The key to fibrinolysis and thrombolysis

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ABSTRACT

This narrative review was written at the occasion of the "**Verstraete Centennial Memorial Lecture**" organized on September 15, 2025 at NEUROMED (Pozzilli, Italy) by Giovanni de Gaetano and Maria Benedetta Donati. It represents a historical account of contributions by the Center for Molecular and Vascular Biology (CMVB), KU Leuven, Belgium, founded by Marc Verstraete, to our understanding of the regulation of fibrinolysis and the development of thrombolytic therapy. This review covers the long trajectory of events which, over almost half a century, have led from basic biochemical discoveries to the development of recombinant tissue-type plasminogen activator (rt-PA) and other clinically valuable drugs to treat thrombotic diseases.

Historical background

Marc Verstraete (1925-2018) (Figure 1) obtained his MD degree from the KU Leuven in 1951. Already in 1947, as a student-researcher, he founded a small Laboratory for Blood Coagulation within the department of General Pathology of the old university hospital St. Raphael. There he developed coagulation tests for clinical and basic investigations, resulting in his first scientific communication at the Société Belge de Pathologie et Médecine Expérimentale (1952; vol 21:321-332). He subsequently specialized in internal medicine and hematology in Leuven, Basle, Oxford and New York. Upon his return to Leuven, he focused his research on hemophilia and von Willebrand Disease, coagulation and platelet disorders and fibrinolysis/thrombolysis. Already in 1957, he published a paper in Circulation demonstrating how the prothrombin time can be used to monitor anticoagulation.[1] Almost 40 years later he still was a member of the steering committee of the multicenter CAPRIE study[2] that established the antithrombotic potential of Clopidogrel; this illustrates his lifelong commitment to research improving patient care.

Figure 1. Marc Verstraete (1925-2018).

In 1970 the lab moved to a small prefab building, previously home to the hospital purchase department. This evolved in 1976 into the Center for Thrombosis and Vascular Research, with 700 m^2 lab space in building O&N1 at Campus Gasthuisberg. In 1994 the lab was renamed into Center for Molecular and Vascular Biology (CMVB) and moved to a new 4000 sq.m^2. fully equipped lab space on the 9th floor of the newly constructed CDG building. Royalty income from the t-PA patent (1988-2006) provided the financial basis for the foundation of Life Sciences Research Partners (LSRP; www.lsrp.be), which in turn was instrumental in financing and equipping the new facilities.

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In addition to the fibrinolysis research group (D. Collen, R. Lijnen), active research groups were further established on platelets (J. Vermylen, J. Arnout) and vascular biology (R. Verhaeghe, M. Verstraete). In 1995, within the CMVB, a new department of the Flemish Institute for Biotechnology (VIB) was established, named Center for Transgene Technology and Gene Therapy, directed by D. Collen. The new lab space comprises a 600 m^2 state-of-the-art "M. Verstraete Specific Pathogen Free Animal Facility", that has been instrumental in the generation of many gene knock-out mouse strains. At this time, the total number of collaborators exceeded 150.

The research labs stayed in close contact with the clinic, as Prof. Verstraete was also director of the department of Bleeding and Vascular Disorders at the University Hospital Gasthuisberg, and he previously (1958) founded the Circle of Friends of Hemophilia. When Prof. Verstraete became Emeritus in 1990, he was succeeded in the university hospital consecutively by J. Vermylen, R. Verhaeghe and P. Verhamme, and in the research lab CMVB by D. Collen, R. Lijnen and K. Freson. The VIB lab, now directed by P. Carmeliet, moved to a new building and was renamed first as Vesalius Research Center and today as Center for Cancer Biology.

The CMVB has significantly contributed to the international recognition and stature of the KU Leuven. Over 250 international collaborators studied and trained in the lab, and for many of those this sabbatical has been a boost to their scientific career. The hospitality extended by the Verstraete family to so many international visitors is still legendary. The family house at Minderbroedersstraat 29 in Leuven was recently acquired and renovated by the Désiré Collen Foundation (https://www.desirecollenstichting.be/). It now comprises studios and meeting rooms intended to foster academic exchange and interactions, in the spirit of the late Prof. Verstraete (https://www.huyzeverstraete.be).

Members of the CMVB served in multiple functions in the university administration, in national institutes such as the Royal Academy of Medicine and the Fund for Scientific Research and played important roles in international scientific organizations such as the International Society on Thrombosis and Hemostasis (ISTH) and the International Society for Fibrinolysis and Proteolysis (ISFP). The CMVB organized the international congress of the ISTH in 1987 (Brussels) and that of the ISFP in 1994 (Leuven). The scientific output of the CMVB in terms of publications, citations and PhD projects ranks among the highest in the field. At present, the CMVB staff consists of 42 full time collaborators of 10 different nationalities, including 20 PhD students and 7 postdocs. They successfully continue the research on thrombosis and hemostasis, vascular biology and risk factors for cardiovascular health (https://gbiomed.kuleuven.be/english/research/ 50000635/CMVB/index.htm). The best-known achievement of the CMVB undoubtedly is the development of tissue-type plasminogen activator (t-PA) from a laboratory concept into a drug that is used worldwide to treat patients with thromboembolic disease. Therefore, this paper will focus on CMVB contributions to fibrinolysis and thrombolysis.

Understanding physiological fibrinolysis

At the beginning of the 1970's, knowledge on the fibrinolytic system was limited to identification of plasminogen that could be converted into plasmin by streptokinase or urokinase, and of the inhibitors α1-antitrypsin and α2-macroglobulin. Studies by Collen and Vermylen on the turnover of radiolabeled plasminogen in patients treated with streptokinase revealed the presence in blood of an inactive complex of plasmin with an unknown protein.[3] After purification, this turned out to be a new member of the serine protease inhibitor (serpin) family, which was named α2-antiplasmin.[4,5] In subsequent years additional components of the fibrinolytic system have been discovered and their mechanism of action elucidated, including Plasminogen Activator Inhibitor-1 (PAI-1), Thrombin Activatable Fibrinolysis Inhibitor (TAFI), and specific cell-associated receptors for urokinase and plasminogen.[6]

Inhibition of plasmin by α2-antiplasmin

α2-antiplasmin is a 70 kDa single-chain glycoprotein containing 464 amino acids.[7,8] Plasmin is a 85 kDa serine protease composed of a heavy chain (N-terminal) containing 5 kringles with lysine-binding sites (LBS), which are required for binding of plasmin(ogen) to fibrin, and a light chain (C-terminal) with the catalytic triad composed of His603, Asp646 and Ser741. Kinetic analysis by Wiman and colleagues revealed the very rapid formation of a 1:1 stoichiometric complex between plasmin and α2-antiplasmin at rate constants approaching diffusion-controlled processes. The time course of inhibition is compatible with a consecutive 2-step mechanism with first a rapid, reversible second-order reaction, followed by a slower irreversible first-order transition. The reaction involves interactions between the LBS in the plasmin kringles and specific Lys residues in the C-terminal end of the inhibitor, followed by cleavage of the Arg376-Met377 reactive site peptide bond in α2-antiplasmin by the plasmin active site, resulting in the formation of a covalent complex with release of a 8 kDa peptide from the C-terminal of the inhibitor.[9] When the active site and the lysine-binding sites in plasmin are not available, inhibition rates are 50- to 100-fold lower.[10] This explains why plasmin in the circulating blood is extremely rapidly inhibited by α2-antiplasmin, whereas it is protected from inhibition when bound to fibrin.

α2-Antiplasmin also binds to fibrin by cross-linking via its N-terminal region, thus impairing endogenous fibrinolysis.[11] Interestingly, an abnormal α2-antiplasmin has been found in a Dutch family with severe bleeding complications (α2-antiplasmin Enschede). It is converted from a plasmin inhibitor into a substrate as a result of insertion of an extra Ala residue in the reactive center loop on the N-terminal site of the P1 residue Arg376.[12]

Production and characterization of t-PA

As early as 1952, T. Astrup extracted a soluble plasminogen activator from animal tissues,[13] followed by purification by others of tissue plasminogen activators from various sources.[14] The first highly purified form of human t-PA was obtained by D. Rijken from uterine tissue (about 1 mg out of 5 kg tissue, representing 5000-fold purification).[15] Looking for a better source of t-PA, we totally serendipitously found that the culture medium of a melanoma cell line, obtained from patient Bowes, contained significant amounts of plasminogen activator. It turned out that, unlike urokinase, this activator had a specific affinity for fibrin. With a modified version of the procedure used by D. Rijken on uterine tissue, it was over a few years possible to purify about 2 g of t-PA.[16]

Large scale use of t-PA would, however, only become possible after cloning and expression of the human t-PA gene by D. Pennica at Genentech.[17] This collaboration was the result of a serendipitous meeting of Collen with Pennica at the occasion of the Fifth Congress on Fibrinolysis (Malmo 1980) where Collen for the first time presented our results with melanoma t-PA, one day after a patent was filed on its preparation and clinical use. The cDNA of human t-PA was first expressed in *E. coli* and subsequently in mammalian cells, yielding a properly processed and glycosylated molecule. The generation of Chinese Hamster Ovary cells capable of producing single-chain human t-PA has allowed the development of large-scale tissue fermentation and purification procedures, yielding recombinant t-PA (alteplase) for commercial purposes (Activase, Genentech/Roche; Actilyse, Boehringer Ingelheim). Their estimated combined market share today amounts to about 45%. After expiration of the t-PA patent in 2006 other companies started to produce rt-PA based drugs, mainly Abbott (Retelex with about 19% market share) and Merck KGaA (about 11%), as well as some smaller companies in India and Russia (source: Coherent Marketing Research 2025).

With the availability of rt-PA it also became possible to better understand its structure-function relations. Human t-PA is a single-chain serine protease of 70 kDa, containing 527 amino acids and 4 structural domains (Figure 2). These include: i) an N-terminal region of 47 residues homologous with the finger domain (F-domain) mediating the fibrin affinity of fibronectin; ii) residues 50 to 87 (E-domain) which are homologous to epidermal growth factor; iii) two kringle regions (residues 87 to 176, K1-domain, and 176 to 256, K2-domain) homologous to the plasminogen kringles, and iv) a serine protease region (residues 276 to 527, P-

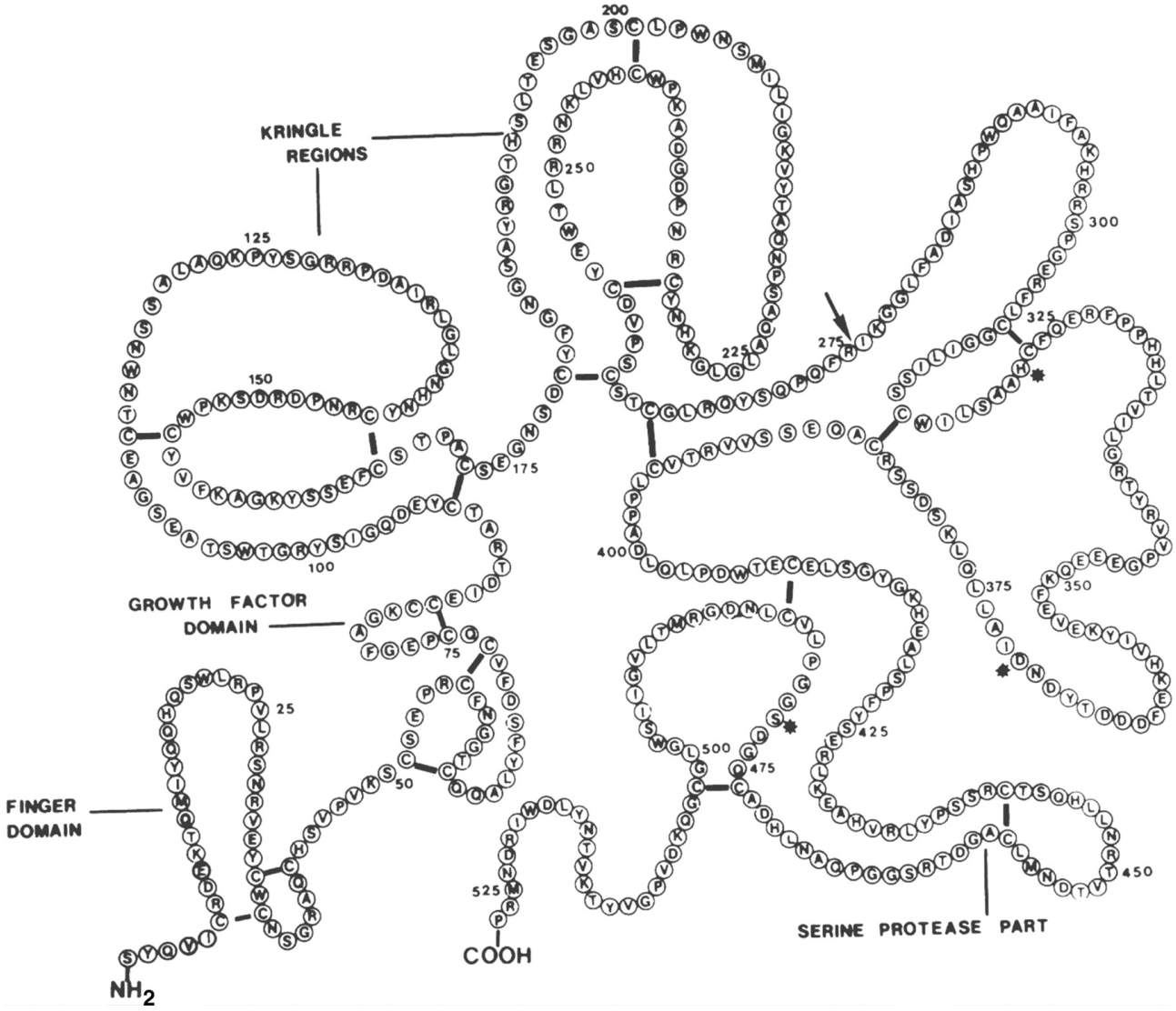

Figure 2. Schematic representation of the primary structure of t-PA. The amino acids are represented by their single letter symbols, and black bars indicate disulfide bonds. The active site residues His322, Asp371, and Ser478 are marked by asterisks. The arrow indicates the cleavage site for conversion of single-chain to two-chain t-PA. Modified from: Pennica *et al.*[17]

domain) with the active site residues His 322, Asp 371 and Ser 478.[17] These distinct domains are involved in several functions of t-PA, including binding to fibrin (mainly F- and K2-domains), rapid clearance *in vivo* with initial half-life of 6 minutes in man (F-or E-domains and carbohydrate side chains), enzymatic activity (P-domain), and rapid inhibition by PAI-1 (sequence Lys296-His-Arg-Arg299). Single-chain t-PA is converted by plasmin to a two-chain form by hydrolysis of the Arg275-Ile276 peptide bond; in contrast to other single-chain precursors of serine proteases single-chain t-PA is enzymatically active.[18]

Based on these structure-function relationships many attempts were made to further improve the functional properties of t-PA. These include mutants and variants, chimeric molecules containing domains of t-PA and urokinase (u-PA) and coupling with fibrin- or platelet-targeting antibodies.[19] Two extensively studied mutants are Reteplase or Retavase (Chiesi Pharma/Wacker Biotech) and Tenecteplase or Metalyse (Genentech/ Roche). Reteplase is a single-chain non-glycosylated rt-PA consisting of the K2- and P-domains (amino acids 1-3 and 176-527), with about 3-fold prolonged half-life as compared to rt-PA.[20] In Tenecteplase (TNK-tPA or Metalyse) replacement of Asn117 with Gln deletes the glycosylation site in K1, whereas substitution of Thr103 by Asn reintroduces glycosylation at a different site in the K1-domain; these modifications decrease the plasma clearance rate (half-life of about 20 min as compared to 6 min for rt-PA). In addition, replacement of the sequence Lys296 to Arg299 by Ala residues confers resistance to inhibition by PAI-1.[21]

Activation of plasminogen by t-PA

As other plasminogen activators, t-PA converts the 92 kDa single-chain zymogen plasminogen into the two-chain active serine protease plasmin by cleavage of the Arg561-Val562 peptide bond. Kinetic analysis with melanoma t-PA revealed that it is a poor plasminogen activator in the absence of fibrin, whereas in the presence of fibrin its activity is two orders of magnitude higher. The kinetic model indicates that both plasminogen and t-PA bind to fibrin in a sequential and ordered way, yielding a cyclic ternary complex in which t-PA has a markedly enhanced affinity for its substrate plasminogen.[22] This model is further supported by the finding that blocking of the lysine-binding sites in plasminogen, e.g. with tranexamic acid, prevents its binding to fibrin and subsequent activation by t-PA.[23] This model for physiological fibrinolysis was presented at the VII International Congress on Thrombosis and Haemostasis (London 1979) and is schematically illustrated in Figure 3.[24] It formed the basis of the concept of the fibrin specificity of t-PA and stimulated great interest in its use for thrombolytic therapy, as alternative to the non-fibrin-specific streptokinase and urokinase that were available at that time.

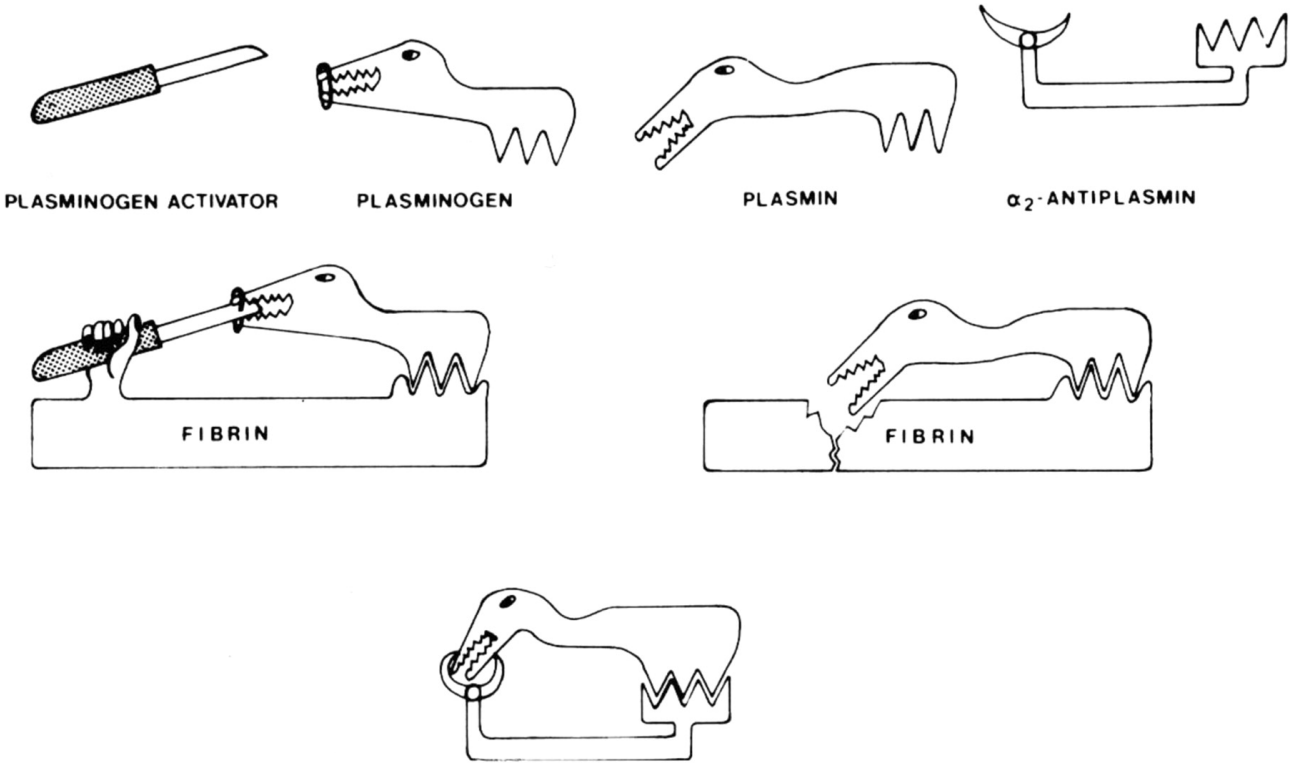

Figure 3. Schematic visualization of the molecular interactions regulating physiological fibrinolysis. Plasminogen is converted to the proteolytic enzyme plasmin by tissue-type plasminogen activator, but this conversion occurs efficiently only on the fibrin surface, where activator and plasminogen are "assembled." Free plasmin in the blood is very rapidly inactivated by α2-antiplasmin, but plasmin generated at the fibrin surface is partially protected from inactivation. The lysine-binding sites in plasminogen (represented as the "legs" of the animal) are important for the interaction between plasmin (ogen) and fibrin and between plasmin and α2-antiplasmin.

Inhibition of t-PA by PAI-1

The serpin plasminogen activator inhibitor-1 (PAI-1), a 52 kDa single-chain glycoprotein with reactive site peptide bond Arg346-Met347, is the main physiological inhibitor of both t-PA and u-PA. PAI-1 plasma levels monitored with specific detection methods vary strongly in many clinical conditions.[25]

PAI-1 inhibits its target proteases by formation of a 1:1 stoichiometric reversible complex, followed by covalent binding between the hydroxyl group of the active site Ser residue of the protease and the carboxyl group of the P1 residue at the reactive site of the serpin. The rapid reaction involves highly positively charged residues in t-PA (296-304) and in u-PA (179-184).[6]

Seminal contributions of the CMVB to understanding the biology of PAI-1 include the identification of vitronectin (multimeric form of S-protein) as a protein that stabilizes PAI-1 activity in plasma,[26] and the detection of a conformationally distinct form of PAI-1 that behaves as a non-inhibitory substrate for t-PA.[27] We have purified and characterized natural and recombinant PAI-1,[28] and used a reactivated form to show in a rabbit model of jugular vein thrombosis that elevated levels of active PAI-1 indeed impair the thrombolytic activity of t-PA *in vivo*.[29]

Gene deficient mice to study the fibrinolytic system

After a postdoc at the Whitehead Institute (Massachusetts, USA) P. Carmeliet introduced transgene technology in the CMVB. His team generated transgenic mice with knock-out of the main components of the fibrinolytic system, thus allowing to study their function in an unprecedented manner.

Plasminogen deficient mice survive embryonic development, but develop spontaneous fibrin deposition due to impaired thrombolysis, and suffer retarded growth, reduced fertility and survival.[30] Restoration of normal plasminogen levels by administration of purified murine plasminogen normalized the thrombolytic potential and resulted in removal of endogenous fibrin deposits.[31]

Inactivation of the t-PA gene in mice impairs clot lysis, whereas inactivation of the u-PA gene results only in occasional fibrin deposition. Mice with combined deficiency of t-PA and u-PA suffer extensive spontaneous fibrin deposition, with associated impact on growth, fertility and survival.[32] Interestingly, it was shown that u-PA but not t-PA mediates arterial neointima formation.[33]

α2-Antiplasmin deficient mice develop and reproduce normally and have an enhanced endogenous fibrinolytic capacity, but without overt bleeding.[34]

Homozygous PAI-1 deficient mice are viable and fertile but present with a mild hyper fibrinolytic state and a greater resistance to venous thrombosis without, however, impaired haemostasis as no spontaneous bleeding or delayed rebleeding was observed,[35] PAI-1 was found to inhibit arterial wound healing and neointima formation.[36]

Mice homozygous deficient for the u-PA receptor display normal lysis of blood clots induced in the jugular vein, not supporting an essential role in fibrinolysis.[37]

Collectively, these studies conclusively established the role of the main components of the fibrinolytic system in fibrin clot lysis. These and other studies also revealed a functional role of the fibrinolytic system in many other biological processes, such as growth and fertility, restenosis, atherosclerosis, neointima formation, angiogenesis, cancer cell invasion and memory.[38,39]

Contributions to thrombolytic therapy

Thrombolysis consists of the pharmacological dissolution of a blood clot by administration of plasminogen activators that activate the fibrinolytic system. Thrombolytic therapy is a potential treatment of conditions caused by an occlusive blood clot (thrombus), such as acute myocardial infarction (AMI), acute ischemic stroke (AIS), acute pulmonary embolism (PE), deep vein thrombosis (DVT) and peripheral arterial occlusion (PAO).

Verstraete was one of the first to show the feasibility of thrombolytic therapy with streptokinase in peripheral arterial occlusions.[40] He also organized the European Working Party on Streptokinase, who performed 3 mortality trials in patients with AMI, demonstrating for the first time that thrombolytic therapy reduces the mortality, presumably by opening the blocked artery.[41,42] This supported the "open artery hypothesis", i.e. blockage of a coronary artery is the cause of heart attack and not the consequence.

Natural melanoma-derived t-PA

In the early 1980's melanoma-derived natural t-PA was available in sufficient amounts to study its thrombolytic potential in small and larger preclinical animal models. The first study, in rabbits with experimental PE, demonstrated a clear superiority of t-PA over urokinase, both in terms of efficacy and fibrin specificity.[43] Also, in closed-chest dogs with coronary thrombosis induced by advancing a copper coil into the left anterior descending coronary artery, t-PA administration elicited prompt thrombolysis without predisposition to systemic bleeding.[44] These and other preclinical studies in dogs and baboons confirmed its coronary thrombolytic potential, clot-specificity and myocardial protection.[45]

The first human being was treated with melanoma t-PA in 1981, because of a serendipitous encounter between Dr. Weimar (Erasmus University, Rotterdam, the Netherlands) and Dr. Billiau (Rega Institute, KU Leuven, who supported us with the melanoma cell culture). Melanoma t-PA (7.5 mg) was given by intravenous infusion to a renal allograft patient who was developing an ascending thrombosis from the iliac vein to the renal transplant. Despite the low dose of t-PA, the clot completely dissolved without side effects and kidney graft function rapidly improved.[46] In 1983, melanoma t-PA was administered for the first time to AMI patients. Intravenously administered t-PA in doses of 0.2 to 0.4 mg/min completely recanalized occluded coronary arteries within 30 to 60 minutes in 6 out of 7 patients without causing a systemic fibrinolytic state.[47] The FDA (Food and Drug Administration) approved t-PA (alteplase) for the treatment of AMI patients in 1987, and the EMA (European Medicines Agency) followed 9 years later. Also, Reteplase was approved for the treatment of AMI by the EMA in 1992 and by the FDA in 1996.

Recombinant t-PA

Experimental animal models of coronary occlusion in dogs and baboons with rt-PA confirmed the clot-specific coronary thrombolytic potential previously demonstrated with melanoma t-PA.[45] These initial promising results stimulated initiation of a multicenter, blinded randomized trial with recombinant t-PA in 50 AMI patients.[48] Intravenous infusion of 0.5 mg/kg body weight

rt-PA over 60 minutes or the same dose followed by 0.25 mg/kg over an additional hour resulted in recanalization of occluded coronary arteries in 75% of patients. This study provided the basis for the NIH Thrombolysis in Acute Myocardial Infarction (TIMI) trials and the European Cooperative Study group (ECSG) led by M. Verstraete. The 6 trials of the ECSG enrolled 2121 patients with AMI and resulted in 6 princeps and 18 ancillary publications. A report on the trials of the European Working Party on Streptokinase and the European Cooperative Study Group on Alteplase in patients with acute myocardial infarction has been published by M. Verstraete on behalf of the steering committee.[49] Numerous other clinical trials have since compared the thrombolytic potential of rt-PA (alteplase) with other agents in megatrials with thousands of AMI patients, such as GISSI and ISIS. Furthermore, several clinical trials such as ASSENT I-IV demonstrated the thrombolytic potential of Tenecteplase in AMI. Collectively, these studies clearly established that thrombolytic therapy reduces mortality in AMI patients, confirming the "open artery hypothesis". With respect to the optimal thrombolytic therapy scheme, there were protagonists of the streptokinase plus aspirin scheme versus defendants of the t-PA plus heparin scheme. In 1993, the GUSTO (Global Utilization of t-PA and Streptokinase for Occluded Arteries) trial and its angiographic substudy[50,51] in over 41,000 AMI patients conclusively showed the potential and limitations of thrombolytic therapy and established t-PA as the leading thrombolytic agent. In the following years, the rt-PA scheme gradually moved from an intravenous infusion (100 mg over 3 hours) to a more effective front-loaded t-PA (GUSTO) or double bolus administration (COBALT), always with intensive heparin anticoagulation. At present, patients with ST-elevation myocardial infarction will receive immediate treatment with percutaneous coronary intervention (PCI) if time from electrocardiogram diagnosis to PCI initiation is 120 min or less. If a longer delay is expected, patients should receive thrombolytic therapy (Tenecteplase recommended) and undergo PCI 2-24h later or rescue coronary intervention if needed.[52]

Beyond the controversies on the use of t-PA or streptokinase in AMI, the superiority of rt-PA was clearly established in eligible AIS patients (with occluding clot confirmed by brain imaging) for whom it has become the standard of care. AIS is according to the WHO the second leading cause of death and disability. In 1996, the FDA approved rt-PA for the treatment of AIS, and in 2019 he WHO declared rt-PA to be an "essential medicine" for AIS. A landmark study by the American National Institute of Neurological Disorders and Stroke (NINDS)[53] had demonstrated the efficacy of rt-PA (alteplase) in improving neurological and functional outcome in AIS patients when administered within 3 hours of stroke onset, a therapeutic window that was later extended to 4.5 hours. As an alternative to IV infusion of alteplase, bolus Tenecteplase is evaluated in several clinical trials with AIS patients. End 2024 a large comparative randomized open-label trial (ATTEST-2) showed non-inferiority of Tenecteplase[54]. Reteplase has also been compared to alteplase in a randomized multicenter open-label non-inferiority trial in over 1400 AIS patients (RAISE trial). Upon administration within 4.5 hours of symptom onset, Reteplase was reported to yield a somewhat better functional outcome but was associated with somewhat higher bleeding risk.[55] These findings remain to be confirmed by further studies. Today, patients with AIS, after clinical assessment will undergo brain imaging (computed tomography or magnetic resonance) and based on time criteria and imaging findings the decision to fibrinolytic treatment is made.[52]

rt-PA was approved for treatment of acute PE by the FDA in 1990 and by the EMA in 2002. Already in 1988 Verstraete et al. studied intravenous (IV) and intrapulmonary (IP) rt-PA in the treatment of acute massive PE.[56] This multicenter trial indicated that the IP infusion of rt-PA did not offer significant benefits over the IV route and suggested that a prolonged infusion over 7 hours (100 mg) is superior to a single infusion of 50 mg over 2 hours. Although since multiple schemes have been studied, it is still debated which therapy should be used for the individual patient with PE and within what timing.

Staphylokinase

Given the high cost of rt-PA (2000$ for 100 mg alteplase in 1988), Collen and the CMVB searched for a cheaper alternative, a "poor man's t-PA" that would be affordable in less affluent countries. That was the start of the staphylokinase (SAK) project, a plasminogen activator derived from *Staphylococcus aureus*. The thrombolytic potential of SAK had been studied in dogs decades ago, but it was found to have poor thrombolytic potency while inducing severe bleeding and complete fibrinogen degradation.[57] In retrospect, these studies have been misleading because the canine fibrinolytic system is unusually sensitive to systemic activation with staphylokinase.[58] We have cloned, purified and extensively characterized staphylokinase, resulting in over 50 publications by CMVB staff.[59,60] SAK is a bacterial protein of about 16 kDa containing 136 amino acids in a single polypeptide chain without disulfide bridges. It is not an enzyme, but it forms a 1:1 stoichiometric complex with plasmin that activates other plasminogen molecules with higher efficiency for plasminogen molecules bound to partially degraded fibrin. The SAK-plasmin complex is rapidly inhibited by α2-antiplasmin in a plasma milieu but associated with fibrin it is protected from inhibition and induces fibrin clot lysis without associated fibrinogen degradation, thus conferring unique fibrin selectivity.[61,62] In several experimental animal models including arterial and venous thrombosis models in baboons, SAK appeared to be at least equipotent to streptokinase and significantly more fibrin specific.[63] The feasibility of fibrin-specific coronary thrombolysis with recombinant SAK (10 mg IV infusion over 30 min) was demonstrated in a small pilot study in patients with evolving AMI.[64] Additional clinical trials included an open multicenter randomized trial comparing staphylokinase with accelerated weight-adjusted rt-PA in 100 patients with AMI,[65] a small pilot study of bolus staphylokinase infusion in patients with myocardial infarction,[66] and a comparative trial of double bolus staphylokinase versus front-loaded rt-PA in 102 patients.[67] These pilot clinical studies indicated that staphylokinase combined with heparin and aspirin is a potent, rapid acting and highly fibrin-specific thrombolytic agent in AMI patients. These findings were confirmed in 30 patients with angiographically documented PAO treated with intra-arterial staphylokinase.[68] However, neutralizing antibodies against staphylokinase were detected from the third week on in all patients.[60] Extensive efforts were undertaken to reduce its immunogenicity by eliminating immunodominant epitopes. A variant with a single substitution of Lys74 with Ala had intact thrombolytic potency and induced significantly less antibody formation in PAO patients than wild type staphylokinase.[69]

Furthermore, derivatization of staphylokinase with polyethylene glycol (PEG-SY161) resulted in up to 20-fold prolonged half-life with preserved thrombolytic potential in AMI patients.[70]

To valorize these promising results, Thromb-X (later renamed ThromboGenics) was founded to develop SAK for thrombolytic therapy. However, to be accepted by regulatory agencies it had to be tested against rt-PA. The costs of these large-scale comparative trials would run in the million dollars, way too high for ThromboGenics. All CMVB patents on staphylokinase expired by 2009, but the extensive preclinical and clinical data remained available to the worldwide scientific community. Over the last years interest in staphylokinase revived in several countries. The China Food and Drug Administration approved SAK for treatment of AMI in 2010. Russian researchers developed Fortelyzin, a recombinant staphylokinase with reduced immunogenicity, by site-directed mutagenesis of charged residues including Lys74 to Ala. It was shown to be non-inferior to alteplase/Tenecteplase in patients with ST-segment elevation myocardial infarction,[71] AIS treated within 4.5 hours of symptom onset,[72] and massive PE.[73]

Single-chain urokinase-type plasminogen activator (scu-PA)

Urokinase (u-PA) is secreted as a 54 kDa single-chain molecule (scu-PA, pro-urokinase) that can be converted to a two-chain form (tcu-PA) by cleavage of the Lys158-Ile159 peptide bond by plasmin. u-PA is a serine protease of 411 amino acids with active site triad His204, Asp255 and Ser356. It contains an N-terminal growth factor domain and one kringle structure homologous to these in plasminogen and t-PA, but without a lysine-binding site.[6] A 32 kDa low molecular weight form of scu-PA (both natural and recombinant) has also been characterized.[74]

Scu-PA has an intrinsic plasminogen activating potential which represents less than 0.5% of the catalytic efficiency of tcu-PA.[75] In plasma, in the absence of fibrin, scu-PA is stable and does not activate plasminogen, whereas in the presence of a fibrin clot it induces fibrin-specific clot lysis although it does not bind directly to fibrin. This was explained by its higher affinity toward plasminogen bound to newly exposed C-terminal Lys residues on partially degraded fibrin.[76] Using specific monoclonal antibodies, it was shown that clot lysis with scu-PA in a plasma milieu does not require extensive conversion of scu-PA to tcu-PA.[77] However, in α2-antiplasmin deficient plasma scu-PA is about 4-fold more potent and causes 3-fold more fibrinogen degradation than in normal plasma, indicating a functional role for α2-antiplasmin in fibrin-specific clot lysis with scu-PA.[78]

Effective fibrin-specific coronary thrombolysis in AMI patients was demonstrated with both natural and recombinant scu-PA in small pilot studies.[79,80] Since, non-glycosylated recombinant scu-PA (Saruplase, Grünenthal) has been studied in many clinical trials in patients with AMI and AIS.[81,82]

We have constructed and characterized several chimeric molecules containing domains of t-PA and u-PA.[19] One such molecule consisting of the K1 and K2 domains of t-PA and the protease domain of u-PA was given to 6 patients with AMI, showing a potential for coronary thrombolysis.[83] In another approach, scu-PA was conjugated to intact monoclonal antibodies or to single-chain Fv fragments of fibrin-specific antibodies.[84-86] All these molecules were evaluated in several animal thrombosis models *in vivo* but were eventually not tested in man.

Microplasmin

Microplasmin is a shorter and more stable derivative of plasmin lacking its kringle domains. Recombinant microplasminogen was produced within the CMVB from the yeast *Pichia pastoris* and activated by staphylokinase (variant SY162 with reduced immunogenicity) into microplasmin.[87] Microplasmin dissolves fibrin clots directly without the need to activate plasminogen by t-PA. Initial animal experiments in ischemic stroke models in mice and rabbits, in an extracorporeal loop thrombosis model in rabbits and in a canine model of copper coil-induced coronary artery thrombosis indicated its potential for thrombolysis in stroke and myocardial infarction.[87,88] Clinical studies with microplasmin in AIS[89] and PAO[90] were promising, and ThromboGenics licensed it for further development. However, preclinical research also revealed the potential of microplasmin to treat back-of-the-eye diseases such as the non-surgical treatment of focal vitreomacular adhesion. ThromboGenics then decided, against the will of its founder who then left the company, to focus on development of microplasmin (Jetrea) for the more lucrative ophthalmology. This did not yield the anticipated return-on-investment, and the company, renamed Oxurion, failed in 2024.

Concluding remarks

Serendipity has played an important role in the t-PA story: Bowes melanoma cells that produce large amounts of t-PA, first patient by Dr. Weimar, fortunate meeting between Pennica and Collen, but also having the right people at the right place at the right time. Without the dedicated team at the CMVB, directed by D. Collen, and the many international collaborators it would not have happened with the same urgency, if at all. A detailed account of the t-PA history can be found in the book "Désiré Collen, Biotech Pioneer" (Lannoo 2018) by P. Huybrechts and F. Van Wijck. An English version of the book can be obtained via the website of the Désiré Collen Foundation. Since 2018, the annual global sales of medicines based on t-PA, mainly alteplase and Tenecteplase, exceeded the billion-dollar threshold and it thus became a "blockbuster". Furthermore, an annual growth rate of sales by about 5% is expected at least until 2032, mainly as a result of increased usage in AIS patients.

Development of t-PA from a laboratory concept into a life-saving drug is an example of translational research avant-la-lettre. It still stands out as one of the fastest drug development projects in history, with only 7 years between the first meeting with D. Pennica and the approval of rt-PA by the FDA as a drug for treatment of acute myocardial infarction. Thus, besides the many seminal studies on hemostasis, coagulation and platelets by CMVB staff, this will always remain an integral part of the legacy of the research center founded by Prof. Em. Marc Baron Verstraete some 75 years ago. Indeed, in 1985 he was awarded hereditary nobility status with the personal title of baron. For his coat of arms, he chose "Finis in Fine" (the finish is at the end of the road), which attests to his tenacious attitude of perseverance. In his honor in 2015 the "Marc Verstraete Foundation" was established at KU Leuven, with the aim to support research on thrombosis and hemostasis, especially for young investigators (see www.desirecollenstichting.be).

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