with many demographic and environmental factors in healthy individuals, such as age, body mass, gender, race, season, smoking, physical exercise, diet and use of several drugs.[3]

Structure of fibrinogen

Fibrinogen molecules are elongated 45 nm structures that consist of identical disulfide-bridged halves, each consisting of three polypeptide chains termed Aα, Bβ and γ.[4] These six polypeptide chains assemble to construct a hexamer $(A\alpha B\beta\gamma)_2$, joined together with 29 disulfide bonds.

The fibrinogen molecule contains at least 12 domains, which are grouped into three major regions named E region, D region and αC region. In the central E region, the amino-termini of the 6 polypeptide chains are connected by five symmetrical disulfide bridges, the nonsymmetrical disulfide bridges formed in this region is called the disulfide ring.[5] The two distal D regions store the carboxy-terminals of the Bβ and γ chain and part of the Aα chain. Both D and E regions contains binding sites for fibrin assembly, cross-linking and platelet interactions.[6] The two αC regions, comprising the flexible carboxy-terminal two thirds of the Aα chain (residues 221–610 in human fibrinogen),[7-9] are involved in fibrin assembly, activation of factor XIII, modulation of fibrinolysis[10-11] and cell adhesion.[12]

The common human fibrinogen molecule contains 2964 amino acids and has a molecular weight of approximately 340 kilo Dalton (kDa). The Aα, Bβ and γ polypeptide chains consist of 610, 461 and 411 amino acids with molecular weights of 68 kDa, 55 kDa and 49 kDa respectively.[13-15]

The 3 polypeptide chains Aα, Bβ and γ are encoded separately by the fibrinogen alpha (*FGA*), fibrinogen beta (*FGB*) and fibrinogen gamma (*FGG*) genes, which are clustered in a region of approximately 50 kilobases (kb) and located on chromosome 4q23-32. The 8.4 kb-*FGG* gene contains 10 exons and is oriented in tandem with the 5.4 kb-*FGA* gene, which contains 6 exons. The *FGG* gene is located 10 kb upstream of the *FGA* gene. Both genes are transcribed in the direction opposite the 8.2 kb--*FGB* gene, which is located 13 kb downstream of the *FGA* gene and contains 8 exons.[16] Assembly of the 6 separate chains takes place sequentially in the endoplasmic reticulum within 5 minutes of synthesis.[17]

Inherited variations in the fibrinogen genes

Numerous polymorphisms have been identified in the three fibrinogen genes. The majority of them are located in the non-coding regions, except for the Arg448Lys polymorphism in the Bβ gene and the Thr312Ala polymorphism

in the Aα gene. An association between the Bβ Arg448Lys polymorphism and variations of clot structure has been suggested,[18] but contradictory results were reported.[19] The Aα Thr312Ala polymorphism is associated with thromboembolic disease.[20-21] The most extensively studied polymorphisms are located in the *FGB* gene, since *in vitro* studies suggested that the synthesis of the Bβ chain is the rate limiting step of the mature fibrinogen production and therefore the *FGB* gene is considered to be involved in determining the fibrinogen plasma levels.[22] A consistent association between *FGB* promoter genotypes and elevated plasma levels of fibrinogen plasma concentrations confirmed this hypothesis.[23-24] On the other hand, the variations of *FGA* and *FGG* genes are reported to play a role in regulating fibrin clot structure.[18]

Noninherited variations

Posttranslational modifications

In addition to the genetic variations in the fibrinogen genes, many variants in fibrinogen are noninherited. They are the result of alternative splicing, posttranslational modifications and proteolytic degradation. Fibrinogen molecules are modified posttranslationally in different degrees as a result of phosphorylation, glycation, glycosylation, oxidization, sialylation and deamidation. These modifications may affect the characteristics of fibrinogen, and affect proteolysis and polymerization.[25]

Proteolytic degradation

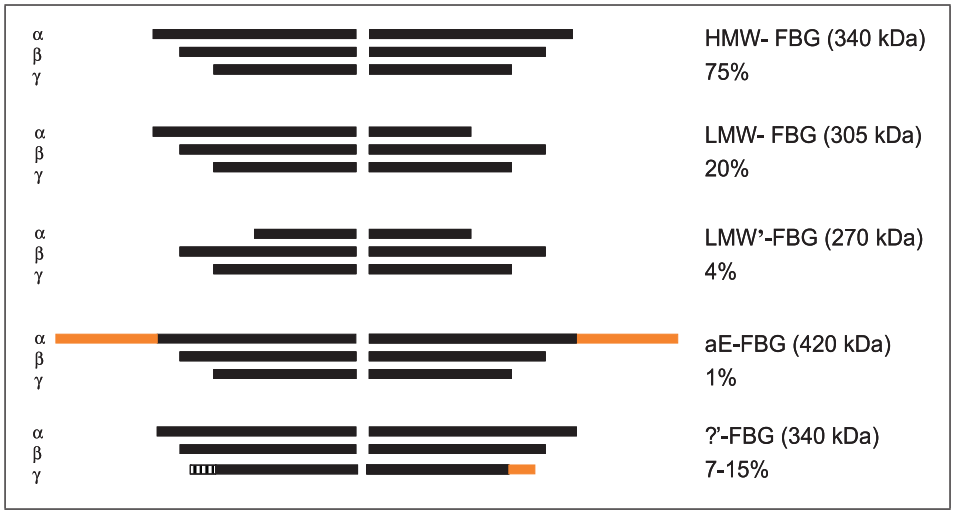
At the carboxyl-terminal of the Aα chains, partial proteolytic degradation can occur, which is the main cause of heterogeneity of the fibrinogen molecule (Figure 1). Fibrinogen is mainly synthesized in the high molecular weight (HMW) fibrinogen with two intact carboxyl ends of the Aα chain (molecular weight [MW] 340 kDa, 70% of total fibrinogen). Partial degradation of one Aα chain results in the low molecular weight (LMW) form (MW 305 kDa, 26% of total fibrinogen) and partial degradation of both Aα chains leads to LMW′ fibrinogen (MW 270 kDa, 4% of total fibrinogen).[26] The ratio of molecular weight variants varies according to the physiological condition, for example, the percentage of HMW fibrinogen increases during an acute phase reaction.[27]

The three molecular weight forms are a mixture of molecules with numerous degradation sites in the carboxyl terminus of the Aα chain. The main termination residues of the degraded Aα chains were Asn-269, Gly--297 and Pro-309.[28] However, no enzyme (included plasmin, gelatinase, trypsin, matrix metalloproteases, and elastase) has been identified that explains the proteolytic cleavage of HMW to LMW and LMW′ fibrinogen.[28] Previous studies showed that the rate of fibrin polymerization, clot stability, fibrin degradation and angiogenesis were influenced in the LMW and LMW′ fibrinogen when compare to HMW fibrinogen. [25,29]

Alternative mRNA processing variants

The extended Aα_E variant (α_E, Fib420)

There are two alternative processing variants from the fibrinogen genes (Figure 1). The first is the result of alternative transcription of the *FGA* gene and includes exon six. This form



Bars indicate relative lengths of fibrinogen Aα, Bβ and γ chains, while the white bars represent elongated sequences of αE and γ′ fibrinogen variants.

Figura 1. Schematic representation of major fibrinogen variants in plasma.

results in the generation of a minor form with an extended $A\alpha_E$ chain (α_E). Only 1 to 2% of the total fibrinogen molecules is α_E.[30] The C-terminus of α_E was reported to have chaperone-like activity,[31] supporting integrin-mediated cell adhesion,[32] mediate leukocyte adhesion and migration via the binding with leukocyte integrins $\alpha_M\beta_2$ and $\alpha_X\beta_2$.[33] However, the physiological role of α_E C has not yet been fully elucidated.

The fibrinogen gamma variants (γ′)

Another natural alternative processing variant is the fibrinogen γ′ variant, which comprises 8% to 15% of the total circulating fibrinogen (Figure 1, reviewed in [34]). Fibrinogen γ′ is a result of alternative messenger RNA processing and polyadenylation at the C-terminal of γA. The last 4 amino acids encoded by exon 10 (γA 408-411 AGDV) are replaced by the 20 unique anionic amino acids encoded by intron 9 (γ′ 408-427 VRPEHPAETEYDSLYPEDDL), leads to the formation of γ′. Approximately 3% to 34% of the γ′ occur as a shortened version, termed γ^{423P}, which probably arises by post-secretary *in vivo* processing of γ^{427L} fibrinogen chains. The γ^{423P} has impaired thrombin binding potential since the 424–427 sequence (EDDL), which is required for thrombin binding, is not included.

The extension of γ′ fibrinogen contains a high affinity binding site for thrombin, which results in antithrombin I activity.[7] In addition, γ′ fibrinogen contains an extra binding site for the factor XIII (FXIII) B subunit and lost its platelet integrin $\alpha_{IIb}\beta_3$ binding site, which results in reduced platelet--fibrin(ogen) interactions. Several studies reported functional and structural differences between fibrinogen γA and fibrinogen γ′ fibrin matrices, such as slower fibrinopeptide B release, slower fibrin polymerization for γ′ fibrinogen, thinner fibers and more branch points with scanning electron microscopy on fibrin γ′ fibrin matrices. The elevated γ′ levels and γ′/total fibrinogen ratio have been reported in cardiovascular diseases,

whereas decreased γ′ levels and γ′/total fibrinogen ratio have been associated with venous disease. Furthermore, the *FGG* haplotypes are associated with thrombotic risk. These associations indicate the γ′ may play a role in cardiovascular events.

FIBRIN POLYMERIZATION

Fibrin formation is initiated by thrombin cleavage of Arg16 at the amino-terminus of the Aα chains, resulting in release of fibrinopeptides A (FpA). Cleavage of FpA results in the exposure of a new polymerization site and fibrin monomers polymerize to form half-staggered oligomers that lengthen into double-stranded protofibrils (Figure 2). Subsequently, thrombin cleavage after Arg14 at the N-terminal of the Bβ chains releases fibrinopeptides B (FpB), promoting the lateral aggregation, resulting in an increase in the fiber thickness.[35]

The fibrin network is stabilized by plasma transglutaminase factor (F) XIII, which is activated by thrombin, and cross-links the fibrin clot covalently via glutamine–lysine bridging between two γ chains.[36] This intermolecular bridging also occurs more slowly between two α chains,[37] and even occurs between one γ and one α chain.[38] These cross-links further strengthen the fibrin clot and protect the clot against mechanical, chemical, and proteolytic degradation.[39]

MODULATORS OF THE FIBRIN STRUCTURE

The fibrin clot structure can be described by the fiber thickness, length,

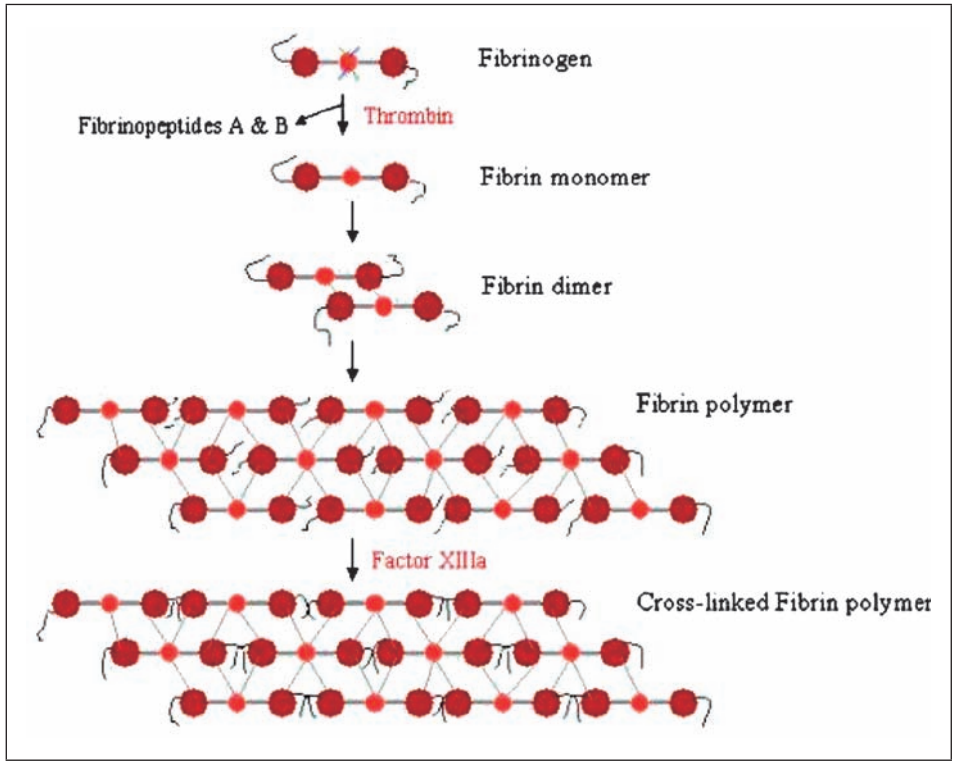


Figura 2. Schematic representation of fibrin monomer and their assembly into polymers.

porosity, permeability, degree of branching, and extent of cross--linking.[40] These variables determine properties of clot, such as stiffness and rate of fibrinolysis. Multiple factors regulate the fibrin clot formation, properties and function, including pH, ionic strength, calcium concentration, fibrinogen concentration and thrombin concentration. For example, an increased fibrinogen concentration and a decreased thrombin concentration lead to thicker fiber and more branch points, resulting in stiffer clots. Moreover, procoagulant activity affects the fibrin clot structure and stability, such as FXIIIa-induced fibrin cross-linking, interactions with platelet and vascular cells, which support the formation of dense fibrin networks that resist fibrinolysis.[41] In addition, fibrinogen polymorphisms (e.g. Bβ Arg448Lys and Aα Thr312Ala polymorphisms), fibrinogen variants (e.g. HMW-fibrinogen, LMW--fibrinogen and γ′ fibrinogen) and pathophysiological condition (e.g. patients with type II diabetes mellitus, premature coronary artery disease or myocardial infarction) also influence polymerization processes and may give an altered fibrin structure.

Taken together, the total effect of genetic and environmental influences determines the structure and function of the fibrin clot. The stability and characteristics of fibrin network play important roles in haemostasis and thrombosis. Alternations in fibrin structure could be a causative factor in the various hemorrhagic and thrombotic disorders, and understanding of the mechanism may lead to the development of therapeutic interventions to manage cardiovascular diseases.

FIBRINOGEN AND WOUND HEALING

Wound healing is a dynamic, interactive and complex process, which consists of three sequential but overlapping phases: (1) inflammation (2) proliferation and (3) remodeling.[42] The wound healing process involves a series of cellular and biochemical events, such as cell adhesion, migration, proliferation, neovascularization, extracellular matrix (ECM) deposition and degradation. These events comprise the intricate interactions between many different cell types, extracellular matrix, soluble mediators (for instance growth factors and cytokines) and proteinases.[43-44]

Fibrinogen is a central protein in the coagulation cascade and it also plays a pivotal role in wound repair. After endothelial injury, a platelets plug is rapidly formed, followed by the formation of a fibrin network. The activated platelets are included in the fibrin network through the binding of fibrin to platelet integrin, the $\alpha_{IIb}\beta_3$ receptors. The resulting insoluble fibrin clot is the first protection against local hemorrhage in wounds. In addition to its function in haemostasis, the fibrin network provides an important provisional, biodegradable ECM that stabilizes the wound and facilitates cell invasion during wound healing process.

One of the important processes of wound healing is angiogenesis, which is the formation of new capillaries from pre-existing vessels in the ECM of the wounded tissue by migration and mitogenic stimulation of endothelial cells (EC).[44] This process consists of several steps, which including the stimulation of ECs by growth factors,

degradation of the extracellular matrix by proteolytic enzymes, invasion of matrix by the migration and proliferation of EC, and finally the formation of capillary tube.[45] It is also highly depends on fibrin structure and the interactions between fibrin and proteins. The rate of fibrinolysis and angiogenesis are affected by the structure of fibrin matrix, for instance a faster fibrinolysis rate was observed in coarse matrix.[46] Furthermore, many haemostasis proteins (such as t-PA, plasminogen and FXIII) bind to fibrinogen and fibrin, which affect the fibrinolysis rate and hence influence the angiogenesis process.[47] The fibrin matrix is also physically associated with other matrix proteins, such as fibronectin and vitronectin, which provide a bridge between smooth muscle cells and fibrin.[6]

Besides being the temporary scaffold to support the wounded tissue, fibrin matrices actively recruit cells to modulate cell-cell and cell-matrix interactions. Previous studies have shown that many cell types have affinity to fibrin matrices, such as EC, smooth muscle cells, fibroblasts and leukocytes. These cells interact with fibrin matrices directly through cell surface integrin receptors (e.g. $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_M\beta_2$, $\alpha_{IIb}\beta_3$) and non-integrin receptors, such as Vascular Endothelial(VE)-Cadherin, intercellular adhesion molecule (I-CAM), P-selectin and platelet glycoprotein Ib-α (GP1ba).[6,48] In addition, fibrin matrices serve as a reservoir for cytokines and growth factors during tissue repairing, in particular the high affinity binding with angiogenic growth factors vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2).[49-50]

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