Combined TGE-SGE Expression of Novel PAI-1-Resistant t-PA in CHO DG44 Cells Using Orbitally Shaking Disposable Bioreactors

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An important modification of thrombolytic agents is resistance to plasminogen activator inhibitor-1 (PAI-1). In previous studies, a new truncated PAI-1-resistant variant was developed based on deletion of the first three domains in t-PA and the substitution of KHRR 128–131 amino acids with AAAA in the truncated t-PA. The novel variant expressed in a static culture system of Chinese Hamster Ovary (CHO) DG44 cells exhibited a higher resistance to PAI-1 when compared with the full-length commercial drug: Actylase. In the present study, the truncated-mutant protein was expressed in CHO DG44 cells in 50 ml orbital shaking bioreactors. The final yield of the truncated-mutant in the culture was 752 IU/ml, representing a 63% increase compared with the static culture system. Therefore, these results suggest that using the combined features of a transient and stable expression system is feasible for the production of novel recombinant proteins in the quantities needed for preclinical studies.

Keywords: Tissue plasminogen activator (t-PA), Chinese Hamster Ovary (CHO), suspension culture, orbital shaker, plasminogen activator inhibitor-1 (PAI-1)

As the leading cause of disability and third major cause of death in developed countries, ischemic stroke constitutes a great socioeconomic burden to society, as clearly reported by the American Heart Association report in 2008 [1]. Coronary heart diseases, including myocardial infarction, are also responsible for a significant percentage (52%) of the deaths due to cardiovascular diseases. Accordingly, the treatment of ischemic stroke is one of the most challenging and crucial areas in medicine today [1, 29]. Plasminogen activators have proven to be of great clinical significance for the management of strokes and myocardial infarctions, where the tissue-type plasminogen activator (t-PA), a glycoprotein consisting of 527 amino acid residues (72 kDa), is generally preferred owing to its better efficacy and safety compared with urokinase and streptokinase. In particular, its enhanced activity in the presence of fibrin is the major advantage of t-PA over other thrombolytic agents [6, 23]. T-PA cleaves the pro-enzyme plasminogen into active plasmin, which then degrades fibrin, the major component of clots, and promotes blood reperfusion. However, type-1 plasminogen-activator inhibitor (PAI-1) and a2-antiplasmin (a2-AP) are potential inhibitors of the thrombolysis cascade [6]. Apparently, complexes of t-PA/PAI-1 and free t-PA compete for the same binding sites on fibrin, thereby inhibiting the fibrinolysis cascade by preventing t-PA from binding to fibrin and greatly impeding the activity of t-PA [10, 31]. Since the recognition that residues 296–304 are critical for the interaction of t-PA with PAI-I, several variants of t-PA have been investigated with mutations or deletions in this domain [12–15, 20, 25]. Yet, Tenecteplase is currently the only FDA-approved PAI-1-resistant thrombolytic agent. Tenecteplase (also called a TNK-mutant of Alteplase) consists of the Alteplase molecule with the exception of two point mutations at positions 103 and 117 that cause a prolonged plasma half-life. Furthermore, at positions 296–299, the amino acids lysine, histidine, arginine, and arginine are replaced by four alanine amino acids to resist the inhibition by PAI-1 [18]. Reteplase is another deletion mutant with a prolonged half-life, in which the finger, EGF, and kringle 1 domains of the full-length molecule are all deleted.

As the finger domain is responsible for fibrin affinity, when compared with Alteplase, Reteplase is characterized by reduced fibrin selectivity and causes more fibrinogen depletion than the full-length forms. In the absence of fibrin, Reteplase and Alteplase are no different in terms of their activity as plasminogen activators or their inhibition by PAI-1 [4, 18, 32].

In previous studies by the current authors, the first three domains of t-PA were deleted and a chimeric tetrapeptide, Gly-His-Arg-Pro (GHRP), with a high fibrin affinity was added upstream of K2S to compensate for the reduced fibrin affinity due to the finger domain deletion. As a result,
a novel truncated form of t-PA with an improved fibrin affinity was expressed in a CHO DG44 expression system [9]. As a further improvement, a PAI-1-resistant novel form of the truncated t-PA was also designed. The truncated mutant variant was then successfully expressed in CHO DG44 and showed an increased resistance to PAI-1 [8].

The culture of suspended mammalian cells in orbitally shaking bioreactors is a preferred method for recombinant protein production, as it facilitates high mass transfers, a low shear force, excellent mixing capacity, low cost, and scalability [34]. Moreover, the majority of therapeutic proteins from mammalian expression systems are currently produced in stably transfected CHO cells [19]. However, conventional approaches of protein expression from stable mammalian cells are laborious and time-consuming. In contrast, transient gene expression (TGE) using suspension cultures of transfected mammalian cells has been successfully used for the rapid generation of reasonable quantities of recombinant proteins for preclinical studies [2, 3, 17, 22, 24, 27, 33].

At present, large-scale mammalian cell cultures are almost exclusively performed in stirred-tank bioreactors at volumetric scales from 1 l to 20,000 l [30]. In order to simplify the technology involved in the production of biopharmaceuticals, disposable-material-based bioprocesses and the use of orbitally shaking bioreactor systems have both been developed. Orbitally shaking disposable bioreactor units up to 1,500 l in volume have already been tested for mammalian cell cultures, resulting in the production of hundreds of milligrams and even gram amounts of the monoclonal antibody IgG when using suspension-adapted mammalian cells, especially HEK 293 EBNA SF cells and NSO cells [28]. Accordingly, this study explored the expression of the truncated-mutant t-PA in CHO DG44 cells when combining features from transient and stable transfection [5] in an orbitally shaking disposable bioreactor (TubeSpin, TPP Trasadingen, Switzerland), plus a rapid approach for producing the truncated-mutant t-PA is developed.

MATERIALS AND METHODS

General Materials
The DG44 transfection kit and Zeocin antibiotic were obtained by cooperation (CA, USA). The Chromolize t-PA Assay Kit was purchased from Biopool (Ireland). The rabbit polyclonal antibody to t-PA was supplied by Abcam (MA, USA), while the goat anti rabbit IgG–HRP conjugate was purchased from Santa Cruz biotechnology (CA, USA).

Plasmid DNA Preparation
Full-length human t-PA (GenBank Accession No. 1 01047) was amplified using the CHO 1–15 cell line (ATCC CRL-9096) genomic DNA and cloned into pTZ57R during our previous work [26]. The deletion mutant gene was then synthesized using 4 sets of primers in a SOEing PCR reaction, as explained in our previous study [8, 9]. The amino acid substitution, KHRR to AAAA, at positions 128–131 was performed using a SOEing PCR vise an appropriate primer design in a three-step reaction utilizing the truncated gene previously cloned in a plasmid as the DNA template for the desired mutations. A detailed explanation of the SOEing procedure has already been given [8, 9]. The SOEIn 1,210 bp gene was then cleaned using a QIAquick PCR Purification kit from Qiagen (Germany) and cloned in an intermediate vector, pJET1.2/blunt Cloning Vector, using a CloneJET PCR Cloning Kit from Fermentas (Lithuania) based on the manufacturer’s procedures. After confirming the proper sequence arrangement by bidirectional sequencing, two upstream and downstream BglII restriction sites of pJET were used for cloning into the EcoRV site in pTracer-SV40 (CHO expression vector). The BglII sticky ends were converted to blunt ends using the CloneJET PCR Cloning Kit DNA blunting enzyme. The recombinant pTracer-SV40-mutated t-PA plasmid was then purified using an EndoFree Plasmid Mega kit from Qiagen (Germany), and the right orientation of the gene was confirmed by Smal restriction enzyme digestion and sequencing.

Cell Culture
The suspension-adapted CHO DG44 cells were cultured in a serum-free chemical defined CD DG44 medium from Invitrogen, Gibco (USA) with 8 mM glutamine, 13.6 mg/l hypoxanthine, and 3.88 mg/l thymidine in the absence of serum, at 37°C. The cells were cultivated in 50 ml disposable bioreactors; TubeSpin, from Sartorius Stedim (Switzerland), 6-well plates, or 24-well plates. The filling volume of the vessels was typically 30% of the nominal volume (50 ml). The disposable TubeSpins were shaken at 140 rpm on an orbital shaker with a shaking diameter of 5.0 cm placed in a 5% CO₂ incubator and 85% humidity. DG44 cells were also statically cultured in flasks (75 cm², 5 ml working volume) in a 5% CO₂ incubator. The cell density and viability were determined using trypan blue staining.

Determination of Zeocin Sensitivity
To generate a stable cell line expressing the truncated-mutant protein, the minimum concentration of Zeocin required to prevent the growth of un-transfected cells (i.e., the parental cell line) was determined using the following protocol: (1) Using a 12-well plate, approximately 2.5 × 10⁶ cells per ml were cultured in the presence of varying concentrations of Zeocin (0, 50, 100, 250, 500, 750, and 1,000 µg/ml) added to each plate (7 plates were prepared). (2) The selective medium was replenished every 3 days, and the percentage of surviving cells was observed. (3) The number of viable cells was also counted every 3 days to determine the appropriate concentration of Zeocin that prevented growth during 3–4 weeks.

CHO Transfection and Expression
A DG44 Transfection Kit from Invitrogen, Gibco (USA) was utilized to transfect the CHO DG44 cells using Lipofectamine 2000 CD based on the manufacturer’s protocol. To obtain a higher efficiency of stable transfectants, the pTracer-SV40 vector was linearized before transfection. This also ensured that the vector did not integrate in a way that would disrupt the gene of interest. Among the enzymes that allow linearization of the pTracer-SV40 construct, BglII (2296) was selected, as co-transfection of this enzyme along with a plasmid has been reported to increase the integration efficiency. After determining the appropriate Zeocin concentration, a stable cell line was established as follows: First, 10⁶ cells were transfected with 20 µg of the vector and DNA (µg):
Lipofectamine ratio of 1:3.3. A plate of un-transfected cells was also included as a negative control. Then 24 h after transfection, the cells were washed once with 1× PBS, and added to a fresh medium containing Zeocin. The cells were fed the selective medium every 3–4 days until foci were identified. The cells were then transferred to 48-well plates, before expanding to Tube-Spins. Following the transfection, the tubes were agitated at 140 rpm in an orbital shaker incubator. The cultures were kept at 37°C under 5% CO₂ and saturated humidity conditions. The clones were tested for their expression of the t-PA protein. Positive clones were expanded into large wells and then into flasks or plates, and re-tested to confirm their expression.

As an alternative approach, the advantages of both TGE and stable cell lines were successfully combined, which resulted in a reasonably high productivity in a shorter period of time. In this procedure, the cells were selected shortly after transfection based on high stringent antibiotic selection. The recombinant proteins were then harvested from the bioprocess roughly three weeks later. According to this procedure, all the cells were subjected to high stringent selection, yet as pools rather than clonal populations. While similar to stable gene expression, pools of cells were investigated comparable to transient gene expression.

**Protein Quantification**
A Biopool Chromolize t-PA Assay Kit is a biofunctional immunosorbent assay, where the sample t-PA is captured by antibodies in the microtest wells. The SP-322 monoclonal antibody used also allows excellent t-PA recovery at pH 5.9 without inhibiting the t-PA activity. After discarding the test plasma, the wells were washed with a mild detergent. The t-PA substrate, consisting of plasminogen, a plasmin-sensitive chromogenic substrate, and t-PA activity promoters in a HEPES buffer, pH 8.5, was then added and the microtest wells were incubated at an ambient temperature (18–25°C) for 90 min with agitation. The samples were read at 405 nm. The amount of color developed was proportional to the amount of t-PA activity in the sample. The t-PA activity standard provided contained human single-chain t-PA and was calibrated against the international standard for t-PA, lot 86/670, distributed by NIBSC, South Mimms, Potters Bar, Hertfordshire, UK. Various dilutions of each sample were assayed as follows:

**Standard and sample incubation.** One hundred µl of the t-PA standards (0, 0.5, 1.0, 1.5, 2.0 IU/ml) and 100 µl of the sample were added to the microtest strip wells and incubated for 20 min on a microtest plate shaker at an ambient temperature at 600 rpm. Various dilutions of each sample were then assayed to bring the t-PA activity into the assay range.

**Washing.** The contents were discarded by tapping onto an absorbent towel or sponge, while the strips were washed 4 times using a repeating pipette, followed by 50 µl of the plasminogen reagent. The plate was then incubated for 90 min at an ambient temperature on a microtest plate shaker at 600 rpm.

**Stop.** Fifty µl of the stop solution (1.7 M acetic acid) was added to each well and mixed on a microtest plate shaker for at least 15 s. The yellow-colored reaction remained stable for 1 h at an ambient temperature.

**Measurement.** The absorbance was measured at 405 nm in all the wells. A second measurement was also taken at 492 nm and subtracted from the reading at 405 nm. Paraoxonasnilide absorbs light at 405 nm, whereas the absorbance due to turbidity is approximately equal at 405 nm and 492 nm. Therefore, the absorbance at 492 nm was measured and subtracted to correct for the background due to turbidity.

**Calibration.** The Chromolize t-PA standard curve was correlated to the International t-PA activity standard lot 86/670 (NIBSC), resulting in a correlation coefficient of 1.0 with a slope of 1.13. A405 or A405-A492 was plotted against each 0, 0.5, 1.0, 1.5, and 2.0 IU/ml standard. A straight line was then fitted to the points using a minimum least-square procedure. The t-PA activity in the samples was determined by interpolation from the standard curve. The amount of color developed was proportional to the amount of t-PA activity in the sample (Fig. 1).

**Gel Electrophoresis**
SDS–PAGE (12%) was carried out under reduced conditions and the Coomassie Blue-stained gel scanned using a densitometer (Bio-Rad) to determine the expression level of the truncated mutant t-PA in the culture supernatant.

**Western Blot**
For a Western blot analysis of the truncated mutant t-PA, the supernatant from 10×10⁶ viable cells was harvested and concentrated using an Amicon filtering system (Millipore, USA) with a 10 kDa molecular weight cut-off. The concentration of total protein was determined using the Bradford method, and 20 µg of total protein was loaded in each lane in a 10-well plate, 12% polyacrylamide gel. The electroblotting was performed in a semidry blotting system. The proteins were transferred to a nitrocellulose membrane, and the antigenantibody complexes visualized using a DAB-HRP system. A rabbit anti-human-t-PA antibody from Abcam (USA) was diluted in a 1/1,000 dilution and used as the primary antibody, while the secondary antibody, an HRP-conjugated goat anti-rabbit antibody from Santa Cruz (USA), was used in a 1/1,500 dilution.

**RESULTS**
**Construction of Eukaryotic Expression Vector**
The PCR product of the amplified truncated mutant t-PA gene cassette (Fig. 2A) was determined to be
1,210 bp (Fig. 2B). The recombinant intermediate vector (Fig. 2C) was then transformed into *E. coli* TOP 10 F' to sequence the correct insert. After digestion with *Bgl*II, the truncated-mutant t-PA gene construct was inserted into the multiple cloning site (MCS) of the eukaryotic expression vector p-Tracer SV40 (Fig. 2D) under the control of the p-SV40 promoter and an expression enhancer of the Simian Virus (SV40) early enhancer sequence.

**Establishing Optimum Cell Culture Process for CHO DG44**
The shaking culture system was able to support cell maintenance for 240 h after the subculture; however, the

![Schematic overview of primer designed for SOEing PCR process](image)

**Fig. 2.** Construction of expression plasmid for truncated mutant t-PA. (A) Domain arrangement of truncated mutant t-PA. (B) PCR product of truncated mutant t-PA gene fragment. (C) Intermediate pJET1.2 vector for blunt-end cloning of PCR product. (D) Final mammalian expression vector design.
The transfected CHO DG44 cells were cultured in a 50 ml TubeSpin (shaking bioreactor) and static T-flask culture in order to compare the cell growth and viability. Whereas the cell viability was found to be similar under both conditions, the cell density varied significantly. A maximal cell density of up to $3.2 \times 10^6$ cells/ml was obtained in the shaking bioreactors, 192 h post culture (Fig. 3). In contrast, the static culture only generated $2.9 \times 10^6$ cells/ml, 72 h post culture.

**Expression of Truncated Mutant t-PA**

The CHO DG44 cells were stably transfected with the truncated mutant t-PA-p-Tracer-SV40 recombinant plasmid based on the optimized Lipofectamine:DNA ratio mentioned above, and the supernatant was harvested on different days (day 3, day 6, day 9, and day 11) after the transfection. The expression of the fusion protein on day 7 was analyzed by SDS–PAGE. A protein band with a molecular mass of approximately 40 kDa was observed in the supernatant of the recombinant plasmid transfected cells. This band was absent in the supernatant of the non-transfected cells (Fig. 4C), which served as a control. To confirm the expression of the recombinant truncated mutant t-PA-p-tracer-SV40, a Western blot analysis with a human t-PA-specific antibody was applied to probe the supernatant products. As shown in Fig. 4B, a truncated-mutant-t-PA-antibody positive protein with an apparent molecular mass of 40 kDa was detected from the recombinant plasmid transfected cells under reduced conditions.

SDS–PAGE and a Western blot analysis using an antibody against t-PA were performed on the purified protein. Fig. 4A shows the bands related to the truncated-mutant t-PA, which matched the theoretically calculated sizes after signal sequence removal; 359 amino acids (39 kDa) and another glycosylated form with 43 kDa. This was also confirmed by the Western blot analysis (Fig. 4B). These findings were similar to those previously reported by Burck et al. [7].

Furthermore, an 86 kDa band was revealed by the Western blotting. However, when the dimer form was converted to a monomer form by dissolving the protein in 8 M urea, just one band at 43 kDa was observed on the Western blot. Thus, the data confirmed that the doubled molecular mass size was due to aggregation of the truncated t-PA. Therefore, when considering the findings, it was concluded that the truncated mutant t-PA was expressed from the transfected CHO DG44 as both a monomeric and dimeric protein linked by potential disulfide bonds.

Fig. 3. Comparison of CHO DG44 cell growth with different culture methods. Viable cell density of cells cultured in 50 ml disposable bioreactors (TubeSpin) and 75 cm$^2$ static culture bottles for 240 h.

Fig. 4. Expression analysis of truncated mutant t-PA protein in supernatant of DG44 cultured in 50 ml disposable bioreactors (TubeSpin). (A) SDS–PAGE analysis for expression of truncated-mutant t-PA; 12% EBT silver stained; Lane 1: Truncated-mutant t-PA transfected cell supernatant; Lane 2: Protein weight marker. (B) Western blot analysis of truncated-mutant t-PA under nonreduced conditions; Lane 4: Truncated-mutant t-PA transfected cell supernatant; Lane 3: Protein weight marker. (C) Western blot analysis of truncated-mutant t-PA under reduced conditions; Lane 5: Truncated-mutant t-PA transfected cell supernatant; Lane 6: non-transfected cell supernatant; Lane 7: Full-length commercial t-PA (Actylase); Lane 8: Protein weight marker.
Quantitative Analysis of Expression

The supernatant from the static and suspension cultures was sampled on different days throughout the experiment to assess the protein production kinetics. An ELISA-based biofunctional immunosorbent assay with a Biopool Chromolize t-PA assay kit was used to quantify the truncated-mutant t-PA. The results showed a volumetric productivity of 752 unit/ml of protein on day 9 in the suspension culture compared with 480 unit/ml for the static culture. Based on Biopool's Chromolize t-PA assay kit, the best expression level was determined to be 752 unit/ml on day 9 of the culture under optimized conditions with a 0.6 million starting cell density, representing a 67.2% increase from 246 unit/ml on day 4. Meanwhile, the maximum amount of expressed protein in the static culture system was determined to be 448 unit/ml. The expressed protein yield decreased on day 11 to 174 unit/ml for the suspension culture and 110 unit/ml for the static culture, due to a reduction in cell viability and the release of cellular proteases.

DISCUSSION

Chinese Hamster Ovary (CHO) cells are the major mammalian host for the production of recombinant proteins that are both compatible and bioactive in humans. The production of t-PA in E. coli, Leishmania tarentolae, and Pichia pastoris was previously tested by the current authors [11, 16, 26]. However, active t-PA was poorly expressed in E. coli (3–7 IU/ml) [21] owing to a lack of posttranslational modifications in this host.

In yeast expression systems, proteins are known to be susceptible to over-glycosylation, which can ruin their bioactivity. Thus, TGE has become an increasingly popular approach for producing recombinant proteins that combines the advantages of both TGE and stable cell lines, along with being time, labor, and cost effective, and having a reasonably high productivity [5]. According to this procedure, the cells are subjected to high stringent antibiotic selection shortly after transfection. The selected clones are then expanded according to the desired volume and concentration, and the proteins harvested on different days post culture. The screening efforts are greatly reduced in comparison with the generation of stable cell lines, as the cells are subjected to selection as pools rather than clonal populations. Owing to this selection procedure, the productivity yield is also increased when compared with the TGE process. However, the potential weakness of this system is unstable transgene expression. Pools are known to be heterogeneous, where cells differ according to their transgene integration sites and, accordingly, expression level. However, with an appropriate gene delivery method and stringent selection, the process can be optimized to alleviate these limitations.

The novel variant suggested in this project is a truncated mutant form of t-PA with 395 amino acid residues, and its theoretical molecular mass is 39 kDa. SDS-PAGE showed two bands (39 and 42 kDa) close to the monomer size of the recombinant, due to glycosylation heterogeneity in the CHO expression system [7]. However, further analysis, such as biological mass spectrometry in combination with a glycosylation pattern analysis, is needed for a better analysis of the glycosylation profile of the new t-PA variant.

The extra 86 kDa bond representing the dimer form was converted to the monomer form by dissolving the protein in 8 M urea. Therefore, the data confirmed that the doubled molecular mass size was likely due to aggregation of the truncated-mutant t-PA.

The results from our previous study suggested that, in the presence of PAI-1, the thrombolytic potency of mutant t-PA mut(K128-R131) is superior to that of full-length t-PA, owing to its resistance to PAI-1 [8].

Whether clinical use of this PAI-1-resistant t-PA mutant will lead to less PAI-1-mediated re-occlusion after thrombolysis or a better thrombolytic potency towards clots remains to be investigated.

REFERENCE


