J. Biochem. **142,** 17–24 (2007)
doi:10.1093/jb/mvm111

Secretion Expression and Activity Assay of a Novel Fusion Protein of Thrombopoietin and Interleukin-6 in *Pichia pastoris*

Hu Zhiyi[1], Lai Wenshan[2], Zhang Wenze[3], Deng Ning[4], Zhang Chi[2], Ye Kenan[3], Wang Ping[3], Wang Qianqian[3] and Zhang Qing[3,*]

[1]National Key Discipline Department Of Internal Medicine, The First School Of Medicine, Guangzhou University of Chinese Medicine 510405, China; [2]School of Pharmaceutical Sciences, Zhongshan (SUN YAT-SUN) University; [3]Biotechnology Research Center, Key Laboratory Of Gene Engineering of the Education Ministry, School of Life Science, Zhongshan (SUN YAT-SUN) University, Guangzhou, 510275, China; and [4]College of Life Science and Technology, Jinan University, Guangzhou, 510632, China

Received March 4, 2007; accepted March 22, 2007; published online May 21, 2007

Thrombopoietin (TPO) is an important haematopoietic factor in megakaryocytic activities as well as in platelet production. Interleukin 6 (IL-6) can co-stimulate TPO-dependent formation of colony forming unit of megakaryocyte (CFU-Meg) growth which could be responsible for residual platelet formation in TPO-deficient or *c-mpl*-deficient animals. In this report, we demonstrated the development of a high-level expression system to produce a 78-kDa human fusion protein IL-6/TPO (named ZH646). This was achieved by constructing the expression vector pPICZα-A-IL-6-linker-TPO, and obtained the recombinant yeast GS115, which then efficiently secreted into a medium with a yield of 30 mg/l from the supernatant of the yeast culture in flask. ZH646 was then purified using two steps via DEAE-Sephacel chromatography and Mono Q columns. Activity assay showed that ZH646 could significantly stimulate the formation of CFU-Meg and the proliferation of Dami cells *in vitro* in a dose-dependent manner. In addition, ZH646 also showed thrombopoietic effect in normal mice, and the ability to enhance recovery of normal platelet counts after myelosuppression mice. These results suggested that ZH646 is a novel protein, and its activities are much stronger than that of TPO or IL-6 alone. ZH646 therefore has a broad spectrum of megakaryopoiesis activity associated with platelet production.

Key words: expression, fusion protein (IL-6/TPO), Pichia pastoris, platelet production, purification.

Abbreviations: CFU-Meg, colony forming unit of megakaryocyte; EPO, erythropoietin; FL, Flt-3 ligand; IL-3, interleukin 3; IL-6, Interleukin 6; IL-11, interleukin 11; SCF, stem cell factor; TPO, Thrombopoietin.

Thrombopoietin (TPO) is an important cloned cytokine with multiple effects in the haematopoietic system (*1*). Its functions include differentiation and proliferation of megakaryocytes as well as platelet production (*2*, *3*). TPO acts synergistically with other haematopoietic cytokines such as stem cell factor (SCF), interleukin 11 (IL-11), interleukin 3 (IL-3), Flt-3 ligand (FL) and erythropoietin (EPO) to promote megakaryocyte development (*4*, *5*). Nevertheless, it is widely accepted that cytokines IL-6 possess a significant megakaryopoietic potential, and IL-6 promotes the *ex-vivo* expansion of haematopoietic stem and progenitor cells in the early stage of megakaryocytic development (*6–8*). Subsequently, some of these cytokines that were thought to promote megakaryocytic differentiation and platelet production such as a number of gp130 protein-signalling cytokines were tested. It was found that only IL-6 co-stimulated TPO-dependent CFU-Meg growth, which was felt to be responsible for residual platelet formation in TPO- or *c-mpl*-deficient animals (*9*). The biological basis for these findings was the capacity of IL-6 to synergistically interact with TPO in megakaryopoiesis and thrombopoiesis. To further explore the feasibility of IL-6 and TPO fusion production, a gene for truncated IL-6, artificial linker and mature TPO was synthesized by using the favoured genetic codons of the yeast *Pichia pastoris*. The fusion gene was then cloned and inserted into the vector pPICZα-A to construct the expression vector and expressed in yeast. The function analysis of this novel fusion protein (ZH646) in megakaryocytic development, cell proliferation and platelet production were carried out both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Plasmids, Strains and Cell Lines—The cloning vector pBluescript SKII, the *Escherichia coli* strains Top10 and the yeast expression system for *P. pastoris* EasySelect™ were purchased from Invitrogen. Dami cell lines were purchased from the experimental animal centre of Sun Yat-sen University. These cells in our laboratory were

*To whom correspondence should be addressed. Tel: 86-20-84113988, Fax: +86-20-84036551, E-mail: lsszq@mail.sysu.edu.cn

free from mycoplasma contamination and maintained routinely.

Reagents and Media—Restriction endonucleases, T4 DNA ligase and DNA polymerase were purchased from Takara Biotech. Yeast extract and peptone were purchased from Clontech. The recombinant human IL-6 and the human TPO were purchased from Pierce and Clonetech, respectively, prepared in 20 mM Tris·Cl (pH 8.0), 0.9% NaCl and 0.25% albumin (BSA). Polyclonal rabbit anti-human IL-6 and TPO antibody were obtained from Jingmei Biotech. Yeast complete medium was YPG (Yeast extract 1%, peptone 1% and glucose 2%). YNBG contained yeast nitrogen base 0.67%, glucose 1% supplemented with 0.5% casamino acids lacking uracil. The YPD/Zeocin plates which contained 1% yeast extract, 20 g/l peptone, 20 g/l dextrose and 100 mg/l of Zeocin. BMGY medium contained 1% yeast extract, 20 g/l peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base with ammonium sulphate, 0.4 mg/l biotin and 1% glycerol. BMMY medium contained the same components as BMGY except for the substitution of 1% glycerol by 0.5% methanol. TBS–Tw solution contained 20 mM Tris–HCI, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 0.5% skim milk, 0.5% BSA.

Animals—Female Bablc/c mice from the experiment animal centre of Sun Yat-sen University, aged 8–12 weeks, were used for this study under Institutional Animal Care and Use Committee-approved protocols. The animals were maintained under depurated laboratory conditions of our laboratory for at least 1 week before use.

The Primers of the Truncated IL-6, the Linker and TPO—The primers were used for the truncated IL-6 cDNA PCR amplification from pCRscript SK (+)-IL-6 (constructed in our laboratory):

P1(5′-CGGAATTCATGATTGACAAACAAATTCGG-3′) and P2 (5′-CGCGGATCCTTACATTTGCCGAAGAG-3′). The linker primers were designed by using the computer software GOLDKEY: P3 (5′-GGCGGTGGCTCTGGTGG ATCCGGT-3′) and P4(5′ACCGGATCCACCCAGAGCCAC CGCC-3′). The primers were used for mature TPO PCR amplification from pBD42-TPO (constructed in our laboratory): P5 (5′-TCTCGAGAAAAGAGCCACCAGAAG ATACTAC-3′) and P6 (5′-A GCGGCCGCTCAGTAGAGGT CCTGTGC-3′).

Transformation and Expression in P. pastoris—The plasmid pPICZα-A-IL-6-linker-TPO was linearized with *Sac*I and transfected into GS115 by lithium acetate method as described in the Invitrogen manual. The transformants were selected on YNBG medium, and the recombinants were selected by plating cells on YPD/Zeocin plates. The high expression strain was cultured in 500 ml of BMGY medium, and shaken at 250 r/min at 30°C until an OD_{600} reached 8. Cells were then harvested by centrifugation at 4500 r/min for 10 min, and re-suspended in 150 ml of the BMMY induction medium. Methanol was added at 24-h intervals to keep a final concentration of 0.5%. Supernatants obtained at 24, 48 and 72 h were assayed for the recombinant protein by SDS–PAGE immediately or stored at –20°C.

Immunological Detection of ZH646—The recombinant protein ZH646 secreted by yeast was analysed by 10% SDS–PAGE, followed by western blotting. Proteins were transferred to polyvinylidene difluoride membranes (Milipore Co.) by a semi-dry transfer (Transblot, Bio-Rad; 15 V, 30 min). The membrane was soaked in TBS–Tw solution at 4°C overnight. After washing, the membrane was incubated with the primary rabbit polyclonal antibody against human IL-6 (2 pg/ml) or TPO (2 pg/ml) in TBS–Tw for 1 h at room temperature. After washing, the membrane was incubated with the secondary HRP-conjugated goat antibody (Sigma Co. 1 : 1000 dilution in 1% albumin/PBS-buffer) in TBS–Tw for 1 h at room temperature.

Purification of ZH646—The broth of the culture was centrifuged to remove yeast cells. The supernatant concentrated sample was then loaded onto a DEAE-Sephacel column (Pharmacia) at a flow rate of 1 ml/min, which was pre-equilibrated with 50 mM Tris–HCl, pH 8.0. The column was developed with a linear 0–1 M gradient of NaCl in 50 mM Tris–HCl buffer, pH 8.0. The concentrated solution was further purified by FPLC on Mono Q column (Pharmacia) using 50 mM Tris–HCl, pH 8.0 buffer. The peak fractions were pooled and analysed by SDS–PAGE. The concentration of the purified protein was determined using the BCA kit (Pierce).

CFU-Meg Colony Assay in vitro—Murine bone marrow cells were harvested from mice by flushing the femoral contents with Iscove's modified Dulbecco's medium (IMDM). Marrow cells (1×10^5) were cultured in 0.20% agar of IMDM in the presence of 0.12 nM ZH646, 0.15 nM recombinant human TPO, 10 nM IL-6 0.15 nM TPO plus 10 nM IL-6, respectively. Cultures were incubated at 37°C in a fully humidified atmosphere supplemented with 5% CO_2. Agar was dried and stained for acetylcholinesterase (AChE) activity to identify and count megakaryocyte colonies. At day 7, groups of 3–50 $AChE^+$ cells clustered together were scored as megakaryocyte colonies (CFU–MK). Colonies that contained >21–50 $AChE^+$ cells were scored separately and reported as large CFU–MK.

Cell Proliferation Assay by MTT—The MTT assay was performed according to the manufacturer's recommendations (Promega). Dami cells were cultured in microplates (6×10^4 cells/well) in 100 μl RPMI-1640 medium containing 0.5% bovine serum albumin (BSA) with various concentrations of ZH646 or rhTPO (10 μM by 2 dilution). The blank control wells had an equal volume of PBS. MTT (5 mg/ml, pH 7.4) was added after 48 h of incubation within CO_2 incubator at 37°C, 5% CO2 and 95% humidity. Ten percent SDS was added to each well and the plates were incubated for 3–4 h. The absorbance at 570 nm was measured with a microplate reader (Model 3550, Bio-Rad).

Administration of the Recombinant ZH646—The 30 μg/kg ZH646, 10 μg/kg TPO plus 10 μg/kg IL-6 and PBS were injected intraperitoneally into normal mice daily for 7 days, respectively. The platelet numbers were measured at a constant interval. The mouse model of cyclophosphamide-induced thrombocytopenia was established according to prior methods described in our lab (*10*). Platelet counts were monitored by F-820

Sysmex (Sysmex Shanghai Ltd) for a 20-day recovery period.

Statistical Analysis—Results were expressed as mean ± SD with data obtained from each experiment conducted in triplicate. Analysis of variance with a one-tailed Student's *t*-test was used to identify significant differences in multiple comparisons.

RESULTS

Construction of Expression Vector Encoding the Fusion Protein IL-6/TPO—As shown in Fig. 1, both the truncated IL-6 cDNA fragment and the linker were ligated and amplified to form the expected 500 bp as determined on 1.0% agarose gel. The PCR product and plasmid pZICZα-A were cleaved by *EcoR*I and *Pst*I digestion, and then collected and linked to form the cloning vector pZICZα-A-IL-6-linker by T4 ligase. The full-length cDNA sequence of TPO was amplified by PCR using *Pfu* DNA polymerase from pBD42-TPO, and the expected 1000 bp as determined by 1.0% agarose gel was then acquired. The TPO PCR product and plasmid pZICZα -A-IL-6-linker were cleaved by *Pst*I and *Xba*I digestion. It was then collected and inserted into the downstream of IL-6-linker as well as linked to form the cloning vector pPICZα-A-IL-6-linker-TPO by T4 ligase. Identification of pPICZα-A-IL-6-linker-TPO was carried out by *EcoR*I and *Xba*I digestion, and the expected 1600 bp as determined by 1.0% agarose gel was then obtained.

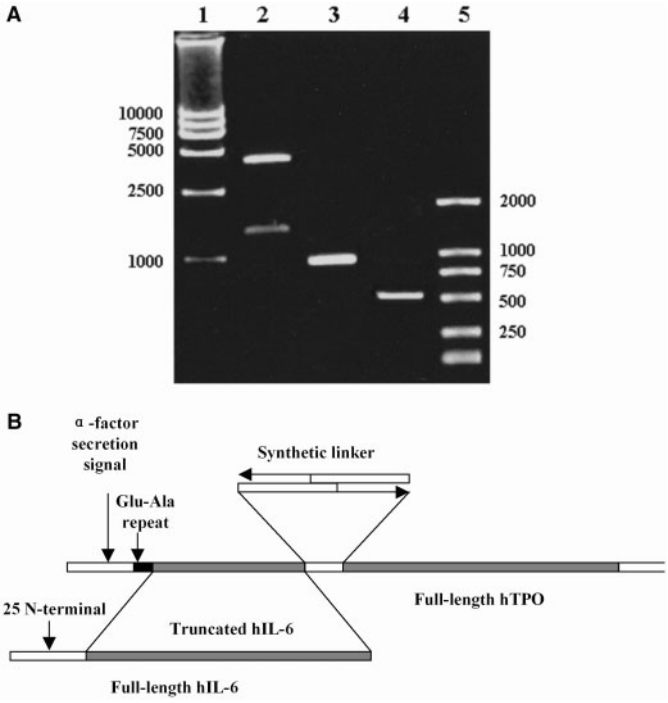

Fig. 1. **Cloning of synthetic IL-6-linker-TPO fusion gene.** (A) Agarose gel analysis of synthetic gene products and restriction digests of IL-6-linker-TPO fusion gene fragment. Lane 1, molecular mass standard 15,000 bp fragment ladder; Lane 2, pZICZα-A-IL-6-linker-TPO purified digested by *EcoR*I and *Xba*I digestion; Lane 3, PCR product of cDNA encoding mature TPO; Lane 4, PCR product of cDNA encoding truncated IL-6 and linker; Lane 5, molecular mass standard 2000 bp fragment ladder. (B) Schematic presentation of the nucleotide sequence of gene fragments (hIL-6, linker, and hTPO) used for final assembly of rhIL-6/TPO fusion gene in plasmid pZICZα-A-IL-6-linker-TPO. The native signal sequences of hIL-6 and hTPO were deleted so that the expression product could be secreted under the mediation of N-terminal α-factor signal peptide. Arrows point at the relative positions of α-factor secretion signal sequence, the Glu-Ala repeat which is removed by the yeast dipeptidyl aminopeptidase, and the lack of 25 N-terminal of IL-6 protein, respectively.

Expression of the ZH646 in P. pastoris—The *P. pastoris* colonies that grew on the YPD/Zeocin plate were tested for expression, in which the ZH646 was produced using the expression plasmid pPICZα-A-IL-6-linker-TPO. This heterologous gene can be integrated into *P. pastoris* chromosome by integrative expression plasmid, which generated genetic stable secretion expression strain. The native signal sequences of IL-6 and TPO were deleted so that the expression product could be secreted under the mediation of N-terminal α-factor signal peptide. As shown in Fig. 2A, SDS–PAGE analysis revealed a new band at 78 kDa after induction, suggesting that the secretion expression protein was isolated from culture supernatant successfully. The maximum yield of ~30 mg/l of culture was obtained at 72 h after induction. As shown in Fig. 2B, the western-blot analysis showed that the positive band with about 78 kDa molecular mass was recognized by TPO antibody and IL-6 antibody, respectively.

Purification of the ZH646 Expressed in P. pastoris—GS115 cells secrete only very low levels of endogenous host proteins, which would simplify purification of the recombinant protein. As shown in Fig. 3, the primary protein (lane 3) was separated through eluting the other yeast-derived protein from the soluble rude proteins (lane 2) by ion-exchange chromatography on DEAE-Sephacel column. The collected ZH646 fractions (lane 4), followed by further purification via a Mono Q column, were pooled and showed a single protein band on

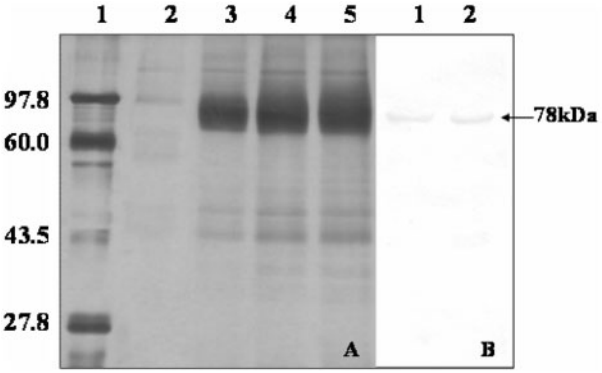

Fig. 2. **Expression and identification of the ZH646. (A) Analysis of induced expression supernatant.** Lane 1, protein marker; Lane 2, supernatant before induction; Lane 3, supernatant at 48 h; Lane 4, supernatant at 60 h; Lane 5, supernatant at 72 h. (B) **Western-blot analysis of the ZH646.** Lane 1, expression supernatant after induction (rabbit anti-serum TPO); Lane 2, expression supernatant (rabbit anti-human IL-6).

a polyacrylamide gel. The final yield of purified protein was 30 mg/l from shake flask.

Stimulates of Proliferation of Human CFU-Meg Progenitors by the ZH646—To understand how ZH646 may affect early megakaryopoiesis, we made an observation of ZH646-stimulated proliferation of mice CFU-Meg progenitor from femur marrow of normal mice, and compared it with that of TPO+IL-6, TPO and IL-6 cytokines, respectively for 7 days. The number of CFU-Meg containing 5–10 and 11–20 cells in the ZH646 group and TPO+IL-6 group was slightly higher than that of the TPO group, although it was statistically insignificant. The proportion of larger colonies (contained ≥20–50 cells) was significantly higher in cultures under ZH646 and TPO+IL-6 treated conditions than those under TPO treated conditions. In the event of affecting slightly CFU-Meg containing 5–10 cells, IL-6 do not show stimulated proliferation of mice CFU-Meg progenitor in CFU-Meg containing 11–20 and 21–50 cells (Table 1). Meanwhile, the sizes of CFU-Meg were measured at 7 days of cultures. ZH646 and TPO+IL-6 also seemed to induce a remarkable increase in the size of these colonies. In fact, at least 50% increase in size and number of cells in the ZH646 and TPO+IL-6 treated colonies were observed when compared with those treated by TPO. TPO is synergistic with IL-6 on day 7 CFU-Mk but did not contain larger and more mature individual megakaryocytes (Fig. 4).

Determination of Effect of ZH646 on the Growth of Dami Cell Line—We further used the MTT assay to compare the effect of ZH646 and rhTPO cytokines on Dami cell proliferation. As shown in Fig. 5, both ZH646 and rhTPO significantly stimulated the proliferation of Dami cells at concentration of 39–10 μM in

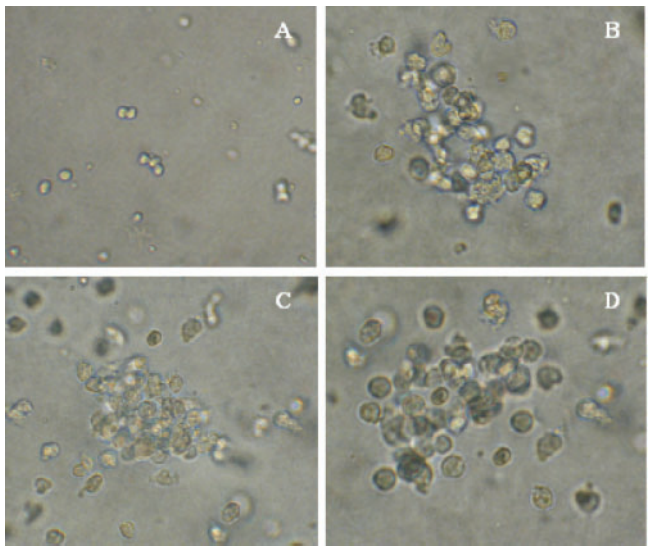

Fig. 4. **Sizes of the CFU-Meg colonies induced by ZH646, TPO+IL-6, TPO and IL-6, respectively after 7 days of culture** (original magnification, ×200. (A) IL-6 group; (B) TPO+IL-6 group; (C) TPO group; (D) ZH646 group).

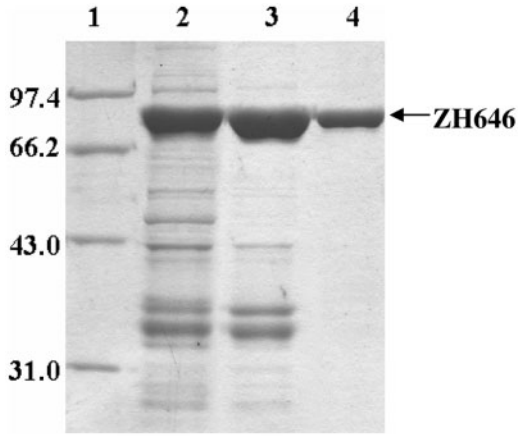

Fig. 3. **SDS–PAGE analysis of purified the ZH646 using 10% polyacrylamide gel.** Lane 1, protein molecular mass standards; Lane 2, crude proteins; Lane 3, proteins purified by DEAE-Sephacel column; Lane 4, fusion protein purified by Mono Q column.

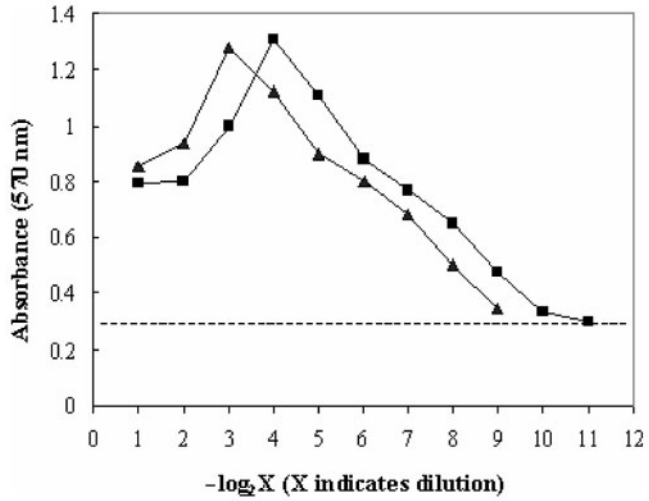

Fig. 5. **Effect of ZH646 or TPO on Dami proliferation respectively.** Dashed line indicated response in the presence of rhTPO or ZH646. (filled triangle) rhTPO, (filled circle) ZH646.

Table 1. **Numbers of CFU-Meg colonies induced by ZH646 and TPO respectively.**

Colony size (no. megakaryocytes)	TPO 7 d culture	IL-6 7 d culture	TPO+IL-6 7 d culture	ZH646 7 d culture
5–10	26.2 ± 2.2	3.3 ± 2.1	30.3 ± 4.1	32.0 ± 4.7
11–20	41.0 ± 3.4	0	44.3 ± 5.1	46.0 ± 2.9
21–50	15.6 ± 5.1	0	18.3 ± 2.2*	21.6 ± 3.1*

Sizes of the CFU-Meg colonies were estimated according to the number of cells included in an individual colony (5–10, 11–20, and 21–50 megakaryocytes). Results represent the mean ± SD of CFU-Meg colonies in three independent experiments. *$P < 0.05$.

a dose-dependent manner, with maximum stimulation occurring at 5.0 μM and 2.5 μM, respectively. The proliferation activity of ZH646 has an effective concentration of 0.2 Mm (EC50 = 0.2 μM), while appeared to be more potent than that of rhTPO (EC50 = 0.3 μM), the mean values on the examined Dami cells was statistically insignificant. Therefore, it is reasonable to conclude that the Pichia-derived ZH646 has a similar specific activity like TPO.

The Thrombopoietic Effect of ZH646 on Normal Mice—Balb/c mice were treated with intraperitoneal injections of 0.5 ml of a vehicle control, ZH646 and TPO+IL-6, respectively, for up to 7 days. As shown in Fig. 6, ZH646 could lead to increased platelet count, which plateau at a level 25–35% higher than that in the control at day 7, with an average platelet count of 2900×10^6/ml. Meanwhile, TPO+IL-6 also increased platelet count to 2050×10^6/ml, which was lower than ZH646 at day 7. Furthermore, ZH646 also maintained a high platelet count in these mice during days 4–10 of the treatment (i.e. count exceeded normal level 6 out of 7 days during treatment) and returned to normal on day 12. TPO+IL-6 only stayed a high platelet count at one day, and rapidly dropped the normal platelet values after day 7.

Normal Platelet Recovery From Cyclophosphamide-induced Thrombocytopenia—We also analysed the recovery of platelet number after myelosuppression induced by cyclophosphamide. As shown in Fig. 7, although the 50% reduction rate of platelets were very similar in the three groups, platelet number returned to pre-treated levels at day 7 after ZH646 and TPO+IL-6 injection, respectively. On the other hand, platelet number exhibited a more pronounced nadir and slower recovery from thrombocytopenia in the control group. ZH646 also maintained a higher platelet count in these mice on days 7–12 compared with those in the control group and TPO+IL-6. On day 12, the average platelet count was 2400×10^6/ml, 2050×10^6/ml in the ZH646 and TPO+IL-6-treated mice, respectively compared with 1600×10^6/ml in the control mice ($P < 0.01$). In addition, TPO in combination with IL-6 do not accelerate platelet recovery by 7–11 days over those stimulated by the control group and an inability to exert return of the platelet count toward normal, except for day 12 ($P < 0.05$).

DISCUSSION

TPO was a major growth factor in the differentiation and proliferation of megakaryocytes as well as platelet production (*1*, *4*). Recently, it was reported that TPO is not essential for the regulation of platelet circulation, as mice with 'knockout' of TPO and TPO receptor (*c-mpl*) do not seem to be more vulnerable to haemorrhage and could retain ~15% of the peripheral platelets (*1*, *11*). It is possible that other ligands or some other cytokines might compensate for TPO deficiency to promote platelet production. Current models suggest that early-acting MK colony-stimulating factors, such as IL-3 or c-kit ligand, are required for expansion of haematopoietic progenitors into cells capable of responding to late-acting MK

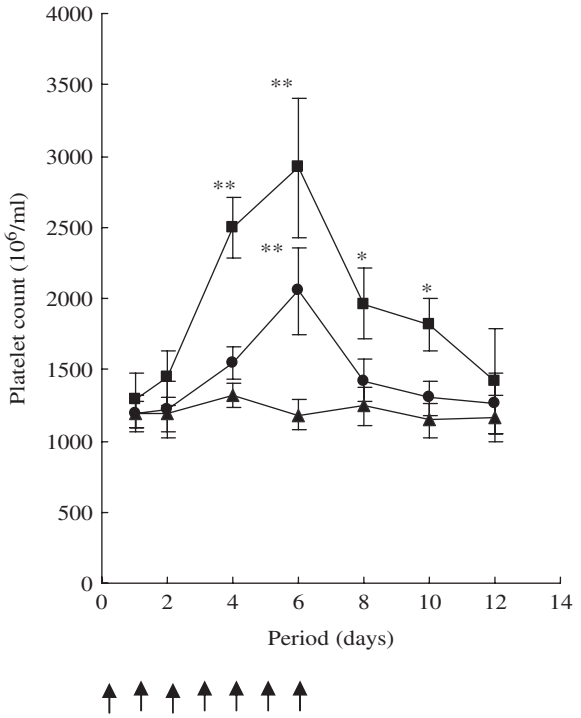

Fig. 6. **Response of normal mice to ZH646 treatment.** Normal mice were treated with ZH646 during the indicated periods (tilled square, treated mice; filled circle, TPO+IL-6; filled triangle, wild-type mice). Arrows indicated the injection of ZH646/TPO+IL-6 once time daily. $^*P < 0.05$; $^{**}P < 0.01$.

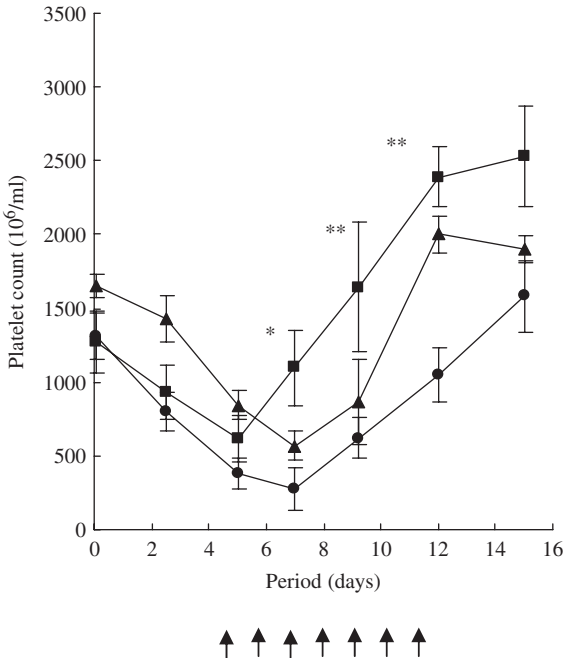

Fig. 7. **Recovery of platelet number after cyclophosphamide injection.** The cyclophosphamide-treated mice were treated with ZH646 for the indicated periods (filled square, treated mice; filled circle, TPO+IL-6; filled triangle, wild-type mice). Arrows indicated the injection of ZH646/TPO+IL-6 once time daily. $^*P < 0.05$; $^{**}P < 0.01$.

potentiators, includng IL-6 and IL-11. The pre-clinical studies indicated that IL-1, IL-3, IL-6, GM-CSF and IL-11 can all stimulate megakaryocyte growth and platelet production (*9*, *12*). Therefore, optimal stimulation of megakaryocyte lineage using various combinations of cytokines is under extensive research (*5*, 13, 14). IL-6, in particular, has been reported to have effects on early haematopoiesis (*15*, *16*) and also megakaryocytopoiesis (*8*, 17, 18). Furthermore, Brakenhoff (*19*) showed that the N-terminal 28 amino acids could be removed without significantly affecting biological activity of IL-6. On the contrary, it was found such deletion could even lead to enhanced IL-6 activity. Nevertheless, the hybrid ZH646 constituting IL-6 and TPO have not been reported to stimulate megakaryopoiesis *in vitro* and platelet production *in vivo*.

With these considerations in mind, we hypothesized that the formation of the IL-6-linker-TPO complex could be enhanced by converting it into a unimolecular protein by using a flexible polypeptide as a linker. We designed the more flexible small molecules by applying conformational changes to keep the epitope characteristics of IL-6 and TPO by using the computer software GOLDKEY (data not shown). Consequently, we used the presumably flexible amino acid with a 13-residue sequence rich in glycine and serine to connect truncated IL-6 and mature rhTPO to produce ZH646 expressed at a high level in the *P. pastoris* expression system. *Pichia. pastoris* is a methylotrophic yeast that has been successfully used to produce large quantities of foreign proteins (*20*). This system has the advantages of an eukaryotic expression system: the presence of an α-factor signal sequence facilitates secretion of the expressed protein into the medium. The yeast strain (GS115) secretes only very low levels of endogenous protein, which simplifies the purification process. In addition, there is the advantage of inducible expression, leading to 10–100-fold higher level of heterologous protein than other eukaryotic systems. We found that the ZH646 secreted by *P. pastoris* can reach a concentration of 30 mg/l. We believe this high secretion level may be due to adapting the codon usage in TPO and IL-6 cDNA to *P. pastoris* codon bias. For the purification of ZH646, we used an anion DEAE exchange system, which recovered the ZH646 from a large volume of culture supernatant. The targeted protein was then further purified via a Mono Q column to complete the two-step purification. To the best of our knowledge, this is the first report on the fusion expression and purification protein of IL-6 and rhTPO in yeast linked by a synthetic linker.

This novel fusion protein, ZH646, appear to exert biological activity in megakaryopoiesis and haematopoiesis. In a semi-solid culture, the megakaryocyte colonies induced by the ZH646 contained larger and more mature colonies consisting of 21–50 megakaryocytes, which is consistent with results obtained by other investigators who suggest that IL-6 promotes cell maturation during later stages of megakaryocytopoiesis (*21*, *22*). Otherwise, TPO in combination with IL-6 induced an increase in colony numbers of at least 20% over those stimulated by TPO and an inability to remain larger and more mature individual megakaryocytes. Although IL-6 has been suggested to have no essential role as a single regulator in normal steady-state megakaryocytopoiesis, and is not required for residual megakaryocyte and platelet production in the *c-mpl*(-/-) mouse (*23*), our results showed ZH646 is much stronger than TPO or IL-6 alone. There may be two important explanations for this observation. The first is that TPO and IL-6 commonly activate signal transducer and activator of transcription3 (Stat3), which has an important role in megakaryopoiesis and thrombopoiesis (*24*). Another possible reason is that long-term administration of IL-6 in mice stimulates megakaryocyte maturation and platelet production with few adverse effects, and maintained the high platelet count for the duration of treatment (*17*). Recently, a novel pathway of TPO regulation by the inflammatory mediator IL-6 is proposed, suggesting that the number of megakaryocytes or platelets by themselves might not be the sole determinant of circulating TPO levels and thus of megakaryopoiesis (*25*). This in turn indicates that IL-6 stimulates thrombopoiesis through TPO, and promotes cell maturation during later stages of megakaryocytopoiesis ZH646, similar to TPO, was also used to effectively expand Dami cells in a dose-dependent fashion, suggesting that it controls early human haematopoietic megakaryocyte line development by means of distinct molecular *c-mpl* targets on Dami. Because of that, it is therefore possible to address the question whether these various effects were mediated by a similar molecular mechanism.

While ZH646 may increase lineage-specific differentiation and nature of megakaryocyte *in vitro*, it would be important to also explore the *in vivo* relevance of these observations. Our study demonstrated that platelet counts increased more in ZH646-induced groups than the controls, and peak value of platelet counts occurred rapidly after intraperitoneal administration of ZH646 in normal mice. It is important that the platelet count exceeded normal levels in 6 out of 7 days of treatment. This could correlate well with a similar observation that IL-6 maintained high platelet count for the duration of treatment that exceeded normal levels in 7 days after 30 days of treatment were stopped (*17*). The increase may reflect that IL-6 plays crucial roles in the proliferation of MK progenitors to increase the peripheral blood platelets. Compared with ZH646, TPO+IL-6 also increased platelet count at day 7 significantly; they only stayed a high platelet count at one day, and rapidly dropped the normal platelet values after day 7.

Although a platelet nadir occurred in myelosuppression groups induced by cyclophosphamide, ZH646-treated groups experienced a much less severe nadir, which occurred earlier and recovered much quicker than the controls. TPO in combination with IL-6 do not show much earlier return of the platelet count toward normal than ZH646 during the ensuing 7 days injection. Some earlier reports also suggested that defective megakaryocytopoiesis/thrombocytopoiesis is not caused by a defect in TPO production but a lack of response to TPO in the signal transduction pathway of *c-mpl* (*26*, *27*). In that regard, IL-6 may contribute to an alteration of normal feedback between TPO and its receptor. Therefore, ZH646 could ameliorate the platelet nadir,

lead to a shorter recovery time and rebound thrombocytosis compared with the controls in chemotherapy-induced thrombocytopenia mice.

In summary, we can conclude that this biological activity may not only translate directly to megakaryocyte in its ability to increase platelet production on normal mice, but also support recovery of platelet counts after myelosuppression in cyclophosphamide-treated mice.

The search for more active stimulation of thrombocytopoiesis has been fostered by the molecular regulation of haematopoietic stem cells, megakaryocytes and platelet production. ZH646 may represent a new therapeutic option *in vivo*, as compared with the combination of TPO or TPO plus IL-6. The reason is that ZH646 does not only offer the convenience of using one single molecule instead of two, but also because it could provide more biological activities than TPO or IL-6 alone.

This work was supported by grants from the National Natural Science Foundation Of China (30570785, 4103250 and 30572453), the National Education Ministry Key Science and Technology Research Project (199177), Guangdong Natural Science Foundation (4203208) and Guangdong Provincial Social Development Plan (2005B20301024).

REFERENCES

1. Cohen-Solal, K., Vitrat, N., Titeux, M., Vainchenker, W., and Wendling, F. (1999) High-level expression of Mpl in platelets and megakaryocytes is independent of thrombopoietin. *Blood* **93**, 2859–2866
2. Bussel, J.B., Mukherjee, R., and Stone, A.J. (2001) A pilot study of rhuIL11 treatment of refractory ITP. *Am. J. Hematol.* **66**, 172–178
3. Borbolla, J.R., Lopez-Hernandez, M.A., De Diego, J., Gonzalez-Avante, M., Trueba, E., and Collados, M.T. (2001) Use of interleukin-11 after autologous stem cell transplant: report of three cases and a very brief review of the literature. *Haematologica* **86**, 891–892
4. Dahlen, D.D., Broudy, V.C., and Drachman, J.G. (2003) Internalization of the thrombopoietin receptor is regulated by 2 cytoplasmic motifs. *Blood* **102**, 102–108
5. Hunnestad, J.A., Steen, R., Tjonnfjord, G.E., and Eqeland, T. (1999) Thrombopoietin combined with early-acting growth factors effectively expands human hematopoietic progenitor cells in vitro. *Stem Cells* **17**, 31–38
6. Sasaki, H., Hirabayashi, Y., Ishibashi, T., Inoue, T., Matsuda, M., Kai, S., Ikuta, K., Yokoyama, K., Yokota, T., Maruyama, Y., and Matsuyama, S. (1995) Effects of erythropoietin, IL-3, IL-6 and LIF on a murine megakaryoblastic cell line: growth enhancement and expression on receptor mRNA. *Leuk. Res.* **19**, 95–99
7. Jacobsen, S.E., Ruscetti, F.W., Okkenhaug, C., Lien, E., Ortiz, M., Veiby, O.P., and Keller, J.R. (1994) Distinct and direct synergistic effects of IL-1 and IL-6 on proliferation and differentiation of primitive murine hematopoietic progenitor cells in vitro. *Exp. Hematol.* **22**, 1064–1069
8. Williams, J.L., Pipia, G.G., Datta, N.S., and Long, M.W. (1998) Thrombopoietin requires additional megakaryocyte-active cytokines for optimal ex vivo expansion of megakaryocyte precursor cells. *Blood* **9**, 4118–4126
9. Majka, M., Ratajczak, J., Villaire, G., Kubiczek, K., Marquez, L.A., Janowska-Wieczorek, A., and Ratajczak, M.Z. (2002) Thrombopoietin, but not cytokines binding to gp130 protein-coupled receptors, activates MAPKp42/44, AKT, and STAT proteins in normal human CD34+ cells, megakaryocytes, and platelets. *Exp. Hematol.* **30**, 751–760
10. Zhang, Q., Peng, H.J., Yu, X.B., and Xu, P.L. (2003) Establishment of a mouse model of cyclophosphamide-induced thrombocytopenia and determination of platelet function in this model. Di Yi Jun Yi Da Xue Xue Bao. *J. First Milit. Med. Univ.* **23**, 1277–1282
11. Gurney, A.L., Carver-Moore, K., de Sauvage, F.J., and Moore, M.W. (1994) Thrombocytopenia in cMpl-deficient mice. *Science* **5177**, 1445–1447
12. Demetri, G.D. (2001) Targeted approaches for the treatment of thrombocytopenia. *Oncologist* **6**(Suppl. 5), 15–23
13. Lazzari, L., Henschler, R., Lecchi, L., Rebulla, P., Mertelsmann, R., and Sirchia, G. (2000) Interleukin-6 and interleukin-11 act synergistically with thrombopoietin and stem cell factor to modulate ex vivo expansion of human CD41+ and CD61+ megakaryocytic cells. *Haematologica* **85**, 25–30
14. Bruno, S., Gunetti, M., Gammaitoni, L., Dane, A., Cavalloni, G., Sanavio, F., Fagioli, F., Aglietta, M., and Piacibello, W. (2003) In vitro and in vivo megakaryocyte differentiation of fresh and ex-vivo expanded cord blood cells: rapid and transient megakaryocyte reconstitution. *Haematologica* **88**, 379–387
15. Yasukawa, K., Tsuchiya, S., Ekida, T., Iida, H., Ide, T., Miki, D., Yagame, H., Murayama, K., and Ishiguro, T. (2003) Fusion protein of interleukin-6 and interleukin-6 receptor without a polypeptide linker. *J. Biosci. Bioeng.* **96**, 38–46
16. Rodriguez Mdel, C., Bernad, A., and Aracil, M. (2004) Interleukin-6 deficiency affects bone marrow stromal precursors, resulting in defective hematopoietic support. *Blood* **103**, 3349–3354
17. Ishibashi, T., Shikama, Y., Kimura, H., Kawaguchi, M., Uchida, T., Yamamoto, T., Okano, A., Akiyama, Y., Hirano, T., Kishimoto, T. *et al.* (1993) Thrombopoietic effects of interleukin-6 in long-term administration in mice. *Exp. Hematol.* **21**, 640–646
18. Lotem, J., Shabo, Y., and Sachs, L. (1989) Regulation of megakaryocyte development by interleukin-6. *Blood* **74**, 1545–1551
19. Brakenhoff, J.P., Hart, M., and Aarden, L.A. (1989) Analysis of human IL-6 mutants expressed in Escherichia coli. Biologic activities are not affected by deletion of amino acids 1–28. *J. Immunol.* **143**, 1175–1182
20. Cereghino, G.P., Cereghino, J.L., Ilgen, C., and Cregg, J.M. (2002) Production of recombinant proteins in fermenter cultures of the yeast *P. pastoris*. *Curr. Opin. Biotechnol.* **13**, 329–332
21. Lazzari, L., Henschler, R., Lecchi, L., and Rebulla, P. (2000) Interleukin-6 and interleukin-11 act synergistically with thrombopoietin and stem cell factor to modulate ex vivo expansion of human CD41+ and CD61+ megakaryocytic cells. *Haematologica* **85**, 25–30
22. Cardier, J.E., Murphy, M.J. Jr, and Erickson-Miller, C.L. (1997) IL-6 interferes with stimulation of HPP-CFC and large CFU-Mk in conjunction with cytokine. *Stem Cells* **15**, 437–442
23. Gainsford, T., Nandurkar, H., Metcalf, D., Robb, L., Begley, C.G., and Alexander, W.S. (2000) The residual megakaryocyte and platelet production in *c-mpl*-deficient mice is not dependent on the actions of interleukin-6, interleukin-11, or leukemia inhibitory factor. *Blood* **95**, 528–534
24. Kirito, K., Osawa, M., Morita, H., Shimizu, R., Yamamoto, M., Oda, A., Fujita, H., Tanaka, M., Nakajima, K., Miura, Y., Ozawa, K., and Komatsu, N. (2002) A functional role of Stat3 in in vivo megakaryopoiesis. *Blood* **99**, 3220–3227

25. Kaser, A., Brandacher, G., Steurer, W., Kaser, S., Offner, F.A., Zoller, H., Theurl, I., Widder, W., Molnar, C., Ludwiczek, O., Atkins, M.B., Mier, J.W., and Tilg, H. (2001) Interleukin-6 stimulates thrombopoiesis through thrombopoietin: role in inflammatory thrombocytosis. *Blood* **98**, 2720–5725
26. Ballmaier, M., Schulze, H., Strauss, G., Cherkaoui, K., Wittner, N., Lynen, S., Wolters, S., Bogenberger, J., and Welte, K. (1997) Thrombopoietin in patients with congenital thrombocytopenia and absent radii: elevated serum levels, normal receptor expression, but defective reactivity to thrombopoietin. *Blood* **90**, 612–619
27. Li, J., Xia, Y., and Kuter, D.J. (2000) The platelet thrombopoietin receptor number and function are markedly decreased in patients with essential thrombocythaemia. *Br. J. Haematol.* **111**, 943–953