Indonesian J. Pharm. Vol. 23 No. 3 : 177 – 182
ISSN-p : 0126-1037

Research Article

EXPRESSION OF RECOMBINANT HUMAN ERYTHROPOIETIN WITH GLYCOSYLATION MODIFICATION IN HEK293T CELLS

Endah Puji Septisetyani, Yana Rubiyana, Popi Hadi Wisnuwardhani, Andri Wardiana, Adi Santoso*

Research Center for Biotechnology, Indonesian Institute of Sciences
Jl. Raya Bogor KM 46, Cibinong, Bogor, 16911, Indonesia

Submitted: 03-06-2012
Revised: 10-07-2012
Accepted: 22-08-2012

*Corresponding author
Adi Santoso

E-mail:
adi_santoso1@yahoo.com

ABSTRACT

Stability of erythropoietin (EPO) depends on its glycosylation states. With more glycosylation sites, the EPO protein will be more stable and also increase its half-life. A construct of recombinant human erythropoietin (rhEPO) which contains 2 additional N-link for glycosylation were designed. Based on translation analysis using ORF (open reading frame)-finder and protein alignment analysis using blast-p of NCBI home page, expected recombinant hEPO with additional 6-histidin tag in carboxyl terminus was expressed. HEK293T cells were transfected with recombinant plasmid containing rhEPO by using calcium phosphate method. Expression of rhEPO was detected by dot blot and Western blot analysis using hEPO antibody as the primary antibody and antirabbit antibody with alkaline phospatase linked as the secondary antibody. The bands were detected by BCIP/NBT color development substrate. The data indicated detection of EPO in culture medium of transfected HEK293T cells.

Key words: HEK293T cell, calcium phosphate transfection, N-linked glycosylation, recombinant human erythropoietin.

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein which is very fundamental for red blood cells maturation (Ashley *et al*., 2002; Durocher and Butler, 2009; Egrie and Browne, 2009). Clinical therapy using EPO, especially recombinant human EPO (rhEPO), is very crucial for the treatment of anemia in the case of chronic renal failure patients (Goldwasser, 1975; Helena *et al*., 2006). EPO consist of 165 amino acids with molecular weight of about 30 kDa. EPO synthesis needs post transcriptional modification for the addition of four carbohydrate chains by glycosylation at N and O sites. Glycosylation of human EPO is modified with addition of sialil groups which are important for *in vivo* stability (Egrie and Browne, 2001; Koury and Bondurant, 1991). So far synthesis or rhEPO for human therapeutic use is produced in mammalian cell, mainly in chinese hamster ovary (CHO) cells.

Recently there are at least three kinds of rhEPO available in the market: epoietin alpha, epoietin beta, and darbopoietin alpha. Those rhEPO are produced by using Chinese hamster ovary (CHO) cells (Lotscher, 2005). Besides mammalian system, another expression system frequently used is a prokaryotic system using bacteria. However, since prokaryotic system does not have post-translational modification capacity, thus, glycosylation moety can not be performed in this expression system (Fischer *et al.,* 1999). Recently, the use of yeast as an eukaryotic expression system has gained a lot of attention. Like other eukaryotic systems, yeast can also perfom post-translational modification including synthesis of glycosylation (Cereghino and Cregg, 2000). However, several studies have proven that the glycosylation pattern of yeast is very different than that of mammalian system (Dasgupta *et al*., 2007; Durocher and Butler, 2009; Lam *et al*., 2007; Moore, 2003). With regard to this, in this current study, we are interested to synthesize rhEPO using mammalian system.

With the long-term goal of producing biosimilar product, we designed a construct of recombinant human erythropoietin (rhEPO) which contains 2 additional N-link glycosylation (Narhi *et al*., 1991). Study on rhEPO demonstrated that increase in sialic acid containing carbohydrate molecule increases its serum half-life and *in vivo* biological activity. To enable easy rapid purification of an expressed

protein product, the coding sequence of this therapeutically important human glycoprotein EPO was fused with a polyhistidine tag. In this research, the modified glycosylation rhEPO was expressed in HEK 293T cells.

METHODOLOGY

Cell culture and reagents

Human embryonic kidney HEK293T (293T) cells were obtained from Prof. Masashi Kawaichi, Nara Institute of Science and Technology (NAIST), Japan. Cells were cultured in DMEM medium (Sigma) supplemented with 10% of fetal bovine serum (FBS, Gibco) and 1% of mixture of antibiotics (penicillin-streptomycin, Gibco). Plasmid construct pJ-601 which contains optimized rhEPO sequence (pJ-rhEPO) was obtained from DNA 2.0, USA. This rhEPO sequence was expected to express 5-N-link rhEPO with 6x histidin tag at C-terminus. Plasmid pEGFP-c1 was from Prof. Masashi Kawaichi.

Calcium phosphate transfection

One day before transfection, $2x10^4$ cells/well were seeded on to 24 well plates. Before transfection, culture medium was changed with 450 uL fresh medium. Transfection reagents were made as follows: 4 tubes of 100 uL of 0.25M of $CaCl_2$ were added with 0.4 ug and 0.8 ug pEGFP-c1, 0.7 ug and 1 ug pJ-EPO respectively. Then, 100 ul of BBS reagent pH 6.95 were added drop by drop with shaking into $CaCl_2$-DNA mixture and incubated for 20 minutes at room temperature. After incubation, 50ul of each mixture were added in to cell culture in each well and mixed properly and then incubated over night. The next day, GFP fluorescence was observed using inverted fluorescent microscope to confirm the transfection method worked well. The cells medium then were changed with fresh medium every day which contain 500ug/uL G418 for selection of successful transfectants.

Dot blotting

The fourth day after transfection, cells were splitted into 24 well plate and incubated for 2 days to check the presence of rhEPO in culture medium by dot blotting. Ten uL of culture medium were spotted into nitrocellulose membrane in laminar air flow hood and let to dry. The membrane was then soaked into blocking solution (5% skimmed milk in TBS) and incubated with shaking for 1 hour then was washed thrice with 0.1% tween in TBS (TBS-T). The membrane was incubated with primary anti-EPO antibody (1:1000) (Calbiochem) in blocking solution for 1 hour. Following TBS-T washing, the membrane was incubated with secondary antirabbit antibody (1:10,000) (Promega) in blocking solution for 1 hour. After washing, the membrane was stained with NBT-BCIP (Promega) in AP-buffer for 10 minutes and then washed with water.

Western blotting

The fourth day after transfection, cells were splitted into 24 well plate and incubated for four days to check the presence of rhEPO in culture medium by western blotting. 15µl of culture medium was added with 5µl of 5× SDS buffer and then boiled for 5 minutes. Epoetin-alpha (Hemapo) as positive control of 3 N-linked rhEPO was from Kalbe Farma. After running of SDS PAGE, polyacrylamide gel then was blotted on to nitrocellulose membrane. The membrane then was soaked into blocking solution (5% skimmed milk in TBS) and incubated with shaking for 1 hour then was washed thrice with 0.1% tween in TBS (TBS-T). Next, the membrane was incubated with primary anti-EPO antibody (1:1,000) (Calbiochem) in blocking solution for 1 hour. Following TBS-T washing, the membrane was incubated with secondary antirabbit antibody (1:10,000) (Promega) in blocking solution for 1 hour. After washing, the membrane was stained with NBT-BCIP (Promega) in AP-buffer for 10 minutes and then washed with water.

RESULTS AND DISCUSSION

Containing 2 additional N-links, recombinant plasmid pJ-EPO was designed to enhance *in vivo* stability of expressed rh-EPO. Analysis of the recombinant plasmid was carried out by using restriction enzyme digestion of pJ-EPO plasmid (Figure 1). The small fragment which is shown after digestion refers to the fragment of interest.

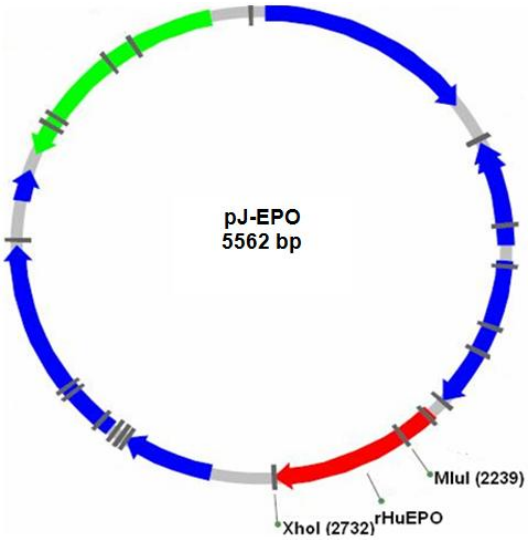

Figure 1. Map of pJ-EPO.

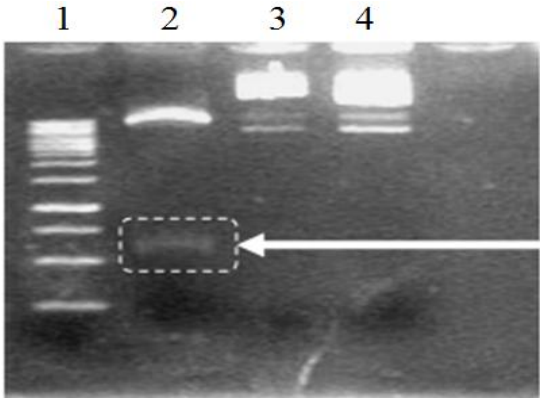

Figure 2. Digestion of pJ-EPO by using restriction enzyme MluI and XhoI. 1) DNA ladder, 2) MluI/XhoI digested pJ-EPO, 3) pJ-EPO 0,5ug, 4) pJ-EPO 1ug. White arrow showed insert fragment around 500 bp.

The result showed that digestion of pJ-EPO using MluI/XhoI resulted in the presence of approximately 500 bp EPO insert (Figure 2).

Efficiency of transfection depends on the amount of plasmid DNA used, number of cells, and time of incubation (Sola and Griebenow, 2009). Confirmation of calcium phosphate transfection method was performed using pEGFP-c1 plasmid. With the presence of *gfp* gene in the pEGFP-c1 plasmid, the transfected 293T cells express green fluorescence protein (GFP) and the GFP was detected using fluorescent microscope. With the use of 0.4 μg of pEGFP-c1 plasmid, green fluorescence was observed 12 hours after transfection (Figure 3).

Transfection of HEK293T cells with pJ-EPO was also done by calcium phosphate method. One day after transfection, cells were incubated with G418 for selection of successful transformants. The next day, cells were passed and grown in new 24 well plate. The remaining cells were grown until 100% confluency. The culture medium was then analyzed by dot blot and western blot. Dot blot data indicated detectable EPO protein in different expression level (Figure 4).

To clarify the detected EPO, SDS PAGE and Western blotting were performed. Epoietin alpha and rhEPO from previous experiment produced in *Pichia pastoris* were used as positive controls. Epoetin alpha possesses molecular weight of about 30 kDa. However, the band of epoietin alpha appeared around 35 kDa (Yoon *et al.*, 2001). As a comparison, rhEPO of *Pichia pastoris* has the molecular weight of about 35 kDa. The increased size of rhEPO of *Pichia pastoris* is due to the presence of fused c-myc and histag at the carboxy terminus of the protein. The band of this rhEPO appeared around 35 kDa (Wardiana and Santoso, 2011). Moreover, the Western blot data showed that the EPO protein was expressed in all four culture medium. In clone 1, a single band with the approximate size of 50 kDa was detected. The two additional N-linked glycosylations of rhEPO contribute to the addition of molecular mass of the protein approximately 6.6 kDa. While 6-histidin tag increase the molecular weight of the protein of about 1 kDa. The expected rhEPO which possesses 5 N-linked glycosylation appeared around 50 kDa (Figure 5). The results shown resemble to the detected rhEPO with 5 N-linked glycosylation state which appeared around 50 kDa (Gross and Lodish, 2006). The bands appeared around 35 kDa seemed to be EPO and its isoforms (Sola and Griebenow, 2010). Eventhough the detected EPO was expected to be rhEPO, further experiments are needed to purify and characterize the rhEPO to confirm additional N-link for glycosylation site.

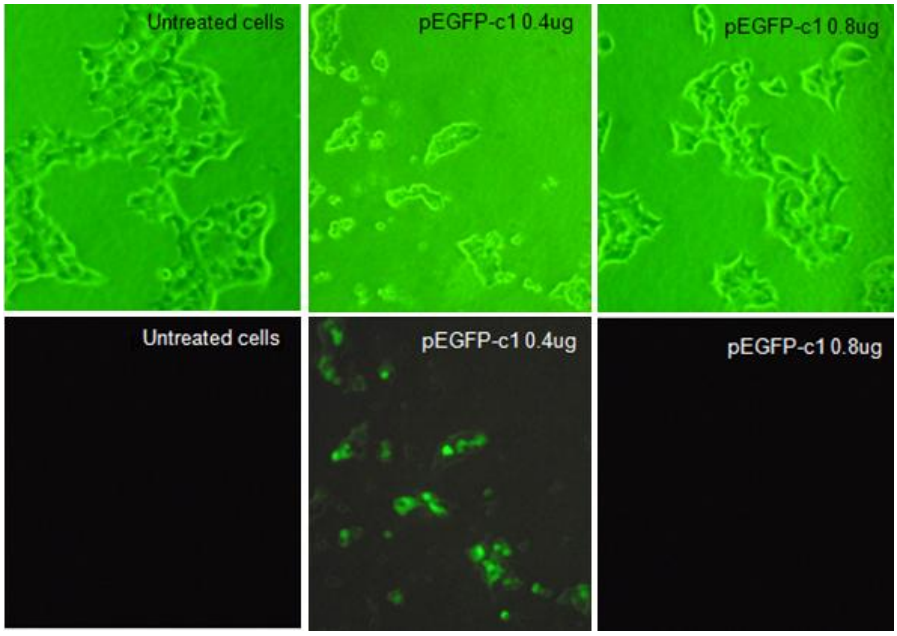

Figure 3. Transfection of HEK293T with pEGFP-c1 by using calcium phosphate method. The transfected cells show green fluorescence observed under fluorescence microscope.

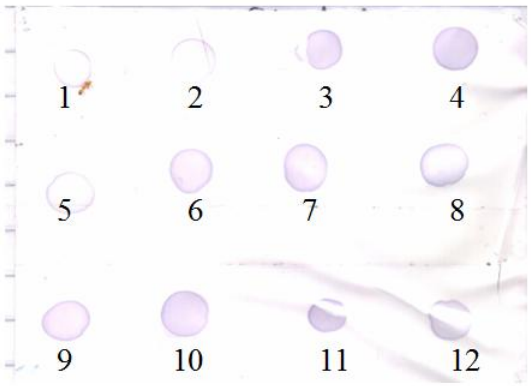

Figure 4. Dot blot analysis of culture medium in 24 well plate after cells selection with G418 showed different detection level of rhEPO.

Based on our translational analysis using ORF (open reading frame)-finder and protein alignment analysis using blast-p of NCBI home page, pJ-EPO would express expected rhEPO with additional 6-histidin tag in carboxyl terminus. rhEPO with modification of its amino acid would performed additional N-link glycosylation in post transcription modification. With more glycosylation sites, the EPO protein expected to be more stable and also increase its half-life (Jordan *et al.*, 1996; Bollin *et al.*, 2011).

Suspension culture generally use for production of recombinant protein to obtain high yields (Mariati *et al.*, 2010). In this experiment, culture of adherent cells was used for detection of EPO. For further EPO

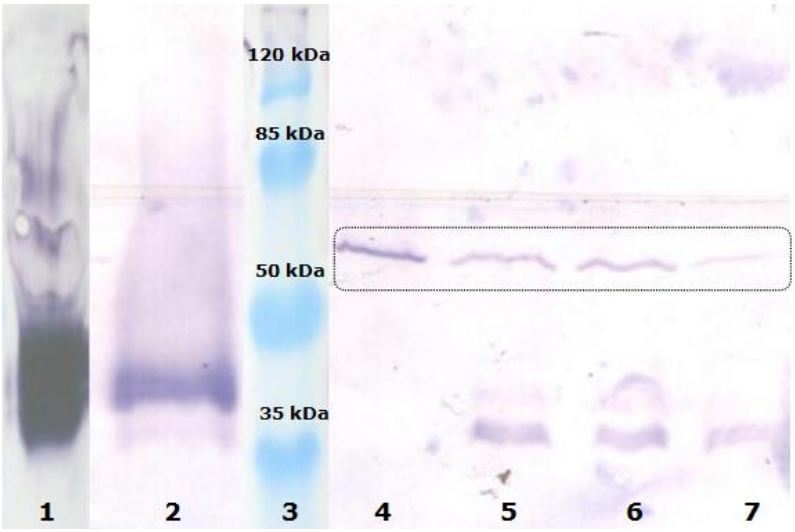

Figure 5. Western blot analysis of culture medium of selected cultures after cells selection with G418. 1) epoetin alpha, 2) rhEPO expressed in *Pichia pastoris*, 3) protein weight marker, 4-7) culture medium of transfected cells (clone 1-4).

production, suspension culture will be use to increase the yields. Transfection can be done by using transient or stable transfection methods (Suzuki *et al*., 2006; Backliwal *et al*., 2008). Transient transfection is not time consuming but it shows low productivity (Loignon *et al*., 2008). However, experiments are set up to develop rapid methods to gain stable transformants to enhance productivity (Strickland, 1999; Pattel, 2009).

CONCLUSION

Dot blot and Western blot data indicated detection of EPO in culture medium of transfected 293T cells.

ACKNOWLEDGEMENTS

This study was supported by the National Innovation Program (SINas), Ministry of Research and Technology (Menristek) (grant No. RD 2012-334). The authors are grateful to Prof. Masashi Kawaichi for providing HEK293T cells and pEGFP-c1 plasmids.

REFERENCES

Ashley, R. A., Dubuque, S. H., Dvorak, B., Woodward, S. S., Williams, S. K., and Kling, P. J., 2002, Erythropoietin Stimulates Vasculogenesis in Neonatal Rat Mesenteric Microvascular Endothelial Cell, *Pedriatic Res.*, **51**,471-478.

Backliwal, G., Hildinger, M., Chenuet, S., Wulhfard, S., Jesus, M. D., and Wurm, F. M., 2008, Rational Vector Design and Multi-pathway Modulation of HEK 293E Cells Yield Recombinant Antibody Titers Exceeding 1g/l by Transient Transfection Under Serum-Free Condition, *Nuc. Ac. Res*., **36**(15), e96.

Bollin, F., Dechavanne, V., and Chevalet, L., 2011, Design Experiment in CHO and HEK Transient Transfection Condition Optimization, *Protein Exp. and Purif.,* **78**, 61-68.

Cereghino, G. P. L. and Cregg, J. M., 2000, Heterologous Protein Expression in the Methylotrophic Yeast *Pichia pastoris*, *FEMS Microbiol. Rev*., **24**, 45-66.

Dasgupta, S., Navarrete, A. M., Bayry, J., Delignat, S., Wootla, B., Andre, S., Christope O., Nascimbeni, M., Jacquemin, M., Martinez-Pomares, L., *et al*., 2007, A Role for Exposed Mannosylations in Presentation of Human Therapeutic Self-Proteins to CD4+ T Lymphocytes. *Proc. Natl. Acad. Sci. USA*, **104**, 8965-8970.

Durocher, Y. and Butler, M., 2009, Expression Systems for Therapeutic Glycoprotein Production, *Current Opinion in Biotechnology*, **20**, 700-707.

Egrie, J. C. and Browne, J. K., 2001, Development and Characterization of Novel Erythropoiesis Stimulating Protein (NESP), *Nephrol. Dial. Transplant.*, **16**[Suppl 3], 3-13.

Fischer, R., Drossard, J., Emans, N., Commandeur, U. and Hellwig, S., 1999, Towards Molecular Farming in the Future: *Pichia pastoris*-Based Production of Single-Chain Antibody Fragments, *Biotechnol. Appl. Biochem.*, **30**, 117-120.

Goldwasser, E., 1975, Erythropoietin and the Differentiation of Red Blood Cells, *Fed. Proc.*, **34**(13), 2285-2292.

Gross, A.W. and Lodish, H.F., 2006, Cellular Trafficking and Degradation of Erythropoietin and Novel Erythropoiesis Stimulating Protein (NESP), *J. Bio. Chem.*, **281**(4), 2024-2032.

Helena, D. A., Jyothsna, N. L., Rao, P. J., Rao, G. V., and Rao, K. R. S. S., 2006, Therapeutic Implications of Recombinant Human Erythropoietin in Anaemic Related Clinical Manifestations, *African J. Biotechnology*, **5**(25), 2503-2509.

Jordan, M., Schallhorn, A., and Wurm, F. M., 1996, Transfecting Mammalian Cells: Optimization of Critical Parameter Affecting Calcium-Phosphate Precipitate Formation. *Nuc. Ac. Res.*, **24**(4), 596-601.

Koury, M. J., Bondurant, M. C., 1991, The Mechanism of Erythropoietin Action, *Am. J. Kidney Dis.*, **18**(4Suppl1), 20-23.

Lam, J.S., Huang, H., Levitz, S.M., 2007, Effect of Differential N-linked and O-linked Mannosylation on Recognition of Fungal Antigents by Dendritic Cells. *PLoS ONE*, **2**, e1009.

Loignon, M., Perret, S., Kelly, J., Boulais, D., Cass, B., Bisson, L., Afkhamizarreh, F., and Durocher, Y., 2008, Stable High Volumetric Production of Glycosylated Human Recombinant IFNalpha2b in HEK293 Cells, *BMC Biotechnology*, **8**, 65.

Lotscher, N. G., 2005, Anaemia Management in Dialysis Patients in Switzerland "AIMS", *Dissertation,* Universitat Basel, Switzerland.

Mariati, Ho, S. C. L., Yap, M. G. S., and Yang, Y., 2010, Evaluating Post-Transcriptional Regulatory Elements for Enhancing Transient Gene Expression Levels in CHO K1 and HEK293 Cells, *Protein Expr. Purif.*, **69**, 9-15.

Moore K.L., 2003, The Biology and Enzymology of Protein Tyrosine O-sulfation. *J. Biol. Chem.*, **278**, 24243-24246.

Narhi, L. O., Arakawa, T. Araki, K. H., Elmore, R., Rohde, M. F., Boone, T., and Strickland, T. W., 1991, The Effect of Carbohydrate on The Structure and Stability of Erythropoietin, *The Journal of Biol. Chem.*, **266**(34), 23022-23026.

Patell, V. M. U. S., Patent No. 2009/0029907 A1, 29 Jan. 2009.

Sola, R. J. and Griebenow, K., 2009, Effects of Glycosylation on The Stability of Protein Pharmaceuticals, *J. Pharm. Sci.*, **98**(4), 1223-1245.

Sola, R. J. and Griebenow, K., 2010, Glycosylation of Therapeutic Proteins: An Effective Strategy to Optimize Efficacy, *BioDrugs*, **24**(1), 9-21.

Strickland T. W., U.S. Patent No. 5856298, 5. Jan. 1999.

Suzuki, J., Fukuda, M., Kawata, S., Maruoka, M., Kubo, Y., Takeya, T., and Shishido, T., 2006, A Rapid Protein Expression and Purification System Using Chinese Hamster Ovary Cells Expressing Retrovirus Receptor, *J. Biotec.***126**, 463-474.

Wardiana, A., and Santoso, A., 2011, Purification and Carbohydrate Analysis of Recombinant Human Erythropoietin Expressed in Yeast System *Pichia pastoris*. *MAKARA SAINS*, **15**(1), 75-78.

Yoon S. K., Ahn Y. and Han K., 2001, Enhancement of Recombinant Erythropoietin Production in CHO Cells in an Incubator without CO_2 Addition, *Cytotechnology*, **37**, 119-132.