The Tissue-Type Plasminogen Activator Story

D. Collen, H.R. Lijnen

Abstract—Milestones in the development of tissue-type plasminogen activator (t-PA) as a fibrin-specific thrombolytic agent include: purification of human t-PA from the culture fluid of the Bowes melanoma cell line, elucidation of the molecular basis of fibrin-specific plasminogen activation, first experimental animal models of thrombosis, first patient (renal allograft) treated with melanoma t-PA, pilot studies in patients with acute myocardial infarction, 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. (Arterioscler Thromb Vasc Biol. 2009;29:1151-1155.)

Key Words: acute myocardial infarction ■ thrombolytic therapy ■ pharmacological reperfusion

As early as 1947 it was reported that animal tissues contain an agent that can activate plasminogen; this factor was originally called fibrinokinase. In 1952, T. Astrup for the first time showed that a soluble fibrinolytic activator could be extracted from animal tissues by using strong chaotropic agents.1

Studies on the turnover of plasminogen in man performed in the early 1970s led to the discovery and identification of the main physiological plasmin inhibitor, α2-antiplasmin.2 In the mid 1970s evidence was gathered, primarily by the group of Ed Reich in New York, that malignant tumors frequently secrete plasminogen activator activity, and that their malignancy correlates with the level of “malignant protease” secreted. In collaboration with A. Billiau (Rega Institute KU Leuven), we confirmed this observation and demonstrated that the protease activity could be inhibited with plasma α2-antiplasmin. Our intention was to develop low molecular weight inhibitors based on the reactive site sequence of α2-antiplasmin (which still had to be determined). To study the kinetics of inhibition of “malignant plasminogen activators,” we looked around for a source and, totally serendipitously, obtained some conditioned culture medium of a melanoma cell line, obtained in 1975 from patient Bowes, which proved to be an excellent producer of “malignant plasminogen activator.” The initial culture medium was obtained via G. Barlow from Abbott but, needing more material, toward the end of 1978 we obtained the cell line from D. Rifkin (New York University Medical School). As our laboratory had no cell culture expertise at that time, A. Billiau took care of the initial culture and generation of conditioned media.

When we sought initially to purify the plasminogen activator from Bowes melanoma cell culture fluid in 1979, we observed that the activator, unlike urokinase, had a specific affinity for fibrin. When mixtures of fibrinogen and plasminogen activator were clotted, the activator remained associated with the clot. However, with the use of purification methods developed previously for t-PA, no homogeneous final product could initially be obtained.

Purification of Natural Human t-PA

Many studies have reported the purification and characterization of plasminogen activators from various sources, including pig heart and ovaries, human postmortem 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.

In October 1979, Dr D.C. Rijken joined us from the Gaubius Institute (Leiden, 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. 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.5 With this material, Drs D. Rijken, M. Hoylaerts, H.R. Lijnen, I. Juhan-Vague, and C. Korninger clarified the molecular basis of fibrin-specific plasminogen activation, first experimental animal models of thrombosis, first patient (renal allograft) treated with melanoma t-PA, pilot studies in patients with acute myocardial infarction, 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. (Arterioscler Thromb Vasc Biol. 2009;29:1151-1155.)

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1151
Mechanism of Action of t-PA

With the availability in the late seventies 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 published in 1978 and was presented at the VIIth International Congress on Thrombosis and Hemostasis (London 1979; Edward Kowalski Memorial Lecture). This comprehensive model, schematically illustrated in Figure 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 nonfibrin specific plasminogen activators that were available at that time (streptokinase and urokinase).

Kinetic analysis revealed that t-PA is a poor plasminogen activator in the absence of fibrin. In the presence of fibrin, however, its activity is two orders of magnitude higher. The kinetic model indicates that both t-PA and plasminogen 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.

Structure-Function Relations in t-PA

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 $M_r$ about 70,000, consisting of 527 amino acids with Ser as the N-terminal amino acid (Figure 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 4 domains: (1) an N-terminal region of 47 residues (residues 4 to 50; F-domain) which is homologous with the finger domain mediating the fibrin affinity of fibronectin; (2) residues 50 to 87 (E-domain) which are homologous with epidermal growth factor; (3) 2 kringle regions (residues 87 to 176, K1-domain, and 176 to 256, K2-domain), which share a high degree of homology with the 5 kringles of plasminogen, and (4) a serine proteinase region (residues 276 to 527, P-domain) with the active-site residues His322, Asp371, and Ser478. These distinct domains in t-PA are involved in several functions, including its binding to fibrin (mainly via F- and K2-domains), rapid clearance in vivo with an initial half-life of 6 minutes in humans (mediated via F- or E-domains and carbohydrate side chains), and enzymatic activity (P-domain). Furthermore, the sequence Lys296-His-Arg-Arg299 was shown to be required for the rapid inhibition by its physiological inhibitor, plasminogen activator inhibitor-1 (PAI-1).
Thrombolytic Properties of Native Human t-PA

The thrombolytic effects of melanoma t-PA were first demonstrated in rabbits with experimental pulmonary embolus in vivo, in collaboration with Dr O. Matsuo in 1980. This study showed a clear superiority of t-PA over urokinase, both in terms of efficacy and of fibrin specificity.

Late in 1981 at an NIH workshop on coronary thrombolysis, Dr B.E. Sobel from 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. These results demonstrated that administration of native human t-PA to animals with induced coronary thrombosis elicited prompt thrombolysis without predisposition to systemic bleeding.

In collaboration with Dr W. Weimar from the Erasmus University in Rotterdam 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 hours. This event was the result of a serendipitous encounter between Dr Weimar and Dr Billiau at an Interferon Conference in Rotterdam on April 2, 1981, where they talked about the t-PA project. Dr Weimar was treating a renal allograft patient who was developing an ascending thrombosis from her iliac vein to her renal transplant. Because there were no other therapeutic options left, it was decided to try the melanoma t-PA. The clot completely dissolved without side effects and kidney graft function rapidly improved; today this first patient is still alive, with a normal functioning allograft. Despite the fact that lysis occurred, the dose was

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**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 reference 11).
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 AMI patients was performed in 1983 with purified melanoma t-PA. Participants included Dr F. Van de Werf and coworkers at the University of Leuven and Dr B.E. Sobel and coworkers at Washington University. Intravenously administered t-PA in doses of 200 to 400 μg/min completely-recanalized occluded coronary arteries within 30 to 60 minutes in 6 of 7 patients without inducing a systemic fibrinolytic state.

**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, Dr D. Pennica from the Department of Molecular Biology at 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. Nowadays the cloning and expression of t-PA would be a relatively trivial accomplishment, but in 1982 this was quite an achievement for which D. Pennica deserves most of the credit.

The cDNA of human t-PA (2530 bp) has first been expressed in Escherichia coli.11 More efficient expression was subsequently obtained in 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. The generation of CHO (Chinese Hamster ovary) 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.; Actilyse, Boehringer Ingelheim).

**Thrombolytic Properties of Recombinant t-PA**

The observations made with melanoma t-PA in experimental animal models were subsequently extended to rt-PA. In a collaborative study between the Cardiology Divisions of the University of Leuven and Washington University, the clot-specific coronary thrombolytic properties of rt-PA were demonstrated with the same experimental animal preparation.17 In a concurrent collaborative study with Dr H.K. Gold, Massachusetts General Hospital, coronary thrombosis was produced between two ligatures of the LAD in open-chest dogs. Infusion of rt-PA elicited clot lysis and myocardial salvage in this preparation as well.18 Subsequently, in a collaborative study with Dr W. Flameng at the University of Leuven, the coronary thrombolytic properties, clot-specificity, and myocardial protection achievable with rt-PA were confirmed in baboons.19

The promising initial observations with native melanoma t-PA in patients with acute myocardial infarction served as a template and stimulated initiation of a multicenter, blinded, randomized trial with rt-PA produced at Genentech. With the approval of the FDA, rt-PA was first administered to a patient on February 11, 1984, by Dr E. Topol, then a Cardiology Fellow at Johns Hopkins. Fifty patients were treated with rt-PA between February 11 and June 20, 1984, at Washington University with Dr Sobel and coworkers, at the Massachusetts General Hospital with Dr Gold and coworkers, and at Johns Hopkins University with Dr 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 rt-PA. Intravenous infusion of 0.5 mg/kg body weight rt-PA over 60 minutes or of the same dose followed by 0.25 mg/kg over an additional 60 minutes resulted in recanalization of occluded coronary arteries in 75% of patients. Fibrinolysis was absent or modest in most but not all of the patients.20 The results obtained in this initial study of rt-PA in AMI patients provided a foundation for the design of both the NIH Thrombolysis in Acute Myocardial Infarction (TIMI) trials led by Dr. E Braunwald in the United States and the European Cooperative Study Group trials initially led by Dr. M Verstraete.

Numerous clinical trials have since compared the thrombolytic properties of rt-PA with those of other agents, culminating in the GUSTO trial and its angiographic substudy,21,22 which conclusively established the potential and limitations of rt-PA for thrombolytic therapy in patients with acute myocardial infarction. In the first large clinical trials, the recommended dose of rt-PA for the treatment of AMI was 100 mg administered as 60 mg in the first hour (of which 6 to 10 mg as a bolus over the first 1 to 2 minutes), 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 minutes and the remaining 35 mg in the following hour.23 In the GUSTO trial, a dose of 15 mg intravenous bolus of alteplase followed by 0.75 mg/kg over 30 minutes (not to exceed 50 mg) and then 0.50 mg/kg over 60 minutes (not to exceed 35 mg) was used. In the COBALT trial, double bolus administration of rt-PA (50 mg given 30 minutes apart) was evaluated in patients with myocardial infarction.24 Whichever regimen is used, it is important to coadminister intravenous heparin during and after rt-PA treatment. To date, rt-PA has been used worldwide in more than 2 million AMI patients. Since its approval for this indication in 1996, rt-PA has also been used in more than 150 000 stroke victims. It is also used in patients with pulmonary embolism, deep vein thrombosis, and peripheral arterial occlusion. Several approaches have been followed to further improve the thrombolytic properties of rt-PA, yielding variants with enhanced plasminogen activating capacity, enhanced fibrin specificity, resistance to inhibition by PAI-1, and reduced plasma clearance.25 Furthermore, the addition of a variety of adjunctive agents to rt-PA and the use of extravascular angioplasty and stenting have been explored.26

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 heart attack patients.

Disclosures
None.

References
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