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Fibrinopeptides and fibrin gel structure

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Abstract

The mechanisms involved in fibrin gel formation are reviewed. Furthermore, a new concept of the role of fibrinopeptide release in this process is presented.

Keywords: Fibrin; Fibrin gel; Clotting time; Fibrinopeptide A; Fibrinopeptide B; Thrombin; Batroxobin

A role of thrombin in fibrin formation was known already at the end of 19th century. It was the main factor causing fibrinogen in blood to clot. The process was an enigma until Bailey et al. in 1951 [1] showed that thrombin initiates the clotting of fibrinogen by proteolysis, thus splitting off fibrinopeptides from the fibrinogen molecule. At this time, Ferry's group had pioneered stages of the polymerization process that follow the action of thrombin. They showed that, on addition of thrombin to fibrinogen, initial polymers or protofibers were formed and after reaching certain lengths gel formation took place due to interaction between the polymers, in three dimensions [2]. In initial polymerization the activated fibrinogen molecules align noncovalently to each other in a half staggered fashion and thus build up two identical polymer strands [3]. Electron microscopy of fibrin fibers visualized this pattern [4]. Ferry and Morrison [5] had already in 1947 published their pioneering work on optical properties of hydrated fibrin gels, as ranging between the extremes transparent and opaque. In transparent gels they deduced the polymer strands being thin and the mesh work fine, whereas in the opaque or turbid gels thick polymer strands are encompassing wide liquid spaces. Thrombin was an important modulator of the process; thus, increasing concentrations led to more transparent gels as clotting time decreased.

The studies by Ferry's group were an important source of inspiration for our group at Karolinska Institutet in Stockholm in the 1950s. Our interest was at that time aimed at clarification of the primary structure of fibrinogen, which we felt necessary for understanding of the polymerization process and, beyond that, the more esoteric question of gelation. Gel formation is the result of nonlinear condensation polymerization [6]. The gel point is of importance since it occurs at a defined stage during polymerization. At that stage soluble polymers suddenly transform into an elastic gel. In our studies on the conversion of fibrinogen to fibrin, we found that the gel point, or clotting time, was the overriding parameter in determining clot structure under shifting conditions of ionic strength, fibrinogen and hydrogen ion concentrations [7,8]. In fact, liquid permeation and opaqueness (turbidity) of fibrin gels revealed changes in clot structure in concert with clotting time. Of particular importance was the finding that the permeability coefficient is directly related to clotting time, strongly suggesting that events, preceding clotting time, are important in the formation of the final gel structure. These findings were confirmed in a study of hydrated fibrin gels by 3D confocal microscopy [9]. The gels displayed straight rod-like elements that often came together at denser nodes and

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encompassed liquid spaces. Increasing thrombin concentration led as expected to shortening of node to node distances, smaller liquid spaces and decrease in turbidity, indicating thinner fibers. The studies suggested that, at gel point, the polymer length together with nonlinear interactions between polymers determined the final gel structure. In order to support this concept, further experiments using recalcified plasma were performed [10]. Addition of thrombin to this plasma is followed by indigenous thrombin generation in the course of gel formation. Confocal 3D microscopy of matured gels, formed at different concentrations of thrombin, showed, like for purified fibrinogen, that the gel structures were related to clotting time (Fig. 1). Thus, the structure created at clotting time prevailed, despite the fact that thrombin concentration increased during gel formation. This means that fibrin polymers formed after the gel point are incorporated into the existing network and do not form de novo net works within fluid spaces of the original net work. The general architecture of the network will remain unchanged during clotting but the strands will grow in thickness during maturation of the gels.

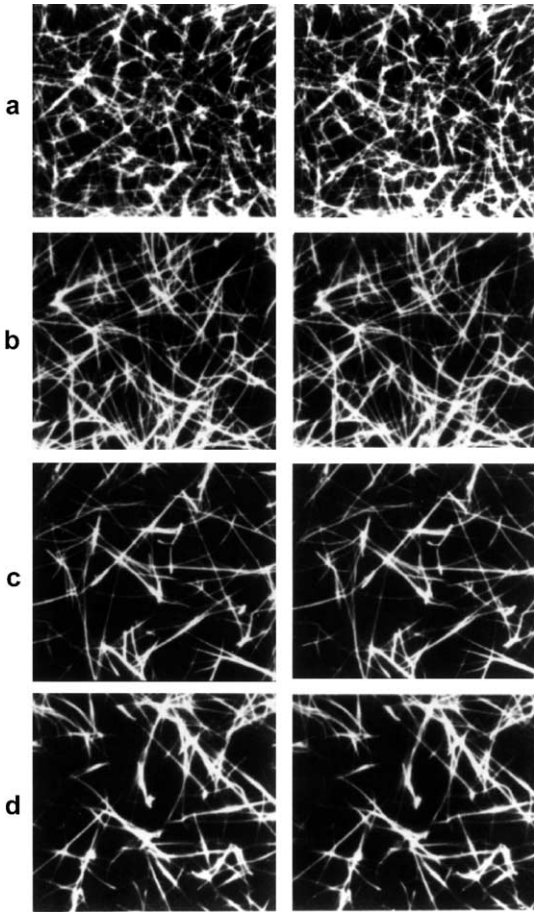

Fig. 1. Confocal laser 3D microscope pictures of fibrin gel networks in recalcified plasma at different thrombin concentrations. Thrombin concentrations: (a) 0.6, (b) 0.1, (c) 0.05 and (d) 0.03 NIH units/ml. From Blombäck et al. [10] with permission from Pergamon Press.

How could the above phenomenon be explained? Thrombin binds to FPA and catalyses its release. Subsequently it appears to bind to two classes of sites on fibrin strands with preserved catalytic activity [11–14]. The location of these sites is apparently in N- and C-terminal parts of the fibrin monomers. It is therefore likely that binding of thrombin to newly formed strands in the network will direct subsequent polymer formation to occur in proximity to the network, thus preserving the general architecture established at the gel point. We may speculate that even the act of gelation may be the result of binding of thrombin to sites on protopolymers. This binding may indirectly lead to unfolding of new complementary binding sites allowing for interaction between polymers in different spatial directions, resulting in a three-dimensional network. It is also possible that one of the sites unfolds as a consequence of FPB release by thrombin. In any case, at high thrombin concentration, complementary sites necessary for interaction would be present at relatively short intervals along the nascent protopolymer, and tight networks are formed. As thrombin concentration decreases, distances between sites necessary for effective complementary interaction between polymers increase and porous networks are formed. Such a scenario would nicely fit our findings.

Branching during polymerization of double-stranded polymers would be a possible way to start gelation [15]. However, it is doubtful whether branching would give the ordered structures, dictated by rate of thrombin activation, we have demonstrated. It has also been suggested that cross-linking by factor XIIIa may play a role in the gelation process proper [15]. However, in our studies we find turbidity profiles of gels being identical whether factor XIII was inhibited or not [16]. We therefore hold that establishment of the three-dimensional network is independent of factor XIII-induced cross-linking, notwithstanding that stabilization of the protopolymers by cross-linking is an important determinant for the visco-elastic properties of fibrin gels [17]. The fact that γ–γ chain cross-linking runs almost parallel with polymer formation is a sign [18].

Release of fibrinopeptide A (FPA) by thrombin in blood is a sine qua non for activation of fibrinogen preceding polymer formation. Thrombin also releases another peptide, fibrinopeptide B (FPB), during clotting of fibrinogen. The role of fibrinopeptide B in fibrin formation has been much discussed. It was early shown that fibrin formation, using purified fibrinogen as substrate, ran parallel with release of FPA, whereas FPB was released at maximum rate when fibrin formation was near completion [19]. Despite this, the general view still seems to be that release of FPB favors side-to-side alignment of polymers being formed. This idea has its origin in studies comparing light scattering and viscosity properties of fibrin formed with thrombin and batroxobin, a snake venom enzyme that only releases FPA [20]. It was concluded that after release of FPA, “fibrinogen polymerized end-to-end while thrombin formed fibrin had a

tendency for lateral aggregation". If true, it is surprising that polymerization and gel formation appear to be quite similar whether induced by thrombin or batroxobin [7,8]. However, although otherwise similar, as shown in Fig. 2, when compared at the same clotting time, tighter, more transparent gels are formed with Batroxobin [8,10].[2] In the abovementioned early study [20], no clotting times for gels formed with the two enzymes were recorded.

The difference in gel tightness at the same clotting time for the two enzymes might indicate that release of FPA and FPB favors gelation at lower degree of activation than when only FPA is released. We have therefore examined previously published data on FPA and FPB release during clotting of fibrinogen [7,8]. These data were transformed as to represent FPA release at the same clotting time for thrombin and batroxobin, respectively. This analysis showed that the amount of FPA released at gel point by batroxobin was in excess of that released by thrombin, in which case also small amounts of FPB were released.[3] We conjecture that release of FPB increases the avidity of the linear polymers for nonlinear polymer interaction and, therefore, gel formation will ensue at a lower degree of FPA release with thrombin than with batroxobin. In the latter case, at same clotting time, a large number of interacting polymer species is required to cause gelation, providing for a tight network. On the other hand, with thrombin, fewer polymers need to be present for interaction to occur and, therefore, a more porous structure with wide liquid spaces is formed. At a shorter clotting time for thrombin, a structure similar to that obtained with batroxobin at a longer time would be formed. After the gel point, with both enzymes, deposition of newly formed polymer strands into the existing network takes place, resulting in increasing strand width as shown by turbidity increase. It is therefore likely that batroxobin, like thrombin, binds to the fibrin network. The relative difference in tightness between the two structures, established at gel point, will remain throughout gel formation. According to this scenario, the role of FPB is to make thrombin more efficient in its clotting action. Side-to-side alignment is a secondary phenomenon and occurs with both enzymes by aligning polymers to the established network.

Although FPB release is much delayed during clotting of purified fibrinogen, its release from fibrinogen in whole blood and platelet-rich plasma is almost as fast as FPA release, and its release seemed to correlate with fibrin formation [21]. However, other studies of fibrin formation in whole blood had indicted that fibrin I (i.e., desFPA fibrin) was the preferred initial species during fibrin formation [18,22]. The use in those studies of inhibitors of the intrinsic pathway of coagulation brought forward a proposal saying that factor XII together with platelets might be of importance for fast FPB release from fibrinogen [23]. These divergent results inspired us to study the release of FPA and FPB in native blood in the absence and presence of an inhibitor of the intrinsic pathway of coagulation. We describe here results of an ongoing study, which will be published in full at a later time. Blood was drawn from an antecubital vein, from one of us, into a siliconized beaker and portions (1 ml) were then immediately transferred to a series of siliconized centrifuge tubes. The tubes were incubated at room temperature and tilted every half minute for observation of clot formation, usually disclosed as small lumps on the vessel wall. At time points in advance of the expected clotting time, at clotting time and after clotting time, tubes were withdrawn and centrifuged for 1 min at $14\,000\times g$. Supernatants were quenched by mixing with an equal volume of ethanol and stored on ice before removal of precipitate by centrifugation in the cold. Supernatants were lyophilized and residue dissolved in ammonium acetate solution for chromatography [24]. Fibrinopeptides, FPA, FPAY(des Ala FPA), FPB and desArgFPB were resolved by reverse phase chromatography on C-18 columns. FPAP (phosphorylated FPA) eluted in front of FPA but could not be identified with certainty.

The blood samples without inhibitor showed already before clotting time release of FPA, FPAY as well as FPB and desArgFPB. FPB plus desArgFPB was, even at that early stage, in quantity slightly larger than FPA plus FPY. In the course of incubation, desArgB was steadily increased at the expense of FPB and the same occurred with FPAY at the

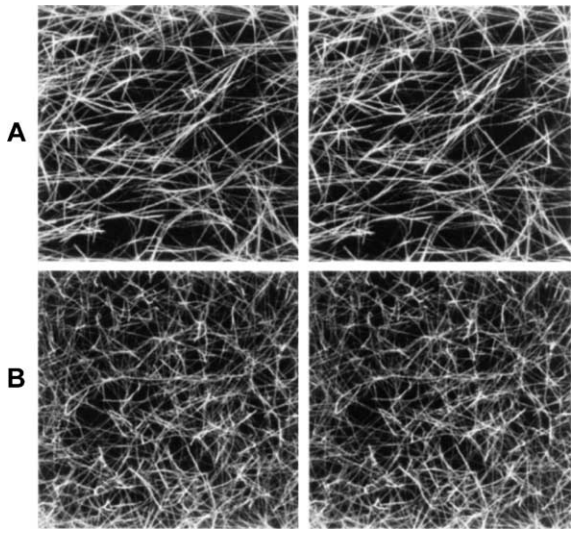

Fig. 2. Confocal laser 3D microscope pictures of fibrin gel networks formed with purified fibrinogen in the presence of batroxobin (0.7 batroxobin units/ml) or thrombin (0.3 NIH units/ml). Clotting time with batroxobin 86 s and with thrombin 90 s. (A) Thrombin. (B) batroxobin. From Blombäck et al. [10] with permission from Pergamon Press.

[2] It should be mentioned here that increase of calcium activity much above physiological will dim the difference in, e.g., permeability between gels formed with the two enzymes at the same clotting time [8].

[3] Treatment of fibrinogen with thrombin or batroxobin was performed at pHs 6.5, 7.4 and 8.2. Excess of FPA, about 40–50% at clotting time 100 s, was noted in the series at pH 6.5 and 7.4. At pH 8.2, FPA values were about the same in the two series.

expense of FPA. Addition of glass beads to the blood samples led to dramatic decrease in clotting time (Fig. 3), but, otherwise, the release and fate of peptides followed the same pattern as for blood that had not been in contact with a charged surface. Addition of aprotinin (Trasylol, Bayer, Germany) to blood (500 KIU/ml) to inhibit the intrinsic pathway of coagulation did, in the presence or absence of glass beads, prolong clotting time (Fig. 3). The release pattern of fibrinopeptides was, however, the same whether inhibitor was present or not (Fig. 4). These results confirm previous results [21], but do not sustain the hypothesis suggesting that fibrin I is initially formed in blood during clotting [18,22] or the proposition that factor XII participates in FPB release [23].

Our experiments have shown that fibrin gel formation in a static system is governed by events that precede clotting time. Although the rate of release of FPA may be the leading event in polymer formation, adjoining release of FPB shortens the time for subsequent gelation. The delayed release of FPB from purified fibrinogen or in platelet-free plasma is in contrast to the greatly increased rate of release in whole blood and in platelet rich plasma, especially in contact with a charged surface. This suggests that conformational change of fibrinogen may occur in the milieu of platelets aggregating on a surface, thus allowing for fast release of FPB. Two adhesive proteins, fibrinogen and von Willebrand factor, are in juxtaposition at a site of vascular injury. von Willebrand factor is present on the subendothelium bound to collagen or other matrix components [25]. Platelets adhere to subendothelium by binding via the GP 1b receptor to von Willebrand factor. Fibrinogen subsequently binds to receptor GPIIb+IIIa on activated platelets. It is possible that the latter binding leads to exposure of a thrombin-susceptible cleavage site, allowing for FPB release. It is likely that a similar conformational change occurs slowly in purified fibrinogen in the course of fibrin formation. In blood, both fibrinopeptides are released in a rapid sequence and polymerization leads to formation of a fibrin network covering the injured area. It is to achieve this efficiently we believe that release of not only FPA but also FPB is important.

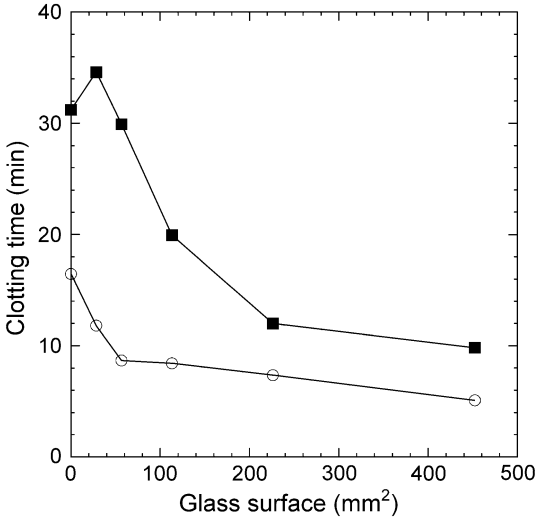

Fig. 3. Clotting time of whole blood with and without contact with glass surface in the absence and presence of aprotinin. Glass beads (3-mm diameter) provided the charged surface. Open circles, without aprotinin. Closed squares, with aprotinin. For further information, see text.

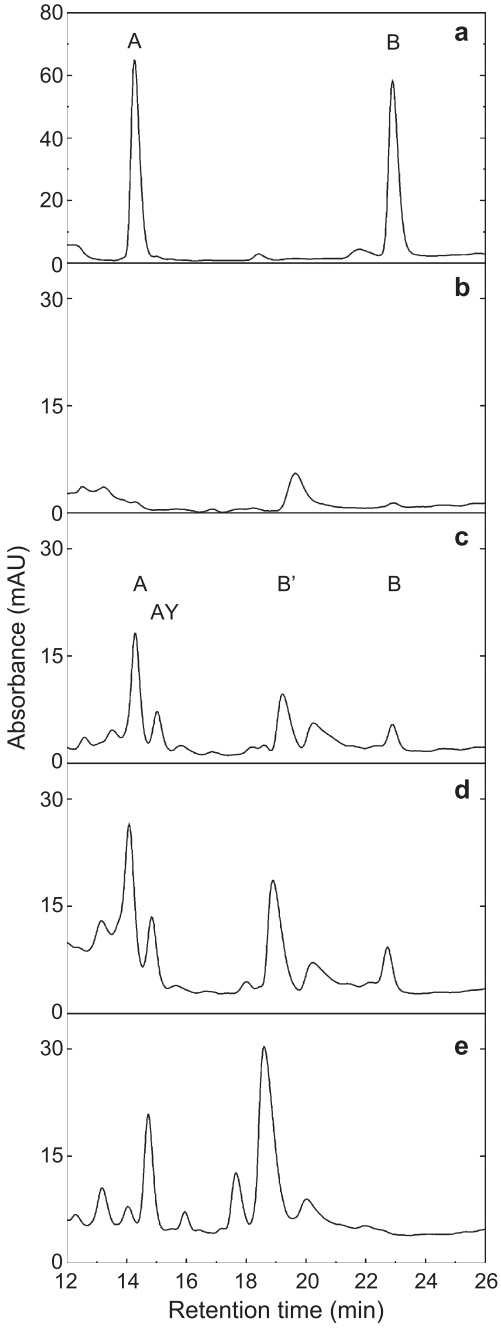

Fig. 4. Chromatogram of fibrinopeptides released from fibrinogen in whole blood at different times of incubation. (a) Purified fibrinopeptide A and B. (b) Blood inhibited with EDTA. (c) Sample quenched at 19 min. (d) Sample quenched at 33 min. (e) Sample quenched at 93 min. Clotting time 36 min. B′ =desArgFPB (identified by its inverse relationship to FPB). For further information, see text.

In flowing blood, especially in arterioles at high shear-force, the network structure is most likely different from that in a static system but the underlying mechanisms for formation of network may still be the same, i.e., polymer interaction, thrombin binding and adherence of newly formed polymers to the existing network. In venules or other places with slow blood circulation, the architecture of clots is likely to be similar to those in the static system. In either system, we perceive how clots with tight structure, thin fibers and small liquid spaces tightly keeping water may be more thrombogenic than gel structures with thick fibers and wide liquid spaces since the latter will easily lose water and synergize on the vessel wall.

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