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Research Article

EXPRESSION OF MODIFIED RECOMBINANT HUMAN ERYTHROPOIETIN IN CHO-K1 CELLS AND ITS *IN VITRO* PROLIFERATION ASSAY IN TF-1 CELLS

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ABSTRACT

Erythropoietin (EPO) is a 30 kDa glycoprotein hormone which is important for red blood cells maturation. EPO consists of 165 amino acids and possesses 3N-linked carbohydrate chains. Recombinant human erythropoietin (rHuEPO), such as epoetin-α and epoetin-β, have been used for many years to treat anemia in patients with chronic renal failure, systolic heart failure, HIV-AIDS, or cancer. *In vivo* stability of rHuEPOs were low due to rapid metabolisms by galactosyl receptor of the hepatocytes. Previously, a novel erythropoiesis stimulating protein (NESP) called darbapoetin-α (DARB) which possesses two additional N-linked glycosylation had been studied. It was observed that NESP showed better *in vivo* stability and biological activity compared to the unmodified form (containing only 3N-linked carbohydrate chains). Based on the above study, we attempted to synthesize recombinant human EPO (rHuEPO) by generating CHO-K1 cell lines expressing codon-optimized human *epo* open reading frame (ORF) in CHO-K1 cells. The ORF was modified to contain 5 N-linked carbohydrate chains. The media obtained from CHO-K1 cell culture was collected and diafiltrated with the use of tangential flow filtration. The rHuEPO protein containing polyhistidine tag was purified using affinity chromatography. An SDS/PAGE and Western blotting analyses confirmed that the purified protein was the modified rHuEPO. MTT based proliferation assay was conducted in TF-1 bone marrow cell line as a model. The result showed that the modified rHuEPO was able to enhance TF-1 cells proliferation.

Key words: CHO-K1, erythropoietin, glycosylation, MTT assay and TF-1 cell line.

INTRODUCTION

Erythropoietin (EPO) is one of protein hormones that commonly used in anemia caused by chronic renal failure (Lotscher, 2005; Pinevich and Petersen, 1991), systolic heart failure (Swedberg *et al*., 2013), HIV-AIDS, and cancer (Helena *et al*., 2006). It supports the production of red blood cells (Durocher and Butler, 2009; Goldwasser, 1975). Despite many efforts to synthesize this protein in many different expression systems, production of recombinant human EPO (rHuEPO) still relies on chinese hamster ovary (CHO) derived cells (Kiss *et al*., 2010).

Endogenous human EPO EPO is a 30-38kDa glycoprotein (exact molecular weight depends on the degree of glycosylation) in which 60% of the molecule composed of 165 amino acid single polypeptide chain containing two disulphide bonds. The remaining 40% of the EPO mass consists of carbohydrate covalently attached at three N-linked sugar chain at Asn 24, 38 and 83, and one O-linked sugar chain at Ser126 (Skibeli *et al*., 2001; Egrie and Brown, 2001).

Glycosylation of EPO does not correspond to its *in vitro* activity, but it significantly contributes to the enhancement of *in vivo* stability and activity (Egrie and Browne, 2001). Functionally, glycosylation has been shown to influence a variety of critical biological processes at both the cellular and protein levels including its stability and long residence in body (Liu, 1992). Structurally,

glycosylation is highly complex due to the fact that there can be heterogeneity with respect to the site of glycan attachment (macroheterogeneity) and with respect to the glycan's structure (microheterogeneity) (Sears and Wong, 1998). With regard to this circumstances, the efforts to synthesize rHuEPO with higher *in vivo* stability and biological activity by modification of its glycosilation pattern was studied (Egrie and Browne, 2001). A novel erythropoiesis stimulating protein (NESP) called darbepoetin-α was synthesized by changing of 5 amino acids (Ala30Asn, His32Thr, Pro887Val, Trp88Asn and Pro90Thr) in the invariant amino acids core (Egrie and Browne, 2001) allowing for additional oligosaccharide attachments at asparagine residues at position 30 and 88. The attachment of these oligosaccharides contributed to addition of molecular weight to approximately 37kDa. With more carbohydrate content, NESP shows higher *in vivo* stability and activity compared to those epoetins (Egrie and Browne, 2001; McDougall, 2002).

In line with the previous study (Egrie and Browne, 2001), in this current study, we report the expression of hEPO in CHO-K1 cells. Under the control of the CMV promoter, codon optimized of human *epo* gene containing five N-linked oligosaccharide chains was subcloned into PJ603 expression plasmid system. Protein rHuEPO secreted into the culture medium was purified using immobilized metal affinity chromatography (IMAC) and analyzed using Western blot. *In vitro* analysis using bone marrow TF-1 cell line was also performed and discussed.

MATERIALS AND METHODS

Cell culture and reagents

CHO-K1 cells were obtained from Prof. Masashi Kawaichi, Nara Institute of Science and Technology (NAIST), Japan. CHO-K1 cells were cultured in Nutrient Mixture F-12 Ham (F12) media (Sigma N6658) supplemented with 10% of fetal bovine serum (FBS, Sigma), 100U of benzylpenicillin and 100μg of streptomycin (Gibco, Invitrogen) in an incubator with addition of 5% CO_2 at 37°C. Cells were passaged every 2-3days. TF-1 cells (EC93022307-FO) were obtained from Saibou, Japan. TF-1 cells were grown in RPMI (Roswell Park Memorial Institute) 1640 media supplemented with 2mM of L-glutamine (Sigma), 10% of FBS (Gibco, Invitrogen), 100U of benzylpenicillin-100μg of streptomycin (Gibco, Invitrogen), and 5ng/mL granulocyte macrophage-colony stimulating factor (GM-CSF, Invitrogen) in an incubator with addition of 5% CO_2 at 37°C. Cells were passaged every 2-3day. Epoetin-α (3000IU/mL, Hemapo) from PT. Kalbe Farma (Indonesia) was used as a positive control. Antibiotics G418 (Geneticin) was obtained from Sigma.

Expression of rHuEPOin CHO-K1 cells

Codon optimized of human *epo* gene containing five N-linked carbohydrate chains was synthesized and subcloned into PJ603 expression plasmid system by DNA 2.0 (pJ-EPO). CHO-K1 cells were transfected with pJ-EPO recombinant plasmidby using Lipofectamin 2000(Invitrogen) according to manufacturer's protocol. Stable expressing transformants were screened by using 1,000ng of G418. In addition, single clone cells were cultured in F12 medium supplemented with 10% of fetal bovine serum (FBS, Sigma), 100U of benzylpenicillin 100μg of streptomycin (Gibco, Invitrogen) and 1.000ng of G418.

Ultrafiltration and purification

About 100mL of supernatant of culture medium was applied on diafiltration by using tangential flow filtration (Pall). Concentrated supernatant was then purified by using Ni Sepharose column (Histrap FF, GE Healthcare) and reconcentrated by using ultra centrifugal filter (Amicon, Merck) for Western blot. The recombinant protein rHuEPO resulted from the purification steps above was used for *in vitro* proliferation assay using TF-1 cell line.

Relative quantitation of rHuEPO

Epoetin-α (45IU) and bovine serum albumin with various concentrations (25, 50, 100, 150 and 200ng) were subjected into a 12% of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel analysis. The gel was stained using CBB (coomassie brilliant blue) solution (Bio-Rad). Area under curve-based analysis was determined by using ImageJ software. Relative concentration of

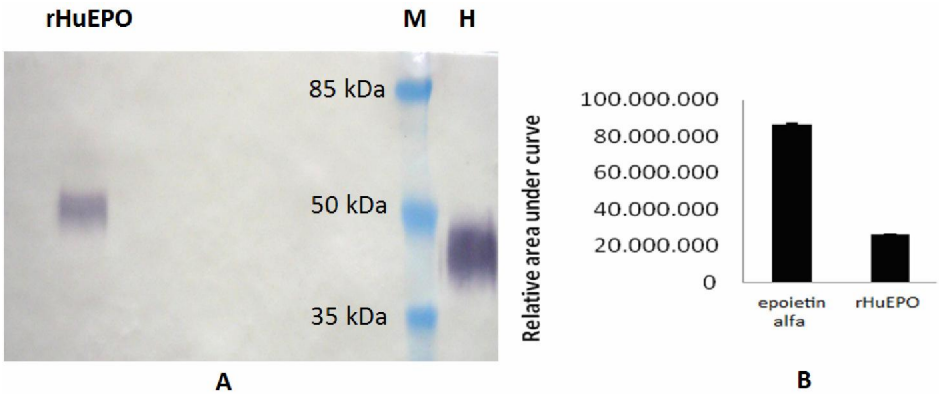

Figure 1. Detection of rHuEPO by Western blot. A) Supernatant of culture medium of CHO-K1 cells transfected with pJ-epo was purified by Ni-NTA agarose beads and concentrated by ultra centrifugal filter. rHuEPO, protein marker (M), and epoetin-α (H) were subjected to SDS PAGE and detected by Western blot. B) Relative area under curve of epoetin-α and rHuEPO bands were analyzed using ImageJ software.

epoetin-α was calculated by using BSA as standard. Relative concentration of epoetin-α was used to determine our modified rHuEPO concentration. As much as 15µL of rHuEPO and epoetin-α (45IU) were analyzed in 12% of SDS-PAGE gel. The proteins then were the transfered into nitro cellulose membrane (GE Healthcare) by wet transfer method. The membrane was soaked in blocking solution containing 5% of skim milk. After washing with tris buffer saline containing 0.1% tween (TBS-T), the membrane was soaked in primary human polyclonal anti-EPO antibodies (1:1000, Calbiochem). Following the next washing step, the membrane was soaked in anti rabbit alkaline phosphatase linkeded antibody as the secondary antibody (3:10,000, Promega) and then visualized by nitroblue tetrazolium chloride-5-bromo-4-chloro-1H-indol-3-yl dihydrogen phosphate (NBT-BCIP, Promega). The same analysis using Image J was performed to obtain relative concentration of modified rHuEPO.

MTT proliferation assay

MTT (methylthiazol tetrazolium) proliferation assay of rHuEPO in TF-1 was done based on Hammerling *et al.* (1996).TF-1 cells were cultured in RPMI 1640 media (Sigma) supplemented with 2mM L-glutamin (Sigma), 10% of FBS (Gibco), 4mM 100U benzyl penicillin- 100µg streptomycin (Gibco), 5ng/mL GM-CSF (Invitrogen) in CO_2 incubator with 5% CO_2 at 37°C. One day before harvesting, the cells were washed with PBS and changed with medium without GM-CSF. As much as 90µL of cells suspension were seeded in RPMI-1640 with serum and antibiotics respectively (without GM-CSF) into 96-well microtiter plates at a density of $2x10^5$cells/mL. Cells were treated with 90µL of serial dilutions of modified rHuEPOand commercial epoetin-α. After 48h incubation, 20µL of 6mg/mL of MTT solution was added to the cells and incubated for 4h. Formazan as a product of MTT convertion was dissolved by 10% SDS in 0,01M HCl. The next day, optical density (OD) of each well was analyzed by microplate reader at 595nm. OD response was analyzed by substracting OD of treated well with OD of blank.

RESULT AND DISCUSSION

Detection of rHuEPO

The PJ603 recombinant plasmid containing codon optimized of human *epo* gene with five N-linked oligosaccharide chains was transfected into CHO-K1 cells using Lipofectamine 2000, and G418 resistance colonies were selected. Individual clones were isolated and screened for the expression of hEPO protein. One clone expressing hEPO

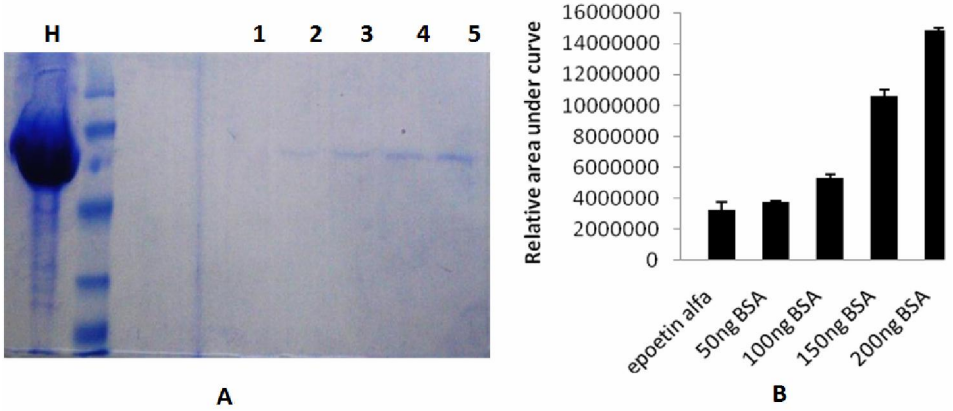

Figure 2. Detection of rHuEPO by CBB staining. A. H = epoetin-α(45 IU), M = protein marker, 1-5 = bovine serum albumin (BSA) 25, 50, 100, 150 and 200 ng respectively; B. Relative area under curve of epoetin-α and BSA bands were analyzed by ImageJ software.

was used for subsequent functional study. The coding sequence of the therapeutically important human glycoprotein EPO was fused with a polyhistidine tag to enable easy, rapid purification. The expression of the human *epo* gene was placed under the control of CMV promoter. Following incubation in the presence of 5% CO_2 at 37°C for 2-3 days, the expressed rHuEPO protein was secreted into the medium. The media were collected by centrifugation at 6000rpm for 5min and the supernatant containing rHuEPO was collected while the cells were removed.

Following purification, the sample was run on SDS/PAGE and detected with Western blot analysis using polyclonal anti human EPO antibody. Eventhough, the molecular mass of the expressed rHuEPO is about 37kDa, in this study, the signal of the expressed protein was detected above 50kDa protein marker due to denaturation and glycosylation (Figure 1), while epoetin-α which was used as positive control detected below 50kDa protein marker.

The remaining 40% of the native EPO mass consists of carbohydrate covalently attached at three N-glycans sugar chains at Asn 24, 38 and 83, and one O-linkeded sugar chain at Ser126 (Browne *et al.*, 1986; Davis *et al.*, 1987; Miyake *et al.*, 1977). Since the consensus sequences for N-linked glycans was known (Asn-X-Ser/Thr where X is any amino acid except proline), we speculate that the increase in size of this molecular mass is attributed to the attachment of two additional oligo-saccharides at asparagine residues positions 30 and 88. The increase of this molecular mass increase the average carbohydrate content of the molecule from 38% to 50% (data not shown) and the maximum number of sialic acids from 14 to 22 (Egrie and Browne, 2001).

Dertermination of rHuEPO concentration

Since we were not able to detect the presence of rHuEPO protein in CBB staining, we modified this detection using combination of Western blot and CBB staining. As much as 45 IU of epoetin alpha was quantified using BSA at concentration of 50, 100, 150 and 200ng as protein control in SDS/PAGE gel (Figure 2). Using ImageJ software, it was found that epoetin-α in Hemapo was about 2.9ng/μL. By knowing weight quantity of epoetin-α, we then quantify the concentration of our rHuEPO sample by running the 10 x concentrated sample in SDS/PAGE using epoetin-α as protein control. Calculated using the software, it is predicted that the concentration of rHuEPO was approximately 0.09ng/μL.

Analysis of rHuEPO *in vitro* proliferation activity

Following characterization of hyper-glycosylated rHuEPO, *in vitro* biological activity was performed. For this purpose the human erythroleukemia cell line TF-1 was employed for determination of proliferative stimulation

Table I. *In vitro* activity of epoetin-α on proliferation of TF-1 cells.

IU/well	ng/well*	OD ± SD**
0.1	0.1	0.037± 0.002
0.2	0.2	0.046 ± 0.006
0.4	0.3	0.051 ± 0.002
0.8	0.8	0.059 ± 0.012

* Calculated based on epoietin-α activity (about 1 IU/ng).
** OD was analyzed by substracting the blank well OD from the treated ones.

Table II. *In vitro* activity of modified rHuEPO on proliferation of TF-1 cells.

µL/well	ng/well*	OD ± SD**
0.33	0.03	0.035 ± 0.002
0.67	0.06	0.048 ± 0.005
1.33	0.12	0.036 ± 0.002
2	0.18	0.046 ± 0.002
2.67	0.24	0.067 ±0.002

* Calculated based on rhEPO concentration (about 0.09 ng/µL).
** OD was analyzed by substracting the blank well OD from the treated ones.

induced by rHuEPO protein. MTT proliferation assay of epoetin-α on TF-1 cells indicated that epoetin-α at concentration below 1 IU enhanced proliferation of TF-1 cells (Table I). However, when concentration of epoetin-α at more than 1 IU the proliferation of TF-1 cells decreased (data not shown).

Purified rHuEPO sample that had been synthesized in CHO-K1 cells showed enhancement of TF-1 proliferation. Moreover, activity of our rHuEPO was comparable to epoetin-α (Table II). Considering the concentration of rHuEPO was about 0.09ng/µL, thus, the activity of 0.18ng of expressed rHuEPO was similar to 2ng of epoetin-α (Table I and II). As additional information, both epoetin-α in Hemapo and our modified rHuEPO sample contain small amount of albumin as stabilizer.

The development of hEPO for the correction of anemia related diseases is a dramatic example of how much hEPO research has been progressing. Initially, it took 20 years to purify a few milligrams of erythropoietin from over 2.5L of urine from patients with aplastic anemia (Miyake *et al.,* 1977). The first replacement therapy for anemia patient with chronic renal failure was to administer native erythropoietin from human urinary. The activity of human urinary EPO is 70,000 IU/mg (Jelkmann, 2006). This massive effort provided the basis for the cloning of the human erythropoietin gene in 1985 and the possibility for large-scale production (Jacobs, *et al.,* 1985). Since then, the use of recombinant hEPO is widely used for treatment of anemia in patients suffer from anemia related diseases (Lotscher, 2005; Pinevich and Petersen, 1991; Swedberg *et al.*, 2013). This epoetin-α shows higher *in vivo* activity than that of urinary EPO, about 200.000IU/mg, probably due to more complete glycosylation (Jelkmann, 2006). In line with this, currently, one of the most significant progresses in EPO research is the development of darbapoietin, an EPO molecule with additional glycosylations resulting in a significantly longer half-life and a reduced dosing frequency.

Related to the previous study (Egrie and Browne, 2001), in this study, we expressed modified rHuEPO with 2 addition of N-linked glycosylations in adherent CHO-K1 cells base. The expressed protein was detected by Western blotting above 50kDa which was higher than that of epoetin-α. Molecular weight of our modified rHuEPO supposed to be similar to NESP, about 37kDa. Various studies have reported that expression of heterologous

glycosylated recombinant protein may result in a greater molecular mass than it should be. This may be caused by the degree in the glycosylation itself or denaturation. This result was similar to that had been reported by Gross and Lodish (2006). The difference of molecular weight of our modified rHuEPO and epoetin-α was about 7.5kDa. This additional molecular weight might be contributed from two additional N-linked carbohydrate chains (about 6.6kDa) and 6xhistag sequence (about 1kDa) (Egrie and Bowne, 2001; Terpe, 2003).

To verify that glycosylation was indeed contributed to an increase of molecular weight, we use enzymatic removal of the glycans moiety of the expressed protein with PNGase F. PNGase F has been utilized extensively for demonstrating shifts in SDS/PAGE mobilities as an indication of N-glycosylation. Digestion with PNGase F resulted in a band with a molecular mass approximately corresponds to the molecular mass of the polypeptide backbone and both our modified rHuEPO and epoetin-α appeared in the same size which is about 20kDa. Thus, it is very likely that our modified rHuEPO was the hyperglycosylated EPO with 2 addition of carbohydrate chains (Santoso *et al*., 2012).

Proliferation assay of our modified rHuEPO and epoetin-α were done in human erythroleukemia TF-1 cells. It has been reported that *in vitro* proliferation assay of EPO in TF-1 cells show high accuracy (Hammerling *et al*., 1996). Treatment of TF-1 cells with EPO in 24h down-regulates the EpoR and reach a peak in 6h. Moreover, in TF-1 cells, EPO only stimulates proliferation for short period of time (Kitamura *et al*., 1989). In this study, the result indicated that our modified rHuEPO showed comparable proliferative activity with that oh epoetin-α. Epoetin-α showed enhancement of TF-1 cells proliferation at concentration below 1IU and its activity declines at concentration more than 1IU. However, concentration of epoetin-α used in this study was remained higher than its physiologic concentration range (Urabe *et al*., 1987).

It has been shown that the addition of carbohydrate is required for secretion, stability, delay clearance and biosynthesis (Sola *et al*., 2010). However, the carbohydrate is not essential for the biological activity of EPO *in vitro*, indicating that only the polypeptide is involved in binding to the EPO receptor. On the other hand, proper glycosilation of EPO is absolutely critical in *in vivo* condition (Egrie and Browne, 2001). Since in our current experiment the functional analysis performed was *in vitro* study, thus the addition of 2-oligosaccharide attachments (at position 30 and 88) has no effect.

Previous study reported that that two additional N-linked glycosylations of NESP causes its molecule to be more bulky. Thus, it makes NESP to have lower *in vitro* activity than that of epoetin-α (Egrie and Browne, 2001). However, excessive amount of NESP shows similar *in vitro* activity with that of epoetin-α (Elliot *et al.,* 2004). Affinity of NESP to its receptor (EpoR) is lower than that of epoetin-α, but activity of NESP *in vivo* is higher than epoetin-α. With lower binding affinity to EpoR, NESP is also released slower (Gross and Lodish, 2006). Moreover, with more glycosyl groups of NESP, the enzymatic digestion rate of NESP by galactosyl receptor in liver is lower than epoetin-α. Altogether, the resultant *in vivo* activity of NESP is greater than that of epoetin-α; and modification of rHuEPO is very important for improving its stability (Jelkmann, 2006).

CONCLUSION

In this study we expressed modified rHuEPO in CHO-K1 cells. The result showed that the modified rHuEPO enhanced TF-1 cells proliferation. *In vitro* proliferation assay indicated comparable values between our rHuEPO and epoetin-α.

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