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Article

Optimization of expression yield in a stable cell line expressing a novel mutated chimeric tissue plasminogen activator (mt-PA)

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The development of stable cell lines producing recombinant proteins is very time-consuming and laborious. One of the practical approaches successfully performed is Fluorescence-Activated Cell Sorting (FACS). A mutated chimeric tissue plasminogen activator (mt-PA) was developed by removing the first three domains of t-PA, insertion of GHRP sequence and mutation toward resistance to plasminogen activator inhibitor-1 (PAI-1). In the current study, a new stable CHO-DG44 cell line producing mt-PA was developed by two sequential clonal selections: FACS and clonal-selection by limiting dilution. Furthermore, the expression was more evaluated using two different expression media. Finally, the high-producing clones were selected based on the dot blot and amidolytic activity test. The transfection efficiency of CHO-DG44 cells was 38% as measured by flow cytometry on green fluorescent protein (GFP). After performing FACS on stable cell pools, the expression yield was increased to fifty-fold. In terms of growth profile, CD-DG44 showed higher viability and cell density results than ProCHO5 medium. The expression of mt-PA was significantly higher in CD-DG44 than in ProCHO5, 765 and 280 IU/mL, respectively. Our data indicated that selection of an appropriate expression medium played a critical role in the development of potent producing stable cells by FACS.

Keywords: CHO-DG44. Fluorescence-Activated Cell Sorting. mt-PA. Stable cell line. Tissue plasminogen activator

INTRODUCTION

Nowadays, biotechnological production has grown significantly, and various techniques have been developed to improve the quality of biopharmaceuticals (Walsh, 2006). Proteins are usually expressed as recombinants by the DNA technology in several hosts such as bacteria, yeast, plant, insect, and mammalian cells. Despite low expression level of foreign proteins in mammalian cells, the necessity of post translational modifications (PTMs) and glycosylation patterns in most recombinant therapeutic proteins has caused manufactures to use such systems for bioactive macromolecules (Browne, Al-Rubeai, 2007). Many investigations have been conducted to optimize and increase expression yield (Wurm, 2004b). For example, production yield has enhanced via media optimization, suitable expression systems, and processes permitting cells to grow at higher densities for long-term cultivation. However, mammalian cell culture has been considered labor-intensive, time-consuming and expensive process. Owing to increasing demands for recombinant drugs at high doses, it is necessary to increase the cell culture capacity by application of high density cell culture system, and to use a fast method of selection to choose the high-producing cell line (Butler, 2005).

Stable producer cell line is used in the pharmaceutical market, since long-term constitutive protein expression with improved properties in terms of protein expression and cell growth is required (Wurm, 2004a). The main disadvantage of this procedure is that it is a time-consuming process (Wulhfard, 2009). Depending on

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the detection technique used, weeks to months should be usually spent on a desired clone isolation procedure (Pendse, Karkare, Bailey, 1992). Despite spending great efforts on the development of high-producing clones, the obtained clones conversely reduced the growth rate and in some cases, the production stopped after several passages (Kim *et al.*, 1998b). The first can be explained by exhausting the cells due to recruitment of energy in production steps, and the later is considered to be caused by position effect (Kayser, Warzecha, 2012). Moreover, contrary to high-throughput screening tools, conventional procedures are practically analyzed using only limited clones, which may lead to missing some high-producing cells (Kim, Kim, Lee, 1998a).

High-throughput screening approaches such as flow cytometry and cell sorting techniques are considered promising tools extensively increasing the total number of screened cells. In such systems, several million cells are analyzed in a short time, and subpopulations and even individual cells with different expression levels are separated (Zang *et al.*, 2012). Practically, two or three sequential steps of cell sorting are required to enrich a population revealing high-level expression (Dean, 1985). However, more steps can be taken, and in some cases have good outcomes have been obtained. For example, Kavathas and Herzenberg (1983) isolated a population exhibiting 40-fold florescent intensity after six cell sorting steps in the case of human T cell differentiation antigen Leu 2.

Improvements of the GFP molecule have made it possible to use it in various biological applications. Therefore, the GFP gene has been used as an important reporter for gene expression, selection of cells expressing inducible gene products, as well as protein labeling localization studies (Welsh, Kay, 1997). In mammalian cell lines, GFP can increase the chance of selecting high-producing clones by co-expression with recombinant proteins and screen based on fluorescence intensity (Meng *et al.*, 2000). Several cell lines expressing recombinant proteins had a correlation between GFP intensity and recombinant protein production. According to a previous study, a recombinant rat serotonin receptor protein was co-expressed with GFP in human embryonic kidney (HEK) cells and showed a six-fold increase in specific production after repeated five times of cell sorting and culturing cycles (Mancia *et al.*, 2004). A selection system based on the surface expression of CD20 molecule linked to the desired protein is established to assort high-level expressing cells (DeMaria *et al.*, 2007). Similar techniques were developed based on this system and used to select high-producing populations such as isolation of Lymphocyte cells producing the high-level α-chain of human IL-2 receptor (Tac protein) (Beckers *et al.*, 1988). Yoshikawa *et al.* (2001) also indicated that cells with the highest gene-amplified product can be sorted by flow cytometry using high fluorescein-labeled methotrexate (F-MTX) intensity. To clone characterization and isolation, a dual-fluorescence approach was also performed using the GFP gene attached to the Discosoma Red fluorescent protein (DsRed) gene to select transfected single cells (Choe, Guo, van den Engh, 2005).

Tissue plasminogen activator (t-PA) is one of the fibrin-specific serine proteases playing a crucial role in the treatment of ischemic stroke. Therefore, it still has indication as a thrombolytic agent in the medical treatment of thromboembolic diseases (Barreto, 2011).

In our mt-PA, the three (F, EGF, and K1) domains of t-PA were removed to decrease the plasma clearance and increase the half-life of recombinant protein in the circulating blood (Davami *et al.*, 2010). Furthermore, a chimeric tetra-peptide Gly-His-Arg-Pro (GHRP) having high affinity for fibrin was inserted to the upstream of K2S to compensate for the effect of F domain deletion (Davami *et al.*, 2011a). Moreover, the four amino acids bound to PAI-1 at positions 296-299 were substituted with four alanine amino acids to prevent PAI-1 inhibition (Davami *et al.*, 2011b; Azarian *et al.*, 2017). In the previous study, we confirmed that the novel mt-PA had better properties than the full-length form, which was performed by transient gene expression (TGE) (Raigani *et al.*, 2017).

Since a small number of clones exhibit expression above the average level, via the limiting dilution technique, tens of thousands of clones need to be evaluated to find a cell line with favorable characteristics (Barnes, Bentley, Dickson, 2001). Therefore, we used the high-throughput screening approach, including the FACS technique, using GFP to screen high-producing cells.

MATERIAL AND METHODS

Preparation of the expression plasmid

The expression vector containing the cDNA of the mt-PA (mt-PA pTracer-SV40) was provided by Davami *et al.* (2010). First, the mt-PA pTracer-SV40 plasmid was confirmed by digestion *Sma*I restriction enzyme (Fermentas, Lithuania), and then purified using the EndoFree Plasmid Giga kit (Qiagen, Germany). To increase the integration efficiency, the plasmid was linearized by *Bgl*II restriction enzyme (Fermentas, Lithuania).

The expression of the recombinant protein was constitutive under SV40 promotor. The Kozak and the signal sequence were inserted upstream of the mt-PA gene. The cDNA of the mt-PA was cloned into the pTracer-SV40 expression vector containing Zeocin selection marker to inhibit the growth of non-transfected CHO cells. The mt-PA pTracer-SV40 was transfected into the suspension-adapted CHO-DG44 cells (GIBCO, Life Technologies, USA) to generate a stable cell line expressing the mt-PA.

Cell culture and Minimal Inhibitory Concentration (MIC) determination

The CHO-DG44 cells were individually cultured in the chemically defined serum-free medium (CD-DG44) from Invitrogen (GIBCO Invitrogen, USA) and ProCHO5 (Lonza, Belgium). The media were supplemented with 13.6 mg/L hypoxanthine, 3.9 mg/L thymidine, and 8 mM glutamine, and incubated at 37 ºC in 5% CO_2.

To generate a stable cell pool expressing the mt-PA, the minimal concentration of Zeocin (GIBCO Invitrogen, USA) must be added to inhibit the growth of non-transfected CHO cells. Therefore, approximately 2×10^5 cells/mL were cultured in 6 well plates in the existence of different Zeocin concentrations (0, 100, 250, 500, 750, and 1000 µg/mL). The medium containing altered Zeocin concentrations was exchanged every 3 days, and the percentage of surviving cells was also counted every day to determine the inhibitory concentration of Zeocin that prevents growth during 10-14 days.

Transfection into CHO-DG44 cells and determination of transfection efficiency

When the cell density reached to 5×10^5 cells/mL in a final volume of 2 ml, the linearized plasmid was transfected to the cells by X-tremeGENE HP DNA transfection reagent with a 1:2.5 DNA/reagent ratio following the manufacturer's recommendations. A negative control formed one plate of non-transfected cells. Transfection efficiency was calculated by flow cytometry (CyFlow, Partec, Germany) and manually by a fluorescent microscope (BEL, Italy). In all the experiments, the intensity of emitted fluorescence was investigated after 48 h of transfection with excitation and emission at 488 and 509 nm wavelengths, respectively. In flow cytometry, viable cells were gated and quantitatively evaluated by the FloMax software for GFP expression. In the manual method, the ten visual fields of each sample were counted by a microscope. Finally, the average fluorescent-counts in all the experiments were compared to non-transfected cells. After 72 h of transfection, the suspended cells were subjected to high stringent selection by adding 250 µg/mL Zeocin (MIC) to each medium, and the selective medium was exchanged every 3-4 days until viability of cells increased. The survived cells were then cultured and propagated for further steps.

Purification

The purification procedure was performed by the HiTrap Benzamidine FF (high sub) column (1 mL) (Pandya *et al.*, 1991). The column is based on affinity to serine proteases. The binding and wash buffers were as follows: 0.5 M NaCl, 0.05 mM Tris-HCL, pH 7.4. Two elution buffers were performed using a step gradient of 1 M NaCl, 10 mM HCl with a pH of 2.0 and 0.05 M Glycine (pH of 3.0). The purification steps were accomplished based on the manual. Briefly, after washing the column with 5-column volumes of distilled water to remove storage buffer, the column was equilibrated with 5-column volumes of binding buffer. Then, cell culture conditioned media was filtered through a 0.45 µm filter and loaded to the column with a peristaltic pump at the 1 mL/min flow rate. The next step, the column was again washed with 10-column volumes of binding buffer until no material appeared in eluted fraction. Finally,

the mt-PA was eluted from the column primarily with 5 mL of 1 M NaCl, 10 mM HCl (pH 2.0) elution buffer in order to remove non-specific attached proteins and then, with 5 mL of 0.05 M Glycine. With the pH and conductivity of these buffers, the t-PA electrostatically bound with the column, while other non-serine protease proteins did not bind with the resin and were removed in the column flow through.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis

After one month, supernatants from the stable transfected CHO culture were harvested and analyzed by electrophoresis on a 12 % polyacrylamid gel using the Coomassie brilliant blue staining method according to Laemmli (1970). Western blotting technique was also carried out according to Sambrook, Fritsch, Maniatis (1989) using a semidry blotting system (Bio-Rad, USA). Primary and secondary antibodies included polyclonal rabbit anti-t-PA antibody (1/1000 dilution, Abcam, USA) and HRP conjugated goat anti-rabbit antibody (1/2500 dilution, Santa Cruz. USA), respectively. Finally, proteins bands were visualized by adding DAB solution (Sigma-Aldrich, Germany).

FACS analysis

Two repeated FACS steps were performed by one month interval. In each step, up to 1×10^6 transfected cells were harvested and considered for fluorescence intensity with a BD FACS Calibur Flow Cytometer (BD Facs AriaII cell sorter. San Jose, CA 95131-1807, USA). Non-transfected cells were used as a negative control. Forward scatter light (FSC) against side scatter light (SSC) was plotted, and the obtained data were analyzed using the WinMDI 12.9 software (freeware from Joe Trotter, La Jolla, CA, USA). The GFP intensity was recognized from 350/30 filter and was excited by a 488 nm wavelength. The cells were then sorted into high and low GFP fluorescence intensity cells by a BD FACS Calibur Flow Cytometer. Finally, the cells exhibiting high GFP fluorescence intensity were sub-cultured into the 48 well microplates for further steps.

Clonal-selection by limiting dilution

First, the completed cloning medium was prepared according to the invitrogen instructions. Briefly, 87 mL of each medium (ProCHO5 and CD-DG44) was supplemented with 3 mL of freshly thawed 200 mM L-glutamine and 10 mL of conditioned medium. The cells were serially diluted by the above-mentioned complete medium to aseptically seed approximately 1–2 cells per 200 µL in a 96-well plate using a multi-channel pipettor. The plates were then incubated for 10–14 days at 37ºC in a 5% CO_2 incubator without shaking. After ten days, the 96-well microplate was visually examined using an invert microscope (Nikon, Japan) to examine growth profile of colonies. Finally, growth positive wells were screened for expression of the protein.

Selection of the high expressing clones

The growing transfected cells were screened for high-expressing clones by the dot blot analysis. Briefly, positive clones were individually cultured in 1 mL desired medium at 37 °C and 48 wells (cell density: 1×10^5 cells/mL) in ProCHO5 and CD-DG44 media. After 9 days, the supernatants of the different clones were harvested and centrifuged. Afterward, they were directly spotted on the nitrocellulose membrane. The procedure was followed as the Western blot protocol. The amount of the mt-PA secreted by various clones was compared using the dot blot analysis. The positive bands were visualized by an enhanced chemiluminescence exposure system (Amersham Life Sciences, Buckinghamshire, UK). The spot intensity was compared using densitometric scanning by the Quantity One 4.62 software (Bio-Rad Laboratories, Hercules, CA, USA). The clones with the highest intensity were selected for quantity analysis.

Amidolytic activity test

The amount of mt-PA in the culture medium was determined by the amidolytic activity test using the Chromolize t-PA Assay Kit (Trinity Biotech plc, Ireland). The test is based on the biofunctional immunosorbent assay during which t-PA is captured by strip-coated with sp-322

monoclonal antibody. The kit was performed according to the manufacturer's manual, which is briefly explained. First, t-PA standards and the samples were inserted into the microtest strip wells. Following the removal of the test plasma and standards, the wells were washed with a mild detergent. Afterward, 50 µl of plasminogen and substrate reagent were inserted into each well, leading to a yellow color, which varied with the amount of t-PA activity. Following reading at 405 and 492 nm wavelengths, absorbance of each sample at 492 nm was subtracted from 405 nm, and the activity (IU/mL) was calculated from standard values (0, 0.5, 1, 1.5 and 2 IU/mL).

Expression analysis in CD-DG44 and ProCHO5 expression media

After twice FACS-sorted and clonal selection for the mt-PA expression, 1×10^5 cells/mL of stable transfected CHO cells for the mentioned statuses, including after the first FACS, the second FACS, and clonal selection, were individually cultured in 10 mL of the CD-DG44 and ProCHO5 media. The cultures lasted for 14 days and were counted every other day. Finally, the supernatant of six cultures was separately harvested and kept at -20 ºC for SDS-PAGE and quantitative analysis.

RESULTS

Preparation of the expression plasmid

According to Figure 1A, the mt-PA pTracer-SV40 plasmid was digested by *Sma*I restriction enzyme; therefore, the presence of the mt-PA gene was confirmed. Digestion by *Bgl*II restriction enzyme resulted in a 5440 bp band of linear plasmid with high integration efficiency (Figure 1B).

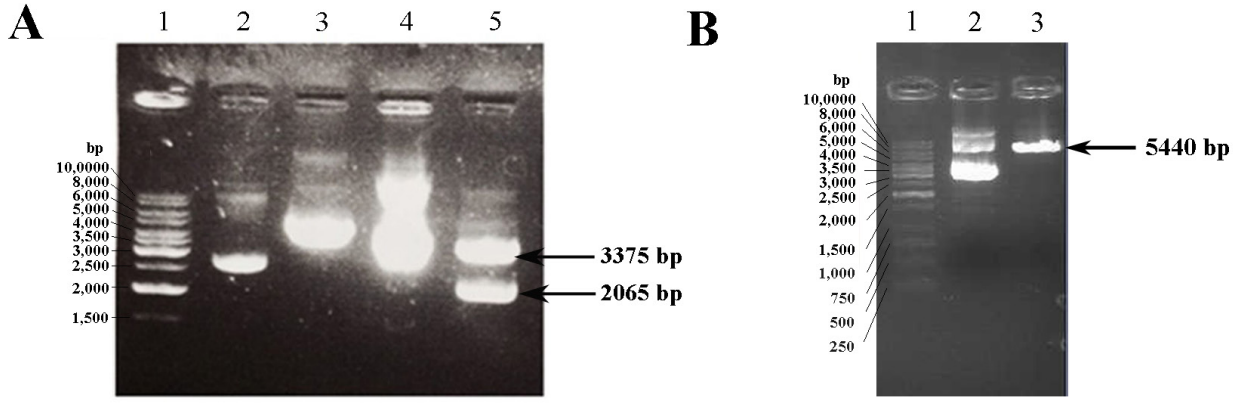

FIGURE 1 - (A) Gel-electrophoresis analysis of the mt-PA- pTracer-SV40 Plasmid by *Sma*I restriction enzyme digestion on 1% agarose gel. Lane 1, DNA Ladder 1 Kb; Lane 2, pTracer-SV40 Plasmid without the mt-PA gene; Lane 3, pTracer-SV40 Plasmid without the mt-PA gene digested by *Sma*I restriction enzyme; Lane 4, pTracer-SV40 Plasmid with the mt-PA gene; Lane 5, pTracer-SV40 Plasmid with the mt-PA gene digested by *Sma*I restriction enzyme. (B) Gel-electrophoresis analysis of the mt-PA-pTracer-SV40 Plasmid by *Bgl*II restriction enzyme digestion on 1% agarose gel. Lane 1, DNA Ladder 1 Kb; Lane 2, pTracer-SV40 Plasmid with the mt-PA gene; Lane 3, pTracer-SV40 Plasmid with the mt-PA gene linearized by *Bgl*II restriction enzyme.

Cell culture and determination of MIC

CHO-DG44 cells were separately cultured in the CD-DG44 and ProCHO5 media. The results (Figure 2) showed higher cell density (cells/ml) and viability of CHO-DG44 cells in CD-DG44 than those in ProCHO5 medium. Before transfection, the MIC of Zeocin for CHO-DG44 cells was determined 250 µg/mL.

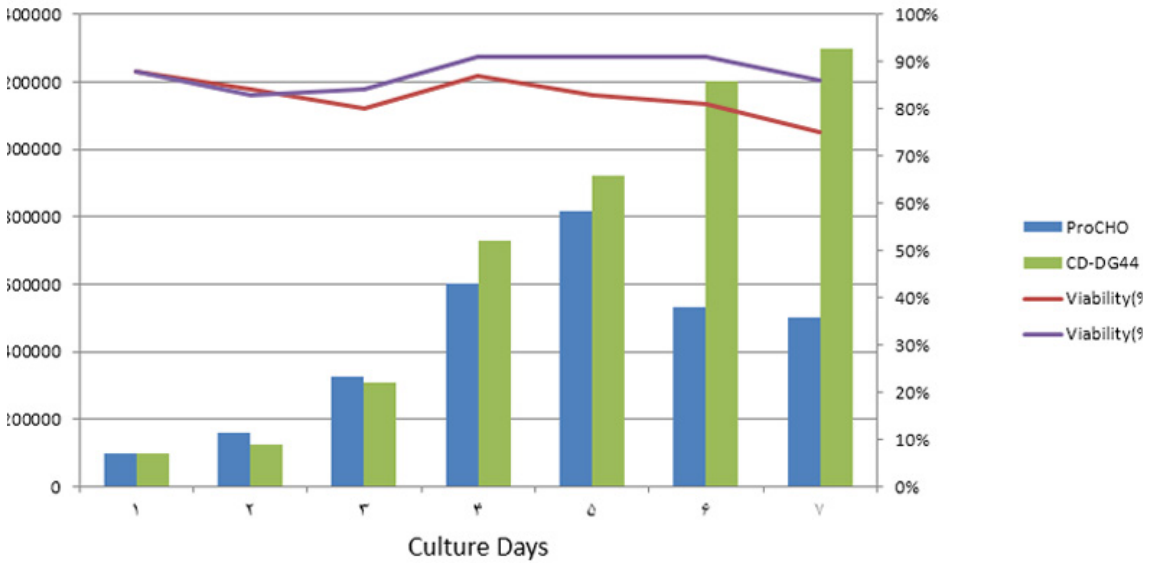

FIGURE 2 - The viability and density of CHO-DG44 cells in CD-DG44 and ProCHO5 media.

Transfection into CHO-DG44 cells and determination of transfection efficiency

The mt-PA pTracer-SV40 plasmid was transfected into CHO-DG44 cells in CD-DG44 and ProCHO5 media individually by X-tremeGENE HP DNA Transfection Reagent. Then, the plates, 48h after transfection, were manually visualized by fluorescent microscope, and transfection efficiency was estimated 35-45% in both media cultures. In flow cytometry, non-transfected cells were first gated and set as the system threshold and 72 h following transfection, the transfection rate was measured 38% on the green fluorescent protein (GFP) basis (data not shown).

Analysis of expression by SDS-PAGE and Western Blotting procedure

The supernatants harvested from stably transfected CHO cells were analyzed by Gel electrophoresis on a 12 % polyacrylamid gel and Western blotting. As Figure 3A shows, the 43 kDa protein bond belongs to mt-PA (lane 1), and no bonds were detected in the negative control as shown in lane 2. Lane 4 indicates the full-length t-PA protein, which is confirmed by the 65 kDa bond. Ultimately, the expression of mt-PA protein was confirmed by the Western blotting analysis (Figure 3B).

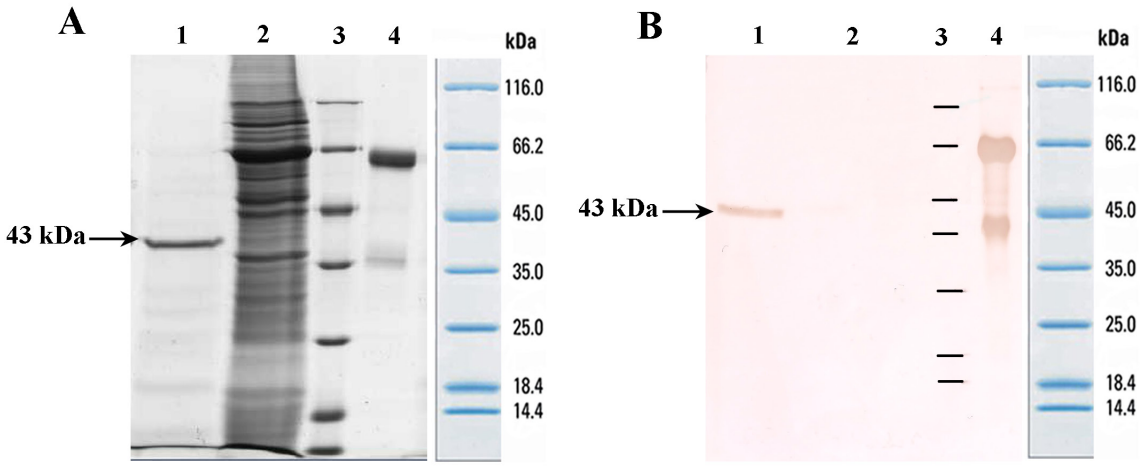

FIGURE 3 - SDS-PAGE and Western blotting analysis of mt-PA after purification. (A) Analysis of mt-PA expression in transfected CHO cells supernatant on a 12% polyacrylamide gel. Lane 1, The mt-PA CHO cell culture medium; Lane 2, Supernatant from non-transfected CHO cell culture medium as negative control; Lane 3, Unstained Protein MW Marker (Thermo Scientific); Lane 4, Alteplase (positive control). (B) Western Blotting analysis of the mt-PA expression in the transfected CHO cells supernatant. Lane 1, The mt-PA CHO cell culture medium; Lane 2, Supernatant from non-transfected CHO cell culture medium as negative control; Lane 3, Unstained Protein MW Marker (Thermo Scientific); Lane 4, Alteplase (full length form).

Fluorescence-Activated Cell Sorting (FACS) analysis

As Figure 4 shows, non-transfected and transfected cells were analyzed for fluorescence intensity. After the first sorting, 1.7-2×10^5 cells were separated to high GFP fluorescence, and then, the second sorting was carried out after one month, based on which 1.3-1.5×10^5 and 6-7×10^5 cells in high and low GFP fluorescence were divided, respectively. Finally, the cells exhibiting high GFP fluorescence intensity were sub-cultured and after one passage, screening of the clones was carried out by limiting dilution.

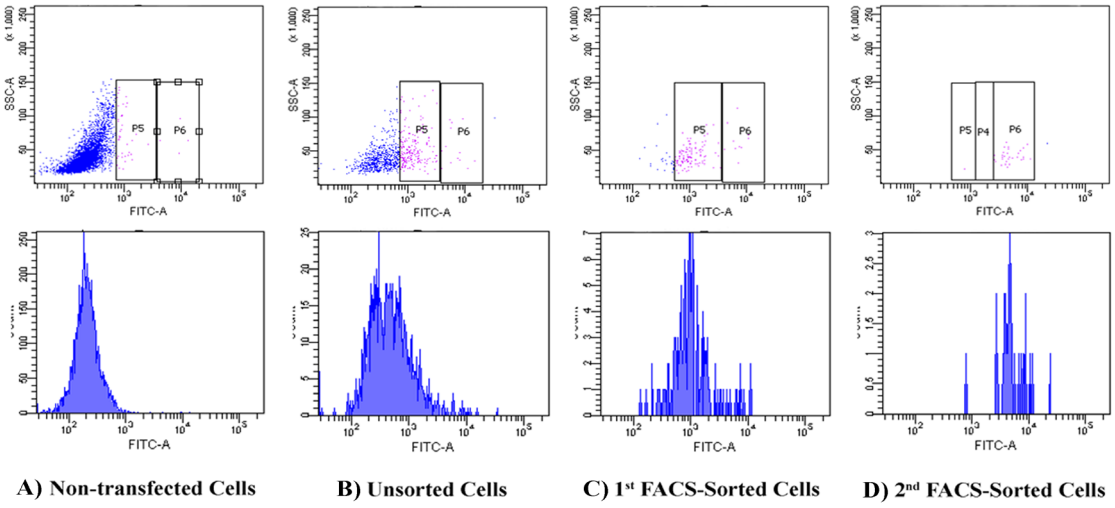

FIGURE 4 - Fluorescence-Activated Cell Sorting (FACS) analysis. (A) Non-transfected CHO cells as negative control. (B) Transfected CHO cells, which have not been sorted by the FACS technique. (C) Transfected CHO cells, which have been first-sorted by the FACS technique. (D) Transfected CHO cells, which have been second-sorted by the FACS technique.

Selection of the high expressing clones

After clonal selection, several wells of 96 plates were screened for growing of clones, and high-expressing clones of the transfected cells were selected using the Western blotting analysis. In this technique, a total of 33 positive clones were individually cultured in a 1 mL desired medium at 37 °C and 48 wells (cell density: 1×10^5 cells/ml) in batch culture. After 9 days, the quantity of secreted mt-PA in each supernatant was monitored by the Western blotting analysis, and the relative expression yield of mt-PA was evaluated by comparative densitometric scanning. Figure 5 shows the relative expression quantity of 16 high-producing clones, identified by the densitometric analysis. These clones with the highest production intensity were selected for quantity analysis.

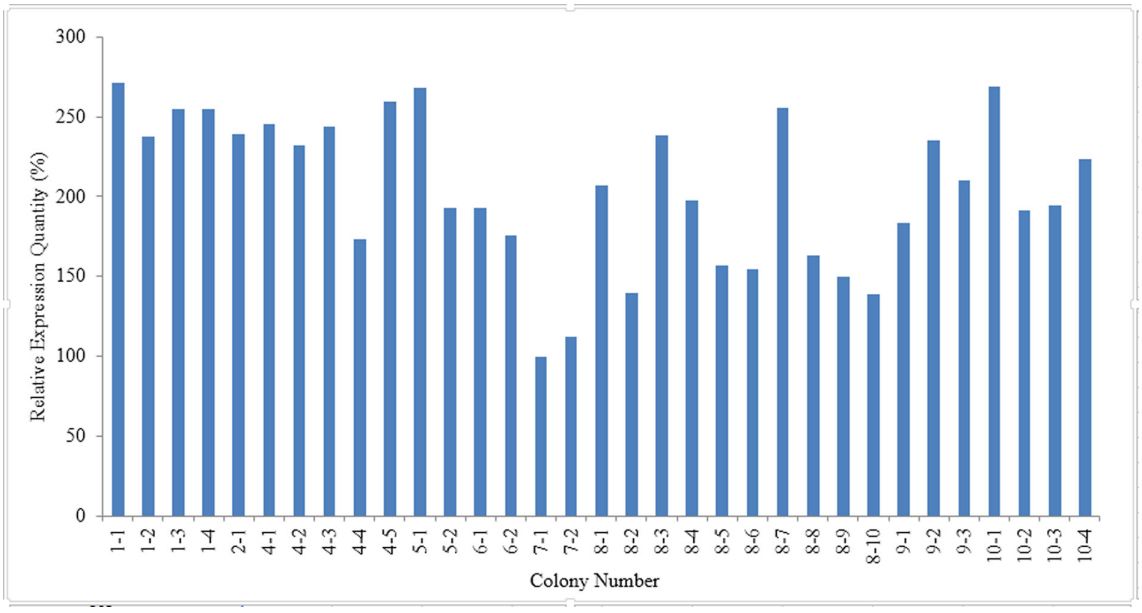

FIGURE 5 - Relative expression yield of mt-PA after clonal selection of different clones. The colonies exhibiting highest productivity were subjected to the Amidolytic activity test.

Expression analysis in CD-DG44 and ProCHO5 expression media

After 14 days of batch cultures, the supernatant of six individual cultures was analyzed by the SDS-PAGE analysis according to Figure 6. As shown, lanes 1-3 belong to the supernatant of the CD-DG44 culture medium of sorted and clonal selected CHO-DG44 cells, which has 43 kDa. In different samples, the results demonstrate that the 43 kDa protein bond of CD-DG44 medium is significantly higher than that of ProCHO5 medium.

According to the quantitative analysis based on the amydolytic test, the volumetric productivity of recombinant protein was determined 280 and 765 IU/mL in ProCHO5 and CD-DG44 culture media, respectively.

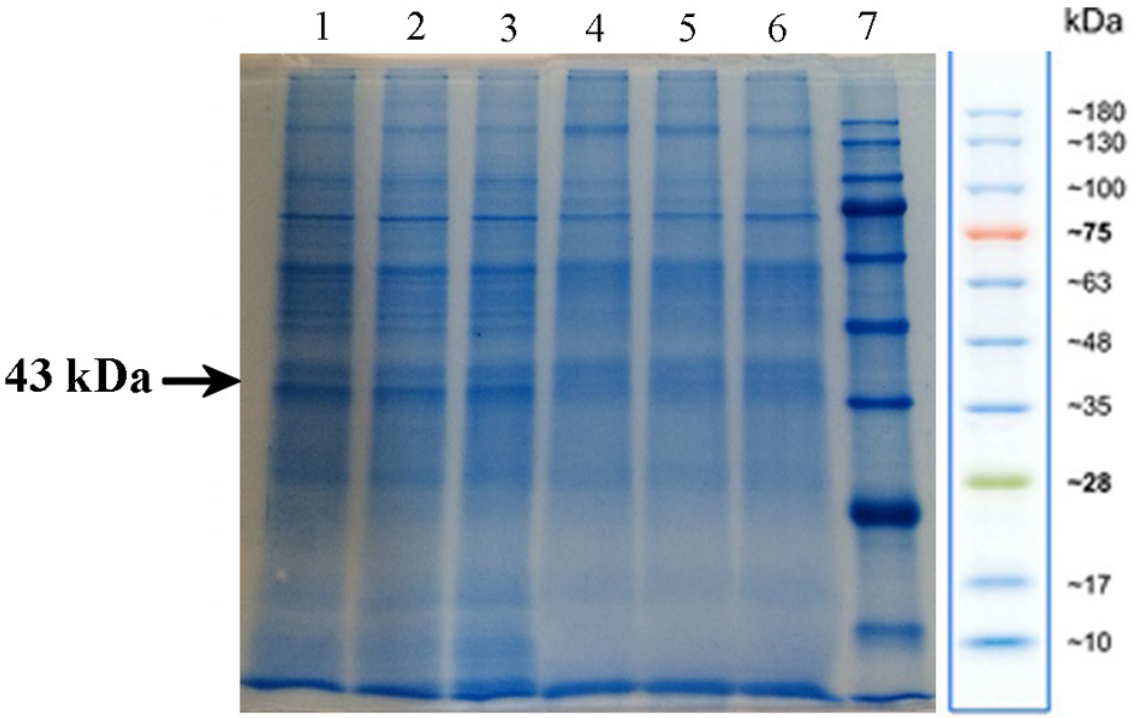

FIGURE 6 - SDS-PAGE analysis on different cultures in CD-DG44 and proCHO5 media. Lane 1, The CD-DG44 culture medium of the first FACS-sorted cells; Lane 2, The CD-DG44 culture medium of the second FACS-sorted cells; Lane 3, The CD-DG44 culture medium of clonal selected cells; Lane 4, The ProCHO culture medium of the first FACS-sorted cells; Lane 5, The ProCHO culture medium of the second FACS-sorted cells; Lane 6, The ProCHO culture medium of clonal selected cells; Lane 7, Prism Ultra Protein Ladder (ab116027).

Amidolytic activity test

The mt-PA activity in the purified supernatant samples of transfected CHO-DG44 cells was quantitatively determined by an ELISA-based biofunctional immunosorbent assay, Biopool Chromolize t-PA Assay Kit. The activity (IU/ml) of the tissue plasminogen activator was calculated from the standard curve. As calculated, the productivity of mt-PA was more increased using CD-DG44 medium up to 765 IU/mL. While the expression was found to be 280 IU/mL for ProCHO5 medium. A fifty-fold increase is observed in the productivity of mt-PA (15 to 765 IU/mL) after 2 steps of FACS and clonal selection in CD-DG44 medium. However, the production yield of mt-PA in ProCHO5 medium was increased from 8 to 280 IU/mL after 2 steps of FACS and clonal selection, a thirty-five-fold increment. As illustrated, the volumetric production of the protein in CD-DG44 medium was higher than that in ProCHO5 medium.

DISCUSSION

The development of a stable, high-expressing cell line is the first step in the construction of a new therapeutic protein (DeMaria *et al.*, 2007). Many efforts have been made to optimize the expression status. Moreover, to achieve stable high producers, it is necessary to screen single cells, but it is impossible by only traditional limited dilution, since low-producing cells' growth rate is higher than that of high-producing cells. In high-producing cells, metabolic resources are directed to protein production; therefore, they have a lower growth rate. Accordingly, the choice of a suitable selection procedure is one of the most crucial points in designing an experiment (Martinez, Schwaneberg, 2013). We used cell sorting to reduce the work load screening cell lines, reduce the time spent, and find the cells with the highest productivity and advantageous properties (Mattanovich, Borth, 2006). The FACS technique can be used to select cells according

to a surface molecule expression by specific ligands. For example, this technique is not able to differentiate between low-producing and high-producing cells, since a large number of cells, without considering their expression level, are rapidly isolated (Carroll, Al-Rubeai, 2005). Therefore, flow cytometry using GFP or markers can be efficiently used for fast-isolation of desired cells.

For instance, Yoshikawa *et al.* (2001) showed that fluorescently labeled MTX could permeate the cell membrane and strongly inhibit DHFR activity. Therefore, high-producing clone can be first sorted by flow cytometry. Selection of a high-producing clone is then performed by traditional methods, including limiting dilution cloning (LDC). Previously, another study was also developed by Beckers *et al.* (1988) that used cell sorting for selection of high-expressing cells. Populations of L cells producing the high level α-chain of human IL-2 receptor (Tac protein) were screened using a fluorescent monoclonal anti-Tac antibody, which specifically binds with the α-subunit and enriches L-cells several-fold.

As Figure 4 shows, the intensity of GFP fluorescence increases after 2 repeated FACS. Therefore, it is guaranteed that the population of cells containing high-level intensity fluorescence of GFP is increased. According to Figure 7, the productivity of mt-PA also increases after the first and second FACS (267 and 673 IU/ml, respectively) in CD-DG44 medium. In ProCHO5 medium, the increased production of protein is observed from 8 to 79 IU/ml after the first FACS and up to 211 IU/ml after the second FACS technique (Figure 7). According to the results, we can confirm that the cells producing approximately average and high amount of protein are selected by flow cytometry. Thus, the FACS screen is more effective in two ways. First, this method effectively predicts the clones that will have high titers of protein production later in the development timeline. Second, we can visually determine quality of clones (DeMaria *et al.*, 2007).

After one month, the second round of cell sorting was carried out to reduce/eliminate cells silencing recombinant gene expression (Figure 4). The expression of the integrated recombinant gene is not always stable over time. Many clonal cells loose or reduce the synthesis of the protein mRNA. The reasons of this phenomenon are not fully illuminated, and degree of this phenomenon differs from one to other clonal cell line (Kayser, Warzecha, 2012). Probably, gene silencing was induced by epigenetic effects and structure of neighboring integrated recombinant gene. In other words, several factors, including the integrated gene copy numbers and the position of gene integration, can also affect the expression level of the recombinant protein (Vinzon *et al.*, 2010). In general, condensed heterochromatin is transcriptionally inactive, whereas relaxed euchromatin is transcriptionally active, which are related to specific histone modifications controlling chromatin condensation and transcriptional activity (Lamond, Earnshaw, 1998).

To screen the highest producing cells with improved growing properties, we also performed traditional limited dilution screening after FACS. Although, LDC requires hard work and is low-throughput, it is still the widely common used approach owing to its relative simplicity and low cost (Puck, Marcus, 1955). Therefore, if this technique is performed following FACS sorting of cells, the selection process of sorted population will be facilitated. Therefore, instead of selection among very low percentage of high/average producing cells, we can screen the highest producing cells between high and/or average producing cells. A total of 33 positive growth clones were selected for subsequent analysis. In this regard, a screening system based on the spot Western analysis was carried out to determine the relative mt-PA expression level in different growth clones (Figure 5). Subsequently, 16 high-producing clones were subjected to quantitatively measure the activity of tissue plasminogen activator, resulting in a yield of 765 IU/mL in CD-DG44 medium. Therefore, the findings indicate that there is a fifty-fold increase in the productivity of mt-PA after two sequential cell sorting steps. Kim *et al.* (2012) achieved a similar outcome by developing an efficient FACS-based selection method to isolate high antibody-producing CHO cells using the split GFP system.

Both antibody chains, the heavy and light chain, were attached to two fragments of GFP as a reporter gene. They found that up to 100-fold increase in the specific antibody productivity (qAb) of FACS-sorted cells with GFP expression was observed compared to the unsorted cells. Kavathas and Herzenberg (1983) also reported a

40-fold increment in the fluorescent intensity of GFP after six rounds of cell sorting steps in human T cell differentiation antigen Leu 2.

Owing to composition of antibody from two fragment proteins (heavy and light chains), Sleiman *et al.* (2008) developed dual color intracellular fluorescent protein- associated FACS to select CHO cells producing high-level heavy and light chain by detecting two reporters, GFP and YFP, respectively. DeMaria *et al.* (2007) showed another selection system based on surface expression of CD20 molecule, which is not normally expressed in CHO cells. This molecule co-expressed with the desired protein, and then the high-producing cells are detected using a fluorescent conjugated anti-CD20 antibody.

We attempted to express our protein in suspended CHO cell line (CHO-DG44) to obtain high-level stable production of mt-PA. As mentioned, a different kind of expression systems exists, but mammalian cells, especially CHO cells, are still the essential hosts for industrial production of therapeutic recombinant proteins. Although other cell lines such as Baby hamster kidney (BHK), human embryo kidney-293 (HEK-293), and NS0 cells have also acquired regulatory approval to produce recombinant protein for pharmaceutical purposes, some components such as antibody derivatives and vaccines have been widely expressed in CHO cells owing to their safety use in humans, PTMs, and glycosylation pattern of the produced proteins, and their easily adaptation to suspension, and large-scale cultivation in bioreactors (Mohan *et al.*, 2008).

In the current study, two different media with particular components, CD-DG44 and ProCHO5 media, were analyzed for the productivity yield of mt-PA. The highly production yield was achieved in CD-DG44 compared to ProCHO5 medium. As Figure 6 shows, the expression level of mt-PA is higher in all the first-sorted, second-sorted, and clonal selected cells developed in CD-DG44 medium. Our finding was confirmed by the quantitative analysis of expressed mt-PA (Figure 7).

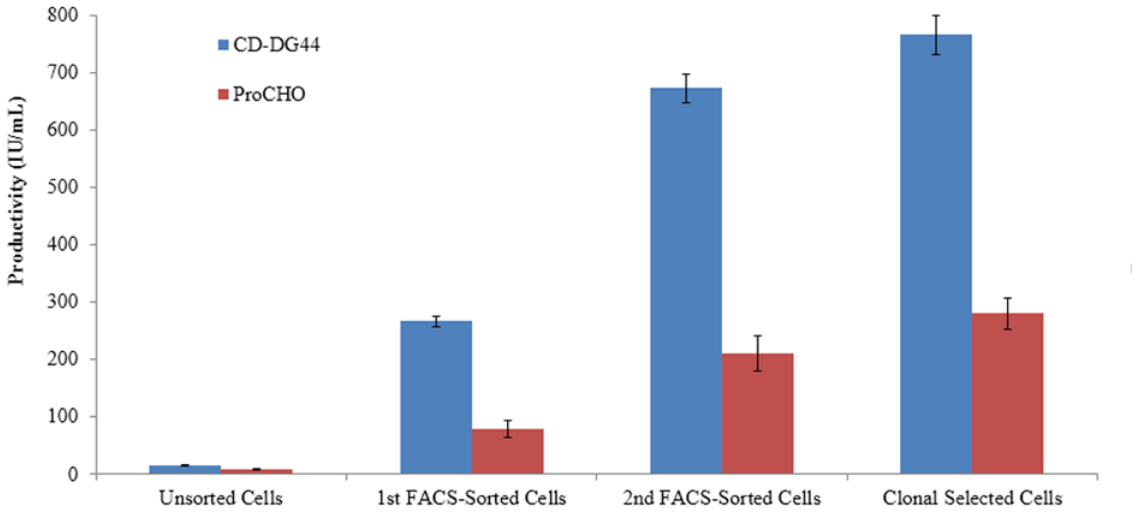

FIGURE 7 - Amidolytic activity test at each step for each medium.

These results also confirm that higher cell viabilities were achieved in CD-DG44 rather than ProCHO5 medium (Figure 2). This may be due to the improved growth compositions of suspension adapted CD-DG44 medium, which can ultimately lead to higher viability and density profile. However, the viability of CHO cells producing mt-PA declined in CD-DG44 earlier than ProCHO5 medium (data not shown). This result can be explained by the higher productivity of the recombinant protein in CD-DG44 medium, while all energy of cells is involved in production

procedures, resulting in the decrease growth profile of the cells (Kayser, Warzecha, 2012).

Varying cell growth rates and the protein productivity profile in pilot or large scale can affect the results obtained. Therefore, to increase the accuracy of the results, it is suggested that these studies be performed on a large scale with different media, a resulting in the highest yield of the protein productivity. This field is open to further future research studies.

In conclusion, the current study results indicate that the high-throughput screening technique, FACS, can reduce the time required to achieve high-producing cell line compared to the traditional approaches, including LDC. Moreover, we indicated that a media with full nutrient concentration had very crucial effects in the productivity yields of recombinant proteins, particularly in a bioreactor for large-scale production. Thus, by optimization of media components, we can achieve increased production of the integrated gene of interest.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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