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

Davami, Fatemeh, Farzaneh Barkhordari, Mahmoud Alebouyeh, Ahmad Adeli, and Fereidoun Mahboudi*

Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

Received: June 1, 2011 / Revised: July 24, 2011 / Accepted: July 25, 2011

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 11 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 (Sweden), 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 via 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 SOEn 1,210 bp gene was then cleaned using a Quick 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 TubeSpin 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. 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):