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Biological Activity of Recombinant Human Thrombopoietin

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To investigate the function and secretion of human thrombopoietin (TPO) in mammalian cells, hTPO cDNA was cloned using human liver cDNA, and recombinant hTPO (rec-hTPO) was produced in CHO cell lines. In addition, six N-linked glycosylation sites were substituted for Ala to elucidate the role of each carbohydrate chain. To analyze the biological activity, rec-hTPO protein was injected subcutaneously. Blood was withdrawn for platelet determination. The metabolic clearance rate (MCR) was also analyzed at the 1, 4, 10 and 24 hr after tail vein injection. Wild-type TPO (WT) was efficiently secreted into the medium. However, a hTPO mutant with 116 deleted nucleotides detected by PCR cloning was not secreted. The N-linked glycosylation sites had nearly the same expression quantity as rec-hTPO WT apart from mutants 3 and 4. The glycosylation site of mutant 4 appeared to be an indispensable site for hTPO secretion. Also characterized was the biological activity through an injection with rec-hTPO (10 ng) to ICR mice (7 weeks). The result of the blood analysis showed a considerable increase in the platelet number six days after the injection. To analyze the pharmacokinetics, rec-hTPO was injected into the tail vein (5 ng). The result was 200 pg/ml 1 hr after this injection. Following this, it dramatically decreased and virtually disappeared 10 hours after the injection. Thus, rec-hTPO may be a treatment for thrombopenia by the production of the high active rec-hTPO. In addition, hTPO can permit the development of potent new analogues that stimulate the platelet value.

Key words : Thrombopoietin, recombinant, biological activity, glycosylation

Introduction

Human thrombopoietin (TPO) has an open reading frame encoding 353 amino acids consisting of a 21 amino-acid signal peptide and a mature human TPO molecule of 332 amino acids composed of two domains [2,4]. An amino terminal domain of 153 amino acids shows 23% identity to erythropoietin (EPO); this value approaches 50% similarity when conservative substitutions are taken into account. This domain also shows a low level of identity to interferon (INF)-α and INF-β [15]. The EPO-like domain of TPO contains four cysteins, three of which are conserved with EPO, including the first and last, which form an essential disulfide in EPO [15]. Similarly, the first and last cysteins of TPO form an essential disulfide [12] (Fig. 1). None of the asparagine-linked glycosylation sites present in EPO is conserved in the EPO-like domain of TPO. However, the EPO-like domain of TPO likely contains two or three O-linked glycosylation sites. The 181-amino-acid carboxy-terminal domain of TPO does not bear homology to any other known protein and contains six potential N-glycosylation sites [29].

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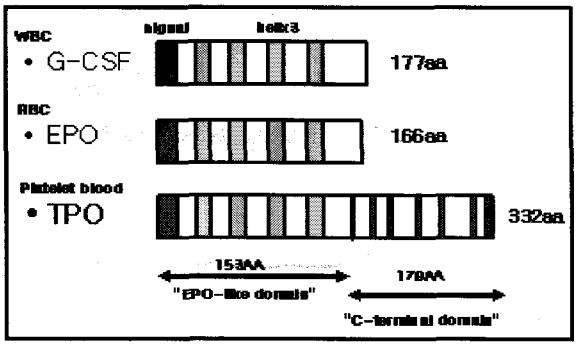

Fig. 1. The domain structures of TPO, EPO and G-CSF. The first amino acids of TPO are partly homologous to the EPO structure. The boxes represent the four predicted α-helical regions in the EPO-like domain. The second domain of TPO shows no obvious homology with any known protein structure. Potential N-linked glycosylation sites are indicated by boxes in the C-terminal domain [6]. Computer modeling suggests that the amino terminal domain (EPO-like domain) forms a four-α -helical bundle configuration similar to that found in other hemopoietic cytokines [3]. This structure would be expected to be stabilized by disulfide bonds between cysteins 1 and 4 and cysteins 2 and 3.

The model studied most thus far has been the response of megakaryocytopoiesis to acute thrombocytopenia. Acute thrombocytopenia has been induced by injection of platelet antiserum [25], by phlebotomy, by exchange transfusion with platelet-poor blood, or by injection of neuraminidase [28]. Hypertransfusing animals with platelets were achieved the decreased platelet demand [11]. Surprisingly, to suppress megakaryocytopoiesis, platelets needed to be sustained at a very high level for several days [25]. The effects of drugs and radiation on platelets and megakaryocytopoiesis have also provided important information relevant to the basic biology and clinical effects of these agents.

In earlier studies, recombinant glycoproteins were prepared in CHO-K1 cells, and it was found that deglycosylated sites varied the expression and biological activity of these recombinant derivatives [14,16-21,24,26]. In the present study, the expressing vector of TPO and its deglycosylated mutants were constructed. The biological activity of rec-hTPO was determined by the platelet value and pharmacokinetics *in vivo*.

Materials and methods

Reagents

The expression vector pcDNA3 was purchased from Invitrogen Life Technologies (CA, USA). CHO-K1 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Ham's F-12, CHO-S-serum free medium II (CHO-S-SFM II), G418 and Lipofectamine were from Gibco BRL (MD, USA). Fetal bovine serum was from Hyclone Laboratories (Utah, USA). Endonucleases were purchased from Takara Korea Biomedical Inc. (Seoul, Korea), Toyobo (Osaka, Japan) and Boehringer Mannheim (MA, USA). The PCR primers were synthesized by CoreBio System (Seoul, Korea), the QIAprep-spin plasmid kit was from QIAGEN Inc. (Hilden, Germany), and the TPO ELISA kit was from R&D systems Inc. (MN, USA). All other reagents used were from Wako Pure Chemicals (Osaka, Japan) or Sigma Aldrich (MO, USA).

Construction of TPO Transfer Vector

The primers [sense: 5'-Tgg TAC CAT ggA gCT gAC TgA ATT gCT CCT C-3' and antisense: 5'-TCT AgA TTA CCC TTC CTg AgA CAg ATT CT-3'] were designed from a previously reported nucleotide sequence of TPO cDNA [4]. A PCR template was used with human liver cDNA from Takara. PCR fragments were ligated into PCR2.1 Vector and sequenced completely to confirm the Kozak site and PCR errors. After they were digested with Kpn I - Xba I, the fragments were inserted into the same sites of pcDNA3 (pcDNA3-hTPO WT).

The mutant vector deleted a 116-nucleotide part was constructed. It cloned into the pcDNA3 expression vector (pcDNA3-deleted hTPO).

Mutagenesis of hTPO in the Glycosylation site

The recognition signal for N-linked glycosylation of protein is the tripeptide sequence Asn-Xaa-Thr/Ser. Site-directed mutagenesis was performed using the overlapping

Table 1. The primer sequences used

primer	Sense(F), Antisense(R)	Codon mutation
TPO cDNA	F 5′ - T ggT ACC ACC ATg gAg CTg ACT gAA TTg CTC CTC - 3′ R 5′ - TCT AgA TTA CCC TTC CTg AgA CAg ATT CT - 3′	
▲1	F 5′ - CTg AAC gAg CTC CCA gCA Agg ACT TCT ggA - 3′ R 5′ - TCC AgA AgT CCT TgC Tgg gAg CTC gTT CAg - 3′	Asn → Ala (197)
▲2	F 5′ - ggA TTg TTg gAg ACA gCA TTC ACT gCC TCA -3′ R 5′ - TgA ggC AgT gAA TgC TgT CTC CAA CAA TCC -3′	Asn → Ala (206)
▲3	F 5′ - ATT CCT ggT CTg CTg gCA CAA ACC TCC Agg -3′ R 5′ - CCT ggA ggT TTg TgC CAg CAg ACC Agg AAT -3′	Asn → Ala (234)
▲4	F 5′ - CAC gAA CTC TTg gCA ggA ACT CgT ggA CTC -3′ R 5′ - gAg TCC ACg AgT TCC TgC CAA gAg TTC gTg -3′	Asn → Ala (255)
▲5	R 5′ - CAg ATT CTg ggA gTg ggT gTA ggA TgT TgC TAg AAg -3′	Asn → Ala (340)
▲6	R 5′ - gA TCT AgA TTA CCC TTC CTg AgA CAg TgC CTg ggA gTg ggT -3′	Asn → Ala (348)

* Bold and underlined indicate the restriction enzyme sites in the primer sequence.
** gCA indicates the sites that changed in the amino acid.

PCR method. To substitute Asn (AAC) at the glycosylation site with Ala (CAG), each individual primer was designed (Table 1). The first PCR was performed using primers of TPO F and TPO ▲1R. The second PCR was amplified using primers of TPO ▲1F and TPO R. Finally, the fragments were purified and subjected to the third PCR using primers of TPO T and TPO R to generate TPO ▲1 cDNA, as shown in Fig. 2. The other mutants (▲2~▲4) were constructed using the same method. Mutant ▲5 formulated by TPO F and TPO ▲5 primers and the second PCR then amplified by TPO F and TPO R primers using the first PCR products. Mutant ▲6 amplified by TPO F and TPO ▲6 primer including the stop codon and glycosylation site in 3′ of the primer. After digestion with Kpn I - Xba I, the fragments were inserted into the same sites of pcDNA3 (pcDNA3-hTPO ▲1~▲6). All mutants were sequenced completely to confirm the Kozak site and PCR errors.

Cell Culture and Functional Expression

The expression vector (pcDNA3-hTPO WT) was transfected into CHO-K1 cells by the liposome formulation (Lipofectamine) transfection method, as previously described [19]. Rec-hTPO protein transfected by transient transfection was collected in the supernatants at 72 hr after transfection. Six or eight pools of stably transfected cells were selected by incubation in a growth medium [Ham's F12 media containing penicillin (50 U/ml), streptomycin (50 mg/ml), and glutamine (2 mM) and 10% FCS] supplemented with G418 (800 ug/ml) for 2-3 weeks of post-transfection according to a reported previously method [19]. After incubation of the selected stable cells (1×10^6) in 20 ml CHO-S-SFM-II at 37℃ for 48 hr, culture media were collected and centrifuged at 100,000x g at 4℃ for 60 min to remove the cell debris. Supernatants were collected and concentrated in an Amicon Stirred cell concentrator. The amount of rec-hTPO protein was quantified using ELISA.

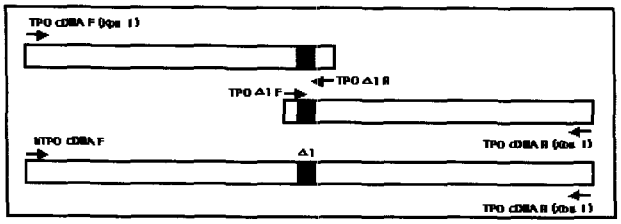

Fig. 2. PCR site-directed mutagenesis diagram. A two-step PCR method was used with site-directed mutagenesis, and two fragments produced were subjected to a third PCR using TPO F and R primers to generate TPO ▲1 cDNA, as described in the *materials and methods* section.

In Vivo Biological Activity and *in vivo* pharmacokinetics

The animal used were 8-9 weeks old mice under controlled lighting of 14 h light: 10 dark (lights on 05:00-19:00) and at a temperature of 22C. Mice were injected subcutaneously with rec-TPO WT (10 ng/200 ul) on days 0 and 2. Blood was withdrawn for platelet number determination at pretreatment and six days after the first injection. The platelet numbers were measured using a blood analysis system.

Before the administration of rec-hTPO, 70-80 ul of blood were collected from the eyes into heparinized micro-hematocrit tubes. Each animal received rec-hTPO via intravenous injections (5 ng/100 ul), and blood samples were obtained at 1 hr, 4 hr, 10 hr and 24 hr *thereafter*. All intravenous injections were performed by skilled technical personnel and monitored independently. The collected tubes were centrifuged, and serum was separated and analyzed by the ELISA method according to the protocol of the supplier (R&D Systems Inc.).

Results

PCR Amplification and Cloning of TPO cDNA

Using the cDNA prepared from human liver cDNA, PCR was used to amplify by the primers designed from sequences published previously [2,3]. The PCR result showed two amplified bands. Each fragment was subcloned into a pCR2.1 cloning vector and sequenced. One fragment was the same size (1,062 bp) as the hTPO cDNA. However, the other fragment had a 116-nucleotide part deleted (946 bp) (Fig. 3). The shift frame was changed by the deletion of the amino acids in the coding region. The stop codon was inserted in 5′ of the hTPO cDNA. The presence of a 116-base deletion that results in a frame shift after amino acid 138 and thereby encodes an entirely unrelated and somewhat truncated carboxyl-terminus was detected. Thus, the function of the deleted mutant should be checked.

Secretion of rec-hTPO Protein in CHO Cell Lines

To study the secretion of TPO WT, the 116-nucleotide deleted TPO part, and each deglycosylated mutant, expressing plasmids were transfected into CHO cell lines.

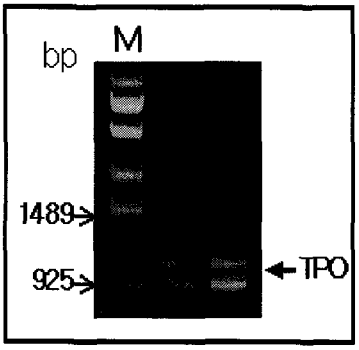

Fig. 3. PCR amplification of hTPO cDNA. The PCR products predicted with primers were analyzed by 1.0% agarose electrophoresis. The two fragments obtained, which comprised wild-type hTPO (1,062 bp) and the deleted mutant (946 bp), were amplified. M indicates markers (bp).

After collecting the supernatant of the cell, rec-hTPO concentration was analyzed by ELISA. WT-TPO was efficiently secreted. However, the deleted-hTPO was not secreted into the medium (Fig. 4). Thus, it was thought that the deleted protein was not secreted in the medium of the cell. Moreover, it was believed to have been inserted into the stop codon in the middle position due to the deletion.

The result of rec-hTPO analysis for six N-linked glycosylated sites was nearly identical in terms of the expression with hTPO WT, apart from mutants 3 and 4 (Fig. 5). However, a slight decrease was shown in the mutant 3 and mutant 4, by nearly 40% compared to that of hTPO WT. Thus, the glycosylated site of mutant 4 is believed to be an essential site for hTPO secretion.

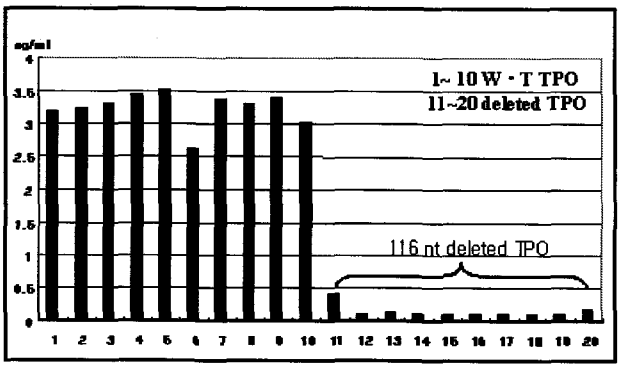

Fig. 4. The quantity of rec-hTPO WT and deleted hTPO expressed in CHO cells. ELISA was analyzed the rec-hTPO expressed by transient method. (1~10: wild-type; 11~20: mutant with 116 nucleotides deleted). Bar was shown the thrombopoietin concentration (ng/ml) of each clone. Rec-hTPO WT and deleted hTPO were replicated 10 times to analysis the expressing concentration of rec-hTPO.

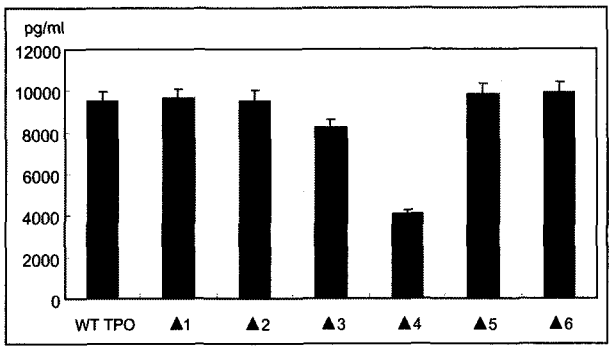

Fig. 5. The transient expressing quantity of rec-hTPOWT and TPO mutants changed by Ala in glycosylated sites. ELISA was analyzed the transient expression quantity of rec-hTPO. Experiments were performed in triplicate (WT: wild-type; ▲1~▲6: for each deglycosylated mutant, as described in the *materials and methods* section). Bar was shown the thrombopoietin concentration (ng/ml). Bars sharing a different letter are significantly ($P<.01$) different from each other.

In Vivo Biological Activity

Rec-hTPO (10ng) was injected on days 0 and 2 into ICR mice. The result of a blood analysis showed a highly increased platelet number six days after the injection. The mean platelet values had increased considerably by 1 - 1.5-fold on day 6 after the injection (Fig. 6). These results indicate that rec-hTPO produced in CHO cell lines has sufficient biological activity *in vivo*. Thus, it is believed that rec-hTPO would be a potent analogue to increase the platelet number.

Pharmacokinetic Analyses

To analyze the pharmacokinetics, rec-hTPO WT was

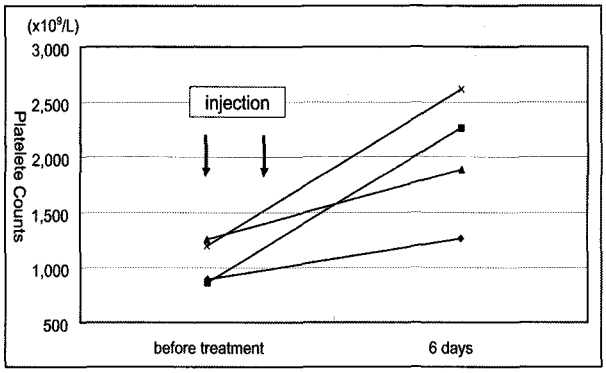

Fig. 6. The change of the platelets by injection of rec-hTPO WR in mice. Four ICR mice were injected subcutaneously with 10ng of rec-wthTPO on day 0 and 2. Platelets were determined at pretreatment (0 day) and at six days after injection. Values are given for each mouse.

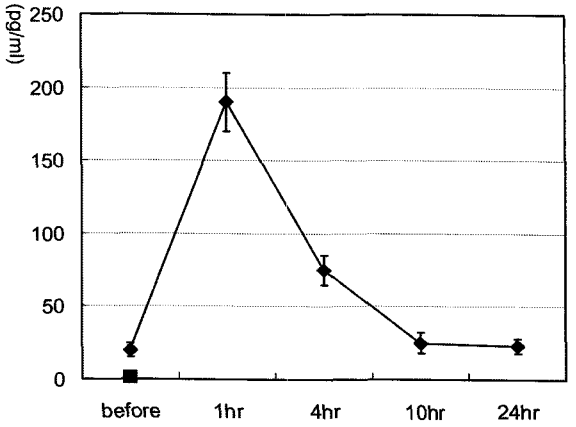

Fig. 7. Pharmacokinetic analysis for wild-type hTPO (5 ng/100 ul). Four ICR mice were injected intravenously with 5ng of rec-wthTPO. At the indicated times, blood samples were obtained, and the plasma concentration of TPO was determined by ELISA. Values were shown the thrombopoietin concentration (pg/ml). Values shown are averages for four mice.

injected into the tail vein (5 ng/100ul). Blood was collected from the eye at 1, 4, 10 and 24 hr after the injection. The findings were 200 pg/ml one hour after the injection. Past this time, this amount dramatically decreased; 10 hours after the injection it had nearly disappeared (Fig. 7). Although it was rec-hTPO WT, the hTPO level was abruptly decreased. Thus, derivative rec-hTPO proteins to address this are a topic of future research.

Discussion

In the present study, it was shown that rec-hTPO introduced into the CHO cell lines enhanced the biological activity *in vivo*. The glycosylated site in the number 4 mutant was shown to plays a role in rec-hTPO secretion. In addition, deleted mutants did not produce any protein in the supernatant of culture cells.

A G → C transversion has been found in the splice donor site of intron 3 of the hTPO gene in all affected family members. This mutation leads to TPO mRNA with shortened 5'-untranslated regions (UTR) that are more efficiently translated compared to normal TPO transcripts. Thus, it was concluded that a splice donor mutation in TPO leads to systemic overproduction of TPO and causes thrombocythaemia [30]. To assess the consequences of the mutation, the deleted mutant was used with transfection. However, rec-TPO protein was not detected in the supernatant of the culture cell. Wiestner *et al.* [30] reported that TPO overproduction by the mutant allele might result from enhanced translation of TPO protein due to the altered signal peptide N terminus. It has been reported that deletion of a single G nucleotide (▲G) in exon 3 of the TPO gene was found to cosegregate with thrombocytosis and elevated TPO serum levels in Japanese kindred with thrombocythemia [13]. The ▲G mutation affects the 5'-untranslated region (5'-UTR) of the TPO mRNA, strongly suggesting that ▲G mutation is responsible for thrombocythemia in this family. However, the molecular mechanism of how this mutation causes TPO overproduction was not elucidated, and in particular, no evidence for increased RNA stability or a more efficient translation of the mutant TPO mRNA was found [13]. Ghilardi *et al.* [7] reported that the 5'-UTR of TPO mRNA exerts a strong inhibitory effect on translation. This inhibition is mediated by the presence of several upstream open reading frames (uORFs) that prevent efficient translation of TPO. The deleted position differs from the findings of other studies. The position is the exon 6, which was deleted 116-nucleotide part. Thus, this frameshift changes and was found to have a very strong inhibitory effect on translation.

The result of a rec-hTPO analysis for six N-linked glycosylated sites showed an expression that was nearly identical to hTPO WT apart from mutants 3 and 4 (Fig. 5). However, mutant 4 showed about 40% compared to that of hTPO WT. Thus, the glycosylated site of mutant 4 is likely an essential for hTPO secretion. Little is known regarding the function and role of each glycosylation site. Thus, it is believed that the glycosylation sites should be elucidated more clearly in order to obtain information regarding protein translation.

Interestingly, a potential dibasic proteolytic cleavage site that is conserved among the various species examined separates the two domains of TPO. Processing at this site may be responsible for the release of the C-terminal region from the EPO-like domain *in vivo* [1]. The 181 amino-acid carboxy-terminal domain of TPO contains six potential N-glycosylation sites. In the case of EPO, unglycosylated forms are fully active *in vitro*, but are relatively inactive *in vivo* due to rapid clearance [29]. Similarly, a truncated form of TPO consisting of only the EPO-like domain is fully functional *in vitro*, but significantly less active *in vivo* when compared with glycosylated full-length TPO [5]. The functional significance of the C-terminal domain is under investigation. No statistically significant structural sim-

ilarities to entries in the GeneBank database have been found for this domain. One notable feature is the abundance of serine, threonine, and proline residues. One function of this domain might to be to stabilize and enhance the circulating half-life of TPO, as has been shown for the glycosylated form of EPO [23].

Hill *et al.* [8] also developed a platelet-production bioassay. Selenomethionine incorporation into platelets was determined after the injection of test materials, partially purified, into mice. Miyake *et al.* [22] reported that the administration of the partially purified protein to this animal model increased 40% after three days. The number of splenic megakaryocytes was also found to increases. The biological activity was not affected by treatment with neuraminidase. TPO purified from thrombocytopenic canine plasma has been shown to stimulate megakaryocytes. In vitro, the plasma-derived TPO increased the number, size, and ploidy of megakaryocytes [10,27].

Pharmacokinetic analyses of full-length, glycosylated TPO versus the EPO-like domain reveal that the former has roughly 10 times the circulating half-life of the latter after subcutaneous administration into rats [6]. The truncated forms of TPO have nearly 20-fold increased specific activity on U/kg basis relative to full-length TPO *in vitro*. These molecules were subcutaneously injected into mice daily for 5 days in graded doses (ug/kg/day). Two days after the last injection, platelet counts were measured in an automatic blood cell analyzer. Full-length glycosylated TPO was significantly more effective at increasing platelet counts compared to the EPO-like domain, with or without glycosylation [6,9]. The greater in vivo activity of PEG-rec-hTPO relative to the unconjugated EPO-like domain is in good agreement with the longer circulating half-life of the former molecule relative to the latter [6]. When pegylated, the *in vivo* activity of this portion of the molecule increases substantially. Nonetheless, the EPO-like domain appears to contain the active portion of the molecule. Thus, the glycosylation site of TPO likely has full biological activity.

Thus, rec-hTPO may be useful as a treatment for thrombopenia due to the production of the high active rec-hTPO, as believed after an analysis of information gained from both genetic and biochemical approaches. It is believed that this can permit thrombopenia development of potent new analogues that stimulate the platelet count. The constructs of the thrombopenia glycosylated mutant molecule will be useful in the study of mutants that affect monomeric association and/or secretion processes. A site-directed mutagenesis analog can be constructed to include additional specific bioactivity, generating potentially efficacious results. Recombinant TPO including mutants that lack oligosaccharides will be useful tools for analyzing the structure-function relationships.

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초록 : 유전자 재조합 인간의 Thrombopoietin의 생리활성

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사람의 혈소판조절인자 (TPO)의 분비와 기능을 분석하기 위하여 사람 간 cDNA로부터 TPO cDNA를 분리하여 동물세포에서 재조합체를 생산하였다. 또한, 당쇄의 기능분석을 위하여 6개의 당쇄첨가부위를 Ala으로 치환하여 각각의 돌연변이체 재조합체도 생산하여 이들의 생리활성분석을 위하여 피하주사하여 혈소판의 증가여부를 분석하였으며, 체내 약동학검사를 위하여 꼬리 정맥에 재조합체를 주사하여 24시간까지 혈액을 채취하였다. Wild-type TPO는 효과적으로 분비하였으나, 크로닝에서 분석되어진 116개 아미노산이 삭제된 돌연변이체는 배양상층으로 분비되지 않았다. N-linked 당쇄첨가 부위는 3번과 4번을 제외하고는 거의 비슷한 발현양상을 나타내었다. 특히 4번당쇄부위는 TPO의 분비에 중요한 역할을 하는 것으로 나타났다. 재조합체 10ng을 피하주사에 의하여 체내 혈소판이 유의적으로 증가하였으며, 5ng을 이용한 약동학 분석결과 1시간에 최대로 증가하였으며 그 이후 급격하게 감소하여 10시간에는 거의 존재하지 않았다. 따라서, 이러한 연구는 고 활성을 가지는 유전자 재조합체 TPO의 생산을 가능하게 하고, 또한 새로운 분자의 TPO를 가능하게 할 것으로 사료된다.