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HISTORICAL SKETCH

Tissue-type plasminogen activator: a historical perspective and personal account

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Summary. Over the past two decades tissue-type plasminogen activator (t-PA), the main physiological plasminogen activator, has been developed as a fibrin-specific thrombolytic agent for the treatment of various thromboembolic diseases. Milestones in this development include: first purification of human t-PA from uterine tissue, elucidation of the interactions regulating physiological fibrinolysis, thus providing a molecular basis for the concept of fibrin-specific plasminogen activation, first animal models of thrombosis and pilot studies in patients supporting the therapeutic potential of t-PA, cloning and expression of recombinant t-PA providing sufficient amounts for large scale clinical use, and demonstration of its therapeutic benefit in large multicenter clinical trials, mainly in patients with acute myocardial infarction (AMI), but also in patients with massive pulmonary embolism, ischemic stroke, deep vein thrombosis and peripheral arterial occlusion. Genetically modified variants of t-PA have been developed for bolus administration in patients with AMI.

History

The fibrinolytic system comprises an inactive proenzyme, plasminogen, which can be converted to the active enzyme, plasmin, which in turn degrades fibrin into soluble fibrin degradation products. Two physiological plasminogen activators have been identified: the tissue-type activator (t-PA) and the urokinase-type plasminogen activator (u-PA). Inhibition of the fibrinolytic system may occur either at the level of the plasminogen activator, by specific plasminogen activator inhibitors (mainly PAI-1), or at the level of plasmin, by specific plasmin inhibitors (mainly α_2-antiplasmin) [1].

As early as 1947 it was reported that animal tissues contain an agent that can activate plasminogen; this factor was originally called fibrinokinase [2]. Since then, many authors have reported the purification and characterization of plasminogen activators from various sources, including pig heart and ovaries, and human post mortem vascular perfusates and postexercise blood. The first highly purified form of human t-PA was obtained from uterine tissue (about 1 mg of t-PA from 5 kg tissue) [3]. Using an antiserum raised against uterine plasminogen activator, it was shown that tissue plasminogen activator, vascular plasminogen activator and blood plasminogen activator are immunologically identical, but different from u-PA [4]. Thus, it was established that the plasminogen activator found in blood represents vascular t-PA that is released, mainly from endothelial cells.

With the availability in the late 1970s of highly purified proteins, biochemical studies, in collaboration with B. Wiman, elucidated the molecular interactions between the main components of the fibrinolytic system that regulate and control physiological fibrinolysis. A model for physiological fibrinolysis was presented at the VIIth International Congress on Thrombosis and Haemostasis (London 1979) [5]. This comprehensive model, schematically illustrated in Fig. 1, formed the basis of the concept of the fibrin specificity of t-PA and stimulated great interest in its use for thrombolytic therapy, as an alternative to the non-fibrin-specific plasminogen activators that were available at that time (streptokinase and two-chain urokinase).

Preparation of t-PA

t-PA has been purified from the culture fluid of a stable human melanoma cell line (Bowes, RPMI-7272), in sufficient amounts to study its biochemical and biological properties [6]. The Bowes melanoma cell line was provided to us by D. B. Rifkin (New York University Medical School) toward the end of 1978. It had been obtained originally from pulmonary, metastatic melanoma cells from a patient named Bowes by G. Moore in 1974 and was maintained and exchanged among investigators because it secreted large amounts of plasminogen activator activity. However, the nature of the activity had not been elucidated. In retrospect, selection of this cell line was fortuitous, because the Bowes cell line is unusually, if not uniquely, efficient in secreting t-PA.

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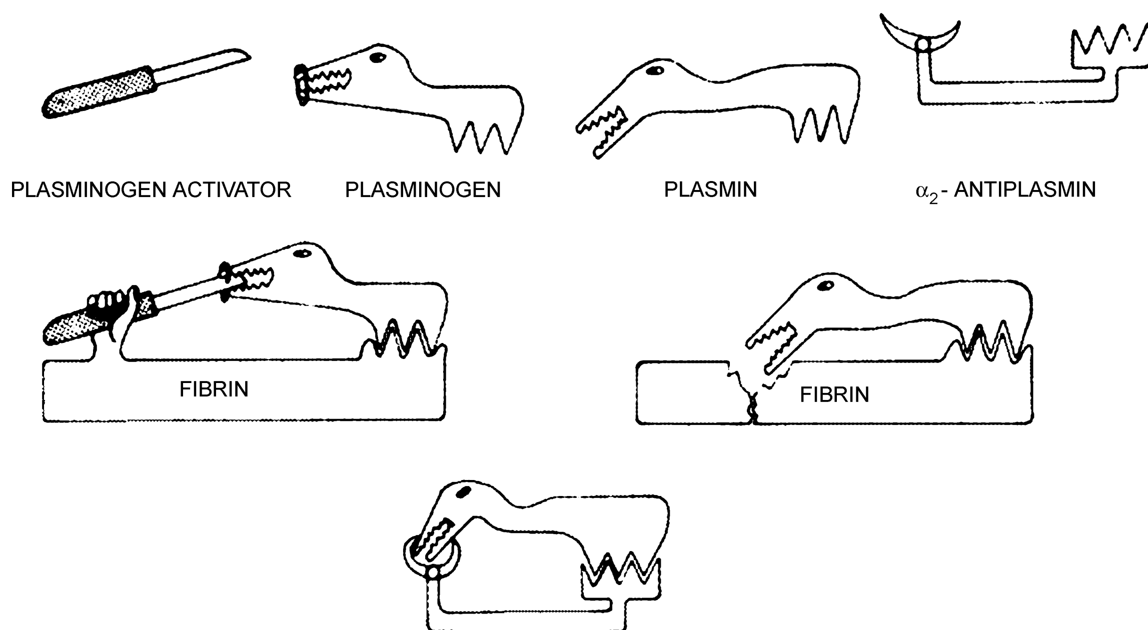

Fig. 1. Schematic visualization of the molecular interactions regulating physiological fibrinolysis. Plasminogen is converted to the proteolytic enzyme plasmin by tissue-type plasminogen activator, but this conversion occurs efficiently only on the fibrin surface, where activator and plasminogen are 'assembled'. Free plasmin in the blood is very rapidly inactivated by α_2-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 α_2-antiplasmin [35].

When we sought initially to purify the plasminogen activator from Bowes melanoma cell culture fluid in 1979, we observed that the activator, like uterine t-PA but unlike urokinase, had a specific affinity for fibrin. When mixtures of fibrinogen and plasminogen activator were clotted, the activator remained associated with the clot. Thus, the melanoma plasminogen activator resembled t-PA. However, with the use of purification methods developed previously for t-PA, no homogeneous, final product was initially obtainable. In October 1979, D. C. Rijken joined us from the Gaubius Institute in the Netherlands. He had developed a method for the purification of the plasminogen activator from human uterus in which adsorption of the activator to surfaces was prevented by the use of Tween 80 and in which zinc-chelate agarose was used in the first chromatographic step [3]. With a simplified version of this purification procedure we were soon able to purify melanoma cell culture fluid plasminogen activator, and to show that it was immunologically identical to the uterine plasminogen activator (t-PA) [6]. With this material, D. Rijken, M. Hoylaerts, H. R. Lijnen, I. Juhan-Vague and C. Korninger clarified the kinetics of plasminogen activation and developed immunoassays for t-PA in plasma [7,8]. Subsequently, the purification procedure was scaled upward to produce a total amount of approximately 2 g of t-PA [9], facilitating systematic characterization of its biochemical, biological, and physiological properties (see below).

Cloning and expression of the human *t-PA* gene

At the Fifth Congress on Fibrinolysis in Malmo, Sweden (1980), where our first results with t-PA were presented, D. Pennica from the Department of Molecular Biology of Genentech Inc. approached us. Collaborations devoted to the cloning and expression of the *t-PA* gene ensued, with results reported at the Sixth Congress on Fibrinolysis in Lausanne, Switzerland (1982), and published in *Nature* in January 1983 [10].

Human melanoma cells actively synthesizing and secreting t-PA were used for the preparation of poly(A)mRNA. This mRNA was size-fractionated using agarose gel electrophoresis and the fractions containing t-PA mRNA were used to prepare a cDNA library, which was hybridized with a pool of radiolabeled synthetic deoxynucleotides potentially complementary to mRNA encoding a known amino acid sequence of t-PA. A recombinant plasmid (pPA25E10) containing a partial-length t-PA cDNA insert was identified and sequenced. The remaining 5′-cDNA sequences were generated by preparing a cDNA library primed with a 16-base long synthetic fragment. A t-PA cDNA clone (pPA17) overlapping with the original clone pPA25E10 and containing the NH_2-terminal and presequence regions was identified after specific hybridization with a DNA fragment prepared from a t-PA genomic clone. The 2530 bp of t-PA cDNA (excluding the poly(A) sequence) code for a polypeptide of 562 amino acids. The 35 amino acids (−35 to −1) preceding the mature sequence probably constitute a 20–23 amino acid long hydrophobic signal peptide followed by a hydrophobic 'pro' sequence of 12–15 amino acids. Nowadays the cloning and expression of t-PA would be a trivial accomplishment, but in 1982 this was quite an achievement, for which D. Pennica deserves much of the credit.

The cDNA of human t-PA has first been expressed in *Escherichia coli* [10]. More efficient expression was obtained in

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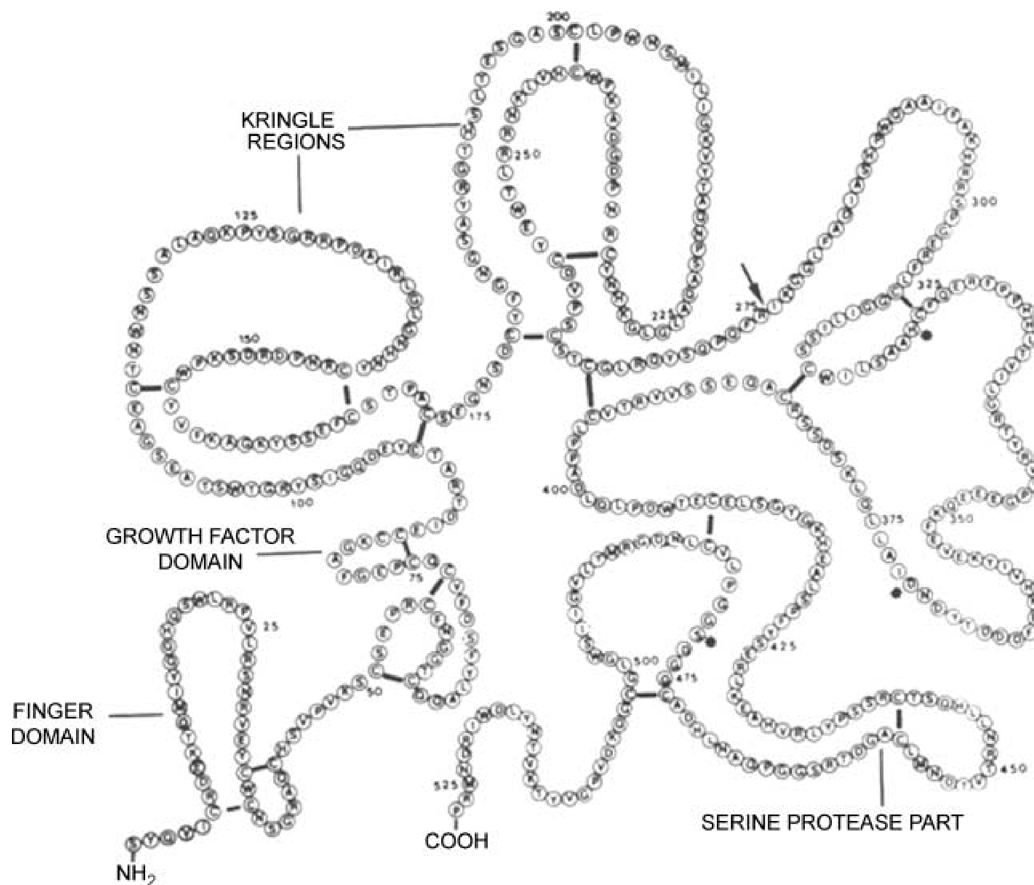

Fig. 2. Schematic representation of the primary structure of tissue-type plasminogen activator (t-PA). The amino acids are represented by their single-letter symbols and black bars indicate disulfide bonds. The active site residues His322, Asp371 and Ser478 are marked by asterisks. The arrow indicates the cleavage site for conversion of single-chain to two-chain t-PA (modified from [10]).

mammalian cells, yielding a properly processed and glycosylated molecule. This recombinant t-PA (rt-PA) was shown to be indistinguishable from the natural activator isolated from human melanoma cell cultures, with respect to biochemical properties, turnover *in vivo*, and specific thrombolytic activity [11]. The generation of Chinese hamster ovary (CHO) cells capable of producing single-chain human t-PA has allowed the development of large-scale tissue culture fermentation and purification procedures, yielding rt-PA (alteplase) for commercial purposes (Activase®, Genentech Inc., South San Francisco, CA, USA; Actilyze®, Boehringer Ingelheim GmbH, Ingelheim, Germany). Biologically active t-PA has also been obtained by expression of cDNA in *Aspergillus nidulans* [12] and in mouse C127 cells [13].

Many investigators, both in academia and industry, have contributed to elucidate the structure–function relationships of t-PA. Human t-PA was first isolated as a single-chain serine proteinase with Mr about 70 000 (pI = 7–8), consisting of 527 amino acids with Ser as the N-terminal amino acid [10] (Fig. 2). It was subsequently shown that native t-PA contains an N-terminal extension of three amino acids, but in general the initial numbering system has been maintained. The molecule has 17 disulfide bonds and an additional free Cys at position 83. Limited hydrolysis of the Arg275–Ile276 peptide bond by plasmin converts t-PA to a two-chain molecule held together by one interchain disulfide bond. The t-PA molecule contains four domains: (i) an N-terminal region of 47 residues (residues 4–50) (F-domain) which is homologous with the finger domain mediating the fibrin affinity of fibronectin; (ii) residues 50–87 (E-domain) which are homologous with epidermal growth factor; (iii) two kringle regions (residues 87–176, K_1-domain, and 176–256, K_2-domain), which share a high degree of homology with the five kringles of plasminogen; and (iv) a serine proteinase region (residues 276–527, P-domain) with the active-site residues His322, Asp371 and Ser478 [10]. The distinct domains in t-PA are involved in several functions, including its binding to fibrin (mainly via F- and K_2-domains), rapid clearance *in vivo* with an initial half-life of 6 min in humans (mediated via F- and/or E-domains) and enzymatic activity (P-domain).

Experimental animal models

The thrombolytic effects of melanoma t-PA were first demonstrated in rabbits with experimental pulmonary embolus *in vivo*, in collaboration with O. Matsuo in 1980 [14].

Late in 1981 at an National Institutes of Health (NIH) workshop on coronary thrombolysis, B. E. Sobel from

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Washington University initiated a collaboration to explore the use of t-PA for the treatment of acute myocardial infarction (AMI). The utility of this approach was demonstrated in closed-chest dogs with coronary thrombosis induced by advancing a copper coil into the left anterior descending coronary artery (LAD). Intravenous infusion of human t-PA purified from melanoma cell culture fluid resulted in prompt coronary recanalization without systemic activation of the fibrinolytic system. Furthermore, it restored myocardial blood flow and intermediary metabolism in the region at risk [15]. These results demonstrated that administration of native human t-PA to animals with induced coronary thrombosis elicited prompt thrombolysis without predisposition to systemic bleeding attributable to a systemic lytic state. Subsequently, these observations were extended to rt-PA. In a collaborative study between the Cardiology Divisions of the University of Leuven, Belgium, and Washington University, the clot-specific coronary thrombolytic properties of rt-PA were demonstrated with the same experimental animal preparation [16]. In a concurrent collaborative study with H. K. Gold (Massachusetts General Hospital), coronary thrombosis was produced between two ligatures of the LAD in open-chest dogs. Infusion of t-PA elicited clot lysis and myocardial salvage in this preparation as well [17]. Subsequently, in a collaborative study with W. Flameng at the University of Leuven, the coronary thrombolytic properties, clot specificity, and myocardial protection achievable with rt-PA were confirmed in baboons [18].

Clinical use of t-PA

In collaboration with W. Weimar (Erasmus University, Rotterdam, the Netherlands) in 1981 two patients with renal vein thrombosis after kidney transplantation were treated with intravenous infusions of 5 and 7.5 mg of melanoma t-PA over 24 h [19]. Despite the fact that lysis occurred, the dose was probably too low for general use judging from the results of subsequent similar patients but without renal dysfunction (unpublished observations).

The first study in which t-PA was administered to patients with AMI was performed in 1983 with purified melanoma t-PA obtained from the Bowes cell line. Participants included F. Van de Werf and coworkers at the University of Leuven and B. E. Sobel and coworkers at Washington University [20]. Intravenously administered t-PA in doses of 200–400 μg min^{-1} completely recanalized occluded coronary arteries within 30–60 min in six of seven patients without inducing a systemic fibrinolytic state. These initial observations with native t-PA served as a template and stimulated initiation of a multicenter, blinded, randomized trial with rt-PA produced at Genentech. With the approval of the Food and Drug Administration, rt-PA was first administered to a patient on 11 February 1984, by E. Topol, then a Cardiology Fellow at Johns Hopkins. Fifty patients were treated with rt-PA between 11 February and 20 June 1984, at Washington University with Sobel and coworkers, at the Massachusetts General Hospital with Gold and coworkers, and at Johns Hopkins University with M. Weisfeldt and coworkers. This rapid progress was possible only because of the efforts of many scientists at Genentech and concomitant investigations elsewhere characterizing some of the biological and thrombolytic properties of the material. Intravenous infusion of 0.5 mg kg^{-1} body weight rt-PA over 60 min or of the same dose followed by 0.25 mg kg^{-1} over an additional 60 min resulted in recanalization of occluded coronary arteries in 75% of patients. Fibrinogenolysis was absent or modest in most but not all of the patients [21]. The results obtained in this initial study of rt-PA in patients with AMI provided a foundation for the design of both the NIH Thrombolysis in Acute Myocardial Infarction (TIMI) trials led by E. Braunwald in the USA and the European Cooperative Study Group trials initially led by M. Verstraete.

Numerous clinical trials have since compared the thrombolytic properties of rt-PA with those of other agents [22,23], culminating in the GUSTO trial led by E. Topol and R. Califf and its angiographic substudy led by A. Ross [24,25], which conclusively established the potential and limitations of rt-PA for thrombolytic therapy in patients with AMI. In the first large clinical trials, the recommended dose of rt-PA (alteplase, Activase®, Actilyse®) for the treatment of AMI was 100 mg administered as 60 mg in the first hour (of which 6–10 mg as a bolus over the first 1–2 min), 20 mg over the second hour, and 20 mg over the third hour. Later, it was proposed to give the same total dose of 100 mg but 'front loaded', starting with a bolus of 15 mg followed by 50 mg in the next 30 min and the remaining 35 mg in the following hour [26]. In the GUSTO trial, a dose of 15 mg intravenous bolus of alteplase followed by 0.75 mg kg^{-1} over 30 min (not to exceed 50 mg) and then 0.50 mg kg^{-1} over 60 min (not to exceed 35 mg) was utilized [24]. In the COBALT trial, double bolus administration of rt-PA (50 mg given 30 min apart) was evaluated in patients with myocardial infarction [27]. Whichever regimen is used, it is important to coadminister intravenous heparin during and after rt-PA treatment. To date, rt-PA is used worldwide in about 300 000 AMI patients every year.

Several approaches have been followed to further improve the thrombolytic properties of rt-PA, essentially by enhancing its plasminogen-activating potency or its fibrin-specificity and by reducing its plasma clearance. These include the construction of mutants and variants (with deletion or substitution of specific amino acids, of entire domains or of carbohydrate moieties), of chimeric molecules (e.g. with domains of u-PA) and of antibody-targeted t-PA (by coupling t-PA to fibrin-specific monoclonal antibodies) [28]. These studies have yielded important insights into the structure–function relationships of t-PA and have led to the development of a few selected mutants which have now been approved for clinical use or which are being evaluated in clinical trials. Such new thrombolytic agents include TNK-rt-PA (tenecteplase) (with Thr103 substituted with Asn, introducing a new glycosylation site; with Asn117 substituted with Gln, eliminating a high mannose glycosylation site; and with the sequence Lys296-His-Arg-Arg299 replaced by Ala, conferring increased zymogenicity and resistance to

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PAI-1), rt-PA E6010 (monteplase, with Cys84 substituted with Ser), n-PA (lanoteplase, with deletion of finger and growth factor domains and Asn117 to Gln mutation), pamiteplase (deletion of kringle 1 and Arg275 to Glu mutation), and reteplase (rt-PA, consisting only of the Ser1-Gln3 and K_2- and P-domains) [29]. Some of these newer derivatives are under clinical evaluation in patients with deep vein thrombosis, peripheral arterial occlusion, ischemic stroke and pulmonary embolism. Their main advantage appears to be their prolonged half-life, allowing bolus administration [reviewed in 30,31]. In patients with AMI reteplase (two boluses of 10 MU given 30 min apart) was found to be equivalent to rt-PA (100 mg over 90 min) in terms of 30-day mortality and frequency of hemorrhagic stroke (GUSTO III trial with about 15 000 patients) [32]. In the ASSENT-2 study led by F. Van de Werf (about 17 000 patients with AMI) 0.53 mg kg^{-1} bolus tenecteplase yielded similar 30-day mortality and rate of intracranial bleeding as front-loaded rt-PA [33]. Furthermore, in patients with AMI the addition of a variety of adjunctive agents to rt-PA enhances benefit with acceptable risk, whereas intravascular angioplasty and stenting increases the potential long-term benefit. Feasibility and timing of prehospital administration of rt-PA variants is also under investigation. In addition, the DSPA-α_1 variant of the plasminogen activator of the vampire bat *Desmodus rotundus* (desmoteplase) is under clinical investigation in stroke patients. This highly fibrin-selective t-PA variant shows 85% homology with human t-PA, but lacks a kringle 2 domain and the plasmin cleavage site [34].

In conclusion, recombinant tissue type plasminogen has become a lifesaving drug for the treatment of evolving acute myocardial infarction and other thromboembolic diseases.

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