What drives „fibrinolysis"?

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Keywords
Fibrinogen, fibrin, fibrinolysis inhibitors, nervous system, plasminogen activators, plasminogen

Summary
The timely removal of blood clots and fibrin deposits is essential in the regulation of haemostasis. This is achieved by the fibrinolytic system, an enzymatic process that regulates the activation of plasminogen into its proteolytic form, plasmin. This is a self-regulated event as the very presence of fibrin initiates plasminogen activation on the fibrin surface due to the presentation of exposed C-terminal lysine residues in fibrin that allow plasminogen to position itself via its lysine binding sites and to be more efficiently cleaved by tissue-type plasminogen activator (t-PA). Hence fibrin, the ultimate substrate of plasmin during fibrinolysis, is indeed an essential cofactor in the cascade. What has now come to light is that the fibrinolytic system is not solely designed to eliminate fibrin. Indeed, it is a broad acting system that processes a variety of proteins, including many in the brain where there is no fibrin. So what drives t-PA-mediated plasminogen activation when fibrin is not available?
This review will describe the broadening role of the fibrinolytic system highlighting the importance of fibrin and other key proteins as facilitators during t-PA-mediated plasminogen activation.

Schlüsselwörter
Fibrinogen, Fibrin, Fibrinolyse-Inhibitoren, Nervensystem, Plasminogen-Aktivatoren, Plasminogen

Zusammenfassung
Die rechtzeitige Auflösung von Blutgerinnseln und Fibrinablagerungen trägt maßgeblich zur Regulierung der Hämostase bei. Erreicht wird dies durch das fibrinolytische System, einen enzymatischem Prozess, der die Aktivierung von Plasminogen zu seiner proteolytischen Form Plasmin regelt. Dies ist ein selbst reguliertes Ereignis, insofern die Plasminogen-Aktivierung auf der Fibrinoberfläche alleine durch die Anwesenheit von Fibrin, aufgrund der Präsentation von exponierten C-terminalen Lysinresten im Fibrin, ausgelöst wird; an diesen kann sich Plasminogen über seine Lysinbindungsstellen selbst anlagern und wirksamer durch den gewebespezifischen Plasminogen-Aktivator (tPA) gespalten werden. Somit ist Fibrin, das letzte Substrat des Plasmins im Verlauf der Fibrinolyse, ein wesentlicher Kofaktor in der Kaskade. Wie sich herausstellt, dient das fibrinolytische System nicht nur dazu, Fibrin zu eliminieren. Vielmehr ist es ein umfassend wirkendes System, das eine Reihe von Proteinen verarbeitet, darunter viele im Gehirn, wo es kein Fibrin gibt. Wodurch also wird die tPA-vermittelte Plasminogenaktivierung vorangetrieben, wenn kein Fibrin verfügbar ist?
In dieser Übersicht beschreiben wir die umfassendere Rolle des fibrinolytischen Systems, wobei wir vor allem die fördernde Bedeutung von Fibrin und anderen Schlüsselproteinen bei der tPA-vermittelten Plasminogen-Aktivierung beleuchten.

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Was treibt die „Fibrinolyse" an?
Hämostaseologie 2015; 35: 303–310
http://dx.doi.org/10.5482/HAMO-14-10-0050
received: October 14, 2014
accepted in revised form: December 15, 2014
epub ahead of print: January 7, 2015

A short history of fibrinolysis

The dissolution of fibrin following coagulation was introduced over a century ago by a French physiologist, Albert Dastre who described the spontaneous lysis of blood clots (1). Dastre was also credited with the first introduction of the term "fibrinolysis" as reported in a 1946 review by Macfarlane and Pilling (2). The enzymatic processes underlying fibrin degradation were being actively pursued at the turn of the 20th century notably by Bertha Barker working at the Rockefeller Institute in New York (3)). Indeed, as early as 1904, a cellular basis for fibrin degradation was suggested by the Belgian scientist Hector Rulot, who reported that fibrin digestion was caused by "*leukocytes imprisoned in the meshes of fibrin*" (4). The fibrinolytic system gained a much stronger foothold in the early 1930's through the efforts of Tillet and Garner at the Johns Hopkins Medical School (5, 6) during their description of the fibrinolytic agent produced by haemolytic streptococci that they referred to as "fibrinolysin". Critical studies by Christensen and colleagues in New York in 1945 revealed that fibrinolysin did not degrade fibrin directly, but by activation of an unknown "zymogen or proenzyme" that existed in human plasma (7, 8). Having "plasma" as the origin of this proenzyme, Christensen named this entity as "plasminogen" and its active form as "plasmin". However, the terms "fibrinolysin" and its pro-form "profibrinolysin" were maintained in the literature for at least another decade before they became more commonly referred to as plasmin and plasminogen, respectively. That plasma also contained an "anti-proteolytic" factor was shown as early as 1903 by Delezene and Pozerski (9) and, as stated in the review by Macfarlane and Pilling (and being influenced by the new nomenclature introduced during this period): "This factor is probably concerned with the

absence of fibrinolysis in normal blood and may be called, for convenience, "antiplasmin" (2). The reader is also directed to a subsequent comprehensive review on the history and understanding of fibrinolysis in 1948 by MacFarlane and Biggs (10).

While the proenzyme (plasminogen) and antiplasmin had been identified relatively early, the endogenous human-derived proteases responsible for the conversion of plasminogen to plasmin were discovered later. Astrup and Permin first described what became known as tissue-type plasminogen activator (t-PA) in 1947 (11), while a "physiological activator of profibrinolysin" was identified in urine by Sobel and colleagues and named by these authors as "urokinase" (u-PA) (12).

Fibrinolysis: a current view

From the very outset, the fibrinolytic system was intrinsically linked with blood and blood clot removal and for good reason given that fibrin was the first substrate for plasmin identified. However, the forefathers of fibrinolysis could not have envisaged the extended and important role of this proteolytic system in areas far removed from fibrin removal. Nonetheless conventional fibrinolysis is still an important aspect of physiology but is beginning to take a back seat with the focus of many current research scientists turning towards these newer "non-fibrinolytic" areas.

Nowadays, the fibrinolytic system is still described in relatively straight forward terms, with the key proenzyme plasminogen still being front and centre together with t-PA and u-PA that both convert plasminogen into its potent fibrinolytic form, plasmin. However, there are many other components that will be outlined later that not only help fine-tune the fibrinolytic system, but also endow it with properties enabling this system to play important roles elsewhere in vivo.

Another important concept that will be highlighted in this review is that fibrin is not only a substrate for plasmin, it is also the initiating event of the fibrinolytic process (13, 14). This is not at all surprising, as fibrin formation under normal circumstances is only designed to provide a temporary matrix, be in it the guise of a blood clot or a fibrin seal during wound repair that ultimately needs to be removed in a timely manner. Mechanistically, this "self-directed" proteolysis is orchestrated by the presence of C-terminal lysine residues that are created on the fibrin surface during its formation. These lysine residues are recognised by lysine binding sites located in most of the kringle domains of plasminogen (15). This convenient arrangement not only brings plasminogen and fibrin together, but also significantly enhances the proteolytic activity of t-PA, as fibrin-bound plasminogen is activated into plasmin orders of magnitude faster in the presence of fibrin than in its absence. Hence fibrin is essentially acting as a co-factor for t-PA. To add further refinement, t-PA itself binds to fibrin (although not only via lysine residues) thereby co-locating itself juxtaposed to plasminogen on the fibrin substrate. The fibrin-selectivity of t-PA distinguishes it from u-PA, which can activate plasminogen with similar efficiency on fibrin or in solution (13).

As with all proteolytic systems, a number of check-points need to be in place not only to restrict plasmin formation, but to allow plasmin sufficient time to perform its proteolytic task. While Delezene and Pozerski first reported the presence of an antiprotease back in 1903, plasmin formation is now known to be controlled at three distinct levels. Two of these involve specific protease inhibitors: plasminogen activator inhibitor (PAI)-1 (described in the early 1980's) (16, 17) and PAI-2 (described in 1970) (18) that target both t-PA and u-PA. As mentioned earlier, antiplasmin targets plasmin directly and is the most influential fibrinolytic inhibitor. Plasma levels of antiplasmin are very high (~1 µmol/l). This, together with its half-life of ~2.6 days (19) ensures that the half-life of plasmin activity is less than 10 ms; see (20) for review. This contrasts with the half-life of it inactive pro-form plasminogen (~2.2 days). It is also interesting to note that the association rate between antiplasmin and plasmin is the fastest described among the serine proteases and serine protease inhibitors.

The third level of plasmin regulation does not require protease inhibition at all, but rather involves a carboxypeptidase (Thrombin activatable Fibrinolysis Inhibitor; TAFI) that functions to stabilise blood clots. TAFI enzymatically removes C-terminal lysine residues from fibrin thereby reducing the capacity of plasminogen and t-PA to bind to the fibrin surface and therefore, indirectly inhibiting fibrin breakdown (21, 22). Being activated by thrombin, TAFI became another link between coagulation and fibrinolysis, i. e. in addition to fibrin (23).

While the importance of plasminogen activation in relation to fibrinolysis is of little doubt, plasmin was also reported over 30 years ago to have important roles in the processing of other substrates, many related to tissue degradation and cancer (24). Furthermore, plasminogen activation was not restricted to the fibrin surface or to the circulation, but, importantly could be focused onto the cell surface in a manner facilitated by receptors for most of the fibrinolytic components. For example, plasminogen receptors were first described over 25 years ago and since that time, ~12 distinct cell surface plasminogen receptors have been described on various cells, nearly all containing C-terminal lysine residues (25). Similarly, receptors for u-PA (uPAR) (26) and t-PA (27, 28) have also been described. Collectively, these receptors provide a means for cell-surface localized plasmin formation where it could not only promote fibrinolysis (29), but also non-fibrinolytic functions related to cell migration (30), wound healing (31), and metastatic spread (32). It is not the purpose of this review to overview all of these numerous receptors, however their very existence underscores the fact that fibrin removal is not the only intention of the fibrinolytic system.

Thrombolysis and lysine based anti-fibrinolytics

The fibrin-dependent nature of t-PA and plasminogen provided the impetus for the pharmaceutical industry to harness this feature for the clinical development of fibrin-specific thrombolytic drugs, with t-PA being the first to reach the hospital pharmacies in the mid-1980's. This period her-

alded a mighty time for t-PA in the thrombolytic treatment of patients with myocardial infarction but this decreased in later years with the advent of angioplasty. t-PA however got an additional boost in popularity in 1995 when the FDA approved its clinical use in patients with acute ischaemic stroke (AIS) (33). Although t-PA is still today (~20 years later) the only approved drug used in AIS, there are a number of restrictions, the most critical being that it can only be administered within 4.5 hours of stroke onset (3 h in some countries, including the USA) due to risks associated with intracerebral haemorrhage and reduced net clinical benefit (34).

While the fibrin-dependency of t-PA proved beneficial for its clinical development, so too did the lysine-dependency of t-PA and plasminogen prove equally beneficial as a means of attenuating fibrinolysis. Indeed, the use of lysine analogues including epsilon-amino caproic acid (EACA) (35) and tranexamic acid (36) have been successfully exploited to limit bleeding in numerous clinical conditions and as a prophylactic treatment in some surgeries. As will be outlined in later sections, lysine-dependent plasminogen activation occurs in many other situations in the absence of fibrin raising the prospect that these treatments may have unintended consequences (see (37) for a recent review).

Non-fibrinolytic roles of the fibrinolytic system

While Albert Dastre (1) provided the first link of the fibrinolytic enzyme system with blood clots, the revolution of recombinant DNA technology and the advent of the gene knock-out mouse provided the fibrinolysis aficionados with some curious results. The t-PA and u-PA knock out (KO) mouse were also among the first KO mice to be generated. While there was an expectation that t-PA deficiency would be prothrombotic due to impaired fibrinolysis, this phenotype was surprisingly mild, with some changes in bleeding times being noted only under challenged conditions (38). The absence of a thrombotic catastrophe in mice deficient in t-PA surprised the scientific community and the field was left pondering as to how important the fibrinolytic system actually was in controlling haemostasis with redundant systems seemingly taking up the challenge.

At about the same time, an unexpected phenotype related to t-PA deficiency was described, in an area distant to haemostasis. A series of landmark publications in the early to mid-1990's showed that t-PA deficiency had a profound phenotype in the central nervous system (CNS) (39–41). t-PA deficiency was linked with the promotion of neurotoxicity (42), learning and memory formation (43), synaptic plasticity (44), the processing of neurotrophic factors (45), the host response to addictive behaviour (46–49) and visual processing (50) just to name a few. These initial reports were published soon after the FDA approval of t-PA for AIS which sent a provocative signal to the stroke community, particularly in relation to potential consequences of t-PA-mediated neurotoxicity. The neurotoxic feature of t-PA was mechanistically linked to NMDA receptor activation (51, 52). t-PA is also involved in other CNS initiated receptor initiated events, include those associated with serotoninergic (53) transmission. The finding that t-PA deficient mice were protected from direct neurotoxic challenges (42, 54), cerebral ischaemia (55) and traumatic brain injury (56, 57) clearly implicated endogenous t-PA as playing a causal role. Consistent with this brain phenotype, t-PA is widely expressed in the CNS, particularly in the hippocampus, amygdala and hypothalamus and t-PA mRNA and activity levels in the brain can be modulated pharmacologically (58). Subsequent reports implicating plasmin with t-PA activity in the CNS also begged the question as to the cellular source of plasminogen. Although initially described as an abundant liver derived plasma protein, it is now clear that many cells in the CNS can actively synthesis plasminogen (59, 60) thereby providing "brain"-t-PA with its substrate locally.

As described above, conventional fibrinolysis in the circulation has a number of well described regulatory events that restrict t-PA and plasmin activity. But how is this regulation achieved in the CNS? While PAI-1 is the most prominent t-PA inhibitor in blood, this particular inhibitor is only weakly expressed in the CNS. Instead, the brain harbours a specific t-PA inhibitor, neuroserpin (61, 62) to control t-PA activity in this compartment. In addition, a_2-antiplasmin is also expressed in various regions in the CNS. Curiously recent studies have suggested that a_2-antiplasmin in the CNS may have some additional role in the CNS unrelated to plasmin inhibition (63, 64) which adds yet another dimension to the complexity of this system.

With the undeniable existence of a regulated t-PA-dependent proteolytic system in the CNS, a key question surrounds the physiological role of t-PA in this setting. Despite the fact that the early reports of the role of t-PA in the CNS tainted t-PA in a deleterious context, it certainly did not evolve in the CNS to promote harm. In recent years, this has become somewhat more topical as some reports have presented evidence that endogenous t-PA can also promote neuroprotection. t-PA was shown to inhibit zinc induced neurotoxicity back in 1999 (65), and more recently to even be protective against excitotoxic or ischaemic challenges depending on the location and concentration (66, 67). Arguably, however, a more clinically relevant role for t-PA in the CNS is its capacity to modulate blood brain barrier (BBB) permeability. This particular feature of t-PA is likely to have a bearing on its limited clinical utility in ischaemic stroke. t-PA was first shown to promote BBB permeability in a mouse model of ischaemic stroke where t-PA deficient mice displayed less parenchymal accumulation of Evans Blue dye after 6 h permanent occlusion (68). That t-PA influences permeability of the BBB (often referred to as the neurovascular unit) has been a consistent finding as reviewed in (69). This is an area of intense research activity and a number of mechanisms have been proposed to explain how t-PA opens the BBB, including interaction with LRP (a member of the low density lipoprotein receptor family), activation of matrix metalloproteinases and processing of platelet derived growth factor (PDGF)-CC and subsequent activation of the PDGFRa receptor (reviewed in (69)). Still, the fundamental question remaining is the biological purpose behind this effect of t-PA. The finding that t-PA could act to

promote glucose uptake following cerebral ischaemia (67) is consistent with a role for t-PA in regulating energy demands with BBB permeability changes complementing this effect. This however requires further exploration.

An interesting element to these effects of t-PA in the CNS is the extent to which t-PA acts alone or via plasmin (70). Plasmin certainly plays an important role in mediating some of these effects of t-PA in the CNS. Indeed, plasmin is one of the proteases (together with furin) that can convert the neurotrophic factor proBDNF into its mature form (45). t-PA-mediated neurotoxicity in vivo also requires plasmin-dependent laminin breakdown (71). While numerous other publications have indicated a requirement for plasmin generation in some of the effects of t-PA in the CNS (72), other reports have provided compelling evidence that t-PA does not require canonical (plasmin-mediated) proteolytic activation or may not require plasmin generation at all. Indeed, t-PA-mediated opening of the BBB via PDGF-CC activation mentioned above occurs in a plasmin-independent manner based on studies using plasminogen deficient mice (73). However, plasmin-dependent pathways however are not fully excluded and this could be influenced by the duration of the ischaemic event (reviewed in (70)). Other reports have indicated that t-PA can directly activate microglial cells essentially acting as a cytokine (74), and also to activate intracellular signalling pathways independently of plasmin upon binding to cell surface receptors (most likely annexin A2; (75)). The neuroprotective effect of t-PA against zinc toxicity referred to above (65) was also reported to be independent of the proteolytic actions of t-PA and was rather mediated by sequestration of zinc (76).

How can t-PA function in the absence of fibrin?

The various non-fibrinolytic effects of the plasminogen activating system are now well entrenched in the literature and have steered the field onto a number of unanticipated courses. However some fundamental questions remain. The previous 30 years of research into the enzymatic basis of t-PA-mediated plasminogen activation in blood had one common yet important thread, that being the requirement of fibrin as an essential cofactor for t-PA to activate plasminogen. Given that the CNS is devoid of fibrin, what provides t-PA with its essential co-factor to allow it to drive plasmin formation? Alternatively, could brain t-PA operate under a different set of rules?

This still remains a pertinent question, although new research has revealed that fibrin is not the only natural cofactor for t-PA. In fact, t-PA can source its cofactor to activate plasminogen from numerous other proteins; see (77) for a review. For example, prion proteins (78, 79), beta-amyloid (80–82) and glycated proteins (83) including albumin were all shown over a decade ago to accelerate t-PA-mediated plasminogen activation, but whether there is a common determinant in these modified proteins remains to be determined. Plasminogen itself was shown to bind to dead or apoptotic cells ~100 times more effectively than live cells in a lysine-dependent manner (84), although the specific cellular binding site was not revealed.

The role of misfolded proteins as a general cofactor for t-PA (and indeed for other proteases) (85) has grown in strength in recent years. While isolated denatured/misfolded proteins have been shown to provide cofactor activity for t-PA, these studies did not report whether this was of any physiological relevance. Samson et al (86) reported the interesting observation that both t-PA and plasminogen both bound to the surface of dead neurons as well as other necrotic cell types, also in a lysine-dependent manner. Moreover this interaction resulted in enhanced plasmin formation and the subsequent breakdown and removal of the cell corpse. Hence, a naturally occurring non-fibrin cofactor for t-PA could indeed occur in cells to facilitate plasmin formation and the subsequent removal of the unwanted tissue. It also seems likely that plasmin-mediated turnover of necrotic tissue is associated with the well described role of plasmin(ogen) in facilitating wound repair (31, 87–89).

More detailed analysis of the role of necrotic cells in facilitating plasmin activation was reported in 2012 (90). Mass spectrophotometric analysis and enzyme kinetic studies revealed that t-PA was binding to an array of misfolded proteins formed during cell death. These proteins were mostly (but not exclusively) cytoskeletal in nature, with the most abundant of these including beta-tubulin and actin. Using a mouse model of traumatic brain injury (TBI) it was also shown that misfolded beta-tubulin

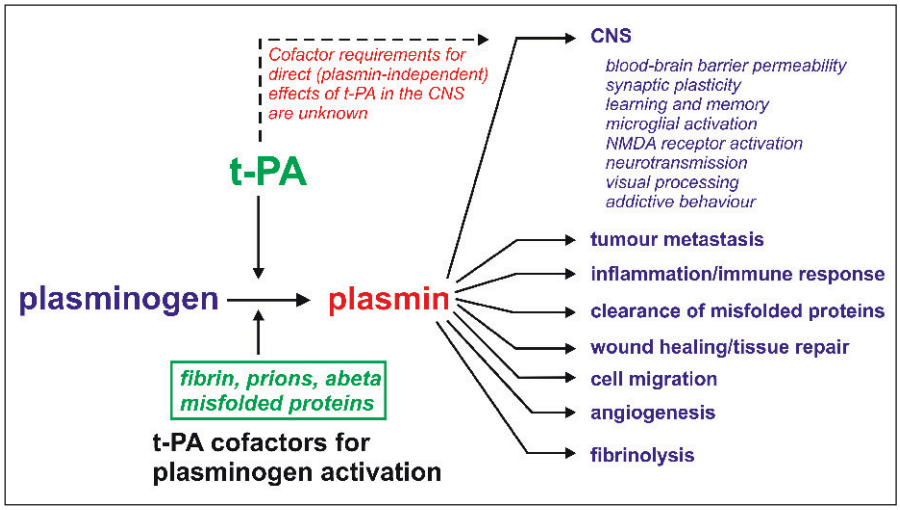

Fig. 1 Pleiotrophic effects of t-PA and plasmin in physiology and in pathophysiology: Many plasmin-dependent effects of t-PA requires a specific cofactor (i. e. the classical cofactor for t-PA in fibrinolysis is fibrin) while other cofactors have also been shown to accelerate t-PA-mediated plasminogen activation in vitro (prion proteins, amyloid beta) or in vivo (misfolded/aggregated proteins). However, it remains to be determined if any of the non-fibrinolytic (plasmin-independent) effects of t-PA (mostly in relation to the CNS) requires cofactor activity.

(as determined by relative solubility) accumulated in the injured brain within 3 h of TBI and these levels subsided after ~72 h. When these experiments were performed in plasminogen deficient mice, misfolded beta-tubulin accumulated to a greater extent and persisted longer in the lesioned brain. This finding provided a strong argument for a role of the fibrinolytic system in the general removal of misfolded proteins. By extension, fibrin itself would fall under this umbrella, itself being an insoluble cross beta structured protein (77). The paper by Samson et al. (90) also described a novel means of intracellular protein aggregation occurring late following cell injury and was reliant on disulphide bonding. This novel form of aggregation was coined "nucleocytoplasmic coagulation (NCC)" (90). NCC was shown to be an injury-induced aggregation event that, akin to fibrin formation in a blood clot, encapsulates the necrotic material and subsequently facilitates their plasmin-mediated removal.

A revised definition of the fibrinolytic system?

So what is the role of the fibrinolytic system really, and what drives it? There is no dispute that this enzyme cascade can remove fibrin, although there is an element of redundancy given that fibrinolysis is largely unaltered in t-PA deficient mice. That fibrin was shown to be the first substrate for plasmin was enough to give the process its current name, but the fact that many misfolded proteins can substitute for fibrin has broadened the scope of this system to one that appears to be a general surveillance system for proteins no longer needed, or for proteins that have evolved to exist temporarily. And what of the CNS? The role of t-PA in learning and memory, synaptic plasticity, modulation of blood brain barrier permeability etc. may have different cofactor requirements or may not be needed at all, particularly in relation to any plasmin-independent effect of t-PA. A summary of the pleiotrophic effects of t-PA and the various co-factor requirements are presented (▶Fig. 1). Nonetheless, the various plasmin-dependent and -independent attributes of t-PA in the CNS has opened up a series of questions that will no doubt be addressed in the near future. Even with our current extended knowledge, it is reasonable to conclude that the original concept for the fibrinolytic system, being focused on fibrin, is a massive understatement.

Conflict of interest

The author declares that there is no conflict of interest in the content of this article

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