Journal of Biochemistry and Molecular Biology, Vol. 31, No. 5, September 1998, pp. 453–458

JOURNAL OF
Biochemistry
and
Molecular Biology

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Secretory Production of Biologically Active Human Thrombopoietin by Baculovirus Expression System

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Received 11 May 1998, Accepted 8 June 1998

Human thrombopoietin (hTPO) was expressed to high levels in insect cells using the baculovirus expression system. Full-length hTPO cDNA containing a native signal peptide sequence was amplified by PCR from a human fetal liver cDNA library and cloned into the *Autographa californica* nuclear polyhedrosis virus (AcNPV) expression vector. Immunoblot analysis with antiserum against hTPO indicated that an approximately 55 kDa protein was produced in recombinant AcNPV infected insect cells. Recombinant hTPO was produced 4-fold higher in *Trichoplusia ni* (Tn5) cells than in *Spodoptera frugiperda* (Sf9) cells, with most of the hTPO produced in Tn5 cells secreted into the culture medium. Addition of tunicamycin in the culture medium resulted in the reduction of the size of hTPO to 35–38 kDa, and most of the protein remained within the cell. These results suggest that N-glycosylation of hTPO is required for the secretion of the protein into the culture medium in insect cells. hTPO produced in insect cells induced proliferation and maturation of megakaryocyte progenitors, indicating that it is in a biologically active form.

Keywords: CFU-Meg., Human thrombopoietin, N-glycosylation, Tn5 cells, Tunicamycin.

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Introduction

Human thrombopoietin [hTPO, also refered to as the c-*mpl* ligand or megakaryocyte growth and development factor (MGDF)] is a recently identified hematopoietic growth factor that induces thrombopoiesis by proliferation of megakaryocyte progenitors of platelets and differentiation of immature megakaryoblasts both *in vitro* and *in vivo*. The clinical applications of the use of hTPO as a therapeutic agent are to treat thrombocytopenia shown in patients undergoing bone marrow transplants or high dose chemotherapy for malignant tumors (Eaton and de Sauvage, 1997).

TPO has been purified from aplastic plasmas from irradiated pigs and dogs (Bartley *et al.*, 1994; de Sauvage *et al.*, 1994). cDNA coding hTPO was cloned by using primers designed either from the N-terminal amino acid sequence or the cDNA sequence of TPO from animal sources (Bartley *et al.*, 1994; de Sauvage *et al.*, 1994; Foster *et al.*, 1994; Lok *et al.*, 1994). The cloned hTPO cDNA contains an open reading frame encoding 353 amino acids including a putative 21 amino acid signal sequence. hTPO has a novel two-domain structure with an amino-terminal domain of 153 amino acids homologous to erythropoietin and a carboxy-terminal domain of 179 amino acids that is rich in serine, threonine, and proline residues. The C-terminal domain contains six potential N-linked glycosylation sites (Eaton and de Sauvage, 1997). Further studies on the structure-function of hTPO and its efficacy on hematopoiesis depend on the availability of a significant amount of hTPO.

Although expression of hTPO in mammalian cells such as BHK or 293 cells was reported, the amount of expressed hTPO was not sufficient to be described in detail (Bartley *et al.*, 1994; Foster *et al.*, 1994). Direct intracellular

expression of hTPO in *Escherichia coli* showed that hTPO was found in insoluble aggregates in the cytoplasm which require complicated denaturation and refolding steps to obtain biologically active soluble forms (Jiang *et al.*, 1996). Several attempts in our laboratory to express hTPO in *E. coli* to high levels as a soluble form, using the OmpA leader secretion system and a thioredoxin fusion system, have not been successful. Since baculovirus has been widely used as a vector for expression of foreign genes in eukaryotic environments (Richardson, 1995), we adopted this system to express hTPO in insect cells.

The aim of this study was to produce and secrete hTPO in functionally active forms in culture medium of recombinant virus-infected insect cells. The influence of N-glycosylation and various insect cell-lines on the secretion of hTPO were also investigated.

Materials and Methods

Viruses and cells Wild-type AcNPV and recombinant virus were grown in *Spodoptera frugiperda* (Sf9) cells using Grace's insect culture medium supplemented with 10% fetal calf serum (FBS, HyClone, Logan, USA). For overexpression of hTPO, Sf9, Sf21, and Tn5 (BTI-TN-5B1-4), cell-lines derived from *Trichoplusia ni* egg cell homogenates were obtained from Invitrogen (San Diego, USA). Cells were maintained in SF900II serum-free media as monolayer cultures at 27°C.

Cloning of the hTPO cDNA hTPO cDNA was amplified from a human fetal liver cDNA library (Clontech, Palo Alto, USA) by PCR. The nucleotide sequence of primers were 5′-GG**TCTAGA**ATGGAGCTGAATTGC-3′ and 5′-TTAAGCTTATCACCCTTCCTGAGACAGATT-3′. The primer containing the *Xba*I site (bold) is complementary to the 5′-end of the hTPO cDNA, and the *Hin*dIII site (dotted) is complementary to the 3′-end of the hTPO cDNA. PCR amplification was carried out in 30 sequential cycles at 94°C for 1 min, 58°C for 2 min, and 72°C for 1 min. The PCR-amplified hTPO cDNA product was digested with *Xba*I and *Hin*dIII, cloned into the pBluescriptII SK(+) plasmid (Stratagene Ltd., Cambridge, UK), and named pBlue404. The nucleotide sequence of the cloned hTPO cDNA was verified by the dideoxy nucleotide sequencing method using a Sequenase version 2.0 DNA sequencing kit (USB, Cleveland, USA).

Construction of recombinant baculovirus The hTPO cDNA fragment generated by digesting pBlue404 with *Xba*I and *Hin*dIII was cloned into the pUC18 plasmid (pUC404). The hTPO cDNA excised from pUC404 by *Bam*HI and *Hin*dIII double digestion was subcloned into the *Bam*HI and *Hin*dIII sites of the pBlueBacIII vector (Invitrogen, San Diego, USA) to generate a baculovirus transfer vector. The transfer vector, pBac404-7, was cotransfected with triple-cut linearized AcNPV DNA, BacPAK6 (Clontech, Palo Alto, USA) into Sf9 cells. At 4 d post-transfection, the culture medium was plaque assayed, and polyhedrin-negative plaques with blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were selected as recombinant viruses. After three rounds of plaque purification, a recombinant virus was obtained and named AcBac404-2.

Expression of hTPO Monolayers of Sf9 cells, Sf21 cells, and Tn5 cells in 35-mm tissue culture dishes (1×10^6 cells per dish) were infected with AcBac404-2 in triplicate and incubated at 27°C. Culture medium and cells were recovered at given time course intervals and centrifuged at $10{,}000 \times g$ for 10 min. Cell pellets were resuspended in 0.5 ml lysis buffer (PBS, pH 7.0 containing 1% Nonidet P-40 and 10 μg/ml phenylmethylsulfonyl fluoride; Sigma, St. Louis, USA), sonicated for 30 sec, and then immunoblot analysis or enzyme linked immunosorbent assay (ELISA) was performed.

Immunoblot analysis and ELISA The protein samples were resolved on either a gradient (10–20%) or 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was treated with 5% skim milk to block nonspecific binding and probed with goat anti-thrombopoietin antibodies (R&D Systems Inc., McKinley, USA). Detection of the protein bands was accomplished by using alkaline phosphatase-conjugated anti-goat IgG (Sigma, St. Louis, USA) and addition of chromogenic substrate. hTPO was quantitated by antigen-capture ELISA using hTPO purchased from R&D Systems Inc. (McKinley, USA) as a standard.

Effect of tunicamycin on the secretion of hTPO Monolayers of Tn5 cells (1×10^6 cells per 35-mm dish) were infected with AcBac404-2 and incubated at 27°C in the presence of different concentrations of tunicamycin (1, 10, and 100 μg/ml) in the culture medium. The cell extracts and the culture medium recovered at 62 h post-infection were separated on SDS-PAGE and immunoblot analysis was performed.

Megakaryocyte colony assay The 8 to 11-wk old female C57BL/6 mice (Charles River, Japan) were sacrificed by rapid cervical dislocation, and bone marrow cells were obtained by flushing the femoral shafts with DMEM (Gibco, Grand Island, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, USA). The bone marrow cells were cultured in a 5% CO_2 incubator for 1–2 h to remove adherent cells. To obtain CFU-Meg progeny, the 35-mm dishes containing bone marrow cells were cultured at a concentration of 5×10^4 cells/well in DMEM supplemented with 1% BSA (Sigma, St. Louis, USA), 10% FBS (HyClone, Logan, USA), 0.1 ng/ml of mIL-3 (R&D Systems Inc., Mckinley, USA), and 0.3% Agar. The cells were incubated at 37°C in 5% CO_2 for 7 d. The agar disks were then removed from the culture dishes, transferred to 25×75 mm glass slides, washed, and dried. The slides were fixed with 5% glutaraldehyde for 10 min and rinsed with distilled water. After drying, the slides were stained for 18 h at room temperature with megakaryocyte-specific enzyme acetylcholinesterase by the method of Jackson (1973) and counterstained with haematoxyline for 10 min. Megakaryocyte colonies containing 3 or more cells were scored under a microscope. The assay was done with quadruplicated dishes and repeated twice. The data were expressed as a mean ± S.E. and statistical significance was determined using the Student's t-test.

Results and Discussion

Construction of the recombinant baculovirus hTPO cDNA synthesized by PCR from human fetal liver cDNA library was cloned into pBluescript(II) (pBlue404) and its nucleotide sequence was determined. The cloned hTPO cDNA is 1079 bp and contains an open reading frame encoding 353 amino acids including a 21 amino acid leader sequence not having any 5′ and 3′ noncoding regions. The nucleotide sequence of hTPO in pBlue404 was identical to that of the previously reported hTPO cDNA (de Sauvage *et al.*, 1994).

hTPO cDNA was subcloned downstream of the strong polyhedrin promoter of pBlueBacIII to make pBac404-7, a baculovirus transfer vector (Fig. 1). pBlueBacIII is a nonfusion vector since the polyhedrin initiation codon was mutated to ATT to prevent translation initiation. A recombinant AcNPV carrying hTPO cDNA (AcBac404-2) was isolated and the presence of hTPO cDNA in AcBac404-2 was confirmed by PCR.

Time course expression of hTPO in Tn5 cells Over the last 10 years, many eukaryotic genes were expressed in large quantities using the baculovirus expression system (Richardson, 1995). Due to the ability of the insect cells to perform proper folding, post-translational modification, and exact signal peptide cleavage, this system is especially useful for the expression of highly modified and secreted proteins. Generally, expression levels of secreted and glycosylated recombinant proteins are much lower (below 5 mg/l) compared to intracellular proteins (Jarvis and Summers, 1989; Murphy *et al.*, 1993).

To confirm that the AcBac404-2-infected insect cells express hTPO, Tn5 cells infected with AcBac404-2 at 10 MOI were harvested at 24, 48, 72, 96, and 168 h post-infection and the expression level of the hTPO was analyzed by ELISA. As shown in Fig. 2, the expressed hTPO appeared in the culture medium as early as 18 h post-infection and steadily increased until it reached the maximal level at 72 h post-infection. The level then decreased rapidly after 72 h post-infection. This diminishing expression level was most probably due to the degradation of the protein by host protease. The effect of MOI on the hTPO production was also examined. The amount of hTPO expressed at an MOI above 10 (Fig. 3, lanes 3 and 4) was higher than at 1 or 5 MOI (Fig. 3, lanes 5 and 6).

Comparison of hTPO expression in Sf9, Sf21, and Tn5 cells In earlier studies, the Sf9 cell was frequently used for the expression of eukaryotic glycoprotein. However, it was reported that Sf9 cells have rate-limiting steps in the translational or post-translational machinery (Jarvis *et al.*, 1990). Therefore, the Tn5 cell-line has been widely used

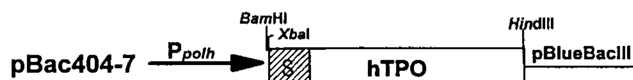

Fig 1. Construction of baculovirus transfer vector, pBac404-7 containing full-length human TPO cDNA. The 1075 bp *Bam*HI/*Hin*dIII fragment encoding full-length hTPO was cloned into the corresponding site of pBlueBacIII. P_{polh} indicates the polyhedrin promoter. "S" in boxes represent the 21-amino acid native signal sequence of hTPO.

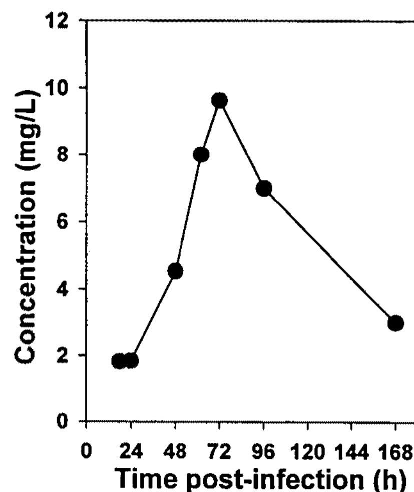

Fig. 2. Time course expression of hTPO in Tn5 cells. Monolayers of Tn5 cells at a density of 1×10^6 cells per 35-mm dish were infected with AcBac404-2 at a multiplicity of infection (MOI) of 10 pfu/cell (●---●). Cell culture media were harvested at given time intervals and the levels of hTPO were analyzed by ELISA.

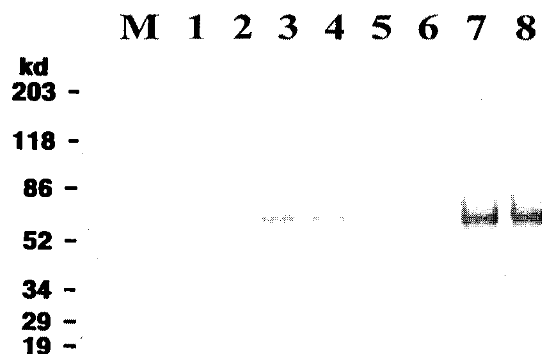

Fig. 3. Effect of multiplicity of infection (MOI) on recombinant hTPO production. Monolayers of Tn5 cells at a density of 2×10^6 cells per 60-mm dish were infected with AcBac404-2 at a different MOI. Ten microliters of culture medium were run on SDS-PAGE and immunoblot analysis was performed. Lane M, prestained molecular weight markers; lanes 1 and 5, 48 and 72 h post-infection at 1 MOI; lanes 2 and 6, at 5 MOI; lanes 3 and 7, at 10 MOI; lanes 4 and 8, at 20 MOI.

for the production of secreted glycoproteins because of higher protein production and secretion efficiency compared to Sf9 (Wickham *et al.*, 1992; Chawla and Owen, 1995; Rupp *et al.*, 1995).

Sf9, Sf21, and Tn5 cell-lines, adapted to the serum-free Sf900II medium, were infected with AcBac404-2 at 10 MOI and harvested at 72 h post-infection. Both the culture media and cell extracts were analyzed by ELISA. As indicated in Table 1, Tn5 cells (13.3 mg/l) produced 2.5-fold and 1.5-fold more hTPO than Sf9 cells (5.3 mg/l) and Sf21 (8.6 mg/l), respectively. Tn5 cells secreted hTPO much more efficiently than Sf9 cells, resulting in about 4-fold higher levels of hTPO secreted into the culture medium.

These results suggest that Tn5 cells are a better choice than Sf9 cells for the mass production of hTPO. About 80% of the hTPO produced in Tn5 cells was secreted into the medium after 72 h post-infection (Table 1). These results indicate that the signal peptide of the hTPO is recognized and is functional in the Tn5 cells.

The effect of N-glycosylation on the secretion of hTPO To determine whether hTPO expressed in insect cells was N-glycosylated, AcBac404-2-infected Tn5 cells were grown in the culture medium containing a series of different concentrations of tunicamycin (1, 10, and 100 μg/ml). The culture medium and cell extracts harvested at 72 h post-infection were separated on SDS-PAGE and immunoblot analysis was performed. No immunoreactive protein bands were detected in the cell extract of the tunicamycin-untreated sample (Fig. 4, Cell fraction, lane 5), while several bands of approximately 35–38 kDa were detected in cell extracts of tunicamycin-treated samples (Fig. 4, Cell fraction, lanes 2–4). However, a single immunoreactive protein band with the size of 58 kDa was detected in the culture medium of the tunicamycin-untreated sample (Fig. 4, Medium fraction, lane 5), while no immunoreactive band was detected in the culture medium of tunicamycin-treated samples (Fig. 4, Medium fraction, lanes 2–4).

Table 1. Expression levels of hTPO in different insect cell-lines[a].

Cell-lines	hTPO[b] (μg/ml)			Protein[c] (μg/ml)		
	medium	cell[d]	total	medium	cell[d]	total
Sf9	2.5	2.8	5.3	61	332	393
Sf21	6.1	2.5	8.6	68	408	476
Tn5	10.5	1.8	13.3	69	483	552

[a] Each cell-line was infected with AcBac404-2 at 10 MOI and samples were harvested at 72 h post-infection.
[b] The amount of hTPO was measured by ELISA.
[c] Protein concentrations were determined by the Bradford assay using bovine serum albumin as the standard.
[d] Cell means the μg of hTPO or proteins produced per 10^6 cells.

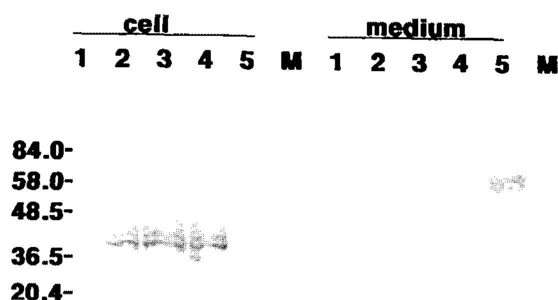

Fig. 4. Effects of tunicamycin on the secretion of hTPO expressed in Tn5 cells. Tn5 cells infected with AcBac404-2 were cultured in SF900II medium containing different concentrations of tunicamycin. The culture medium and cell extracts harvested at 72 h post-infection were analyzed on SDS-PAGE and subjected to immunoblot analysis. Lane 1, uninfected Tn5 cells; lanes 2 to 4, AcBac404-2 infected Tn5 cells treated with 1, 10, and 100 μg/ml of tunicamycin, respectively; lane 5, Tn5 cells infected with AcBac404-2; lane M, prestained molecular weight markers.

These data indicate that the hTPO expressed in insect cells is N-glycosylated. Most of the N-glycosylated form is present in the culture medium whereas non- or partially-glycosylated forms remain within the cell. These results also suggest that N-glycosylation of hTPO is required for secretion from the cell into the culture medium. The role of N-glycosylation in the secretion of glycoprotein is not yet clearly explained. In most studies using tunicamycin, N-glycanase, or deletion or insertion of N- or O-glycosylation sites, N-glycosylation is required directly or indirectly in baculovirus-infected insect cells or mammalian cells for the secretion of glycoproteins (Jarvis and Summers, 1989; Vernet and Tessier, 1990; Rothwell *et al.*, 1993). Further work should be performed to elucidate the role of glycosylation in the secretion of hTPO.

The molecular weight of the hTPO expressed in insect cells was estimated to be 55 kDa. Since the molecular weight of the hTPO calculated from the deduced amino acid sequence is approximately 35 kDa, about 36% (20 kDa) of the molecular weight should be derived from post-translational modification. Because the molecular mass of recombinant hTPO expressed in mammalian cells is reported to be about 60–80 kDa (de Sauvage *et al.*, 1994; Lok *et al.*, 1996), it is thought that the hTPO expressed in insect cells is not completely modified. Reduction of the molecular weight of most mammalian glycoproteins expressed in insect cells is generally thought to be due to the low degree of glycosylation (Davidson *et al.*, 1990).

Megakaryocytopoietic effect of hTPO expressed in Tn5 cells. In order to determine if hTPO expressed in insect cells is bioactive, the effect of hTPO expressed in Tn5 cells on the formation of colony forming unit-megakaryocyte

(CFU-Meg) was investigated. Since IL-3 acts additively with hTPO to stimulate proliferation of megakaryocyte progenitors in murine or human megakaryocytopoiesis assays, 0.1 ng/ml of mIL-3 was added into the culture medium. In the presence of hTPO expressed in the culture medium from Tn5 cells, large numbers and greater size of megakaryocytes which stained positive for the megakaryocyte marker, acetylcholinesterase, in a dose response pattern (Fig. 5) were seen as compared to 10 ng/ml or more of hTPO expressed in Tn5 cells.

The size of acetylcholinesterase-stained megakaryoctyes in the presence of hTPO in the medium (Fig. 6, Panel B) was larger than in cells cultured in the absence of hTPO in the medium (Fig. 6, Panel A). This result suggests that hTPO produced from insect cells has a similar *in vitro* ability to differentiate the progenitor cells compared to that produced from mammalian cells (Kaushansky *et al.*, 1994). These results show that hTPO expressed in insect cells is bioactive and can induce proliferation and maturation of progenitor megakaryocytes.

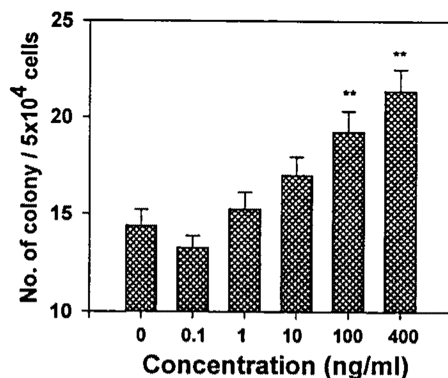

Fig. 5. Effect of hTPO expressed in Tn5 cells on CFU-Meg in normal bone marrow cells. Female C57BL/6 mice were used for experiments. Megakarytocytic colony assay was performed by the agar culture method in quadraplicate. Bone marrow cells were cultured in 35-mm tissue culture dishes in DMEM supplemented with 1% BSA, 10% FBS, and made semi-solid with 0.3% agar. After 7 d in culture at 37°C, the agar disks were transferred to glass slides and fixed with 5% glutaraldehyde. Colonies were enumerated after histochemical staining for acetylcholinesterase and counterstaining with hematoxyline. Colonies containing more than 3 cells were counted. The data were expressed as standard error of mean. hTPO was quantitated by direct ELISA. ** $p < 0.01$ (vs mIL-3 group).

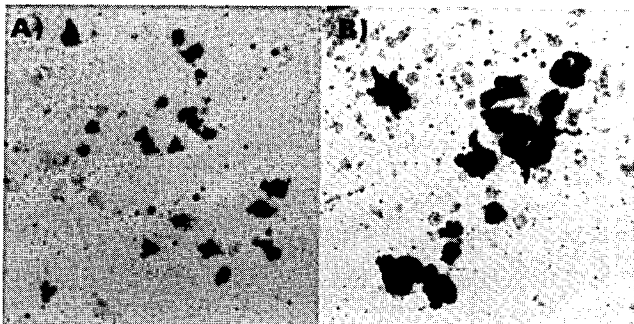

Fig. 6. Megakaryocyte colony from murine normal bone marrow cells in semi-solid agar cultures after 7 d of incubation. Panel A represents mIL-3 only and panel B is hTPO expressed in Tn5 cells with mIL-3 (×63).

Acknowledgments We thank Mr. Seok Won Yoon for his help on the initial cloning experiments. This study was supported in part by a grant from the Ministry of Science and Technology of Korea.

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