DARU Vol. 16, No. 2 2008

Comparison of real time RT-PCR and flow cytometry methods for evaluation of biological activity of recombinant human erythropoietin

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Received 12 Aug 2007; Revised 27 Oct 2007; Accepted; 3 Nov 2007

ABSTRACT

Background: Evaluation of bioactivity of recombinant erythropoietin is essential for pharmaceutical industry, quality control authorities and researchers. The purpose of this study was to compare real time RT-PCR and flow cytometry for the assay of biological activity of recombinant erythropoietin.

Methods: Three concentrations of recombinant erythropoietin BRP (80, 40 and 20 IU/ml) were injected subcutaneously to mice. After 4 days the blood was collected and used for reticulocyte counts by flow cytometry and also for the RNA extraction. Real time RT-PCR amplification was carried out for β-globin.

Results and conclusion: There was a significant correlation between the total RNA amounts (R^2= 0.9995), relative quantity of β-globin mRNA (R^2= 0.984) and reticulocyte counts (R^2= 0.9742) with rhEpo concentrations. Total RNA and quantitative RT-PCR showed significant dose dependent results as well the reticulocyte counts by flow cytometry for the biological activity assay of rhEpo and so these methods could be considered as alternatives for flow cytometry.

Keywords: Reverse Transcription Polymerase Chain Reaction (RT-PCR), Recombinant Human Erythropoietin (rhEpo)

INTRODUCTION

Erythropoietin (Epo) is the main regulator for red blood cell maturation and release (1-2) and consists of 165 amino acids that form a single polypeptide chain containing two intra-chain disulfide bonds (cys $^{7-161}$ and cys $^{29-33}$). There are four potential glycosylation sites on Epo, of which three are N-linked (Asn^{24}, Asn^{38} and Asn^{83}) and one is O-linked (Ser^{129}) (3). The molecular mass of this sialoglycoprotein hormone is 30-34 kDa, and contains around 40% carbohydrates, mostly in the form of N-linked (1, 4).

Recombinant human Epo (rhEpo) is produced commercially by the expression of Epo cDNA clones in eukaryotic cell lines, usually in Chinese hamster ovary (CHO) or baby hamster kidney cells (BHK). rhEpo is the same as natural Epo in peptide sequences but it is different with respect to glycosylation (2-3). While such differences may result in different specific activities, most studies have shown that biological activity of rhEpo and EPO are equivalent (2, 4).

Different methods have been used to determine bioactivity of rhEpo. Currently normocythemic and polycythemic mice bioassays of the European Pharmacopeia which is based on a single injection to normocythemic mice (2, 5) and counting reticulocytes is one of the most valuable tests to assess the bone marrow erythropoietic activity. The reticulocyte counting is carried out manually and automatically. The automated flow cytometery is based on fluorescence (Thiazole orange) or absorbance (methylene blue) (5).

Reverse transcription combined with the polymerase chain reaction (RT-PCR), is one of the most powerful methods for quantification of gene expression (6). It has been reported that a significant increase in amounts of beta globin mRNA can be detected by RT-PCR in whole blood samples for rhEpo abuse in athletes (7).

The aim of the present study was to evaluate real time RT-PCR as a sensitive and accurate alternative method for evaluation of biological activity of rhEpo by the normocythemic mice bioassay and to compare it with other methods.

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MATERIALS AND METHODS

Laboratory animals

Female B6D21F1 mice, 8 weeks old with a body weight range 18-22g, were obtained from Pasteur Institute and kept under controlled conditions at 22°C, 70% humidity, and 12 hrs light/darkness for five days before injection.

Normocythemic mice bioassay

Erythropoietin BRP (Batch No.1.E.P.Comission, 32500 IU/vial) was dissolved and diluted in phosphate albumin buffered saline (pH 7.2, 0.1M) to obtain activity of 80, 40 and 20 IU/ml respectively. Mice were distributed randomly into cages of minimum of 9 mice per cage and were injected subcutaneously 0.5 ml of prepared solution. Phosphate albumin buffered saline was injected to one group as control.

Blood collection

Ninty six hours after injection, 300 µl of the blood was collected into tubes containing 10 µl of 0.5M EDTA in phosphate buffered saline of pH 8.2 and gently mixed. A part of the collected blood was used for flow cytometry assay and other part was subjected to RNA extraction.

RNA extraction

RNA extraction was carried out by High pure RNA isolation kit (Roche), according to the manufacturer's instruction. The concentrations of extracted total RNA was measured by reading the absorbance at 260 nm in the Biophotometer (Eppendorf).

Reverse transcription

Reverse transcription was performed by Expand Reverse transcription kit (Roche) with 1 µg of total RNA and 20 pmol of oligo $(dT)_{15}$ primer at 42 °C for 60 minutes.

PCR and quantification

The primers for PCR were designed with respect to the β-globin cDNA sequences in Gene Bank (accession no.V00722). The designed forward primer for β-globin was 5' TTA GTG GTA CTT GTG AGC CA 3' and the reverse primer was 5' ATG GTG CAC CTG ACT GAT GC 3'.

The primers for β-actin gene as internal standard were 5'CAG GGT GTG ATG GTG GGA A 3' for forward primer and 5' GCT CAT TGT AGA AGG TGT GG 3' for reverse primer.

The PCR amplification was carried out in a Stratagene Real Time PCR instrument using Taq DNA polymerase (Promega) in the presence of SYBR® Green (Roche). Resulting Ct values were normalized according to β-actin and the relative quantification was obtained by the reported method using $2^{-\Delta\Delta Ct}$ (6) and SPSS 11 software.

Reticulocyte counting

The number of reticulocytes was determined by using thiazole orange dye in flow cytometer equipped with a 488nm laser FACS CAN™ system (Partec PAS). A 5 µl of the collected blood sample was added to 1 ml of thiazole orange solution (100 ng/ml thiazole orange in PBS) and incubated at room temperature for 30 min in the dark. Then the solutions were aspirated into automated flow cytometer, and the results reported as the absolute numbers or presence of reticulocytes.

RESULTS

Nine mice of each group were subjected for total RNA extraction, RT-PCR and reticulocyte counts. The electrophoresis of RT-PCR products on agarose gel is represented in Figure 1. A distinct bond around 500bp for β-globin and a 230bp fragment for β-actin are observed clearly in this figure.

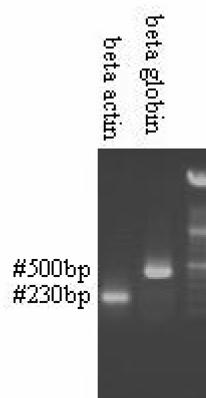

Figure 1. Electrophoresis of RT-PCR products on 1% agarose gel

Figures 2, 3 and 4 show correlation between concentration of rhEpo and reticulocyte counts (Fig.2, R^2= 0.9742), total RNA (Fig.3, R^2= 0.9995), and relative increase in β-globin mRNA level in rhEpo injected mice (Fig.4, R^2= 0.984).

The correlation of total RNA and reticulocyte counts is represented in Fig.5 (R^2= 0.9741). Fig.6 shows correlation of relative increase in β-globin mRNA level and total RNA (R^2= 0.9785) and Fig.7 represents correlation between reticulocyte counts and relative quantitative RT-PCR of beta globin (R^2=0.9411).

DISCUSSION

Erythropoietin enhances the release of some RBC progenitors as erythrocytes. Several methods based on automated analyzer instruments have

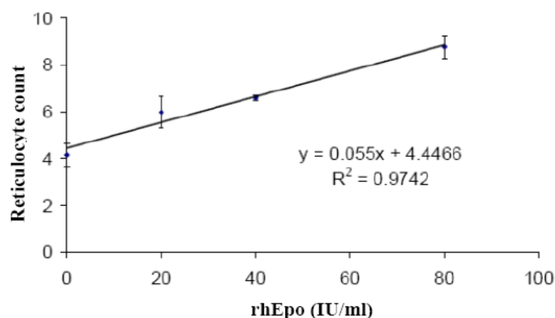

Figure 2. Correlation between reticulocyte counts and rhEpo concentration

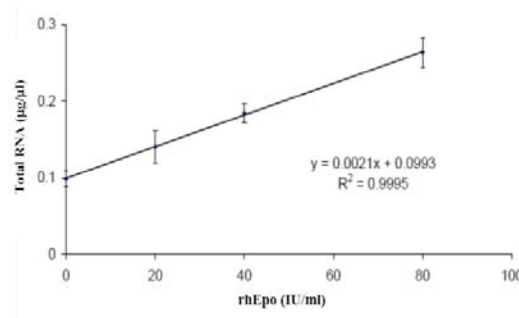

Figure 3. Correlation between total RNA and rhEpo concentration

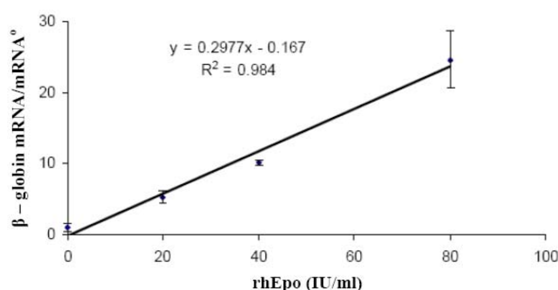

Figure 4. Correlation between relative increase in β-globin mRNA level and rhEpo concentration

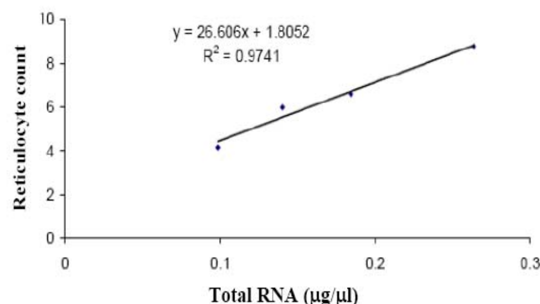

Figure 5. Correlation between total RNA and reticulocyte counts

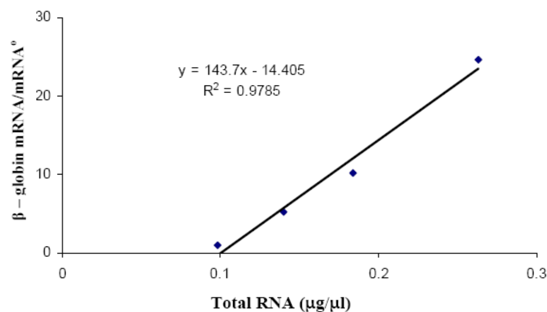

Figure 6. Correlation between total RNA and relative increase in β-globin mRNA level

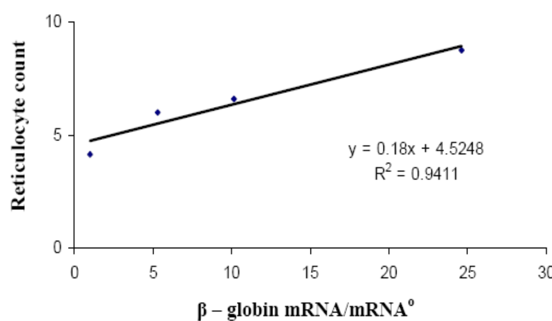

Figure7. Correlation between reticulocyte counts and relative increase in β-globin mRNA level

been developed for erythrocyte count as a marker for biological activity of injected recombinant erythropoietin. The correlation between these methods has been reviewed (8), and only flow cytometry method has been accepted by European pharmacopoeia. Staining of erythrocytes nucleic acid by Thiazole orange is the basis of recommended Flow cytometry method (5). Erythrocytes don't have nucleus but contain mRNA for synthesis of essential proteins for mature RBCs. Increase in β-globin during exogenous erythropoietin abuse by athletes has been reported (6).

The results of our study showed a good correlation between total RNA and the injected concentrations of rhEpo (R^2=0.9995) which might be due to the dose dependent increase in RNA containing cells during injection of rhEpo. As a result, the amount of total RNA could be a marker for rhEpo biological activity. However, there are some difficulties in normalization of blood samples for total RNA extraction which could be overcome by using commercial RNA extraction kits for extraction of RNA from whole blood. Also a significant correlation was found between the increase in amount of β-globin mRNA and injected concentrations of rhEpo (R^2= 0.984), and it may be concluded that there is a significant and dose dependent elevation of β-globin mRNA level in these samples.

In addition, there is a significant correlation between elevation of β-globin mRNA level and reticulocytes counting by the flow cytometry method. It could be concluded that injection of rhEpo results in increase of peripheral reticulocytes which contain mRNA for β-globin.

On the basis of findings of this study, using total amount of RNA or quantitative RT-PCR for β-globin mRNA which are simple, easy and sensitive methods may be considered for the assay of biological activity of rhEpo.

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