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Development of Recombinant Chinese Hamster Ovary Cell Lines Producing Human Thrombopoietin or Its Analog

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Abstract Recombinant Chinese hamster ovary (rCHO) cell lines expressing a high level of human thrombopoietin (hTPO) or its analog, TPO33r, were obtained by transfecting expression vectors into dihydrofolate reductase-deficient (dhfr$^-$) CHO cells and subsequent gene amplification in media containing stepwise increments in methotrexate (MTX) level such as 20, 80, and 320 nM. The parental clones with a hTPO expression level >0.40 μg/ml (27 out of 1,200 clones) and the parental clones with a TPO33r expression level >0.20 μg/ml (36 out of 400 clones) were subjected to 20 nM MTX. The clones that displayed an increased expression level at 20 nM MTX were subjected to stepwise increasing levels of MTX such as 80 and 320 nM. When subjected to 320 nM MTX, most clones did not display an increased expression level, since the detrimental effect of gene amplification on growth reduction outweighed its beneficial effect of specific TPO productivity (q_{TPO}) enhancement at 320 nM MTX. Accordingly, the highest producer subclones (1-434-80* for hTPO and 2-3-80* for TPO33r), whose q_{TPO} was 2- to 3-fold higher than that of their parental clones selected at 80 nM MTX, were isolated by limiting dilution method and were established as rCHO cell lines. The q_{TPO} of 1-434-80* and 2-3-80* was 5.89±0.74 and 1.02±0.23 μg/10^6 cells/day, respectively. Southern and Northern blot analyses showed that the enhanced q_{TPO} of established rCHO cell lines resulted mainly from the increased TPO gene copy number and subsequent increased TPO mRNA level. The hTPO and TPO33r produced from the established rCHO cell lines were biologically active *in vivo*, as demonstrated by their ability to elevate platelet counts in treated mice.

Key words: CHO, clonal selection, gene amplification, methotrexate, thrombopoietin

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Thrombopoietin (TPO) is a hematopoietic growth factor that induces thrombopoiesis by proliferation of megakaryocyte progenitors of platelets and differentiation of immature megakaryocyte [5, 10, 17, 28]. The cloned human TPO (hTPO) is a polypeptide composed of 353 amino acids, including a 21 amino acid secretory leader sequence [1, 5] and two disulfide bonds necessary for biological activity [11]. The mature form of hTPO has a highly complex glycosylated structure consisting of N-terminal and C-terminal domains. The glycosylation of hTPO accounts for more than one-half of its observed 80-100 kDa molecular mass in human plasma [15]. The clinical applications of hTPO as a therapeutic agent are to treat thrombocytopenia of patients undergoing bone marrow transplants or high dose chemotherapy for malignant tumors [6].

Recently, it was observed that the addition of two N-linked glycosylation sites by amino acid substitutions at specific sites of erythropoietin (EPO) gave rise to the striking increase in its *in vivo* biological activity [7]. This result suggests that N-linked sugar chain may have a significant effect on the activity of the glycoprotein. In an effort to improve the *in vivo* biological activity of native hTPO, a variety of hTPO muteins were generated by creating N-linked glycosylation sites at specific positions of hTPO. Among 66 muteins, TPO33r was selected in view of its elevated thrombopoietic activity as compared to hTPO (J. Y. Chung *et al.* 1999. PCT/KR99/00347).

For high-level expression of recombinant proteins, the most popular mammalian expression system in both research and industrial applications uses expression vectors containing the dihydrofolate reductase (dhfr) gene as the selective gene and dhfr-deficient (dhfr$^-$) CHO cells as the host cell line [16, 22, 27]. For the establishment of recombinant CHO (rCHO) cell lines, dhfr$^-$ CHO cells are first transfected with expression vectors including the dhfr and the foreign gene, and subjected to drug selection and several rounds of

gene amplification with methotrexate (MTX) [2, 29]. However, clonal variations of rCHO cells in regard to recombinant protein productivity are significant, because clones acquire MTX resistance by mechanisms other than dhfr-mediated gene amplification despite the stepwise selection [18, 19]. Furthermore, rCHO cells with increased foreign gene copy numbers suffer from metabolic and growth decrements [8, 9, 25] and are prone to die of environmental stress [4], resulting in a low product titer. Therefore, a tedious and labor intensive effort during dhfr-mediated gene amplification is required to obtain the desired rCHO cell line that has a high specific productivity and growth rate.

Although many reports on the development of rCHO cell lines with dhfr-mediated gene amplification are available, only a few reports on rCHO cell lines producing highly glycosylated proteins such as TPO are available. Lucas *et al.* [23] and Kaszubska *et al.* [14] reported on TPO expression in CHO cells, but the expression level of constructed cell lines are low (1.6–4.0 μg/ml) and the details of cell line development are lacking.

In the present study, the development of the CHO cell lines producing a high level of hTPO and its analog, TPO33r, is reported. Highly producing parental clones were selected from a large population, and their degree of gene expression increase was analyzed along with the MTX concentration increment. In addition, a clonal lineage obtained during dhfr-mediated gene amplification was characterized by Southern and Northern blot hybridizations. Finally, the biological activity of TPO produced by the established rCHO cell lines was also examined.

MATERIALS AND METHODS

Expression Vectors

The expression vector for hTPO was constructed from pSV2-dhfr (37146, ATCC, Rockville, MD, U.S.A.) and pCDT, which was built by inserting the cDNA fragment encoding hTPO into a unique *Kpn*I and *Eco*RI site of pcDNA3.1/Zeo(+) (Invitrogen, Carlsbad, CA, U.S.A.). For enhancement of gene amplification at a low MTX level [24], the enhancer element from the SV40 early promoter in pSV2-dhfr was removed as described previously [3]. The crippled dhfr gene from pSV2-dhfr was subcloned into the *Bgl*II site of pCDT, resulting in pDCT (Fig. 1). The expression vector for TPO33r, an analog of hTPO with the enhanced biological activity, was constructed in the same manner.

Cell Lines and Cell Culture

Schematic representation of the procedure for the establishment of cell clones at various MTX levels is shown in Fig. 2.

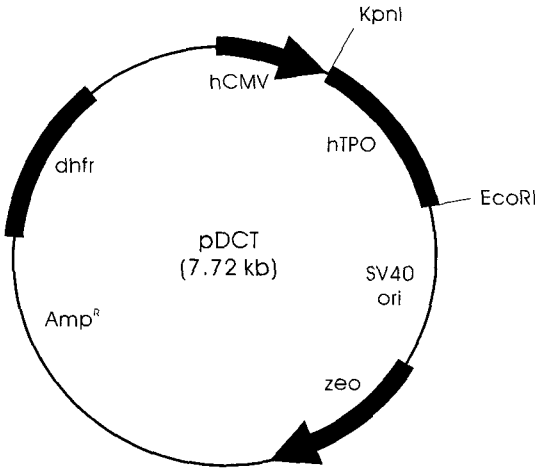

Fig. 1. Expression vector for human thrombopoietin.
hCMV, Human cytomegalovirus promoter; hTPO, human thrombopoietin; SV40 ori, Simian virus 40 origin of replication; zeo, Zeocin resistant gene; Amp^R, ampicillin resistant gene; dhfr, dihydrofolate reductase.

medium (IMDM) supplemented with 10^{-4} M hypoxanthine, 1.6×10^{-5} M thymidine and 10% dialyzed fetal bovine serum (dFBS, all from Life Technologies, Rockville, MD, U.S.A.). Transfection of expression vector (2 μg) into dhfr$^-$ CHO cells was performed using LipofectAMINE™ (12 μg, Life Technologies) according to the manufacturer's protocol. Drug selection was carried out by seeding 10^3 cells per well in 96-well tissue culture plates (Nunc, Roskilde, Denmark) containing IMDM supplemented with 10% dFBS and 500 μg/ml of Zeocin (Invitrogen). Zeocin was used only in the first selection. Cells were re-fed with fresh medium every 3–4 days for approximately 3 weeks until the size of the single colony reached near confluency. The TPO titer in the culture supernatant of Zeocin-resistant clones isolated on 96-well tissue culture plates was determined by an enzyme linked immunosorbent assay (ELISA) kit (R&D System, Minneapolis, MN).

On the basis of the TPO titer in the supernatant, Zeocin-resistant clones with high-level expression were transferred to T-25 cm^2 flasks (Nunc) containing 5 ml of IMDM supplemented with 10% dFBS for cell expansion. For determination of TPO productivity of Zeocin-resistant clones, cells were seeded at 2×10^5 cells per well containing 3 ml of medium in 6-well tissue culture plates (Nunc) and, after 4 days of cultivation, the TPO titer in the culture supernatant was determined by ELISA. Zeocin-resistant clones (parental clones) producing more than 0.4 μg/ml for TPO and 0.2 μg/ml for TPO33r were selected for gene amplification and were subjected to successive rounds of selection in media containing stepwise increments of MTX (Sigma, St. Louis, MO, U.S.A.) (20, 80, and 320 nM). Cells were re-fed with fresh medium every 3–4 days and

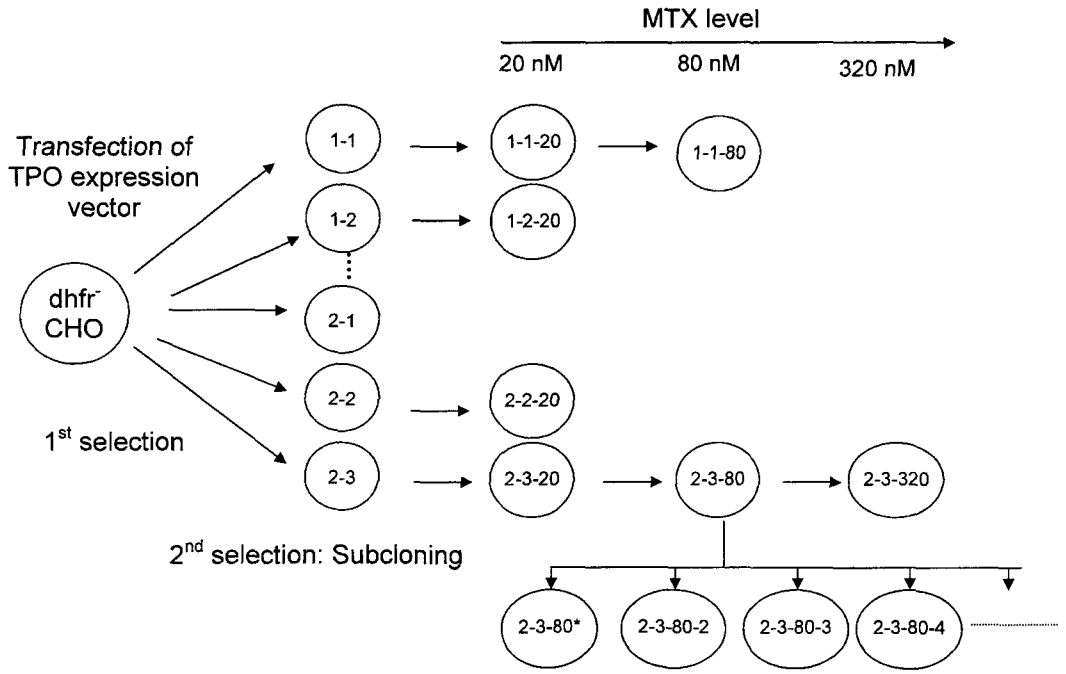

Fig. 2. Schematic representation of the procedure for the establishment of hTPO producing cell lines.

at 2×10^5 cells per well in 6-well tissue culture plates (Nunc). After 4 days of cultivation, the TPO titer in the culture supernatant was determined by ELISA.

To isolate the highest producer, the clones producing the highest level of TPO and TPO33r at 80 nM MTX were subcloned by plating 0.3–0.5 cells per well in 96-well tissue culture plates containing 0.1 ml IMDM supplemented with 10% dFBS and 80 nM MTX, respectively. When the subclones reached 60–70% confluency, they were replenished with 0.1 ml of medium per well. After 3–4 days, the culture supernatants of individual subclones isolated on 96-well tissue culture plates were tested for TPO production by ELISA. Subclones producing the highest level of TPO and TPO33r were selected, expanded, and investigated for specific TPO productivity (q_{TPO}) in 6-well tissue culture plates (Nunc).

To characterize 2-3 clonal lineage producing the highest level of TPO33r (Fig. 2), batch cultures of 2-3-1-80* subclone and 2-3 clones were carried out in 6-well tissue culture plates containing 3 ml of IMDM medium with 10% dFBS and the corresponding level of MTX. The initial cell concentration was approximately 2×10^5 cells per well. One well was sacrificed every 48 h for determination of cell concentration. The supernatant was aliquoted and kept frozen at −20°C for TPO assay. The cultures were performed 3 separate times in a humidified 5% CO_2 incubator at 37°C.

Southern and Northern Blot Analyses

Southern blot analyses of genomic DNAs from each amplified clone were performed as described by Kim *et al.* [20], except for the preparation of the hybridization probe. The hybridization probe was prepared by *Kpn*I and *Eco*RI enzyme digestion of pDCT vector and subsequently the extraction of 1.1 kb hTPO gene fragment was performed. The probe was radioactively labeled by random primed incorporation of [α-^{32}P] dCTP (Amersham, Amersham, UK).

For Northern blot analysis, cytoplasmic RNA was isolated from late-exponential-phase culture of each clone by RNAwiz (Ambion, Austin, TX) according to the manufacturer's recommendation. After electrophoresis of cytoplasmic RNAs (5 μg) from each sample on a 1.0% formaldehyde gel, hTPO mRNAs were characterized by Northern blot hybridization with the same probe used in Southern blot analysis. The procedure for membrane transfer, prehybridization, hybridization, and subsequent colorimetric detection was the same as described in Southern blotting, except that the prehybridization and hybridization were performed at 68°C. The relative hTPO mRNA contents were determined by quantitating the band intensities using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Biological Activity

To examine the biological activity of hTPO and TPO33r produced from established rCHO cell lines, exponentially growing cells were seeded at a concentration of 2×10^5 cells/well in 6-well tissue culture plates containing 3 ml of IMDM supplemented with 80 nM MTX and 10% dFBS. After 3 days of cultivation, culture medium was replaced with 3 ml of a serum-free medium containing DMEM/F12

(Invitrogen) supplemented with Yeastolate (1×) (Invitogen). After 3 days of cultivation, culture supernatants containing secreted TPO were aliquoted and kept frozen at 70°C for *in vivo* biological activity assay.

The *in vivo* biological activities of hTPO and TPO33r were tested in mice. Female Balb/c mice (20-22 g) were administered with the concentration of 10 μg/kg as a single subcutaneous injection. Each test group consisted of five mice. On each sampling day, the mice were anesthetized with diethylether. About 200 μl of peripheral blood was obtained from the abdominal artery for measuring the hematological parameters into ethylenediaminetetracetic acid (EDTA) tubes. The blood cell counts were determined using a Cell-Dyn 3500 analyzer (Abbott Laboratories, Abbott Park, IL, U.S.A.). Statistical significance of difference in platelet levels between the vehicle and treated groups was assessed by Student's t-test.

Evaluation of Specific Growth and Production Rates

The specific growth rate (μ) and q_{TPO} were based on data collected during the exponential growth phase and were evaluated as described earlier [19, 21]. When the plot of TPO titer versus time integral of viable cells is fitted with a straight line with a regression coefficient close to 1, the slope represents q_{TPO}.

Results and Discussion

Development of CHO Cell Lines Producing hTPO and TPO33r

As described in Materials and Methods, the TPO and TPO33r cDNAs were cloned into enhancer crippled pSV2-dhfr vectors, respectively (Fig. 1). Their nucleotide sequences were confirmed by restriction mapping and direct sequencing. Among three promoters tested, a CMV promoter was selected for TPO expression in these constructs, because the CMV promoter (0.09 μg/ml) mediated higher expression of TPO than SV40 (0.05 μg/ml) and SRα promoters (0.07 μg/ml). To confirm the TPO expression of the constructed vector, transient expressions of TPO vector were performed with dhfr$^-$ CHO cells. The expression level of TPO in culture supernatant collected 3 days after transfection was 0.05-0.10 μg/ml. Western blot analysis showed that the molecular mass of TPO was approximately 90 kDa, indicating that transfected CHO cells fully expressed glycosylated TPO.

To develop rCHO cell lines expressing a high level of hTPO or TPO33r, dhfr$^-$ CHO cells were transfected with TPO expression plasmids. The dfhr-positive (dhfr$^+$) transformants were selected in a nucleoside-free IMDM containing 500 μg/ml of Zeocin. The dhfr$^+$ parental clones with an hTPO expression level >0.40 μg/ml (27 out of 1,200 clones) and the dhfr$^+$ parental clones with a TPO33r expression level >0.20 μg/ml (36 out of 400 clones) were subjected to 20 nM MTX (Fig. 2). The clones that displayed an increased expression level at 20 nM MTX were subjected to stepwise increasing levels of MTX, such as 80 and 320 nM. When subjected to 320 nM MTX, most clones did not display an increased expression level and displayed a reduced growth rate. Accordingly, clones producing the highest level of hTPO (1-434-80 clone with 6.52 μg/ml) and TPO33r (2-3-80 clone with 3.83 μg/ml) were selected at 80 nM MTX.

To isolate the highest producer (HP) subclones, 1-434 and 2-3 clones were subjected to subcloning in 96-well tissue culture plates containing IMDM supplemented with 10% dFBS and 80 nM MTX, respectively. The HP subclones were selected and named 1-434-80* and 2-3-80*, respectively. Their growth and TPO production characteristics were investigated in 6-well tissue culture plates. Their growth characteristics in terms of μ and maximum viable cell concentration were similar to those of their parental clones. On the other hand, their q_{TPO} was 2- to 3-fold higher than that of their parental clones (1-434-80 and 2-3-80). Furthermore, these HP clones were quite stable in regard to TPO production during long-term cultures. After they were maintained as monolayer cultures in the presence as well as absence of 80 nM MTX for 2 months, they still retained more than 80% of their initial q_{TPO} regardless of the presence of MTX. Cell growth and TPO production characteristics of the HP subclones and their parental clones are summarized in Table 1.

The expression level of hTPO obtained from the established rCHO cell line (1-434-80*) was 11.40 μg/ml, which was 3- to 6-fold higher than the values reported in the literature [14, 23]. When compared with the expression level of humanized antibody from rCHO cell lines, the expression level of hTPO was not that high despite the extensive efforts to establish high producing rCHO cell

Table 1. Values for μ, maximum viable cell concentration, q_{TPO}, and maximum TPO titer in cultures of established rCHO cell clones.[a]

Clone (protein)	μ (/day)	Max. viable cell conc. (10^6 cells/ml)	q_{TPO} (μg/10^6 cells/day)	Max. TPO titer (μg/ml)
1-434-80* (hTPO)	0.49±0.04	0.55±0.12	5.89±0.74	11.40±1.83
1-434-80 (hTPO)	0.46±0.02	0.57±0.14	2.11±0.54	6.52±1.43
2-3-80* (TPO33r)	0.78±0.08	1.85±0.49	1.02±0.23	5.56±1.54
2-3-80 (TPO33r)	0.64±0.0	1.64±0.13	0.50±0.23	3.83±1.59

[a]Means±SD (n=3). The experiments were performed three separate times.

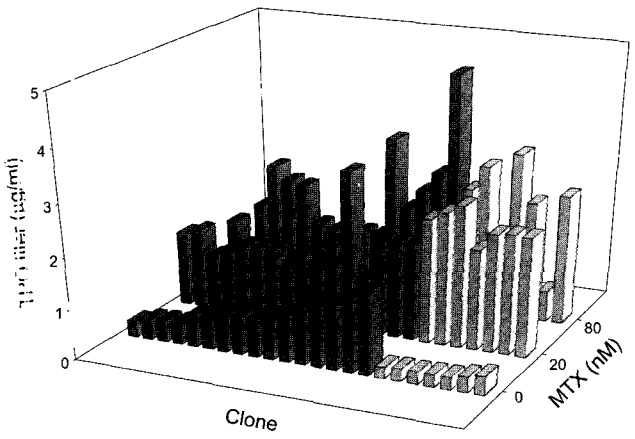

Fig. 3. Expression levels of 23 clonal lineages during MTX-mediated gene amplification up to 80 nM MTX.
The dark gray bar represents clonal lineages for hTPO expression and the bright gray bar represents clonal lineages for TPO33r expression.

lines. The relatively low expression level of TPO may be due to its highly glycosylated complex structure with six N-linked glycosylation sites, twenty O-linked glycosylation sites, and two disulfide bonds.

Analysis of TPO Expression Levels of Clones Subjected to Stepwise Increments in MTX Concentration

Development of rCHO cell lines with high-level expression requires time-consuming and tedious screening procedures. Systematic characterization on the changes in the expression level of clones during MTX-mediated gene amplification may reveal an efficient way of establishing rCHO cell lines with high-level expression. Thus, 23 hTPO-expressing parental clones that displayed a high expression level of hTPO (>1.1 μg/ml, 16 clones) or TPO33r (>2.0 μg/ml, 7 clones) at 20 nM MTX were selected and their expression levels at 80 nM MTX were estimated.

Figure 3 shows the expression levels of 23 clonal lineage during MTX-mediated gene amplification up to 80 nM MTX. Clonal variations in regard to TPO expression at 20 nM MTX was found to be significant. When subjected to 20 nM MTX, the parental clones with the highest expression levels (1-434 clone for hTPO and 2-16 clone for TPO33r expression) did not show the highest expression level at 20 nM MTX. Furthermore, the degree of enhancement of expression levels at the higher MTX level varied significantly among clones regardless of their expression level. At 20 nM MTX, 2-26 clone showed the highest increase in the expression level (11.5-fold increase). However, its expression level was rather decreased at 80 nM MTX. Thus, no correlation between clones at the higher MTX level and their parental clones was found in regard to the expression level.

Characterization of a Clonal Lineage Exhibiting the Highest Expression Level of TPO During MTX-Meditated Gene Amplification

When subjected to 320 nM MTX, most clones did not display an increased expression level of TPO. To understand the cells behavior in regard to growth and TPO production during MTX-mediated gene amplification, clones in a 2-3 clonal lineage such as 2-3, 2-3-20, 2-3-80, and 2-3-320 clones were cultivated in 6-well plates containing IMDM with 10% dFBS and the corresponding level of MTX.

Cell growth and TPO production characteristics of the 2-3 clonal lineage are summarized in Table 2. In the 2-3 clonal lineage, μ of clones was inversely related to the MTX level. The μ of 2-3 clone was 1.05±0.09 (±standard deviation, SD)/day, whereas the μ of 2-3-320 clone was 0.54±0.06/day. The maximum viable cell concentration of 2-3 clone was (2.19±0.51)×10^6 cells/ml, whereas that of 2-3-320 clone was (1.34±0.12)×10^6 cells/ml. As expected, q_{TPO} rapidly increased with MTX level up to 80 nM, and thereafter, a slight increase in q_{TPO} was observed. The q_{TPO} of 2-3-320 clone was 0.59±0.19 μg/10^6 cells/day which is 18% higher than that of 2-3-80 clone. Accordingly, since growth reduction outweighed q_{TPO} enhancement at 320 nM MTX, the expression level of TPO at 320 nM MTX was not higher than that at 80 nM.

To investigate whether the increased q_{TPO} of clones at an elevated MTX level was related to changes in TPO33r gene copy numbers, Southern blot hybridization was carried out. The relative gene copy numbers of clones in the 2-3 clonal lineage, which were determined by comparison of band intensities on Southern blots, are plotted as a function of MTX concentration in Fig. 4. The TPO33r gene copy number of amplified clones was normalized by that for 2-3 clone. Like q_{TPO}, TPO gene copy number rapidly increased with increasing MTX level up to 80 nM, and it slightly

Table 2. Values for μ, maximum viable cell concentration, q_{TPO}, and maximum TPO33r titer in cultures of clones in a 2-3 clonal lineage.[a]

Clone (MTX conc, nM)	μ (/day)	Max. viable cell conc. (10^6 cells/ml)	q_{TPO} (μg/10^6 cells/day)	Max. TPO33r titer (μg/ml)
2-3 (0 nM)	1.05±0.09	2.19±0.51	0.08±0.02	0.75±0.14
2-3-20 (20 nM)	0.83±0.04	1.66±0.42	0.24±0.07	2.43±0.62
2-3-80 (80 nM)	0.64±0.04	1.64±0.13	0.50±0.23	3.83±0.59
2-3-320 (320 nM)	0.54±0.06	1.34±0.12	0.59±0.19	3.53±1.13

[a] Means±SD (n=3). The experiments were performed three separate times.

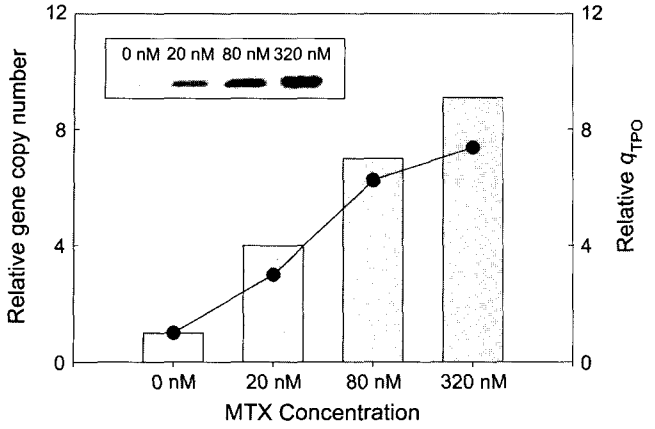

Fig. 4. Relative TPO33r gene copy numbers and q_{TPO} of clones in a 2-3 clonal lineage as a function of MTX concentration.
The band intensity in autoradiogram obtained by Southern blot analysis indicates the relative TPO33r gene copy numbers of clones in a 2-3 clonal lineage. The line indicates the relative q_{TPO} and the bar indicates the relative gene copy number.

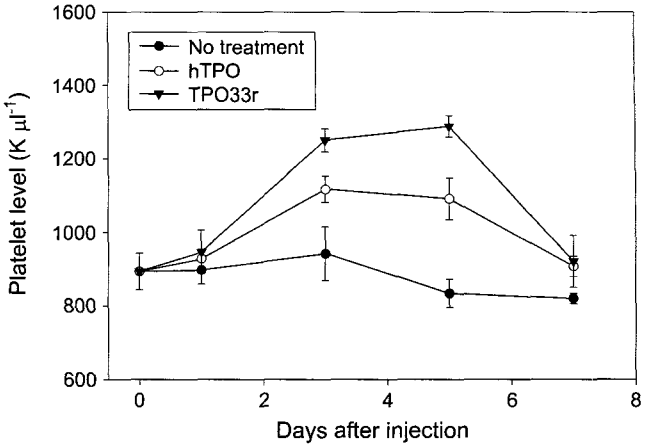

Fig. 6. Effect of hTPO and TPO33r on platelet elevation in normal female mice.
Normal female Balb/c mice were subcutaneously single-injected with the concentration of 10 μg/kg. Blood analysis was performed with a cell dyn 3500 eletronic blood cell analyzer. Data are represented as means±standard error of mean (n=4 or 5).

increased at 320 nM MTX. Accordingly, the increased q_{TPO} of clones at an elevated MTX level was related to changes in TPO33r gene copy numbers.

Northern blot hybridization was also carried out to quantify the relative TPO33r mRNA contents of clones at each MTX level. The mRNA level of amplified clones was normalized by that for 2-3 clone, and plotted as a function of MTX concentration in Fig. 5. A trend obtained in the quantitation of TPO33r mRNA content of clones in the 2-3 clonal lineage was similar to that in Southern blot analysis. Furthermore, q_{TPO} at 320 nM MTX was not enhanced as much as TPO33r mRNA content, suggesting that there might be translational and/or post-translational limitations in 2-3-320 clone. Compared with 2-3 clone, the q_{TPO} and

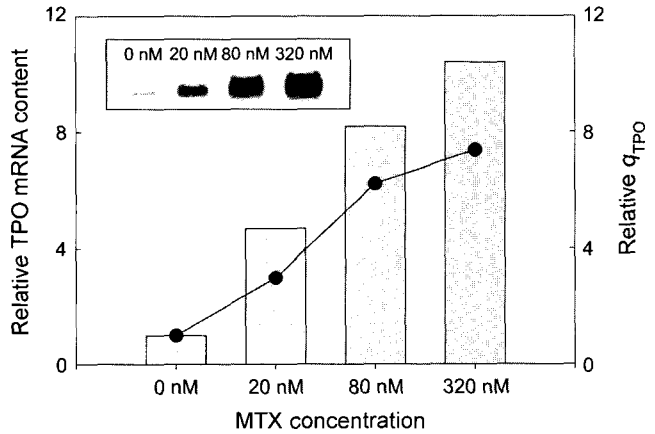

Fig. 5. Relative TPO33r mRNA levels and q_{TPO} of clones in a 2-3 clonal lineage as a function of MTX concentration.
The band intensity in autoradiogram obtained by Northern blot analysis indicates the relative TPO33r mRNA levels of clones in a 2-3 clonal lineage. The line indicates the relative q_{TPO} and the bar indicates the relative mRNA level.

TPO33r mRNA content at 320 nM MTX (2-3-320 clone) was increased by 640% and 810%, respectively.

Biological Activity of hTPO and TPO33r Produced from Established rCHO Cell Lines

As observed in rCHO cells producing human antithrombin III [26], overexpression of recombinant proteins with a very complex structure like TPO in CHO cells may result in the production of inactive recombinant proteins. Accordingly, to investigate the biological activities of hTPO and TPO33r produced from the established rCHO cell lines (1-434-80* and 2-3-80*), the *in vivo* mouse assay was performed. The TPOs produced from 1-434-80* and 2-3-80* exhibited a full biological activity which was determined by platelet elevation in mice upon TPO treatment.

Figure 6 shows the changes in platelet levels of mice after a single injection of hTPO or TPO33r at a concentration of 10 μg/kg. Both hTPO and TPO33r treatment elevated platelet levels in mice, but to a different degree. When mice were injected with hTPO or TPO33r, platelet counts started to increase rapidly one day after injection and reached the maximum levels 3-5 days after injection.

The sugar chain in a glycoprotein exerts various effects on the physical, chemical, and biological properties such as protein stability, secretion, *in vivo* biological activity, and pharmacokinetic properties [12, 13]. In order to improve the *in vivo* biological activity of native hTPO, a variety of hTPO muteins with one or more additional N-linked glycosylation sites were produced by substituting amino acids at specific positions in a hTPO protein. However, the introduction of additional sugar chains into hTPO was not always accompanied with an increase in its biological activity of hTPO (J. Y. Chung *et al.* 1999. PCT/KR99/

00347). In fact, the biological activities of most hTPO muteins with additional sugar chains were rather reduced, when compared with native hTPO. Furthermore, it was found that the specific glycosylation site rather than the number of sugar chains is crucial for elevating its biological activity. The TPO33r is an hTPO mutein in which one N-linked glycosylation site is introduced by substituting Arg to Asn at amino acid position 164 of hTPO (J. Y. Chung *et al.* 1999. PCT/KR99/00347). The biological activity of TPO33r was found to be superior to that of hTPO. Compared with the control group without TPO treatment, there was a 1.3-fold increase in the hTPO group and a 1.6-fold increase in the TPO33r group on the fifth day after injection.

In conclusion, rCHO cell lines that express a high level of fully biologically active hTPO or its analog, TPO33r, were established by transfecting expression vectors into dhfr CHO cells and subsequent two rounds of MTX-mediated gene amplification followed by limiting dilution. The strategy of establishing rCHO cell lines described here will be useful, particularly for the development of rCHO cell lines expressing a high level of highly complex glycoprotein.

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