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Pharmacokinetics and peripheral platelet counts after a single dose of recombinant human thrombopoietin in rhesus monkeys

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KEY WORDS *recombinant proteins*; pharmacokinetics; *Macaca mulatta*; enzyme-linked immunosorbent assay; thrombopoietin; platelet count

ABSTRACT

AIM: To study the pharmacokinetics and the change of peripheral platelet counts after a single dose of recombinant human thrombopoietin (rhTpo). **METHODS**: After iv or sc injections of rhTpo in 12 rhesus monkeys, rhTpo concentration in serum was determined by ELISA. Platelets were counted by automatic microcell counter. **RESULTS**: The terminal half-lives of rhTpo were 12 - 18 h. AUC following sc were linearly increased with dose, while Cl_s were 0.061, 0.08, and 0.07 $L \cdot kg^{-1} \cdot h^{-1}$ in sc 0.5, 2, and 8 $\mu g \cdot kg^{-1}$ groups, respectively. Bioavailability was 0.50 ± 0.18 after sc. Single dose of rhTpo was associated with an increase in platelets (55.9 % - 107.4 %, $P < 0.05$) in a dose-related manner. The peak response and the sustained days of platelet increase were dose-related. The degree of platelet increase (% × time) correlated with the systemic exposure to rhTpo (C × time). **CONCLUSION**: rhTpo behaved as a linear pharmacokinetics in the monkey within dose range of 0.5 - 8 $\mu g \cdot kg^{-1}$.

INTRODUCTION

Thrombopoietin (Tpo), the ligand for the proto-oncogene *c-mpl*-encoded receptor (*c-mpl* receptor), was identified and cloned in 1994[1]. *In vitro* and *in vivo* experiments showed it to be a primary regulator of thrombocyte production[2,3]. Chinese hamster ovary (CHO) cells-expressed recombinant human thrombopoietin (rhTpo) had been produced by Sunshine Pharmaceutical Co Ltd (SP), Shenyang, China. This article attempted to reveal the pharmacokinetics, the change of peripheral platelet counts, and their relationship after a single dose of rhTpo in monkeys.

MATERIALS AND METHODS

Subjects Twelve rhesus monkeys, ♂ 6 and ♀ 6; weighing 3.5 kg ± 0.2 kg, were provided by Animal Center of Chinese Academy of Military Medical Sciences (Certificate No 97083).

Equipments and reagents The automatic microcell counter (F-800, Sysmex). Wellscan MK-2 microplate reader (Denley Dragon). The rhTpo was provided by SP. This rhTpo was a full-length, about 67 % glycosylated molecule expressed in CHO cells and purified by standard procedures. The molecular mass, determined by size exclusive HPLC and SDS-PAGE, was about 9 - 10 kDa. It was mixed with preservative-contained saline as a diluent and stored at 4 ℃ before use. Reagent were all AR.

Experimental design Eight monkeys were randomly divided into 2 subcutaneous groups of sc 0.5 and 8 $\mu g \cdot kg^{-1}$. Other 4 monkeys were in a cross-over designed group, in which each monkey was firstly sc or iv injected with rhTpo 2 $\mu g \cdot kg^{-1}$ by random and received the same dose by the other route for the second time after 16 -

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18-d washing out time, respectively.

Sample assay Serum samples were collected before and at 1, 5, and 10 min in iv group and 30 min, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, 96, 120, and 168 h in all groups ($n = 4$ in each group) after medication. Samples were stored at 4 ℃ until assay. The rhTpo level was quantitated by ELISA kits (R&D Systems, lot 9725274) in duplicate. The assay was guided by the procedures specified by the manufacturer. A series of calibration standards provided by SP was set up in each microplate.

The peripheral blood samples of each monkey ($n = 3 \times 2$ from cross-over group and $n = 4 \times 2$ from the other two groups) were collected before and 2, 4, 6, 8, 10, 12, 14, and 16 d after administration of rhTpo. The platelet counts in the samples were evaluated by the microcell counter.

Data analysis The pharmacokinetic parameters were obtained by program 3p87. Statistical inferring was obtained by t test or chi-square test. The linear ($\hat{Y} = A + B \cdot X$) or hyperbola ($\hat{Y} = E_{max} \cdot X/[K + X]$) regression of the results was proformed by Origin (MicroCal Software Inc).

RESULTS

Determination of rhTpo in monkey serum Within the dose range of 4 – 1 275 ng·L^{-1}, the rhTpo standard in kits (*Sf*21-expressed) and rhTpo standard provided by SP (CHO-expressed) appeared as 2 parallel lines. The slopes of them were 0.996 ± 0.008 and 0.996 ± 0.011, respectively, indicating that their immunologic responses to the antibody of kits were similar. Coefficients of variation (CV) of intra-assay and inter-assay were all within 10 %, and the recovery rate of rhTpo from serum was 84 % – 114 % within the same dose range. The minimal detectable level of rhTpo was 7 ng·L^{-1}. The endogenous Tpo level observed in monkeys was (19 ± 7) ng·L^{-1} ($n = 12$).

Pharmacokinetics of rhTpo in monkeys Concentration-time curves after iv and sc rhTpo were all best fitted by 2-compartment model (Fig 1).

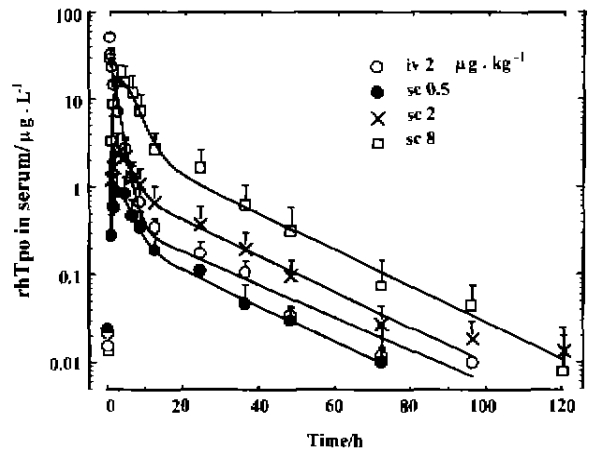

Fig 1. Serum rhTpo concentration after iv & sc a single dose of rhTpo in monkeys. $x \pm s$.

The terminal half-lives ($T_{\frac{1}{2}\beta}$) were 12 – 18 h. The absorption half-lives and time to peak (T_{peak}) had a tendency of prolonging with the increase of dose in 3 sc groups ($P > 0.05$). Apparent volume of distribution of central compartment (V_c) following iv approximated to the volume of blood plus interstitial liquid. AUC following sc 0.5, 2, and 8 μg·kg^{-1} were linearly increased with dose ($n = 3$, $r = 0.998$, $P < 0.05$), while systemic clearances (Cl_s) among groups were similar, indicating that rhTpo had a linear pharmacokinetics in the dosage range studied. Bioavailability (F) after sc was 0.50 ± 0.18 (Tab 1).

Platelet count Injection of a single dose of rhTpo aroused increase of platelets in normal monkeys (baseline $4.2 \times 10^{10} \cdot L^{-1}$; maximal $7.9 \times 10^{10} \cdot L^{-1}$, $P < 0.01$). Although observed T_{peak} of rhTpo reached at about 1 min (iv) to 2 – 4 h (sc), the increase of platelets began to be seen on d 4, and approached the top by d 7 – 8. After maximal response, platelets gradually declined to baseline, and were back to normal on

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Tab 1. Pharmacokinetic parameters of recombinant human thrombopoietin after iv and sc injection in monkeys. $n = 4$ monkeys. $\bar{x} \pm s$.

Parameters	iv 2 $\mu g \cdot kg^{-1}$	sc 0.5 $\mu g \cdot kg^{-1}$	sc 2 $\mu g \cdot kg^{-1}$	sc 8 $\mu g \cdot kg^{-1}$
$T_{\frac{1}{2}\alpha}$/h	1.00 ± 0.07	2.2 ± 0.8	2.1 ± 0.7	2.6 ± 0.6
$T_{\frac{1}{2}\beta}$/h	12.6 ± 1.9	18 ± 5	15.7 ± 1.8	18.3 ± 1.0
$T_{\frac{1}{2}Ka}$/h		1.3 ± 0.6	1.5 ± 0.6	2.0 ± 0.4
K_{21}/h^{-1}	0.070 ± 0.010	0.11 ± 0.09	0.11 ± 0.05	0.050 ± 0.010
K_{10}/h^{-1}	0.56 ± 0.04	0.18 ± 0.11	0.16 ± 0.03	0.17 ± 0.10
K_{12}/h^{-1}	0.12 ± 0.04	0.10 ± 0.06	0.13 ± 0.07	0.05 ± 0.04
$C_{max}/\mu g \cdot L^{-1}$	50 ± 5	0.8 ± 0.5	2.2 ± 1.2	13 ± 8
T_{peak}/h		2.7 ± 0.8	2.8 ± 1.0	3.4 ± 0.6
V_c or V_c/F, $L \cdot kg^{-1}$	0.070 ± 0.010	0.6 ± 0.7	0.53 ± 0.29	0.34 ± 0.16
$CL_c/L \cdot kg^{-1} \cdot h^{-1}$	0.037 ± 0.005	0.061 ± 0.019	0.08 ± 0.04	0.07 ± 0.03
$AUC_{0-\infty}/\mu g \cdot h \cdot L^{-1}$	59 ± 6	10 ± 3	30 ± 12	163 ± 74
F			0.50 ± 0.18	
MRT/h	4.9 ± 1.0	10.8 ± 1.0	16 ± 5	11.7 ± 2.7

d 16 after injection in all monkeys.

Increase of platelets from baseline of 94.3 % ($P < 0.05$) was seen in the iv group, and 55.9 % ($P < 0.05$), 75.8 % ($P < 0.01$), and 107.4 % ($P < 0.01$) in groups of sc 0.5, 2, and 8 $\mu g \cdot kg^{-1}$, respectively. In the highest dose group, the platelets increased to above 1 million, a level thrombosis effect might be induced, in two of four monkeys ($1.1 \times 10^{12} \cdot L^{-1}$ in one and $1.3 \times 10^{12} \cdot L^{-1}$ in another).

The maximal increase of platelets and the sustained days of platelet increase were in dose-related manner. The degree of platelet increase (increased percentage of platelets times time, % × T), was dose-related too. (Tab 2)

Relation between systemic exposure to rhTpo and the platelet increase Neither maximal concentration nor lasting time of increased rhTpo level was directly related to the degree of platelet increase. While the systemic exposure to rhTpo (serum rhTpo concentration times time, $C \times T$) was related to the degree of platelet increase with r of 0.68 ($n = 14$, $P < 0.01$). The platelets increased as a linear or hyperboic function of systemic exposure to rhTpo.

Tab 2. Peripheral platelet kinetics after injection of recombinant human thrombopoietin in monkeys. n = monkeys. $\bar{x} \pm s$. $^bP < 0.05$ *vs* sc 0.5 $\mu g \cdot kg^{-1}$ group. $^fP < 0.01$ *vs* sc 8 $\mu g \cdot kg^{-1}$ group

Parameters	n	Sustained days/d	Maximal reponse/%	Degree of platelet increase/% × d
iv 2 $\mu g \cdot kg^{-1}$	3	7.3 ± 0.5^b	102 ± 77	705 ± 464
sc 0.5 $\mu g \cdot kg^{-1}$	4	3.8 ± 2.5	54 ± 37^f	276 ± 207^f
sc 2 $\mu g \cdot kg^{-1}$	3	8.7 ± 2.1^b	67 ± 13^f	400 ± 85^f
sc 8 $\mu g \cdot kg^{-1}$	4	6.5 ± 1.7^b	150 ± 10	975 ± 116

The sum of residual squares (789 842) and the P value (0.0024) of hyperbolic regression were relatively less than those of linear regression (950 787 and 0.0080, respectively), but in the same order of magnitude. (Fig 2)

DISCUSSION

As guideline of International Conference on Harmonisation (ICH-S6) pointed out, due to the species specificity of many biotechnology-derived pharmaceuticals, it is important to select relevant animal species for preclinical testing [Preclinical safety evaluation of biotechnology-derived

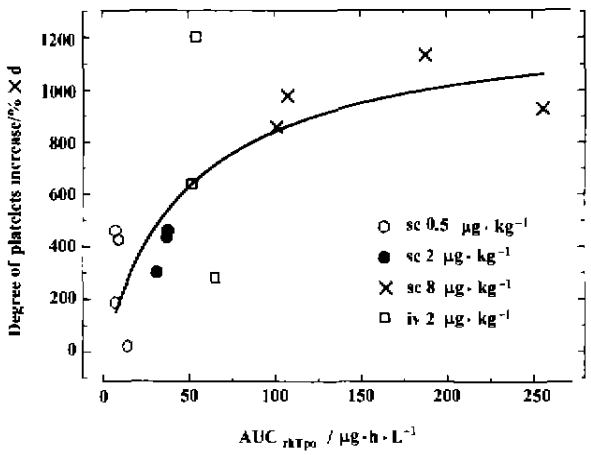

Fig 2. Correlation between systemic exposure of recombinant human thrombopoietin (serum concentration times time, $C \times T$) and the degree of platelets increase (increased percentage of platelets times time, % × T). The best fitted hyperbolic curve was $\hat{Y} = 1273.6(\pm 324.0) \cdot X/[51.5(\pm 33.0) + X]$. $n = 14$. $P < 0.01$.

pharmaceuticals. International conference on harmonisation (ICH) steering committee, ICH-S6; 1997 Jul 16 – 18; Brussels, Belgium. p 103]. There is marked conservation of amino acid sequences of Tpo across species[4,5]. This conservation is reflected in the considerable species cross-reactivity reported for the Tpo molecule[1]. Results in the study indicated that the selection of non-human primate was appropriate.

The most prominent pharmacokinetics behavior of rhTpo in monkeys was its prolonged terminal half-life and remaining in the circulation for 4 – 5 d, which had never been seen in other recombinant cytokines. Based on this phenomenon and the data accumulated in our laboratory, we assumed that terminal half-life of a polypeptide (in the monkey, determined by ELISA) appeared to be related to its m and degree of glycosylation. The m of rhTpo is about 9 – 10 kDa, and its degree of glycosylation is approximately 67 %, its terminal half-lives following sc were 16 – 18 h. The terminal half-life of recombinant erythropoietin (baby hamster kidney cell expressed, m about 35 kDa, approximately 39 % glycosylated) was about 7 – 11 h. Other two smaller nonglycosylated proteins from *E coli* (fusion protein of recombinant human interleukin-3 and granulocyte-macrophage colony stimulating factor, 30 kDa, and interleukin-6, 23 kDa) had shorter terminal half-lives (approximately 1.5 – 3.3 h and 1.5 – 2.5 h, respectively). However, further investigation is needed to verify this assumption.

There was a delay of 4 d between administration of rhTpo and the beginning of platelet increase, suggesting that megakaryocytopoiesis was a cellular development process[6]. Further, in the research, we found that the same administration of rhTpo did not arouse similar platelet increase in some individuals (for example, in the sc 0.5 $\mu g \cdot kg^{-1}$ group, the degree of platelet increase of an individual was 25 % × d, while the mean value of the whole group was 276 ± 207). So we preferred to say that individual variation played a very important role in the reaction of platelets increase.

In this study, we found that neither maximal concentration nor lasting time of increased rhTpo level directly influenced the degree of platelet increase. While systemic exposure, a parameter considered both of concentration and time, was generally the most appropriate measure of serum exposure of the drug[7]. The relation between systemic exposure of rhTpo and the course of platelet increase was evaluated in the study, suggesting that appropriate concentration and enough sustained time of drug should be considered at the same time when conducting an reasonable dosage regime in clinical trial. But the precise funcitonal relationship between them needed further research in future.

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猕猴单次注射重组人血小板生成素后的药动学及外周血小板计数的变化

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关键词 重组蛋白；药物动力学；猕猴；酶联免疫吸附测定；血小板生成素；血小板计数

目的：研究猕猴单次注射 CHO 细胞表达的重组人血小板生成素（rhTpo）后药动学及血小板计数变化．**方法**：酶联免疫吸附测定法（ELISA）检测血清中 rhTpo 含量．血细胞计数仪进行血小板计数．**结果**：rhTpo 末端相半衰期（$T_{\frac{1}{2}\beta}$）12 - 18 h．各 sc 组间 AUC 依赖于剂量，而 Cl_s 相近．生物利用度 0.50 ± 0.18．rhTpo 引起猕猴与剂量相关的血小板增加（平均增加率 55.9 % - 107.4 %，$P < 0.05$）．增加峰值及持续天数与剂量有关．**结论**：rhTpo 在猕猴体内表现为线性药动学．rhTpo $T_{\frac{1}{2}\beta}$ 长与其分子量大，高度糖基化有关．血小板增加程度（血小板净增率 × 持续时间）与 rhTpo 全身暴露强度（血药浓度 × 时间）呈正相关．

（责任编辑 杨如华）