

# Novel Aspects of Fibrin(ogen) Fragments during Inflammation

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Coagulation is fundamental for the confinement of infection and/or the inflammatory response to a limited area. Under pathological inflammatory conditions such as arthritis, multiple sclerosis or sepsis, an uncontrolled activation of the coagulation system contributes to inflammation, microvascular failure and organ dysfunction. Coagulation is initiated by the activation of thrombin, which, in turn, triggers fibrin formation by the release of fibrinopeptides. Fibrin is cleaved by plasmin, resulting in clot lysis and an accompanied generation of fibrin fragments such as D and E fragments. Various coagulation factors, including fibrinogen and/or fibrin (fibrin(ogen)) and also fibrin degradation products, modulate the inflammatory response by affecting leukocyte migration and cytokine production. Fibrin fragments are mostly proinflammatory, however, B $\beta$ 15–42 in particular possesses potential anti-inflammatory effects. B $\beta$ 15–42 inhibits Rho-kinase activation by dissociating Fyn from Rho and, hence prevents stress-induced loss of endothelial barrier function and also leukocyte migration. This article summarizes the state-of-the-art in inflammatory modulation by fibrin(ogen) and fibrin fragments. However, further research is required to gain better understanding of the entire role fibrin fragments play during inflammation and, possibly, disease development.

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## INTRODUCTION

Inflammation is a complex response to infection or injury with the aim to (i) confine inflammation and/or infection to a limited area, (ii) eliminate noxious stimuli and (iii) restore homeostasis. However, this process is associated with the activation of the coagulation cascade. A wide range of inflammatory conditions including sepsis (1), rheumatoid arthritis (2), Alzheimer disease (3), and multiple sclerosis (4) are not only attributed to an uncontrolled inflammatory response, but also to the disturbance of coagulation. Thus, when coagulation is compromised, it can contribute to the pathogenesis of various inflammatory conditions via fibrin deposition and microvascular failure;

as well as by enhancing the inflammatory response (5,6). The contribution of fibrinogen and/or fibrin [fibrin(ogen)] to inflammation has been recognized, while the role of fibrin degradation products is still under investigation. However, in septic patients with organ dysfunction serum levels of fibrin(ogen) degradation products, D-dimers, B $\beta$ 15–42-related peptides and soluble fibrin are increased (7,8). The entire cross-talk of inflammation and coagulation is reviewed extensively (1,6,9,10). This article will highlight some aspects of the interaction between inflammation and coagulation while focusing on the inflammatory potential of fibrin(ogen) and their degradation products.

## Cross-talk between Coagulation and Inflammation

An inflammatory response shifts the hemostatic system toward a prothrombotic state, while coagulation also affects inflammation. Two coagulation factors stand out during this cross-talk: tissue factor (TF) and thrombin. TF, the initiator of the coagulation cascade, is strongly induced during inflammation in endothelial cells (ECs) and leukocytes (11), which in turn activates thrombin. Blocking TF using neutralizing antibodies abrogates the inflammatory and coagulopathic response in two experimental *in vivo* models of sepsis (12) and ischemia/reperfusion (13). When TF is blocked, thrombin generation is also compromised. This is associated with less activation of the inflammatory and coagulation system, suggesting that thrombin is one of the major players in this process. Thrombin activates endothelial and immune cells by binding mainly to protease-activated receptor (PAR)-1, -3 and -4. It induces a strong inflammatory

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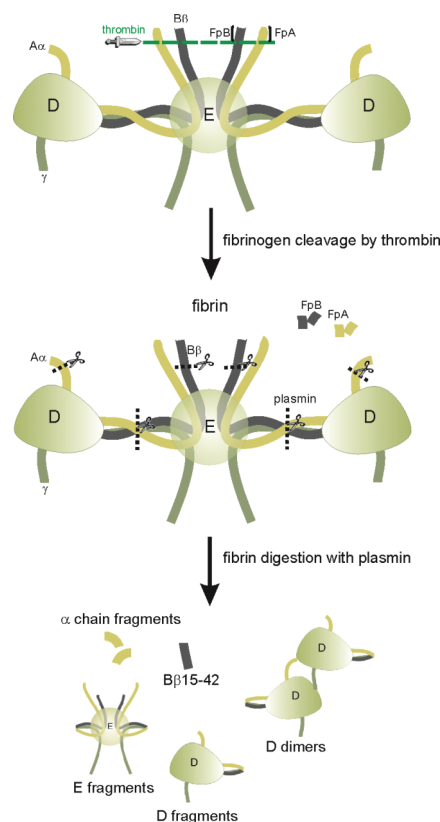
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response by enhancing cytokine and chemokine expression as well as by increasing leukocyte recruitment, mainly via PAR-1, but also PAR-4 signaling (6,14–16).

### Fibrinogen and Fibrin Structure

Fibrinogen is a 340 kDa glycoprotein synthesized in the liver with corresponding plasma levels of 1.5–3 g/L. The protein complex consists of two sets of three polypeptide chains, namely the  $\alpha$ ,  $\beta$  and  $\gamma$  chains. The chains are joined together by disulfide bridges of their N-termini forming the central E globule,



**Figure 1.** Structure of fibrin(ogen) and its cleavage sites. During coagulation, thrombin cleaves fibrinogen releasing the fibrinopeptides A and B ( $\alpha$ 1–16,  $\beta$ 1–14) therewith triggering fibrin polymerization. Following proteolysis of fibrin by plasmin generates various fibrin fragments such as D-dimers, D and E fragments,  $\beta$ 15–42 and  $\alpha$  chain fragments. The scissors mark plasmin cleavage sites, while the knife marks thrombin cleavage sites.

whereas the C-termini of  $\beta$  and  $\gamma$  form the two outer D domains connected to the E domain by coil-coiled rod-like structure of the three polypeptide chains.

Coagulation is initiated by conversion of fibrinogen to fibrin. Thrombin cleaves within the N-termini of the  $\alpha$  and  $\beta$  chains releasing the fibrinopeptides A (FpA,  $\alpha$ 1–16) and B ( $\beta$ 1–15), thereby exposing polymerization sites. The activated fibrin monomers polymerize by binding the exposed polymerization sites to complementary binding pockets within the D domains, therewith forming protofibrils. Finally, thrombin activates coagulation factor XIIIa (FXIIIa) and stabilizes the fibrin clot by catalyzing the formation of isopeptide bonds between the  $\gamma$  chains of two fibrin molecules (reviewed in 17,18).

Fibrinolysis is facilitated by plasmin. It is synthesized as plasminogen and activated by proteolysis via tissue-type (t) or urokinase-type (u) plasminogen activator (tPA or uPA). Activated plasmin in turn cleaves fibrin at various cleavage sites resulting in X and Y fragments, D-dimers, D and E fragments,  $\beta$ 15–42 and smaller fragments mostly derived from the  $\alpha$  chain (19–21). Figure 1 schematically depicts fibrinogen, fibrin, its domains, cleavage sites and the resulting plasmic derivatives.

### Inflammatory Potential of Fibrinogen and Fibrin

During the 1960–70s the first evidence of a potential inflammatory role of fibrin(ogen) *in vivo* was found. It was demonstrated that fibrinogen and fibrin contribute to inflammation by inducing leukocyte migration (22–24). Later, *in vitro* and *in vivo* studies demonstrated that fibrin(ogen) alters inflammation not only by affecting leukocyte migration, but also by directly modulating the inflammatory response of leukocytes and ECs via an increased cytokine/chemokine response. Exposure of ECs to fibrin induces the expression of interleukin (IL)-8 mRNA and protein (25). Fibrin(ogen) has been shown to cause an inflammatory response in peripheral blood mononuclear cells (PBMCs) induced by high levels of

reactive oxygen species (ROS) (26), increased cytokine (for example, tumor necrosis factor- $\alpha$ , IL-1 $\beta$  and IL-6) (27,28) and chemokine expression (for example, macrophage inflammatory protein-1 and -2 [MIP-1 and -2] and macrophage chemoattractant protein-1 [MCP-1]) (29). Fibrin(ogen)-induced chemokine expression was associated with toll-like receptor 4 (TLR-4) signaling, since this effect was absent in C3H/HeJ mice, which express a TLR-4 (29) mutant. In contrast, others have excluded the involvement of TLR-4 signaling pathway in response to a fibrin(ogen) challenge (4). Kaneider *et al.* showed that fibrinogen reduces clotting time in monocytes and induces matrix metalloproteinase-9 (MMP-9) (30). Analysis of underlying mechanisms revealed that fibrin(ogen) modulates mitogen-activated protein kinase (MAPK) signaling, protein kinase C (PKC) and nuclear factor  $\kappa$ B (NF $\kappa$ B) (30–33), a key transcription factor during inflammation (34).

Fibrin(ogen) also binds to various integrins and adhesion molecules such as  $\alpha_v\beta_3$  or  $\alpha_x\beta_2$  (CD11c/CD18) (35,36). Current data suggest that the integrin  $\alpha_M\beta_2$  is the “main” fibrin(ogen) receptor involved in (i) ROS production (26), (ii) cytokine expression (28,30–33) and (iii) leukocyte migration.  $\alpha_M\beta_2$  interacts with fibrin(ogen) by recognizing specific sequences (so called P1: $\gamma$ <sup>190–202</sup> and P2: $\gamma$ <sup>377–395</sup> and P2 core recognition motif P2-C: $\gamma$ <sup>383–395</sup>) within the  $\gamma$  chain of the D nodule (37–41). Recently, the role of  $\alpha_M\beta_2$  and fibrin(ogen) interaction was addressed under *in vivo* inflammatory conditions. Converting the  $\alpha_M\beta_2$ -binding motif of fibrinogen (fibrinogen- $\gamma$ <sup>390–396A</sup>) abolished leukocyte adhesion *in vitro*, while migration in response to *S. aureus*-induced peritonitis was not affected. However, bacterial clearance in fibrinogen- $\gamma$ <sup>390–396A</sup> knock-in mice was impaired significantly, indicating the importance of fibrinogen (42). This is further supported by the fact that fibrinogen deficiency and also fibrinogen- $\gamma$ <sup>390–396A</sup> knock-in significantly reduced disease severity in a mouse collagen-induced arthritis model. Interestingly, this effect

was associated with lower cytokine expression, whereas leukocyte trafficking was unchanged (2). Adams *et al.* demonstrated that fibrinogen activates microglia via  $\alpha_M\beta_2$  but not the TLR-4 pathway. In an experimental autoimmune encephalomyelitis model, inhibition of fibrin(ogen)- $\alpha_M\beta_2$  interaction with the peptide  $\gamma^{377-395}$  reduced microglia activation, coagulation was not affected (4). In summary, fibrinogen- $\alpha_M\beta_2$  interaction strongly modulates the inflammatory response by leukocytes, while trafficking seems to be compensated by other integrins and adhesion molecules.

The intercellular adhesion molecule-1 (ICAM-1) of ECs is another important fibrinogen receptor. By binding to ICAM-1, fibrinogen acts as a bridging molecule enhancing leukocyte-endothelium interaction (43). This interaction is augmented by the vascular endothelial-cadherin (VE-cadherin)-dependent induction of ICAM-1. The exposed B $\beta$ 15-42 sequence was essential for VE-cadherin binding, since blocking FpB release also prevents binding and ICAM-1 induction (44). Even though there is lot of evidence for fibrin(ogen)-dependent leukocyte adhesion, *in vivo* studies revealed that leukocytes and platelets do not readily accumulate in fibrin clots (45,46).

Undoubtedly, fibrin(ogen) plays a major role during inflammation. The thrombin inhibitor refudan reduced macrophage adhesion, while fibrinogen knock-out additionally reduced MCP-1 and IL-6 expression in response to thioglycolate (TG) (47). Cunningham *et al.* demonstrated in a mouse crescentic glomerulonephritis model that administration of the thrombin inhibitor hirudin afforded greater protection against renal injury and inflammation than did PAR-1 deficiency (14). This might indicate the involvement of fibrin fragments inducing inflammation, although, hirudin also inhibits thrombin-dependent activation of PAR-4. Therefore, it cannot be excluded that some protective effects of hirudin in this animal model are due to abrogated PAR-4 signaling. Moreover, platelet PAR-4 plays an essential role in TF-mediated inflammation (16).

Depleting fibrinogen with anicrod showed similar results in a TF-induced inflammation model; TF increased IL-6 levels. This effect was abolished completely when thrombin or FVIIa were blocked or when fibrinogen was depleted by anicrod (16). In a myocardial ischemia/reperfusion model, fibrinogen knock-out mice showed a significant reduction in infarct sizes, when compared with littermates (48). Fibrin deposition seems to contribute to the pathogenesis of Alzheimer disease by increasing blood brain-barrier (BBB) damage and neuroinflammation (3).

In conclusion, fibrin(ogen) augments the severity of various inflammatory conditions. Even though fibrinogen depletion reduces inflammation, it remains unclear if this is solely due to fibrin or also to fibrin(ogen) degradation products. We hypothesize that fibrin fragments also contribute to the pathophysiology of inflammation.

### Fibrin Fragments

Various fibrin(ogen) degradation products are generated during coagulation and fibrinolysis. Initiation of clotting involves the release of FpA and B. Plasmin leads to the generation of D-dimers, D and E fragments and also smaller fragments such as the peptide B $\beta$ 15-42. These fragments, and especially D-dimers, are used as biomarkers for increased fibrin(ogen) turnover. Under various inflammatory conditions, such as sepsis or rheumatoid arthritis, fibrin degradation product levels are increased (7,49), therefore assuming the important role of the coagulation/fibrinolysis system under these conditions. Besides fibrin fragments, fibrinogen degradation products also seem to modulate inflammation. In 1989, Kazura *et al.* reported an inhibitory effect of fibrinogen degradation products on the oxidative burst and concomitant bactericidal activity of peripheral mononuclear cells, probably by inhibiting PKC activation (50).

**Fibrinopeptides A and B.** Various groups reported proinflammatory effects

of fibrinopeptides, in particular its function as a chemoattractant for neutrophils, monocytes and macrophages (51-53). Analysis of exudates from animals injected with purified FpA and FpB revealed that the numbers of leukocytes and MCP-1 levels were increased in rat air pouches. Since both fibrinopeptides have been injected in combination, single effects of each peptide remains to be elucidated (54). In contrast, one study revealed opposite effects of fibrinopeptides. In a carrageenan-induced inflammatory rat model, FpA and FpB reduced paw swelling, suggesting anti-inflammatory potential. In this particular study, thrombin-dependent effects were abolished by the use of heparin (55). Since Singh *et al.* showed that FpB but not FpA attracts macrophages (53), involvement of thrombin to the proinflammatory response could be excluded. As a result of these conflicting reports, further investigation is warranted.

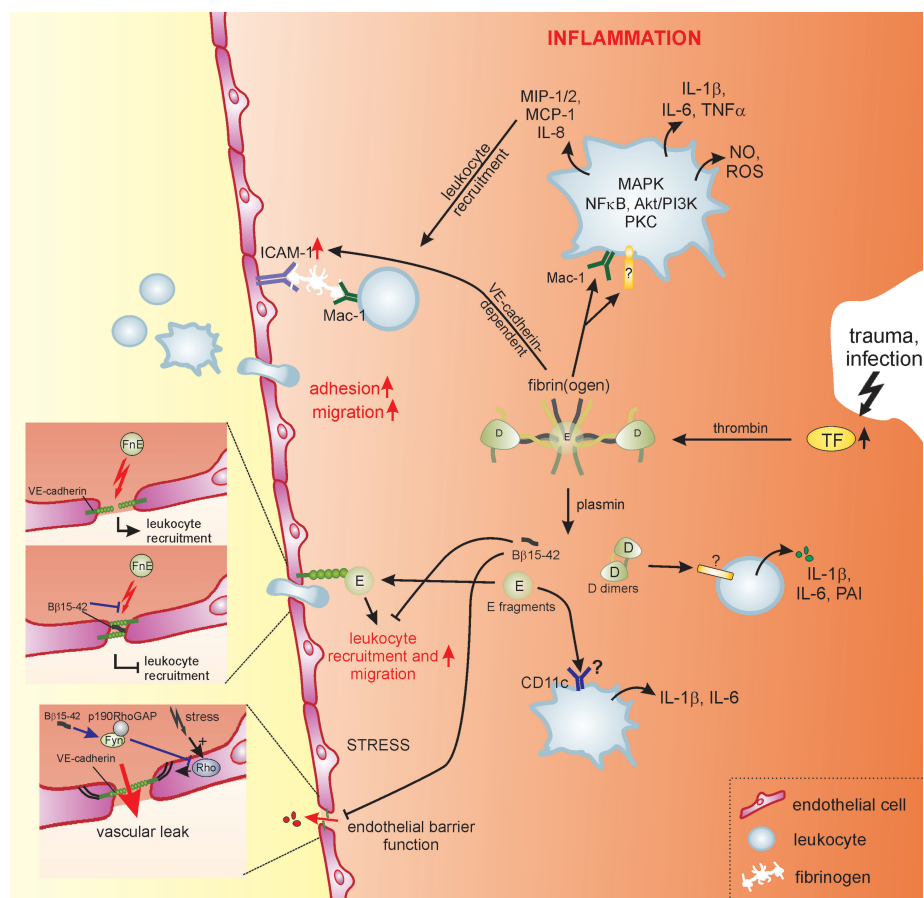
**D fragments.** D-dimers generated by plasmin digestion of fibrin are used as markers for fibrinolysis and disseminated intravascular coagulation (DIC) in man. Exposure of D-dimers or D monomers on the human promonocytic leukemia cell line, NOMO-1, increased the levels of IL-1 $\alpha$  and  $\beta$ , uPA, TF and plasminogen activator inhibitor-2 (PAI-2) (56). In addition, D-dimers triggered the release of IL-1 $\beta$ , IL-6 and PAI in peripheral blood monocytes (57). Others have shown that D fragments have neither pro- nor antiinflammatory effects on monocytes/macrophages (58,59).

**Fibrin fragment E (FnE).** So far, little is known regarding the inflammatory potential of FnE. Various groups reported that FnE has angiogenic activity (60-62), while fibrinogen fragment E (FgnE) inhibits angiogenesis (62,63). In rat peritoneal macrophages, FnE as well as FgnE induced IL-6 production, which was supposed to be mediated by binding to CD11c (58). In the human monocytic cell line, THP-1-induced IL-1 $\beta$  production was increased by adherent FnE, but not by fragment D or FgnE (59). In addition to the studies on physiological FnE, the

biological activity of FnE is often associated with a fragment named NDSKII (N-terminal disulfide knot II). NDSKII is generated synthetically via the cleavage of fibrinogen by cyanogen bromide and thrombin digestion. The resulting product is similar—but not identical—to the physiological FnE (FnE: A $\alpha$ 17–78, B $\beta$ 15–122 and  $\gamma$ 1–62; NDSKII: A $\alpha$ 17–51, B $\beta$ 15–118,  $\gamma$ 1–78) (19,64). Bach *et al.* studied the interaction of NDSKII using human umbilical vein endothelial cells (HUVECs). Binding assays demonstrated that the interaction of NDSKII is dependent on the B $\beta$ 15–42 region, since NDSK (generated after cyanogen bromide digestion without thrombin cleavage) does not expose B $\beta$ 15–42 and therefore did not show any affinity to HUVECs. Moreover, NDSKII binds to VE-cadherin but not to PECAM-1, ICAM-1 or various integrins (65) and facilitates leukocyte migration (48). In detail, lymphocyte migration depends on VE-cadherin and is inhibited by B $\beta$ 15–42, while monocyte and neutrophil migration is mediated by the binding of the  $\alpha$  chain (NDSKII to CD11c). ICAM-1 antibody only partially inhibited migration of all three cell types (48).

**B $\beta$ 15–42.** B $\beta$ 15–42, a fragment of the N-terminal  $\beta$  chain, is generated by plasmin cleavage of fibrin. In contrast to later studies, Skogen *et al.* demonstrated that the fragment B $\beta$ 1–42 is a potent chemoattractant for neutrophils and fibroblasts, independent of the FpB sequence (66). B $\beta$ 15–42 also was capable of inducing IL-8 expression in human oral squamous cell carcinoma cells. Antibodies against B $\beta$ 15–42 inhibited fibrin-induced IL-8 expression, while a peptide representing a sequence of the N terminus of the region (GHRP) mimicked fibrin exposure (67). However, later studies mainly reported the immunosuppressive potential of B $\beta$ 15–42 being protective in various pathologic conditions such as ischemia-reperfusion injury.

Thus, B $\beta$ 15–42 protects the myocardium against ischemia-reperfusion injury by reducing infarct size and leukocyte accumulation. Interestingly, the protective effect was abrogated in fibrinogen-



**Figure 2.** Modulation of inflammation by fibrin(ogen) and their degradation products. Following trauma and/or infection, tissue factor is released and induces coagulation. Fibrinogen is converted to fibrin, which in turn is degraded by plasmin. Fibrin(ogen) modulates the inflammatory response by affecting leukocyte migration, but also by induction of cytokine/chemokine expression mostly via Mac-1 signaling. Fibrin fragment E also induces cytokine expression and leukocyte recruitment/migration by binding to VE-cadherin, which is inhibited by B $\beta$ 15–42. Furthermore, B $\beta$ 15–42 preserves stress-induced endothelial barrier function by inhibiting Rho-kinase activation and subsequent junction opening. NO, nitric oxide; Mac-1, macrophage antigen 1; PI3K, phosphoinositide-3-kinase; TNF $\alpha$ , tumor necrosis factor- $\alpha$ .

deficient mice, suggesting that B $\beta$ 15–42 reduces fibrinogen-dependent inflammation. Furthermore, B $\beta$ 15–42 reduced NDSK II-induced leukocyte recruitment by competing with it for binding to VE-cadherin (48). This work has been translated into a multicenter phase IIa clinical trial investigating the effects of B $\beta$ 15–42 (FX06) on myocardial infarct size. In summary, B $\beta$ 15–42 significantly reduced the size of the necrotic core zone of infarcts while total late enhancement was not significantly different between control and B $\beta$ 15–42-treated groups (68).

In a pig model of hemorrhagic shock, B $\beta$ 15–42-treated animals showed improved pulmonary and circulatory function. B $\beta$ 15–42 reduced plasma IL-6 and neutrophil influx into the myocardium, liver and small intestine, protecting these organs from shock (69). Moreover, B $\beta$ 15–42 functions as a signaling molecule. In two different shock models—Dengue shock syndrome and LPS-induced shock model, B $\beta$ 15–42 preserved endothelial barrier function by inhibiting stress-induced opening of EC adherens junctions (70). Cell-to-cell contact between ECs is formed

mainly by VE-cadherin, which in turn is under the control of RhoGTPases regulating actin dynamics and junction stability. Rho-kinase is activated in response to stress, and causes loss of function of the endothelial barrier. B $\beta$ 15–42 prevented Rho-kinase activation by dissociating Fyn from VE-cadherin, which in turn associates to p190RhoGAP. B $\beta$ 15–42-treated animals had improved survival rates and reduced hemoconcentration and fibrinogen consumption (70). In a cardiac transplant model, B $\beta$ 15–42 also attenuated the ischemia-reperfusion injury (71). Thus, B $\beta$ 15–42 is a promising therapeutic agent; however, the full mechanism of action is still under investigation.

**Other fragments.** Staton *et al.* proposed a potential role for a 24-amino acid fragment derived from the N terminus of the  $\alpha$  chain of FgnE named alphastatin. This peptide exhibited antiangiogenic effects by inhibiting growth factor-mediated migration, proliferation and tubule formation of human dermal microvascular ECs. In a syngeneic tumor model, alphastatin disrupted endothelium layers, caused widespread thrombosis and attenuated tumor growth (72).

Recently, a novel fragment of the B $\beta$  chain (B $\beta$ 43–63) was identified. The fragment released after plasmin cleavage of fibrin had no effect on EC proliferation or migration, but inhibited tubule formation partly owing to cytotoxic effects on ECs. Moreover, B $\beta$ 43–63 reduced adhesion of ECs to the extracellular matrix by binding to and blocking integrin  $\alpha_v\beta_3$ , exhibiting antiangiogenic and anti-tumor properties (73).

However, further fragments such as  $\alpha$  chain fragments are generated during fibrinolysis while their function still remains elusive. Figure 2 depicts a schematic overview of the inflammatory effects of fibrin(ogen) and fibrin degradation products.

## CONCLUSION

It is undisputed that inflammation activates the coagulation system and *vice versa*. Our current knowledge suggests that most players within the coagulation

cascade have proinflammatory properties. In contrast, the peptide B $\beta$ 15–42 mediates potent antiinflammatory effects. Therefore, any modulation of the coagulation system may reduce inflammation on one side, but on the other side may induce bleeding. Thus, targeting the proinflammatory aspects of coagulation, without affecting coagulation itself, might represent a novel therapeutic strategy within the treatment of inflammatory conditions.

High levels of fibrin fragments in septic patients with organ dysfunction suggest an increased fibrin turnover. Disease severity and outcome have not been correlated with fibrin fragments. Therefore, it might be of further interest to evaluate potential implications of fibrin degradation products as biomarkers of inflammatory conditions.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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