Effect of IRES Controlled Reporter Gene on Screening and Production of Recombinant Human EPO Proteins from Cultured CHO Cells

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ABSTRACT

This study was conducted to examine the effect of IRES controlled reporter gene on screening and production of recombinant human erythropoietin (EPO) proteins from cultured CHO cells. The cDNA was cloned for EPO from human liver cDNA. Using site-directed mutagenesis, we generated recombinant human EPO (rhEPO) with two additional N-glycosylations (Novel erythropoiesis-stimulating protein: NESP). Wild type hEPO and NESP were cloned into expression vectors with GFP reporter gene under regulatory control of CMV promoter and IRES so that the vectors could express both rhEPO and GFP. The expression vectors were transfected to cultured CHO-K1 cells. Under microscopy, expression of GFP was visible. Using supernatant of the culture, ELISA assay, immunocytochemistry and *in vitro* assay using EPO dependant cell line were performed to estimate biological activity to compare the production characteristics (secretion levels, etc.) between rhEPO and NESP. The activity of NESP protein, obtained by mutagenesis, was described and compared with its rhEPO counterpart produced under same conditions. Although NESP had less secretion level in CHO cell line, the biological activity of NESP was greater than that of rhEPO. These results are consistent with previous researches. We also demonstrated that rhEPO and GFP proteins expressed simultaneously from transfected CHO cell line. Therefore we conclude that use of GFP reporter gene under IRES control could be used to screen and produce rhEPO in cultured CHO cells.

(Key words : hEPO, Glycosylation site, Site-directed mutagenesis, IRES, GFP, CHO cell)

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein with molecular mass of approximately 30 kDa with 165 amino acids, which circulates in plasma of human with three N-linked and one O-linked oligosaccharide side chains in the protein (Fisher, 1997). EPO protein contains three N-glycosylation sites(Asn-24, Asn-38, and Asn-83) and one O-glycosylation site (Ser-126) and is involved in the regulation of the level of red blood cells. EPO is primarily produced in adult kidney and fetal liver cells and its production are regulated at the level of its gene by tissue oxygenation; hypoxia or anemia (Sasaki *et al.*, 1987). The plasma EPO concentration reflects EPO production (Spivak, 1993). The function of EPO is to stimulate the proliferation and differentiation of erythroid ancestor cells (Goldwasser and Kung, 1968). EPO also regulates the production of erythrocytes operating under a feedback mechanism whereby elevated serum oxygen levels result in lower EPO expression and low oxygen levels result in increased EPO production (Macmillan *et al.*, 2001). The plasma EPO concentration reflects EPO production (Spivak, 1993). The function of EPO is to stimulate the proliferation and differentiation of erythroid ancestor cells (Goldwasser and Kung, 1968). The EPO gene has been cloned and sequenced in a number of species including mouse, monkey, rat, human, sheep, pig and cat (McDonald *et al.*, 1986; Shoemaker *et al.*, 1986; Nagago *et al.*, 1992; Wen *et al.*, 1993). The overall scope of glycoforms can have a variety of different biophysical and biochemical properties, although details of structure-function relationships are poorly understood due to the micro heterogeneity of biological samples (Macmillan *et al.*, 2001). The presence of sialic acid on the oligosaccharide chains is essential for the full *in vivo*, but not *in vitro*, biological activity of EPO (Takeuchi *et al.*, 1989). Elliott *et al* reported that EPO has the important regions for its biological activity: amino acids 11 to 15, 44 to 51, 100 to 108, and 147 to 151 (Elliott *et al.*, 1997). The N-linked carbohydrates chain at position 38 is critical for the polarized secretion (Kita-

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gawa *et al.*, 1994). The carbohydrate of glycosyl groups is different because of their various monosaccharide compositions, sequences, branching sites, linkages, and modifications involving phosphate and sulfate groups (Kawasaki *et al.*, 2001). Nowadays, only a few types of recombinant human EPO (rhEPO), produced in cultured cells, are vastly used in therapy to cure severe anemia.

Three N-glycosylation sites (38, 83 and 126) of EPO are essential for *in vivo* biological activity (Macmillan *et al.*, 2001) and are necessary for *in vitro* activity (Takeuchi *et al.*, 1990). The elimination of all three N-glycosylation sites decreases EPO production up to 10% of the wild-type EPO. Mutant proteins lacking glycosylation at each of these three sites are either mainly associated with membrane components or degraded rapidly (Dube *et al.*, 1988). The mean terminal half-life of intravenous NESP was three fold longer than that of intravenous EPO (Macdougall *et al.*, 1999).

Internal ribosome entry sites (IRESs) occur in the 5'-UTR of numerous viral genomic RNAs as well as some eukaryotic cellular RNAs (Kieft *et al.*, 2001). Many of IRES elements initiate translation of mRNAs encoding proteins that protect cells from stress (Komar *et al.*, 2005). It is internal mRNA sequences on the control of eukaryotic translation initiation by a cap-independent recruitment of the 40S ribosomal subunit (Vagner *et al.*, 2001). The translation can be much more complex and the balance between the cap-dependent and IRES-mediated expression can result in the production of several proteins "isoforms" (Komar *et al.*, 2005).

In this study, we examined the expression of the genes for rhEPO or NESP with enhanced green fluorescence protein (EGFP) that is separated with the IRES under regulatory control of CMV promoter. Here we demonstrated that both target and reporter genes are simultaneously expressed from transfected CHO cell line without hindering quantity or activity of target protein.

MATERIALS AND METHODS

Cloning of EPO cDNA and Site-directed Mutagenesis

A 582 bp-long hEPO cDNA was amplified from human liver cDNA library (TaKaRa, Japan) using primers EPO-F(5'-tgaattcaccatgggggtgcacgaatgtcct-3') and EPO-R (5'-gagtcgacctcatctgtcccctgtcctgca-3') by PCR. The reaction (25 μl) of PCR contained 30 pM sense and anti sense primers; 10 ng of EPO cDNA; 0.4 mM dNTP; 10×Buffer; 1 unit rTaq DNA polymerase (Toyobo, Japan). They were subjected to 1 cycles of 2 min predenaturation at 94℃, 30 cycles (1 min denaturation at 94℃, 1 min annealing at 55℃ and 1 min extension at 74℃). To construct NESP, we introduced site-directed mutations to hEPO cDNA to change the codons at amino acid positions 30~32 from Ala-Glu-His to Asn-His-Thr positions 87~90 from Pro-Trp-Glu-Pro to Val-Asn-Glu- Thr.

Expression Vector Construction

rhEPO and mutant EPO were digested with EcoR I and Sal I. Each fragment was ligated into 5.3 kb of pIRES-EGFP expression vector (ClonTech, USA) that contains the IRES of the encephalomyocarditis virus (ECMV) between the MCS and the EGFP coding region. This permits both the gene of interest (cloned into the MCS) and the EGFP gene to be translated from a single bicistronic mRNA.

Transfection

Transfections were performed according to published protocols for lipofectamin 2000 reagent (Invitrogen, USA). Cells were seeded at 6×10^5 per well in a 60 mm dishes to obtain 50~80% confluence the following day. Complexes were prepared with 6 μg (50 μl) DNA and lipid solution 10 μl in 600 μl serum-free medium. The two solutions were mixed gently and incubated at room temperature for 15 to 45 min to allow DNA-lipid complexes to form. While complex formation, the dishes were rinsed once with 4 ml of serum-free medium that added the complexes (1.8 ml) directly to each dish and mixed gently. The transformants were incubated at 37℃ in 5% CO_2 for 5 to 24 hrs. Following incubation, 2.4 ml of serum (twice as much as normal concentration) was added without removing the transfection mixture. And the medium changed with fresh, complete medium at 24 hrs following the start of transfection. The supernatants were collected after 72 hrs and stored at −20℃ until analysis.

Erythropoietin ELISA

The amount of EPO protein in culture supernatant was measured by sandwich type ELISA (R&D systems). For ELISA, two monoclonal antibodies that recognize different epitopes of EPO were used. The supernatant samples were diluted with cold 1×DPBS (1:200, Invitrogen). EPO concentrations were measured at 450 nm with a microplate reader (Model 550, BioRad).

In Vitro Assay

To evaluate the biological activity, effects of rhEPO and NESP on the F36E cell line were measured as follow. Growing cells (5×10^4 cells/ml) were transferred into 96-well culture plates containing 100 μl of Dulbecco's modified Eagle's medium (DMEM, Invitrogen). Each supernatant was added to the culture medium (final concentration 5U/ml EPO), and incubated for 5

days. Then, MTT assay (Roche, Germany) was performed with culture supernatants and percent of the cell viability (%CV) was calculated using a formula (%CV = mean OD of treated cells/mean OD of control cells).

Immunocytochemistry

Three days after transfection, we harvested and seeded glass coverslips in 12-well plates with cells ($1x10^5$). Cells were fixed in 3.7% (w/v) paraformaldehyde in phosphate-buffered saline (PBS). After quenching in 2% BSA PBS, permeabilization, and saturation were performed in PBS supplemented with 0.2% bovine serum albumin (BSA) and 0.3% (w/v) Triton X-100. Cells were incubated with primary antibodies for 1 hr and with secondary antibodies for 30 min. The coverslips were mounted in glass slides and analyzed with a FV300 scanning laser confocal microscope (Microscope confocal Olympus Fluoview FV300).

RESULTS

PCR Mediated Site Directed Mutagenesis

We PCR-amplified a 180 bp region upstream and a 442 bp region downstream of the target mutation using "central primers" and "side primers" and then conducted PCR using two mutants and only "side primers" that incorporated the glycosylation site into their oligonucleotides. As a result, we produced Ala-30 to Asn-30 mutant. Serially 32nd, 87th, 88th and 90th amino acid was mutated by such method. Positions 30~32 was changed from Ala-Glu-His to Asn-His-Thr. Positions 87~90 was changed from Pro-Trp-Glu-Pro to Val-Asn-Glu-Thr. We have constructed pIRES2-EGFP-rhEPO (Fig. 1B-1) and pIRES2-EGFP-NESP (Fig. 1B-2) expression vectors.

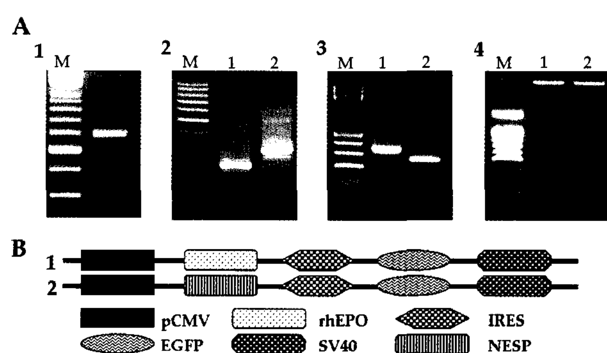

Fig. 1. Cloning of hEPO cDNA and construction of hEPO/NESP expression vectors. 10$\mu\ell$ of PCR product were visualized on a 2% agarose gel, stained with ethidium bromide. A: Expression vectors cloning. a: human EPO cDNA (582 bp) amplified from human liver cDNA library. Panel A-2, -3: Site directed mutagenesis 5' and 3' products (250 bp) amplified from PCR product (Panel A-1). Panel A-4: pIRES2EGFP with rhEPO (lane 1) and NESP (lane 2). M, 100 bp Ladder. B: Diagrammatic representations of expression vectors.

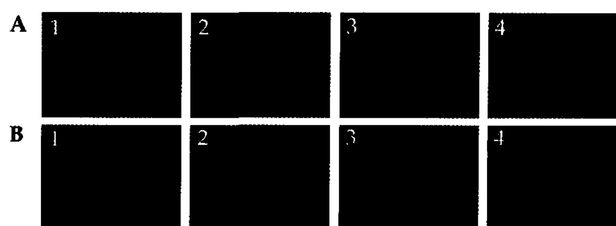

Fig. 2. The expression and distribution of EGFP in CHO-K1 cell line visualized by fluorescent microscopy. A: Transmission image. B: fluorescent image. 1: Untransfected CHO-K1 cell line as negative control. 2~3: CHO-K1 cell line transfected with pIRES2-EGFP plasmid (2), pIRES2-EGFP-rhEPO (3), pIRES2-EGFP-NESP (4).

Expression of EGFP in CHO-K1 Cell Line

To investigate the transfection efficiency of CHO-K1 cell line, expression of EGFP proteins were observed by fluorescent microscopy. EGFP was expressed in similar levels for mock, rhEPO and NESP, respectively (Fig. 2B-1~-3).

Comparison of EPO Secretion Levels and Biological Activities

We analyzed EPO secretion level at day 4, using harvested media with expressed EPO proteins by ELISA assay (Fig. 3). As a result, rhEPO (35.99± IU/ml) showed higher expression level than NESP (18.69± IU/ml). To measure biological activity of each EPOs produced in CHO cell that were transfected with rhEPO and NESP, EPO dependent cell line F36E was used (Fig. 4). In contrast, biological activities of supernatants taken from NESP-transfected cells were higher than that of supernatants from rhEPO-transfected cells.

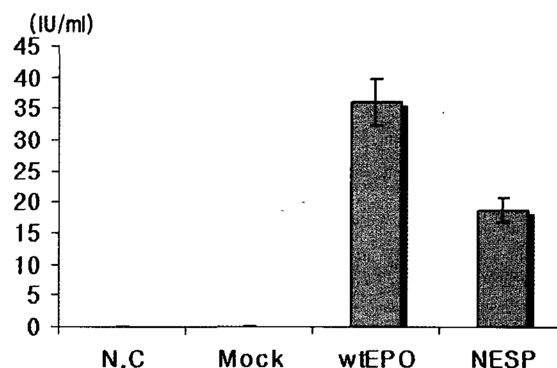

Fig. 3. Comparison of EPO secretion level between NESP and rhEPO. Mock: pIRES2-EGFP, wt: wild type, pIRES2-EGFP-rhEPO.

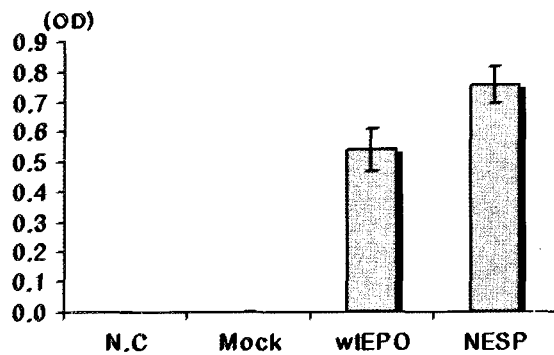

Fig. 4. Comparison of EPO biological activities between NESP and rhEPO. Mock: pIRES2-EGFP, wt: wild type, pIRES2-EGFP-rhEPO.

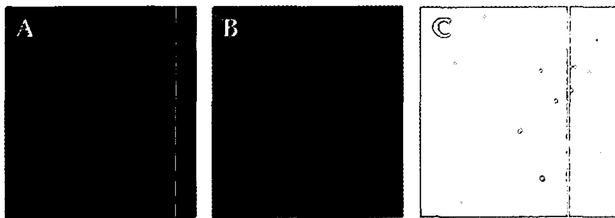

Fig. 5. Immunocytochemistry with anti-human EPO antibody. Representative confocal microscopic images are shown CHO-K1 cells that are transfected after 4 days. A: fluorescent image of EGFP (green), B: CHO-K1 cells were immunostained for EPO (red) with NESP transformants. C: transmitted image.

Co-localization of EGFP and EPO

The cellular localization of two proteins were confirmed in CHO cell lines by immunocytochemistry using EPO antibodies. As a result, EGFP protein expression was observed in whole cell (Fig. 5A) while NESP was observed only in endoplasmic reticulum (Fig. 5B).

DISCUSSION

The rhEPO has been used to the treatment of anemia of chronic renal failure which is often associated with anemia, mainly because of insufficient renal synthesis of EPO (Urena, 2002). There have been many reports about efficient protein productions including recombinant erythropoietin using site-directed mutagenesis. The mutants were constructed with deleted or added glycosylation sites. All mutations that removed N-glycosylation sites did not impair binding of EPO to the receptor, and some mutations even increased binding affinity to the receptor (Yamaguchi *et al.*, 1991). Browne (2001) reported that NESP has a longer serum half-life and greater *in vivo* biological activity compared to rhEPO. Numerous researchers have made efforts to improve the short half life of recombinant erythropoietin. As a result, the novel glycoprotein, NESP was produced (Macdougall, 2001). According to Vagner et al. (2001), IRES controls eukaryotic translation initiation. This study was conducted to confirm if IRES-controlled NESP has normal secretion level and biological activity. While secretion level of NESP was lower than wild type EPO, the biological activity of NESP was higher than wild type. These results suggest that changes in glycosylation of EPO greatly affected the biological activity and that the changes make biological activity of NESP higher than that of wild type EPO (Macdougall, 2001). Also, IRES-controlled NESP expression showed similar pattern in expression from cultured CHO cells.

REFERENCES

1. Chris D, Paul D, Sarah B, Diana W, Judy F, John C (2001): Gene targeting in primary fetal fibroblasts from sheep and pig. Cloning and Stem Cells 3: 221-231.
2. Cointe D, Beliard R, Jorieux S, Leroy Y, Glacet A, Verbert A, Bourel D, Chirat F (2000): Unuaual N-glycosylation of a recombinant human erythropoietin expressed in a human lympholastoid cell line does not alter its biological properties. Glycobiology 10:511-519.
3. Dube S, Fisher JW, Powell JS (1988): Glycosylation at specific sites of erythropoietin is essential fer biosynthesis, Secretion, and function. J Biol Chem 263: 17516-17521.
4. Elliott S, Lorenzini T, Chang D, Barzilay J, Delorme E (1997): Mapping of the active site of recombinant human erythropoietin. Blood 89:493-502.
5. Egrie JC, Browne JK (2001): Development and characterization of erythropoiesis stimulating protein(NESP). Nephrol Dial 3:3-13.
6. Fibi MR, Hermentin P, Pauly JU, Lauffer L, Zettlmeissl G (1995): N- and O-glycosylation muteins of recombinant human erythropoietin secreted from BHK-21 cells. Blood 85:1229-1236.
7. Fisher JW (1997): Erythropoietin : physiologic and pharmacologic aspects. Proc Soc Exp Biol Med 216: 358-369.
8. Fukuda MN, Sasaki H, Lopez L, Fukuda M (1989): Survival of recombinant erythropoietin in the circulation: the role of carbohydrates. Blood 73:84-89.
9. Goldwasser E, Kung CK (1968): Progress in the purification of erythropoietin. Ann NY Acad Sci 29: 49-53.
10. Goodman M, Bunn HF (1993): Erythropoietin function relationships : High degree of sequence homology among mammals. Blood 82:1507-1516.

11. Kawasaki N, Haishima Y, Ohta M, Itoh S, Hyuga M, Hyuga S, Hayakawa T (2001): Structural analysis of sulfated N-linked in erythropoietin. Glycobiology 11:1043-1049.
12. Kieft JS, Grech A, Adams P, Doudna (2001): Mechanisms of internal ribosome entry in translation initiation. Cold Spring Harb Symp Quant Biol 66: 277-283.
13. Kitagawa Y, Sano Y, Ueda M, Higashio K, Narita H, Okano M, Matsumoto S, Sasaki R (1994): N-glycosylation of erythropoietin is critical for apical secretion by Madin-Darby canine kidney cells. Exp Cell Res 213:449-457.
14. Komar AA, Hatzoglou M (2005): Internal ribosome entry sites in cellular mRNAs: mystery of their existence. J Biol Chem 280:23425-23428.
15. Macmillan D, Bill RM, Sage KA, Fern D, Flitsch SL (2001): Selective *in vitro* glycosylation of recombinant proteins: semi-synthesis of novel homogeneous glycoforms of human erythropoietin. Chem Biol 8:133-145.
16. Macdougall IC, Gray SJ, Elston O, Breen C, Jenkins B, Browne J, Egrie J (1999): Pharmacokinetics of novel erythropoiesis protein compared with epoetin alfain dialysis patients. J Am Soc Mephol 10:2392-2395.
17. Macdougall IC (2001): An overview of the efficacy and safety of novel erythropoiesis stimulating protein (NESP). Nephrol Dial Transplant. 3:14-21.
18. McDonald JD, Lin FK, Goldwasser E (1986): Cloning, sequencing, and evolutionary analysis of the mouse erythropoietin gene. Mol Biol 6:842-848.
19. Nagago M, Suga H, Okano M, Masuda S, Narita H, Ikura K, Sasaki R (1992): Characterization of erythropoietin isolated from rat serum : biochemical comparison of rat and human erythropoietins. Biochim Biophys Acta 1171:99-102.
20. Narhi LO, Arakawa T, Aoki KH, Elmore R, Rohde MF, Boone T, Strickland TW (1991): The effect of carbohydrate on the structure stability of erythropoietin. J Biol Chem 266:23022-23026.
21. Sasaki H, Bothner B, Dell A, Fukuda M (1987): Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. J Biol Chem 262:12059-12076.
22. Shoemaker CB, Mitsock LD (1986): Murine EPO gene: cloning, and human gene homology. Mol Cell Biol 6:849-858.
23. Spivak JL (1993): Recombinant erythropoietin. Ann Rev Med 44:243-53.
24. Takeuchi M, Inoue N, Strickland TW, Kubata M, Wada MR, Hoshi S, Kozutsumi H, Takasaki S, Kobata A (1989): Relationship between sugar chain structure and biological of recombinant human erythropoietin produced in Chinese ovary cells. Proc Natl Acad Sci USA 86:7819-7822.
25. Urena P (2002): Treatment of anemia in chronic renal failure by a long-activing activator of erythropoiesis. Presse Med 31:505-514.
26. Vagner S, Galy B, Pyronnet S (2001): Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO Rep 10:893-898.
27. Wasley LC, Timony G, Murtha P, Stoudemire J, Dorner AJ, Caro J, Krieger M, Kaufman RJ (1991): The importance of N-andoligosaccharides for the biosynthesis and *in vitro* and in biologic activities of erythropoietin. Blood 77:2624-2632.
28. Wen D, Boissel JP, Tracy TE, Gruninger RH, Mulcahy LS, Czelusniak J, Goodman M, Bunn HF (1993): Erythropoietin function relationships : high degree of sequence homology mammals. Blood 82: 1507-1516.
29. Yamaguchi K, Akai K, Kawanishi G, Ueda M, Masuda S, Sasaki R (1991): Effects of site-directed removal of N-glycosylation sites in human erythropoietin on its production and biological. J Biol Chem 266:20434-20439.

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