Recent Developments in Thrombin-Activatable Fibrinolysis Inhibitor Research

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Abstract: Thrombin-activatable fibrinolysis inhibitor (TAFI) provides an important molecular link between the coagulation and fibrinolytic systems. In this review, recent major advances in TAFI research, including the elucidation of crystal structures, the development of small inhibitors and the role of TAFI in systems other than hemostasis, are described and discussed.

Key Words: TAFI, carboxypeptidase B, carboxypeptidase U, carboxypeptidase R, fibrinolysis, inflammation.

THE BASICS ABOUT TAFI

The coagulation system is a strictly regulated series of enzymatic reactions that prevents blood loss after vascular injury. The reactions ultimately lead to the formation of thrombin. Thrombin converts soluble fibrinogen into a fibrin network, which is subsequently removed by the fibrinolytic system during the healing process.

Thrombin-activatable fibrinolysis inhibitor (TAFI, recent reviews include: [1-10]) is a glycoprotein with a molecular mass of 55 kDa that is synthesized in the liver and secreted into the bloodstream in a zymogen form. TAFI is best known for its function in bridging the coagulation and fibrinolytic cascades. TAFI is activated by the key component of the coagulation system thrombin, either free or – more likely [11] – in complex with thrombomodulin [12]. Alternative activators are plasmin [13-15] and neutrophil-derived elastase [16]. The active form, the enzyme TAFIa, attenuates premature breakdown of the fibrin clot. Hence its name with the accompanying acronym TAFI was chosen.

TAFIa functions by removing C-terminal lysine residues from partially degraded fibrin, which act as binding sites for plasminogen and tissue-type plasminogen activator. This binding facilitates the conversion of plasminogen into plasmin, the enzyme that degrades the fibrin network of the blood clot.

Besides a function in fibrinolysis, TAFI also plays a role in inflammatory processes by hydrolysis of bradykinin, osteopontin and the anaphylotoxins C3a and C5a (reviewed elsewhere [10]). An overview of TAFI activation and TAFIa's substrates, functions and inactivation process is provided in Fig. (**1**).

Due to more or less simultaneous discovery in various laboratories, the enzyme TAFIa was also given different names, based on the biochemical features of the protein. TAFIa is a member of the metallocarboxypeptidase subfamily, which is characterized by the presence of a zinc atom in the active site that is required for the catalytic mechanism of the enzyme. Metallocarboxypeptidases are further divided according to their substrate specificity into the carboxypeptidases A (CPA), which preferentially hydrolyze aliphatic residues, and carboxypeptidases B (CPB), which preferentially hydrolyze basic residues. TAFIa belongs to the latter subfamily and is therefore sometimes referred to as plasma carboxypeptidase B. The finding that TAFIa prefers to hydrolyse arginine residues, prompted other researchers to call it carboxypeptidase R, where R stands for Arginine. Finally, TAFIa is a very labile enzyme, hence it is also known as carboxypeptidase U, where the U stands for unstable.

THE AUTO-REGULATION MECHANISM OF TAFIa

Notwithstanding the high degree of homology between TAFIa and other members of the carboxypeptidase B family (approximately 45%), TAFIa distinguishes itself clearly *via* an auto-regulation mechanism which accounts for the enzyme's short half-life. One of the first observations regarding TAFIa inactivation was that TAFIa is not inactivated by proteolysis [17,18], and second that the catalytic zinc ion is not released in the inactivation process [19]. A third possibility was that the bond between the activation peptide and the catalytic domain, amino acids 92 and 93, is cleaved during activation, but that the activation peptide remains attached to the remainder of the protein. The actual release of the activation peptide could then account for loss of activity. However, recently we were able to show that the activation peptide is not required for TAFIa activity and is not involved in stabilization of TAFIa, excluding a role for the activation peptide in the inactivation mechanism [20].

In the past decade, numerous studies were conducted to reveal the mechanism of TAFIa inactivation by engineering more stable variants [17, 18, 21-26]. Extensive mutagenesis studies revealed that all mutations that influence TAFIa stability are located in one segment of the protein covering β-sheet 9 and α-helix 11 (residues 297-335). The most stable

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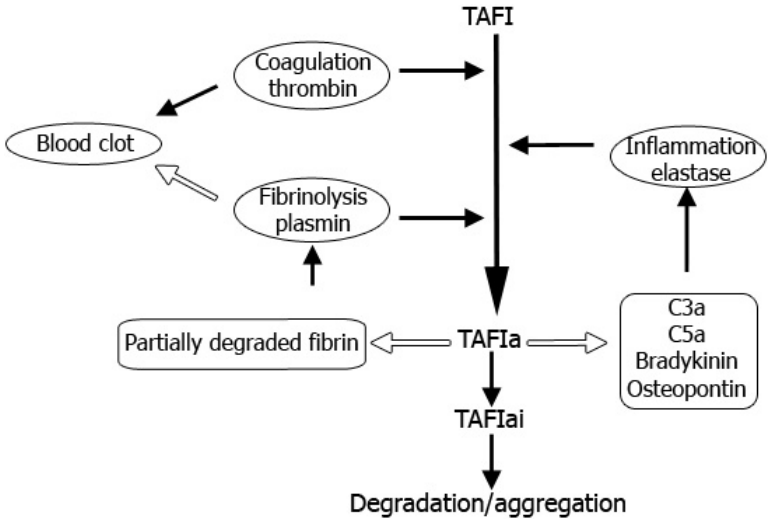

Fig. (1). Diagram of TAFI activation, TAFIa substrates, TAFIa functions and TAFIa inactivation. TAFI is activated (closed arrows) by thrombin generated by the coagulation cascade, plasmin generated by the fibrinolytic system, and elastase, that is released from neutrophils during inflammation, into TAFIa. TAFIa, the active enzyme, converts several substrates (partially degraded fibrin, C3a, C5a, bradykinin and osteopontin) to attenuate (open arrows) fibrinolysis or inflammatory processes. TAFIa inactivates rapidly into TAFIai due to its structural instability after which it is proteolytically broken down and prone to aggregation.

variant generated today contains five point mutations (T325I, T329I, S305C, H333Y and H335Q) and it has a half-life of 180 times that of wild type TAFIa [25]. Remarkably, several of the more stable mutants have an anti-fibrinolytic capacity that is less than expected from their increase in half-life [21,25,26]. Although the reason for this observation is unknown, suboptimal fitting of larger substrates due to structural changes distinct from the active site, may explain this phenomenon.

CRYSTAL STRUCTURES EXPLAIN THE MECHANISM OF TAFIa AUTO-REGULATION

Recently a breakthrough in the understanding of the autoregulatory mechanism was made when the crystal structures of various TAFI forms were solved [27-29]. Obtaining crystals suitable for structure determination was a time consuming process due to the glycosylation extent of the protein. TAFI has five putative N-glycosylation sites which account for the heterogeneous appearance of the protein. Expression of TAFI in a particular cell line – HEK293ES, which lacks N-acetylglucosaminoyltransferase-I – yielded a recombinant TAFI form with homogeneous N-linked glycans. This engineering trick made it possible to grow properly diffracting crystals and to solve the TAFI structure [27].

Similar to other members of the procarboxypeptidase A and B families [30-34], TAFI consists of two structural domains, the activation peptide (first 92 amino acid residues) and the catalytic domain [27]. In the zymogen, the activation peptide covers the active site preventing substrates to enter the catalytic cavity and stabilizing a dynamic segment of the enzyme moiety (residues 296-350). Proteolytic activation by for example thrombin, results in release of the activation peptide and concomitant increase in dynamic segment mobility. The increased dynamics lead to conformational changes that disrupt the catalytic site and exposure of a cryptic thrombin-cleavage site at Arg302. An overall structure of TAFI is given in Fig. (**2**). In agreement with this model, introduction of the stabilizing mutations T325I, T329I, H333Y and H335Q, which results in a 70-fold more stable TAFIa form, or binding of the reversible inhibitor GEMSA, which also stabilizes TAFIa, reduced the mobility of the dynamic segment (Fig. **3**). Earlier research had already shown that Arg302 is the main site for proteolytic breakdown of the enzyme moiety after it had lost the active conformation [17,18]. Our structural data [27] were confirmed by two other studies published shortly after on the crystal structures of bovine TAFI and TAFIa [28,29]. Recently, we observed that the inactivated species, TAFIai, is prone to aggregation, forming large, insoluble protein aggregates that are easily removed by centrifugation [20].

SUGARS: IMPORTANT POST-TRANSLATIONAL MODIFICATIONS

The crystal structures also provided more information on the glycosylation status of TAFI. Four N-linked glycans were observed in the structure, all located in the activation peptide, e.g. Asn22, Asn51, Asn63, and Asn288. The fifth reported N-linked glycosylation site at Asn219 [35] is entirely buried, excluding glycosylation. Usage of different sources of TAFI, recombinant or plasma-derived, may explain differences in glycosylation pattern, although the fact that Asn219 was completely buried within the structure indicates that the physiological significance of glycosylation of this residue is most likely limited. The role of the four glycans is probably to increase the solubility of the protein, as non-glycosylated TAFI, as well as TAFIa, which contains no sugars, have a poor solubility [35], and to ensure proper folding and secretion of the protein. In addition, contacts between the glycans could play a role in stabilizing the dynamic flap. In particular a complex *N*-glycan attached to Asn22 seems sufficiently close to the dynamic flap to establish direct interactions [27]. A recent study showed that the replacement of this Asn22 resulted in an increased intrinsic activity of the zymogen [36], indicating that this particular

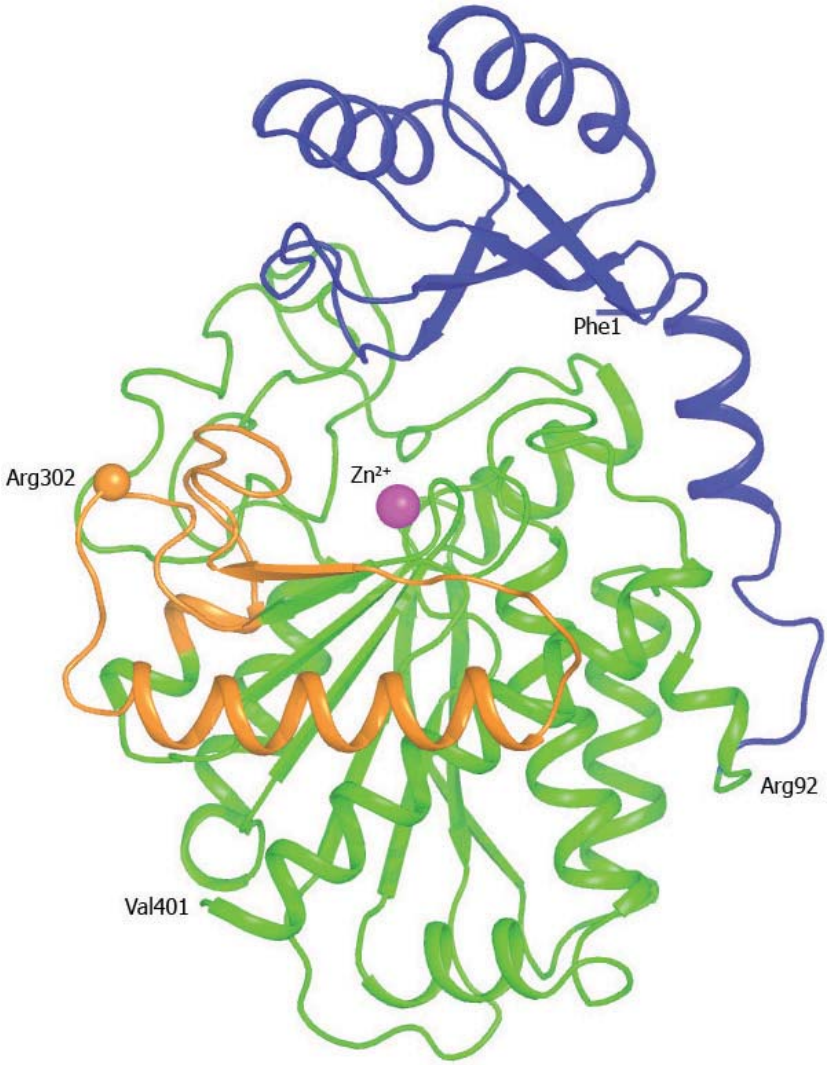

Fig. (2). **Ribbon drawing of TAFI.** TAFI (401 amino acid residues) has two structural domains, the activation peptide (blue) and the catalytic domain (green), including the catalytic zinc ion (magenta sphere) and the highly dynamic 'flap' (residues 296-350, orange).The dynamic region provides an explanation for the instability of the enzyme TAFIa. As a result of proteolytic activation of TAFI at Arg92 and the ensuing release of the activation peptide, the activation peptide is no longer capable to restrict the dynamics of the flap. Increased dynamics lead to loss of structural integrity and consequently to TAFIa inactivation. Inactivated TAFIa (TAFIai) is prone to proteolytic breakdown at Arg302 and aggregation.

glycan indeed forms interactions within the TAFI molecule. It is however unclear if it interacts with the dynamic flap directly. Slight changes in catalytic efficiency of the active form and anti-fibrinolytic potential of this glycosylation mutant as well as one other, Asn63Gln, were also reported [36].

INTRINSIC ACTIVITY OF THE ZYMOGEN: NO ROLE IN FIBRINOLYSIS

As mentioned earlier, the glycosylated activation peptide is cleaved off during the activation process, but a recent paper suggests that not only the enzyme TAFIa, but also the zymogen TAFI, exerts catalytic activity [37]. However, although it was shown that the zymogen displays enzymatic activity towards small molecular substrates [37,38], it does not add significantly to the attenuation of fibrinolysis [39, 40].

TAFI AS THERAPEUTIC TARGET: INHIBITION VERSUS STABILIZATION

The crystal structure provides not only information on the TAFIa inactivation process, it also paves the way for the development of rationally designed inhibitors and stabilizers for TAFIa that can be used in a clinical setting in the future. Inhibition of TAFIa is an attractive new concept of antithrombotic therapy as it is based on enhancing fibrinolysis rather than direct inhibition of the coagulation cascade, thus limiting the adverse hemorrhagic side effects seen with anticoagulant drugs. It may also find application as an adjunct to thrombolytics. Ideally, a useful inhibitor is not only a potent inhibitor of TAFIa, but is also strongly selective for this particular enzyme. The major blood component of interest in this respect is carboxypeptidase N (CPN). CPN is a constitutively active enzyme circulating in the bloodstream that

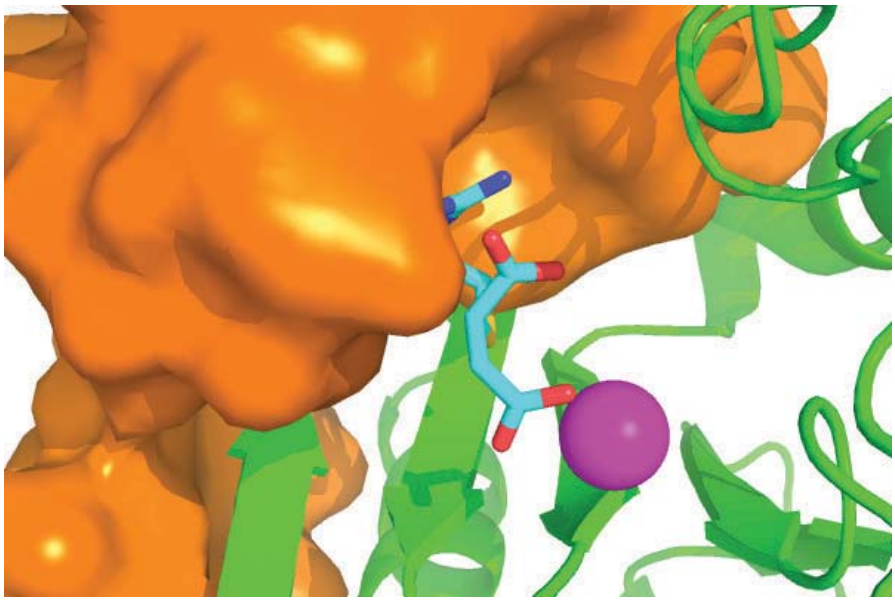

Fig. (3). Inhibitor binding stabilizes TAFIa. Crystallographic data provided an explanation for the stabilizing effect of reversible inhibitors, like 2-guanidino-ethyl-mercaptosuccinic acid (GEMSA), on TAFIa. GEMSA binds in the catalytic cleft S1' pocket where the carboxy-terminal arginine or lysine residue of the substrate would bind. One carboxylate group of GEMSA coordinates the catalytic zinc ion and the second carboxylate is coordinated by catalytic site residues Arg217 and Arg235. Additional hydrogen bonds are formed with Asp348 and Asp349, while hydrophobic interactions are formed with residues 299 and 340-349 that are all part of the dynamic flap of TAFI. The stabilizing effect of GEMSA on the flap region supports the idea that flap dynamics and the instability of TAFIa are directly linked. Dynamic flap, orange; catalytic domain, green; catalytic zinc ion, magenta sphere; GEMSA, cyan; oxygen, red; nitrogen blue.

shares TAFIa's specificity for C-terminal basic residues. Although commonly regarded as an inhibitor of inflammatory processes – among CPN substrates are the anaphylotoxins C5a and C3a – an anti-fibrinolytic function was recently ascribed to CPN [41]. Simultaneous inhibition of both TAFIa and CPN may have adverse effects.

Some encouraging efforts were made in developing TAFIa inhibitors as well as in *in vitro* and *in vivo* testing of the efficacy of TAFIa inhibitor therapy [42-48]. An alternative to the structure-based design of small inhibitory molecular component is the production of inhibitory antibodies and fragments thereof [49].

Among the inhibitors commonly used in *in vitro* experiments, and some also in animal studies, are the carboxypeptidase inhibitor derived from potatoes (CPI), ε-amino caproic acid (ε-ACA), guanidinoethyl-mercaptosuccinic acid (GEMSA), dithiothreitol (DTT), DL-2-mercaptomethyl-3guanidino-ethylthiopropanoic acid (MERGEPTA) and zinc-chelators. One of the major findings on TAFIa inhibitors is that reversible TAFIa inhibitors can both stimulate and inhibit fibrinolysis [50,51]. A potential mechanism explaining this observation is that substrate-bound TAFIa inactivates at a lower rate than free TAFIa [50,51] as the flexibility of the dynamic loop is limited by interactions of the inhibitor with the dynamic flap, the catalytic residues and the zinc ion [27] (Fig. **3**).

In contrast to thrombotic episodes, in the case of excessive bleeding, stabilizers of TAFIa would increase the stability of a blood clot and prevent premature lysis. With the discovery of TAFIa's threshold mechanism of action [52,53] and the TAFI-325 polymorphism – either an Ile residue or a Thr residue at position 325 – the impact of TAFIa stability became apparent [54,55]. The TAFIa-325Ile variant is more stable than the more common TAFIa-325Thr variant and this is also reflected in its antifibrinolytic potential [55]. Stabilization of TAFIa would therefore potentially be a good therapeutic strategy for the treatment of bleeding disorders.

THE TAFI GENE, POLYMORPHISMS AND TAFI EXPRESSION

Besides the TAFI-325 polymorphism (1057C/G), two other polymorphic sites in the coding sequence of TAFI have been identified, TAFI-147 (505A/G, Ala or a Thr residue), which has no known functional consequences, and a silent variation at position 678. In contrast, in the promoter region of the TAFI gene and the 3'UTR, numerous additional single nucleotide polymorphisms (SNPs) have been identified [56], many of which are in strong linkage disequilibrium and some are in or in the proximity of potential transcription factor binding sites [56,57]. Some polymorphisms are associated with clinical outcome, such as blood pressure [58], angina pectoris [59], meningococcal disease [60], splanchnic vein thrombosis [61], recanalization resistance [62] and recurrent pregnancy loss [63].

The gene encoding the 423 amino acids of pre-TAFI, CPB2, is located on chromosome 13q14.11 [64,65]. The 11 encoding exons stretch over approximately 48 kb of DNA [66] TAFI is produced in the liver and seems to be under control of liver-specific transcription factors. Research using the liver cell line HepG2 showed the importance of binding of nuclear factor Y and hepatocyte nuclear factor α for TAFI expression [67]. In mice, mRNA was detectable in the liver, in a hepatocyte-specific, pericentral lobular distribution pat-

tern [68]. TAFI was also detected in human platelets and may have been produced in megakaryocytes rather than taken up from the plasma [69].

A large number of studies over the years have been dedicated to the determination of the plasma TAFI concentration in health and disease. In normal individuals, mean TAFI levels were reported between approximately 75-275 nM [70-73], with a considerable variation of 45%-150% of the mean value. The variation can in part be explained by the presence of different genotypes, both because the variants are expressed at different levels and because some of the polymorphisms affect the assays to detect TAFI. Because of the latter phenomenon – discussed in more detail below – also the data on the influence of age, gender, ethnicity, disease etc. and TAFI levels, is confusing and warrants further analysis in the near future. For now it seems that approximately 25% of the variation can be explained by SNPs in the TAFI gene [74], leaving a large percentage for non-genetic factors. Glucocorticoids were shown to upregulate TAFI expression *in vitro*, whereas the interleukins IL-1β and IL-6 could down regulate expression [75]. Since there are essential differences in the promoter sequences of the human and mouse TAFI gene the mouse is not an optimal model system to study TAFI gene regulation [2]. This may hamper progress in revealing the role of inflammation in regulation of the CPB2 gene.

TAFI ASSAYS: NOT ALL ASSAYS MEASURE THE SAME

Although it is not quite clear yet what the impact of the polymorphisms is on development and progression of various disorders, it is fact that many assays to measure TAFI and/or TAFIa are compromised by the presence of various TAFI forms, especially the 325 polymorphism, in the general and patient population [76,77]. Antibody-dependent assays suffer from affinity differences between the TAFI-325-Thr and Ile form, and activity-based assays from a difference in half-life. Nevertheless progress in this area over the past few years resulted in a polymorphism-independent activity-based assay [78,79], an assay for the direct measurement of functional TAFIa in plasma [80], the development of various new ELISAs specific for the various TAFI fragments (zymogen, activation peptide, TAFIa/TAFIai) [81] and TAFI from different species (human, mouse, rat) [82-84], and (global) fibrinolytic assays [85-87]. Also, testing of novel substrates resulted in more selective TAFIa substrates that distinguish between TAFIa and CPN [88]. Although not all these techniques are widely available, they are valuable tools in TAFI research.

TAFI FROM DIFFERENT SPECIES

The above mentioned assays for measuring TAFI of animal origin are important since the use of experimental animals can yield valuable information. Also, TAFI from different species has been cloned and characterized. The deduced amino acid sequence of rat TAFI is 83% [82] identical to human TAFI. For mice this is 85% [68] compared to human, whereas the protein sequences of rat and mice share 95% identity [82]. Although rat, mouse and human TAFI share similar biochemical properties, the half-life of the rodents' TAFI is shorter than the half-life of their human counter part [68,82,89]. Also the plasma concentration of TAFI is lower in these animals [68,82,90].

Some of the crystal structures of TAFI were solved using bovine TAFI, which has a sequence identity of 77% with human TAFI, although little characterization of the functionality of this protein in cows has been reported so far. Furthermore, the presence of TAFI was established in pig, guinea pig, rabbit, dog, and baboon [90].

FUNCTIONS: THE INTERFACE BETWEEN COAGULATION, FIBRINOLYSIS, INFLAMMATION AND MORE

Besides in experimental animals, relations between TAFI levels and diseases were investigated in human subjects. Recent advances on the role of TAFI in bleeding and thrombotic disorders included the discovery that increased plasma TAFI concentrations are associated with an increased risk for venous thrombosis and coronary artery disease [91,92] and associations were found between TAFI levels and a number of disorders such as type-2 diabetes mellitus [93-96], hypertension [97,98], obesity [99], stroke [100-106], sepsis [103,107-109], liver cirrhosis [110] and glomerulonephritis [111].

Patients with type 2 diabetes mellitus showed significantly higher TAFI levels compared to non-diabetics [93] and TAFI levels were correlated with the urinary albumin excretion rate in normotensive diabetes mellitus patients [94,96]. However, fasting TAFI levels were decreased in normotriglyceridemic patients with type 2 diabetes compared to non-diabetes patients and TAFI levels decreased postprandially in both groups [112].

The risk for ischemic stroke was also associated with elevated TAFI levels [101,102,105]. These patients showed elevated TAFI levels during the acute phase [100,106] and significantly higher levels of TAFI were observed in stroke patients after recanalization by tissue-type plasminogen activator infusion [103]. The baseline levels of TAFI, together with plasminogen activator inhibitor 1, can predict the risk of symptomatic intracranial hemorrhage after tissue-type plasminogen activator infusion [113].

In contrast, septic (both severe sepsis and septic shock) patients had significantly decreased TAFI levels compared to controls [108]. Moreover, associations were found with TAFI and mortality of meningococcal sepsis [107].

With the engineering of TAFI knockout animals [84,114-116], the *in vivo* role of TAFI advanced rapidly in the past few years. Compared to wild type animals, these mice were normal in many respects, including survival, development, and fertility. Mao *et al*. reported that mice lacking TAFI indeed have an enhanced endogenous fibrinolysis [117] and Wang *et al*. [116] demonstrated the protective effect of TAFI deficiency in a ferric choride-induced occlusion model of the vena cava. Similar results were obtained when TAFIa was inhibited by treatment with carboxypeptidase inhibitor (CPI) [48,116]. Previously, enhanced *in vivo* thrombolysis was already observed for TAFI deficiency in a background of plasminogen deficiency [118].

Besides the anti-fibrinolytic function of TAFIa, TAFI is also involved in inflammation and wound healing [84]. The role of TAFI in inflammation is for example illustrated by the observation that TAFI knock out mice, in contrast to control mice, were protected from liver necrosis after intra peritoneal injection with *Escherichia coli* [119]. In another inflammation model, this time with TAFI/plasminogen double knock out mice, the migration of leukocytes towards the peritoneum was increased in the deficient animals compared to the wild types showing the importance for TAFI in (plasminogen-dependent) cell migration *in vivo* [118]. Lately, we reported the binding of TAFI to the surface of a group A streptococci (M41 serotype) and subsequent activation at the bacterial surface *via* plasmin and thrombin-thrombomodulin [120]. Furthermore, activation of TAFI on the surface of *Streptococcus pyogenes* evoked inflammatory reactions by modulating the kallikrein/kinin system [121].

Additional *in vivo* experiments showed that the TAFI-deficient mice have a wound healing problem [84], which may be related to the cell migration process mentioned above. In a skin wound model [84], the keratinocyte migration pattern was disturbed, again pointing to a role for TAFI in cell migration. Subsequent *in vitro* studies showed that TAFI inhibits endothelial cell movement and tube formation [122].

However, it will take another while before the exact (patho)physiological roles of TAFI are revealed, partly because interpretation of the data is difficult due to the genotype sensitivity of many assays used in the past and still in use at the moment, and partially because some studies were contradictory.

CONCLUDING REMARKS

As outlined above, the TAFI research field has developed swiftly in the past few years and now expands beyond hemostasis. The interest for the protein has increased since it is recognized as a potential therapeutic target for novel intervention strategies. Inhibition of TAFIa is expected to increase the efficacy of fibrinolytic therapy in thrombotic disorders. Conversely, agents that improve or stabilize TAFIa, thereby down-regulating fibrinolysis, may be useful for the treatment of bleeding disorders. In addition it may prove to be a target to treat inflammatory or wound healing disorders.

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