

## TISSUE-TYPE PLASMINOGEN ACTIVATOR: CHARACTERISTICS, APPLICATIONS AND PRODUCTION TECHNOLOGY

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### ABSTRACT

Plasminogen activators have immense clinical significance as thrombolytic agents for management of stroke and myocardial infarction. Tissue-type plasminogen activator (tPA) is generally preferred as being effective and safer than either urokinase or streptokinase type activators. Large-scale production of tPA became possible through groundbreaking developments in cell lines and bioprocess technology. Nevertheless, at thousands of dollars per treatment, tPA remains expensive. Enhancing cellular productivity and downstream product recovery through new approaches continue to be major challenges as discussed in this review. Recent clinical experience suggests the need for yet better fibrinolytic agents and attempts are underway to modify the tPA molecule to second generation products. Emerging trends in this field are outlined.

**Key words:** Tissue plasminogen activator, streptokinase, urokinase, thrombolytics, thrombosis, fibrinolysis, myocardial infarction.

### INTRODUCTION

Plasminogen activators are a group of proteolytic enzymes that are used in treating cardiovascular and cerebrovascular obstructions (i.e., heart attack and stroke, respectively). The thrombolytic role of plasminogen activators is of immense healthcare significance as thrombosis remains a major cause of death in the Western world. Plasminogen activators convert the blood protein plasminogen to plasmin. Plasmin dissolves fibrin, the insoluble matrix of clots. Thus, circulatory blockages due to fibrin clot or thrombus can be cleared with plasminogen activators.

Two classes of human plasminogen activators are generally recognized: tissue type (tPA) and urokinase type (uPA). These two types are biologically and immunologically

different [1]. A plasminogen activator of bacterial origin, streptokinase, produced by various strains of *Streptococci*, has also been used in clinical practice. Streptokinase is relatively inexpensive, but because of non-human origin its use is associated with undesired immune responses and pyrogenic reactions. Furthermore, streptokinase activates not only fibrin-bound plasminogen but also that in circulation, leading to serious risk of hemorrhage. Unlike streptokinase, human urokinase is not antigenic or pyrogenic, but its extraction from urine is expensive and supply is limited for any extensive therapeutic use. Although production of urokinase in recombinant microorganisms is feasible, it is unlikely because therapeutic qualities of uPA are generally inferior to those of tPA. Like streptokinase, urokinase activates both the circulating and the fibrin-bound plasminogen. In contrast, tissue plasminogen activator is more specific in its action, binding relatively strongly to fibrin clots and preferentially activating the plasminogen entrapped in the clots. Tissue plasminogen activator has little effect on circulating plasminogen or other blood clotting factors. The basal level of tPA in circulation is about 1 ng/mL rising to as much as 100 ng/mL during periods of stress such as intense exercise. The elevated levels do not last long, being rapidly removed in the liver. Circulation half-life of tPA is about 2–6 minutes. Therapeutic doses can be kept within physiological limits [2], but 60–100 mg per dose is the norm (equivalent to about 1,100–1,800 ng/mL). Because of these advantages, tPA is regarded as a more effective and safer thrombolytic agent than urokinase and streptokinase.

Bowen melanoma derived tissue plasminogen activator consists of a single polypeptide chain that has 527 amino acid residues (Figure 1). Seventeen disulfide bonds between 34 cysteine residues contribute to the characteristic folding of the chain. The protein is glycosylated; approximately 7% of the total molecular weight is carbohydrate. Its isoelectric pH is 7.5. It is a serine protease with a molecular weight of approximately 70,000 daltons [3]. It is synthesized within the cell as a polypeptide chain and released as a one-chain enzyme. Limited proteolytic action by plasmin in the extracellular medium cleaves the chain between arginine 275 and isoleucine 276, converting it to a two-chain form. The chains are held together by a single disulfide bond [4]. The one- and two-chain forms of tPA show little difference in fibrinolytic performance.

Several functional regions have been identified in the tPA molecule (Figure 1): a fibrin-binding 'finger' domain near the N-terminus; an epidermal growth factor (EGF)

domain that is seen in several serine proteases; two disulfide looped 'kringle' structures similar to those found in plasminogen, prothrombin and urokinase; and a carboxy-terminal serine protease domain associated with the proteolytic activity [5]. The molecule has four potential glycosylation sites at asparagines 117, 184, 218 and 448 (Figure 1). The site 218 never appears to be glycosylated [6]. The site 184 is usually not glycosylated. Thus, depending on the number of carbohydrate groups, tPA can be divided into two types: type I contains three carbohydrate groups (at positions 117, 184 and 448), and type II has only two groups (positions 117 and 448). The finger domain and the double kringle regions are associated with fibrin binding, hence being important to clot-specific therapeutic function.

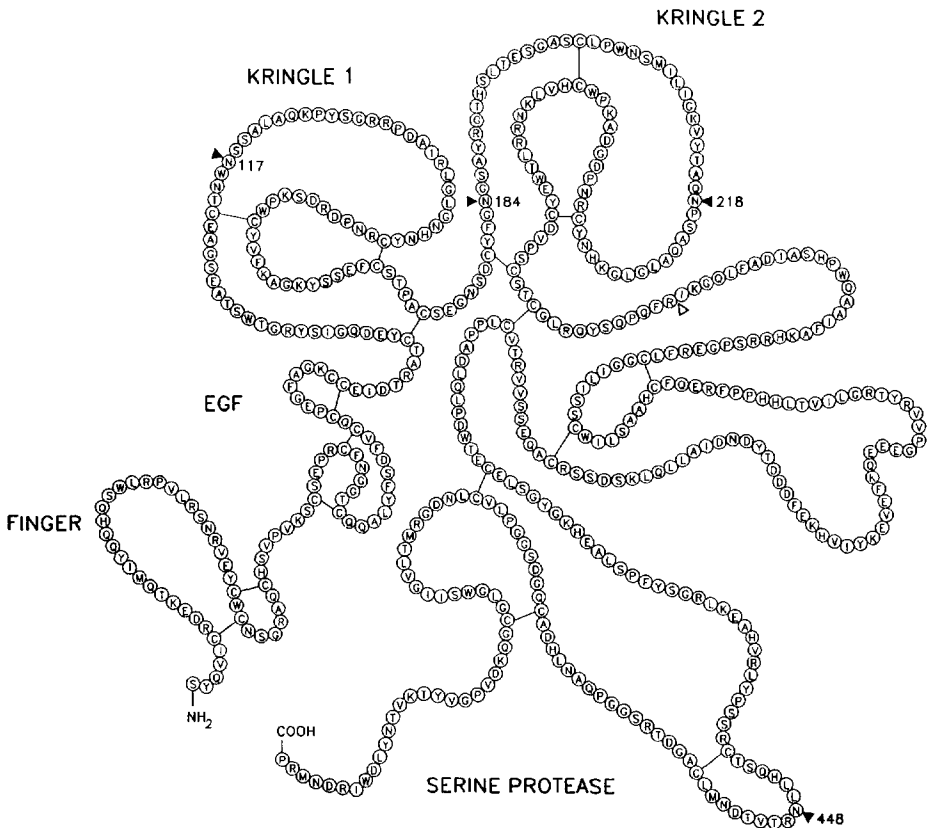


Figure 1. The tPA molecule. Amino acid residues are represented by one-letter codes. The potential disulfide bridges between cysteine residues are based on homology with other serine proteases. Open arrowhead indicates the potential cleavage site that generates the two-chain molecule from the one-chain form. The solid triangles indicate potential glycosylation sites. Based on Klausner [6] and Pohl et al. [7].

## PRODUCTION OF tPA

Therapeutic potential of tPA as a fibrinolytic agent drove the development of its large scale production. Clinical use required a consistent and efficient production method that could provide high purity protein in good yield. Insufficient supplies impeded initial clinical studies. Early trials employed material produced in Bowes melanoma cells in relatively large amounts. Current production methods rely on higher yielding recombinant cells; however, despite enhanced yields, tPA remains expensive selling for \$ 22,000 per gram, 20-fold higher than streptokinase [6]. Improving productivity of cell lines is paramount for reducing costs. The producer cells are discussed in the following sections.

### Mammalian Cells

#### Non-recombinant producers

In view of the widespread occurrence of tPA in human (Table 1) and animal tissue, cells derived from various tissues and species were the natural focus of the vast effort devoted to producing this protein. A clear correlation was observed between the plasminogen activator content of an organ and the level of activator produced by primary cultures of cells originating from that organ. Malignant cells were generally significantly better producers than normal cells.

**Melanoma cells.** The Bowes melanoma cell line is perhaps the best known producer of tPA. This line is very stable and produces relatively large amount of a tPA that is very similar to that found in normal human tissue. Material for characterization and *in vivo* trials came from this line. Bowes melanoma cells were generally grown on collagen coated microcarriers. Cell attachment was critically pH dependent and the presence of serum (0.5%) was essential [4]. Melanomas could be cultured in free suspension but the tPA expression levels were lower [8]. The productivity of carrier-bound Bowes melanoma cells exceeded 30 IU per  $10^6$  cells per day (0.1 mg/L conditioned medium). Although equivalent to production from ten human uteruses (one of the richest sources of tPA), this level was too low for industrial scale processes [4]. Furthermore, use of cells that originated in a malignant human tumor for producing the drug for wider use did raise considerable concern.

Table 1. Plasminogen activator activity in extracts of human tissue [9].

Tissue	Activity (units/g fresh tissue)	Tissue	Activity (units/g fresh tissue)
Uterus	720	Pituitary	140
Adrenal	410	Kidney	119
Lymph node	378	Muscle	110
Prostate	334	Heart	82
Thyroid	325	Brain	35
Lung	223	Testis	25
Ovary	210	Liver	0

**Epithelial cells.** A variety of normal epithelial cells have been shown to produce tPA. Cultures of guinea pig ear keratocytes (GPK) and human breast epithelial cells are two examples [10]. Growth of these cells generally requires serum-supplemented media. Yield of tPA declines with increasing passage number [4]. High density microcarrier culture is the preferred cultivation method for these anchorage-dependent lines. Cell growth and the tPA production phases are sequentially separated, hence allowing the use of serum-lean medium for the production phase. Yields of tPA have ranged over 5–10 mg per liter of conditioned medium from high density perfusion cultures having  $(7-10) \times 10^6$  anchored cells per milliliter [4]. In terms of units of tPA, the productivity has been similar to that of the melanoma system (Table 2).

Biochemical properties of the epithelial cell derived tPA are not the same as that obtained from Bowes melanoma [4]. The specific activity of epithelial cell tPA is only 12,500 IU/mg [10] compared to a value of 90,000 IU/mg for the melanoma tPA [4]. Similarly, the isoelectric pHs are different at 4.7 and 7.8 for the epithelial and melanoma derived material respectively. Despite these differences, the thrombolytic activity on human blood clots is similar for the two products [4].

Table 2. Comparative yields of tPA from various cell lines expressing melanoma tPA [4].

Cell line	Production rate (pg/cell-day)	Production rate (U/10 <sup>6</sup> cells-day)
Bowes melanoma	0.25–0.31	15–18
rBowes melanoma	3.10	180
Epithelial cell (GPK)	0.38	3
GPK (+ azacytidine)	1.26	10
GPK (+ concanavalin-A)	5.80	46
rCHO	0.86	50
rCHO (+ methotrexate)	8.61	500

**Fibroblasts.** Diploid human fibroblasts such as IMR-90 and WI-38 have been adapted for production and secretion of large amounts of tPA. Phenotypic stimulation of these cells has enhanced the enzyme secretion level by 20-fold. Highest productivity (100–300 U per 10<sup>6</sup> cells per day) was achieved by pre-coating the culture substrate with poly-D-lysine and using horse serum instead of fetal calf serum [11]. Production returned to the normal low level (1.0 U per 10<sup>6</sup> cells per day) when the cells were returned to non-permissive conditions [4].

**Endothelial cells.** Although a rich source of tPA, vascular endothelial cells have not been considered for large-scale production because they produce a fast acting plasminogen activator inhibitor in excess of the free tPA, making tPA measurements impossible under certain conditions [12]. Nonetheless, such cells have been used as *in vitro* models for studying the effects of various stimuli on tPA synthesis and secretion.

### Recombinant mammalian cells

Recombinant DNA technology made commercial production of tPA feasible. Mammalian cells inserted with copies of human tPA gene have generally been better producers than other cell lines. Nevertheless, a variety of other hosts have been investigated including insect cells, bacteria, yeasts and filamentous fungi. While expression has been possible in many

cases, the productivity has not matched that of recombinant mammalian cells. Research into developing superior producers continues. Recombinant producers are discussed in the following sections.

Suitably engineered mammalian cells will produce human tPA. Cloning and expression have been successful in mouse C127 fibroblasts, rat myeloma, and CHO cells as well as in recombinant human melanoma cells containing additional copies of the tPA gene. CHO cells are generally superior producers than most other host cells studied (Tables 2 and 3). The mouse C127 line is also a relatively good producer (Table 3), but it is anchorage dependent, hence processing is more complex, expensive and less easily scaled-up. Generally, recombinant cells are substantially more productive than the best known non-recombinant systems such as Bowes melanoma (Table 3).

The commonly used expression vectors for tPA production have depended on strong constitutive promoters and enhancers such as virus-derived or immunoglobulin regulatory elements to drive tPA expression [14]. Thus promoters from simian virus SV<sub>40</sub>, bovine papilloma virus (BPV), Epstein-Barr virus (EBV), Rous sarcoma virus (RSV) and adenovirus type 2 (Ad2) have been used [14]. Gene amplification has often been achieved

Table 3. Reported yields of recombinant tPA in animal cells [13].

Cell type	System	Peak titer (mg/L)	Specific productivity (mg/10 <sup>9</sup> cells·day)
Rat	Fed batch airlift	40	4 <sup>a</sup>
Myeloma	Fed batch airlift	52	4 <sup>a</sup>
CHO/SV <sub>40</sub>	Petri dish	—	26–49
CHO/SV <sub>40</sub>	Perfused matrix	65	20
CHO/Ad2	Petri dish	—	10
Mouse	Petri dish	0.3	20
C 127	Perfused microcarrier fermentor	55	25 <sup>a</sup>
Human melanoma	25 cm <sup>2</sup> flask	8	3.1
Bowes melanoma <sup>b</sup>	25 cm <sup>2</sup> flask	1	0.3
Bowes melanoma <sup>b</sup>	Perfused microcarrier fermentor	0.1	0.1

<sup>a</sup> Averaged over entire fermentation; <sup>b</sup> Non-recombinant cells shown for comparison.

through the use of methotrexate (MTX), a cytotoxic agent that inhibits dihydrofolate reductase (DHFR) that is essential for deoxythymidylate synthesis. Cells that survive increasing concentrations of MTX do so by amplification of the dihydrofolate reductase structural gene. An appropriate gene construct placed next to the DHFR gene may be co-amplified. This technique was first used by Genentech to enhance the yield of tPA [14]. Nevertheless, use of MTX is not free of pitfalls and cells show extended doubling times and increased fragility. The latter factor can have severe implications on the type of bioreactor that may be used in processing [15]. Airlift bioreactors that have been proven in large scale suspension culture of animal cells may be particularly advantageous [16].

In yet another approach to using animal cells, Cartwright and Crespo [17] developed several CHO/mouse myeloma hybrids that produced tPA. The best hybrid could be adapted to grow in serum free medium in stirred bioreactors. Production levels in batch culture plateaued at 100 mg tPA per liter per  $10^9$  cells after 6 days of initiation.

Commercial production of tPA by Genentech relies on methotrexate-amplified recombinant CHO cells in free suspension culture (see Table 2). The general processing scheme is similar to that described by Chisti [18] for producing monoclonal antibodies. Bioreactors up to 10,000 liter are employed [13]. Nonetheless, in attempts to minimize the size of bioreactor high density culture has been evaluated by several groups. Unlike free suspension culture where cell concentrations do not typically exceed a few million per milliliter, the concentrations in high density cultivation can exceed  $10^8$  per mL. One example of such a system was the reactor developed by the now defunct Verax Corporation. Cells were entrapped in weighted beads of a collagen-based matrix. The beads were fluidized by continuous recycle flow of a serum free medium in the reactor. Fresh medium was added continuously. A steady state tPA concentration of 65  $\mu\text{g/mL}$  was obtained, corresponding to a daily output of 21.4 g of tPA from a 24 liter reactor. The specific yield was estimated at 45  $\mu\text{g}$  per  $10^6$  cells per day. This level of production was stably maintained for at least 10 days [19]. Other high density culture systems have achieved similarly high productivities although specific productivities are usually no better than in free suspension culture. Some of these examples are the perfused hollow fiber reactors [20] and perfused chambers containing ceramic [21] or other matrices for cell immobilization.

Despite excellent volumetric productivities, immobilized high density culture systems have not gained industrial acceptance for any significant product. Verax's failure is a more recent example of other similarly rejected technologies. High density culture systems take significant time to reach stable production and once contaminated must be reestablished. Furthermore, the environment of the cells in such systems is highly heterogeneous and poorly defined. Live and dead cells coexist and cell mass characterization is difficult. Similarly, process control is not at all easy. Large volumes of culture medium must be continuously perfused, yet nutrient limitations are commonly encountered. Oxygen supply is a particular problem.

A more successful alternative to extremely high density culture is perfused suspension culture in which the cells are retained by self cleaning rotary 'spin filters.' Such devices are in commercial use with a variety of cell lines. A tPA-like thrombolytic from CHO cells has been produced in such reactors [22]. The cell density in the continuously perfused state was  $10 \times 10^6$  cells/mL, compared to  $2 \times 10^6$  cells/mL in the batch mode of operation. Optimal production occurred at a cell density of approximately  $5 \times 10^6$  cells/mL. At higher cell densities, a perfusion rate of 4 reactor volume per day was insufficient to maintain the maximum productivity. Availability of nutrients and energy controlled the productivity rather than concentration of any inhibitor. In the perfused mode (4 reactor volumes/day), the volumetric productivity of the tPA variant, was 15- to 18-fold higher than that of batch operation [22].

A practical culture option for anchorage dependent cells is microcarrier suspension culture. Cell retention in the bioreactor is relatively easier with such systems than in perfusion operation with freely suspended cells. Although for batch and fed-batch processes, the additional processing associated with microcarriers is a disadvantage. Semi-continuous culture of microcarrier-supported CHO cells for the production of tPA has been evaluated using carriers made of gelatin and dextran [23]. Higher specific and total production rates were observed with cells on gelatin microcarriers. Addition of 6-aminohexanoic acid to the medium improved the specific production rate in both cases. Human fibroblasts anchored on Cytodex microcarriers have also been used [24-26].

With anchorage dependent lines, another potentially useful approach is the use of a packed bed of cell-supporting *macrocarriers* located in the downcomer of an airlift

bioreactor [27]. Evaluations suggest that such a device should be substantially more productive than comparable microcarrier suspension culture in stirred vessels [27].

Irrespective of the specifics of the culture method, attention to sterile operation, prevention of contamination and clean-in-place procedures is essential to successful commercial production of cell culture derived products. These aspects have been discussed by Chisti [28,29].

### **The culture medium**

In addition to the cell line, the culture medium is the most important influence on productivity. Formulation of medium may affect synthesis of the product, its stability in the extracellular fluid, ease of downstream recovery and the overall cost of production. Generally, the tPA producing cells have been grown in conventional tissue culture media supplemented with various components. Serum has usually been found essential for cell growth and has often been added to a concentration of 10% (vol/vol) to obtain maximal cell proliferation [4]. Once a high cell density is attained, the serum concentration may be reduced to less than 1%, or entirely eliminated to facilitate downstream recovery [4]. While melanoma cells survive in serum-free media for several weeks [30], such media do require additional components such as transferrin, insulin and trace elements.

The tPA in culture medium is susceptible to protease activity that may be suppressed by supplementing the medium with aprotinin [31]. Another process consideration is the tendency of tPA to adhere to surfaces. This property has in the past hampered its purification and characterization. Supplementing the culture medium and the purification buffers with the nonionic detergent Tween-80 to 0.01% overcomes this problem [32–34]. Besides these essential components, stimulants are usually added to the media to improve productivity. Stimulants are discussed below.

**Stimulants.** Cultured cells generally produce exceedingly low levels of tPA. Yields can be improved substantially by chemical stimulation of expression. Griffiths and Electricwala [4] list a variety of stimulants that have been used. Tumor inducing agents such as phorbol esters, retinoids and mitogenic lectins are well known stimulants. These compounds attracted attention because cancerous cells and transformed lines had been observed to

produce much higher levels of tPA than equivalent normal cells [4]. Studies of stimulatory role of a number of phorbol esters have shown varying levels of yield enhancement. The most active are 12-O-tetradecanoyl phorbol-13-acetate and phorbol myristate acetate [4]. Among retinoids, retinoic acid is more active than both retinol and retinyl acetate [4].

Certain steroids are known to increase vascular tPA activity *in vivo*. Similar hormones have been used to stimulate tPA production in culture. Substances, such as poly-D-lysine and sera that alter the culture environment for higher expression of plasminogen activator have also been investigated [4]. Hypomethylating agent azacytidine, epidermal growth factor and concanavalin-A are effective stimulants (Table 2) because of their impact on the regulation of cellular metabolism [4].

In comparing the effects of concanavalin-A and its derivatives on tPA production by epithelial and melanoma cells, Clarke et al. [35] reported that for epithelial cells, con-A at a concentration of 50 µg/mL enhanced production by 10-fold relative to unstimulated cells. The yields of tPA with succinyl con-A and acetyl con-A as stimulants, were 8.5- and 9-fold the unstimulated level, respectively. The optimum stimulatory concentration for both those compounds was 100 µg/mL. Long term cultures of epithelial cells treated with con-A showed initial increased yields of tPA followed by reduced production levels. Whereas, cultures treated with con-A derivatives maintained increased level of production. Moreover, con-A altered the morphology and reduced the viability of cells. The cells became rounded, ragged and permeable. The change in morphology and the loss in viability were suggested as probable reasons for declining productivity with con-A stimulated cells [35]. Conversely, con-A and its derivatives did not enhance the production level of the Bowes melanoma cells. This led to the assumption that the pathways by which con-A like molecules stimulated tPA production were already fully active in the melanoma line. However, morphological changes, similar to those noticed with epithelial cells, were induced in the melanoma. Consequently, morphological changes were concluded to be unrelated to stimulation of tPA production [35]. For the recombinant cell lines, it remains to be shown if the tPA yield could be further enhanced with the use of stimulants proven with non-recombinant lines [36].

Besides chemicals, physical stimuli may be used to enhance tPA production. Stimuli such as exercise, stress and electric shock are known to increase plasminogen activator

activity *in vivo*. Similar stress factors are effective stimulants in cell culture. Thus, in one study application of arterial level of fluid shear stress (25 dynes/cm<sup>2</sup>) increased tPA secretion from endothelial cells (human umbilical vein) by 300% over unsharped cells [37].

### **Cellular control of tPA synthesis**

Production of tPA in cultured cells is negatively affected by its concentration in the extracellular medium. For an extracellular product, this negative feedback control is unusual. The control mechanism and other observations suggest that extracellular tPA may re-enter the cell. To minimize productivity loss due to this effect, the production process must maintain a low concentration of tPA in the culture fluid. Either a high ratio of medium volume to cell number may be used, or the cells may be continuously perfused with fresh medium. In the latter method the rate of perfusion is kept so high that the critical inhibitory concentration of tPA is not reached. However, because the animal cell culture media are expensive, perfusion significantly affects the cost of production. Furthermore, the product needs to be recovered from a very dilute stream. An analysis of production costs has shown that as the productivity of a perfused reactor increases, the cost of the medium becomes the overriding cost component [11]. A more economical alternative is to recycle the medium through a high affinity adsorption column capable of in-line removal of tPA. Results with this type of operation have shown that medium recycling does not adversely affect the production of tPA by the cells [11].

## **Other Recombinant Cultures**

### **Bacteria**

The bacterium *Escherichia coli* has been studied as a host for recombinant tPA production [4,6,14]. The tPA gene from Bowes melanoma cell line has been expressed in *E. coli*. Expression in bacteria eliminates the risk of expressing tumor associated proteins and the risk of contamination by animal viruses. However, pyrogenic and immunogenic reactions may occur due to bacterial contaminants unless the product is highly purified.

The tPA produced in *E. coli* is a non-glycosylated single chain polypeptide which is retained within the cell as insoluble denatured inclusion bodies constituting 5–10% of total

cell protein [6]. Lack of glycosylation may not be drawback because glycosylation is non-essential to biological function. Nevertheless, a production process based on *E. coli* does not seem feasible because of low recovery of active tPA from inclusion bodies under any practical conditions [6].

When compared with commercially available rtPA from CHO cells, the bacterial tPA possessed equivalent specific activity against clots. The affinity for fibrin and degree of stimulation of plasminogen activator were similar for the two tPAs. The bacterial tPA was specific in its action on plasminogen; however, its half-life was four-fold longer than the CHO product [6]. Similarly, the clearance rate was longer compared to CHO rtPA. Hence the non-glycosylated tPA may have an advantage in therapy where a lower dose could maintain an extended thrombolytic state.

Although extraction and renaturation from inclusion bodies add complexity and cost to producing any protein [38], *E. coli* fermentations are far less demanding than animal cell culture. In addition, bacterial culture media are relatively inexpensive. Hence, tPA production in bacteria remains desirable and may indeed become feasible with advances in renaturation methods.

### **Yeasts and fungi**

Production of tPA in *Saccharomyces cerevisiae* has proven possible, but the yields have been low [39]. The yeast product is extensively hyperglycosylated and molecular weight heterogeneity has been observed. In addition, extracellular secretion does not occur, hence the cells must be disrupted to recover the tPA. Although large-scale cell disruption methods are well established [40], purification from cell homogenates is complicated [38].

Synthesis of recombinant tPA in the filamentous fungus *Aspergillus nidulans* has been reported [41]. Active, single chain form of tPA was secreted into the culture medium. The product was very similar to mammalian tPA suggesting absence of glycosylation beyond what is normal for the protein. The yield was approximately 100 µg per liter of culture medium, but could be enhanced to 1 mg per liter with an improved promoter [41]. Expression levels of mammalian cells are about 20-fold higher, but potentially within reach of microbial systems.

### **Insect cells**

Recombinant human tPA has been expressed in a cell line derived from the fruit fly *Drosophila melanogaster* Schnieder #2 [42]. The rtPA isolated from this cell line was identical to that expressed in CHO cells. It had a specific activity ( $4.1 \times 10^5$  IU/mg) similar to rtPA from CHO ( $5.4 \times 10^5$  IU/mg). The enzyme was expressed mostly in the single chain form, unlike the CHO rtPA which is a mixture of one- and two-chain types. Amino acid analysis showed close correspondence among tPAs produced in CHO, *Drosophila* and Bowes melanoma. The specific glycosylation sites for *Drosophila* rtPA were consistent with recombinant mammalian tPAs [42]. The product expressed in *Drosophila* cells was as effective as the CHO rtPA, but the carbohydrate side chains of the two products were not identical. Based on limited pharmacokinetic data, the *Drosophila* rtPA cleared from circulation a little faster than the CHO product. This difference was attributed to differences in carbohydrate side chains. The *Drosophila* rtPA was more resistant to proteolysis [43].

### **Expression in Transgenic Animals and Plants**

Several proteins have been successfully expressed in domesticated animals [44]. The desired protein is generally secreted in milk. Even at a production level of 1 mg per liter of milk, such a system would be substantially less expensive than methods available today [45]. Expression in plants is another possibility that has been proven with other proteins. If feasible, the latter system has many potential advantages. However, at present there seem to be no reports of transgenic expression of tPA.

## **DOWNSTREAM RECOVERY AND PURIFICATION**

The methods used in recovering and purification of tPA depend to some extent on the source material, whether tissue, animal cell culture or microbial broths [46]. Commonly used laboratory purification schemes have been summarized by Griffiths and Electricwala [4]. Recovery from tissue involves extraction in buffers and removal of solids [1,9,47]. Precipitation with ammonium sulfate [48] or polyethylene glycol [49] has been employed, but precipitation is usually not necessary. A series of chromatographic steps follow. Commercial therapeutic tPA is obtained exclusively from animal cell culture, but

microbial culture remains a potential alternative source. The following sections focus on tPA recovery from these systems.

### **Animal Cell Culture**

As a first step, cells are separated from cooled culture broth. Cross flow microfiltration is used. Plate and frame filter configuration is the norm [15]. Because tPA is susceptible to proteolysis, aprotinin (a protease inhibitor) is usually added to the process fluid to suppress formation of the two-chain molecule [32–34]. Surface adherent tendency of tPA is commonly overcome by supplementing process fluids with the surfactant Tween-80 (polyoxyethylene sorbitane monooleate). A typical concentration of 0.01% is used during most processing steps [32–34,47]. Cross flow ultrafiltration may be used to concentrate the broth [10], but zinc chelate chromatography is preferred. In the latter case, a concentrated tPA stream is obtained by gradient or step elution with imidazole-containing buffers [36]. Subsequent purification is achieved through chromatographic steps [33,34,48,50]. Purification schemes vary in the specifics of chromatography, but a gel filtration step is invariably used in final polishing.

Fibrin adsorbed on Celite (a diatomaceous earth) has been used for affinity chromatography to preferentially retain tPA while non-fibrin binding proteins such as urokinase-like plasminogen activator are washed away [31,51]. Immuno-adsorption on antibodies has been used as the principal purification step [33,34,50]. A one step purification using immunoaffinity chromatography has been described [52]. Immunopurification methods tend to be highly selective. In another approach, tPA's affinity for serine protease inhibitors has been used to advantage. Thus *p*-benzamidine-Sepharose has been used to preferentially retain tPA [53]. Lysine affinity adsorption has been employed in which lysine binding regions in the kringle structures interact with lysine attached to chromatographic matrix [33,51]. Lysine or arginine containing buffers are used to displace the tPA, but lysine is a better displacing agent [33]. Several other chromatographic options have been employed, including ion exchange, zinc or other metal chelate, hydroxyapatite [49], and concanavalin-A bound to agarose [32].

As a specific example, Cartwright and Crespo [17] purified tPA from recombinant CHO supernatants. Batch purification on SP-Trisacryl ion exchange resin was followed by

affinity chromatography on lysine-Sepharose. A polishing step on phenyl-Sepharose was used. Purity of the product exceeded 99%. Single chain tPA predominated, but lower molecular weight fragments were present at roughly 0.5%.

Other chromatographic purification schemes have adapted the procedure developed by Rijken and Collen [54] for tPA recovery from cultured human melanoma cells. This method has been applied also to tPA produced in bacteria, yeast and animal cells [55]. Three column chromatographic steps are used. Affinity chromatography on zinc chelate agarose is followed by a concanavalin-A affinity separation. The final step employs gel filtration on Sephadex G-150. Tween-80 is used in each step and the pH is held at 7.5.

Higher purities can be attained using methods such as preparative reverse phase HPLC or preparative electrophoresis; however, these techniques are not suitable for large scale use. Industrial processes should avoid such sophisticated methods [36,56].

### **Microbial Culture**

Microbial cells are not used commercially to produce tPA. Nonetheless, given continual improvements, production of human tPA in recombinant microorganisms cannot be ruled out, particularly if the yields can be increased to compensate for the losses that may be incurred in any additional recovery steps. Recovery from microbial systems depends on location and state of the product. If the product is secreted, as by the mycelial fungus *A. nidulans* [41], recovery from culture broth is quite similar to that from animal cell culture fluids. Additional process steps are needed if the product is retained within cells. If the product is soluble and active as in the recombinant yeast *S. cerevisiae* [39], cell disruption and debris removal are required prior to purification [15,40]. In such cases, mechanical disruption is preferred to using chemicals or enzymes [57]. When the product is produced as insoluble 'inclusion bodies' in recombinant bacteria such as *E. coli* [4,6,14,58], recovery becomes substantially more complicated than any of the other production scenarios. Not only must the cells be disrupted and debris separated from the insoluble denatured inclusion body, but the protein must be renatured in a series of low-yield steps [38].

In a typical recovery sequence the inclusion body fraction may be washed with a solution of 5 M urea and a surfactant such as Triton (2%) followed by dissolution in a denaturant such as guanidine hydrochloride (7 M). The dissolution buffer may contain a

reducing agent such as  $\beta$ -mercaptoethanol (50 mM) to ensure cleavage of all disulfide bonds [38,45,58]. Renaturation is achieved by reducing the concentration of chaotropic agents by dialysis or dilution. The renaturation buffer may have a small amount of urea (2.5 M), 10 mM lysine and a redox couple to aid regeneration of the disulfides [58]. Incubation temperature of 15 °C has been reported in an air-free environment. The polypeptide chain refolds into a thermodynamically stable configuration with reformation of the correct disulfide linkages [17,58,38].

Renaturation must be carried out in dilute solutions to prevent interchain aggregation of proteins [38]. Yield of active tPA tends to be low and concentration from very dilute solutions becomes necessary. In addition, renaturation is a slow process taking up to two days. Recovery of active proteins from inclusion bodies is discussed in greater detail by Chisti and Moo-Young [38].

Purification schemes such as the one outlined here have been shown to recover 3 mg of fully active tPA per liter of bacterial broth. In one case the product was more than 95% pure and the single chain tPA constituted over 95% of total. The product's specific activity was identical to the international tPA reference preparation isolated from Bowes melanoma. A variety of assays confirmed this equivalence. The purification process removed more than 99.999% of endotoxin; the endotoxin level of the final preparation was within the acceptable limits for parenterals [17].

In comparing production of tPA in animal cells and *E. coli*, Datar et al. [6] concluded that animal cell culture was clearly more economical. Whereas *E. coli* fermentation was less expensive than animal cell culture, this advantage disappeared when recovery costs were considered. Low tPA yield in *E. coli* and the expense of renaturation in dilute solutions contributed to costs. Disposal of recovery chemicals was an additional problem. In comparison, for mammalian cells, cost of the culture medium represented a major cost factor.

### PRODUCT QUALITY

As a parenteral therapeutic, tPA preparations must be purer than 99.99%. However, because of complex physicochemical properties, a high molecular weight, variations in glycosylation patterns and existence of one- and two-chain forms, undesired variants of tPA are potential

impurities in the final product. Even highly purified preparations will likely contain multiple variants because of the inherent heterogeneity of the molecule or because of degradative reactions such as deamidation and proteolysis. Natural variants of tPA are type I and type II glycosylated forms and the one- and two-chain forms. The relative amount of these variants in a preparation depends on the cell line, culture conditions and the recovery process.

A variety of approaches are used to characterize the product. Variations in glycosylation pattern can be demonstrated with isoelectric focusing gel electrophoresis (IEF) because of the electric charge differences in differently glycosylated forms. Gel permeation chromatography (GPC) can be used to determine the amounts of one- and two-chain variants. Isoelectric focusing will provide information on presence of products of degradation reactions [59]. Other analytical techniques used for the detection of contaminants in tPA are noted in Table 4. A masterly review has been presented by Anicetti et al. [59].

Undefined impurities, such as microbial contaminants, also pose a serious risk for the biologicals manufactured from genetically engineered mammalian cells [60]. Potential contaminants include pyrogens, other proteins and chemicals, mycoplasma,

Table 4. Quality assurance methods used in characterization of potential impurities and contaminants in biotechnology-derived therapeutic proteins [61].

<b>Impurity or contaminant</b>	<b>Analytical technique</b>
Protein contaminants (e.g., host cell proteins)	SDS-PAGE electrophoresis, HPLC, immunoassays (ELISA, etc.)
Endotoxin	Rabbit pyrogen test, LAL <sup>a</sup>
DNA	DNA dot-blot hybridization
Proteolytic degradation products	IEF <sup>b</sup> , SDS-PAGE, HPLC, N- and C-terminus analysis
Presence of mutants and other residues	Tryptic mapping, amino acid analysis
Deamidated forms	IEF
Microbial contamination	Sterility testing
Virus	Viral susceptibility tests
Mycoplasma	21 CFR <sup>c</sup> method
General safety	As per 21 CFR 610.11

<sup>a</sup> Limulus amoebocyte lysate; <sup>b</sup> Isoelectric focusing; <sup>c</sup> Code of Federal Regulations.

and viruses. Viral contaminants may be introduced during processing or they may come from the producer cell line or animal sera. Therefore, control and characterization of cell bank and all process additives are required. Safeguards must be in place to prevent introduction of viruses and other contaminants during processing. The product purification scheme must demonstrate viral inactivation or removal.

A further contaminant is residual DNA from established cells. Such DNA fragments were once thought to be potentially oncogenic. Hence a DNA contamination level of no more than 10 pg DNA per dose was recommended by the Food and Drug Administration. Subsequent work could not demonstrate oncogenic events following injection of large doses of DNA. Consequently, less restrictive limits are now accepted. Nonetheless, DNA is a contaminant and demonstration of its satisfactory clearance is essential to quality assurance of the product. DNA removal is usually achieved through anion-exchange.

### FUTURE TRENDS

Increasing experience with tPA suggests that its clinical efficacy relative to other thrombolytics may not be significantly superior. In comparing effectiveness of recombinant tPA, microbial streptokinase and anisoylated plasminogen activator-streptokinase complex (APSAC), the third International Study of Infarct Survival (ISIS-3) noted insubstantial differences in patient mortality. Mortality was 10.3 percent in patients receiving tPA, compared with 10.5 percent in APSAC treated individuals and 10.6 percent in those given streptokinase [62]. The study further revealed that three out of 1,000 patients receiving streptokinase suffered stroke, compared with six receiving APSAC and seven receiving tPA [62]. Fewer cases of allergy, hypotension and bleeding complexities accompanied the use of tPA. Overall, the differences were not significant enough to justify the high price of tPA (\$ 2,200 per dose) relative to streptokinase (\$ 300 per dose) and APSAC (\$ 1,700 per dose). In view of these results, a substantial potential market for thrombolytics, and the fact that commercial production of tPA from CHO cells is patent protected, incentive for developing an improved tPA remains strong. The aim is to develop rival substances, modified 'second generation' tPAs, producing mutants, and to examine the use of adjuvants to enhance performance. Because of the potential market reward, research in this area is highly

competitive [5]. Some of the approaches to a second generation tPA are detailed in the following sections.

## Improving Efficacy

### Protein engineering

Protein engineering is potentially a highly promising method of improving the tPA. Therapeutic characteristics may be modified through alterations to the molecule. Principal focus of this effort are the finger, kringle and the epidermal growth factor domains of the tPA molecule (Figure 1). Deletion of the kringle structure has been found to increase *in vitro* stability in the presence of inhibitors [5]. This suggests a potential for increasing the half life *in vivo* and hence, the therapeutic effectiveness. Structural modifications that better expose such likely fibrin binding sites as the finger domain may enhance specificity. Similar results may be achieved by inserting additional binding sites into the molecule. Elimination of non-essential parts to reduce the size of the molecule is another objective. Changes to one region can profoundly alter the activity of another, but these interactions are not fully understood. A summary of how alterations to the principal domains affect biochemical function of tPA is presented in Table 5.

Table 5. Structure–property relationships in the tPA molecule [14].

TPA domains deleted <sup>a</sup>	Biological effects			
	Fibrin binding	Fibrin enhancement of plasminogen activation	Binding to lysine Sepharose	Initial plasma half- life
F	Reduced	Unchanged	Unchanged	Increased
E	Reduced	Unchanged	NA	Increased
F, E	Reduced	Unchanged	Unchanged	Increased
F, E, K1	Reduced	Reduced	Unchanged	Increased
F, E, K2	Abolished	Abolished	Unchanged	NA
K1, K2	Greatly reduced	Abolished	Abolished	NA
F, E, K1, K2	Abolished	Abolished	Abolished	NA
Glycosylation site(s)	Unchanged	Unchanged	Unchanged	Increased

<sup>a</sup> E = epidermal growth factor domain; F = finger domain; K1 = kringle 1; K2 = kringle 2.

### Cell line development

Development of cell lines is vital to producing modified plasminogen activators. A cell line has been used to prepare a kidney plasminogen activator (kPA), that seems to be a precursor of urokinase [5]. Unlike tPA, kPA circulates in blood as an inactive zymogen that becomes active only upon contact with fibrin-bound plasmin at the site of a blood clot [5].

Cell lines may be altered to manipulate glycosylation patterns to prolong *in vivo* stability of tPA. Moreover, selection of cells, media, stimulants and improved bioreactors continue to be important in substantially reducing production costs.

### Synergism strategies

Another proven approach to enhancing clinical efficacy of tPA is its administration with synergistic substances. Several companies are developing such complementary products. Secretion of a recombinant hybrid plasminogen activator (tPA/uPA) by mouse myeloma cells has been reported [63]. Yet other work has shown that a reduced dose of 10 mg tPA given with 3 mg kPA can be successful in treating heart attack patients [5]. Results may be further improved by modifying the dispensing protocol to sequential administration. Among other molecules that may be synergistic with tPA are protein S and protein C. Protein S is a cofactor to the anticoagulant protein C [5]. Protein S localizes protein C's activity to where it is needed [5]. In combination, these two proteins are known to accelerate the action of tPA *in vitro* [5]. Another synergistic approach being considered uses monoclonal antibodies to localize the action of tPA to fibrin clot [5]. Attempts are underway to engineer the thrombolytic agent into the antibody or to create an antibody with simultaneous specificity to the clot and the thrombolytic agent. Treatment with the anabolic steroid stanozolol (Stromba<sup>®</sup>) before administration of tPA has been known to improve thrombolytic efficacy [64].

## CONCLUDING REMARKS

Tissue-type plasminogen activator is a proven therapeutic thrombolytic, but room remains for substantial enhancements to its effectiveness through a variety of approaches. Moreover, the production technology—cell lines, media, bioreactors and culture schemes, and downstream recovery methods—needs to be significantly improved to ultimately reduce the price per dose to as low as a tenth of the existing value. Hence, a 10-fold increase in yield

over the best current cultured animal cells may be an immediate and quite reasonable goal. Improvements must come primarily through genetic enhancement of cells.

Production in microorganisms remains a realistic possibility considering the current infancy of the protein renaturation technology. Major increases in secretion of active tPA from microorganisms cannot be ruled out. Transgenic expression in animals and plants remains untested. Even with the existing production methods, integrated process optimization can help to reduce the cost of manufacture. Expensive products such as tPA generally hold a greater promise for cost reduction. Computer simulations based on realistic process models are likely to play a significant role in such optimizations. In view of the many untried cost-reduction options, the price per dose of tPA is likely to fall substantially in the future.

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