Thrombopotein: its ups and downs

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Chapter 2

Sensitive Measurement of Thrombopoietin by a Monoclonal Antibody-Based Sandwich Enzyme-Linked Immunosorbent Assay

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Sensitive Measurement of Thrombopoietin by a Monoclonal Antibody-Based Sandwich Enzyme-Linked Immunosorbent Assay

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ABSTRACT
In this report a sensitive enzyme-linked immunosorbent assay (ELISA) for the measurement of plasma thrombopoietin (Tpo) is described that is solely based on monoclonal antibodies (MoAbs).

The assay has an intra- and inter-assay variance of 5-7% and 7-13%, respectively. Native and recombinant human Tpo (rhTpo) were recognized equally well, no cross-reactivity with other cytokines was found and rhTpo added to plasma and serum was completely recovered. With the ELISA, Tpo concentrations in EDTA-anticoagulated plasma of all controls (n=193) could be determined, since the limit of detection (2 ± 0.8 A.U./ml, mean ± SD) was lower than the concentration found in controls (11 ± 8 A.U./ml, mean ± SD; 2.5th-97.5th percentile: 4-32 A.U./ml). Tpo levels in serum were on average 3.4 times higher than in plasma.

We showed in vivo that Tpo is bound by platelets, as in thrombocytopenic patients (n=5) a platelet transfusion immediately led to a drop in plasma Tpo level, whereas in patients receiving chemotherapy the induced thrombocytopenia was followed by a rise in plasma Tpo levels.

In summary, these results indicate that this ELISA is a reliable tool for Tpo measurements and is applicable for large scale studies.
INTRODUCTION

Since the cloning and isolation of thrombopoietin (Tpo), the primary regulator of megakaryocyte growth and development [1-6], there is a need for a sensitive and reproducible method to measure this cytokine. Among the currently used assays, a cell-line-based bioassay is the most applied one [1-11]. Several Tpo-dependent cell lines have been generated by transfection of cell lines, such as Baf3 [1,3-8] and 32D [2,9], with c-mpl, the receptor for Tpo. A variety of read-out systems to measure the proliferation of these cells in response to Tpo have been described. Although the presence of biologically active Tpo can be accurately measured with such bioassays, they are not sensitive enough to measure low to normal Tpo levels. In addition, bioassays are relatively cumbersome and time consuming. Moreover, when used for diagnostic purposes, they are not completely reliable since the presence of toxic substances in blood, such as cytostatics, might affect cell growth. Therefore, for diagnostic and research purposes, a reliable immunoassay would be a good alternative.

Recently, three reports have been published in which an enzyme-linked immunosorbent assay (ELISA) for the measurement of Tpo levels is described [12-15]. Although two of these assays were sensitive enough to measure Tpo concentrations in healthy individuals [14,15], all of the assays are dependent on the use of a polyclonal antiserum. A disadvantage of such a system is that the production of a new batch of antiserum with the same quality is not guaranteed.

In this report we describe a sensitive and reliable sandwich ELISA based solely on monoclonal antibodies (MoAbs), for capturing as well as for detection. Sensitivity, specificity and reproducibility were assessed and the concentration of Tpo was measured in both plasma and serum of healthy individuals to establish reference values from which to compare diagnostical relevant Tpo measurements. In addition, with this ELISA, Tpo levels were assessed in patients receiving a platelet transfusion and patients receiving chemotherapy. The results indicate, in concordance with previous studies [10,16], that platelets are able to bind Tpo, since Tpo levels drop after platelet transfusion and rise after chemotherapy.

MATERIALS AND METHODS

Cytokines

Recombinant human Tpo was a kind gift from Genentech Inc. (South San Francisco, CA) and Zymogenetics (Seattle, WA) and was purchased from Research Diagnostics Inc. (Flanders, NJ). Truncated rhTpo comprising 174 amino acids of the N-terminal part of Tpo was purchased from PBH (Hannover, Germany). Erythropoietin was purchased from Connaught Laboratories Inc. (Ontario, Canada), IL-3 from R&D (Abingdon, UK) and SCF and G-CSF from the National Institute for Biological Standards and Control (NIBC ampul 91/682 and 88/502, respectively; Potters Bar, UK). GM-CSF was kindly provided by Sandoz (Basel, Switzerland), and IL-6 was produced at our own institute (CLB, Amsterdam, The Netherlands).
Preparation and selection of MoAbs against Tpo

MoAbs directed against Tpo were prepared according to the procedure previously described for IL-6 [17]. Briefly, Balb/c mice were immunized with 7.5 μg full-length rhTpo and hybridoma cell lines were obtained after fusion of SP2/0 myeloma cells with spleen cells of these mice. Hybridoma cell lines were screened for the production of Abs against rhTpo by means of a radioimmunoassay (RIA) in which culture supernatant (50 μl) was incubated overnight at RT under rotation with 50 μl (=1 ng) of 125I-labeled Tpo (Genentech Inc., South San Francisco, CA), iodinated by way of the Chloramine-T method [18], and 500 μl 0.5 mg/ml CNBr-activated Sepharose beads (Pharmacia LKB, Uppsala, Sweden) to which rat Abs directed against the mouse kappa light chain had been coupled (Euroclone 226; CLB, Amsterdam, the Netherlands). MoAbs that were not bound to the sepharose beads and/or free 125I-labeled Tpo were washed away and the radioactivity was measured with a gamma counter (1260 multigamma II, LKB Instruments Inc. Gaithersburgh, MD). Binding was calculated as the percentage of bound 125I-Tpo as compared to the total amount of Tpo added. From two fusion experiments seven clones that stably produced MoAbs directed against rhTpo were selected. The clones were designated αTpo4, αTpo5, αTpo11, αTpo12, αTpo13, αTpo14 and αTpo15, respectively.

Purification of MoAbs

Supernatants of stable hybridomas that were cultured in roller bottles (Becton Dickinson, Lincoln Park, NJ) in Iscove's modified Dulbecco's medium (IMDM) supplemented with 2.5% heat inactivated fetal calf serum (Gibco BRL, Life Technologies LTD, Paisley, Scotland), penicillin (100 μg/ml, Gibco BRL) and streptomycin (100 U/ml, Gibco BRL), were collected and concentrated over a Hemoflow F5 capillary dialyser (Fresenius AG, Bad Homburg, Germany). MoAbs were purified by means of Protein A-Sepharose (Pharmacia) chromatography, and the eluates were extensively dialysed against phosphate-buffered saline. Isotypes of the different MoAbs were determined with the Mab Mouse Isotyping Kit (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions.

Biotinylation of MoAbs

Antibody was diluted in 0.1 M NaHCO3 at a concentration of 1 mg/ml. Sulfo NHS LC biotin (Pierce, Rockford, IL) was added (125 μg/mg MoAb) and the solution was incubated for two hrs at RT. Finally, the buffer was changed and excess biotin was removed by dialysis against PBS. Biotinylated MoAbs were stored at -20°C.

Enzyme-linked immunosorbent assay (ELISA)

Initially, various combinations of capture and detecting MoAbs were tested as described in the Results section. In the final ELISA, a 96-well plate (Nunc Immunoplate Maxisorp, Rockslide, Denmark) was coated overnight at RT with a combination of αTpo5 and αTpo14, both in a concentration of 2 μg/ml in 100 μl of 0.1 M carbonate buffer pH 9.6. Coated plates could be stored at 4°C for at least one week. Before use, plates were washed twice with PBS / 0.02% Tween (v/v) and non-specific binding sites were blocked by incubation at RT for 30 min. with 150 μl of PBS containing 2% (v/v) pasteurized cows' milk. Subsequently, plates were washed 5
times with PBS / 0.02% Tween, and 50 µl of biotinylated MoAb (1 µg/ml) in High Performance Elisa buffer (HPE; CLB, Amsterdam, The Netherlands) was added together with 50 µl of standard, blank or sample. The plate was incubated for two hours under shaking conditions. Then the plates were washed five times with PBS / 0.02% Tween, and 100 µl of streptavidin polyHRP (CLB, Amsterdam, The Netherlands) in a dilution of 1:10000 in PBS/2% milk (v/v) was added. Plates were incubated for 30 min. at RT under shaking conditions and after washing five times with PBS / 0.02% Tween, the substrate TMB (0.1 mg/ml) was added diluted in substrate buffer (0.11 M NaAc pH 5.5 and 0.003% H₂O₂). After 30 min of incubation in the dark, the colorimetric reaction was stopped by addition of 100 µl of H₂SO₄ and the absorbance at 450 nm was measured in a Titertek multiscan ELISA reader (Flow Laboratory, Rockville, MD). On each plate a standard curve was incorporated, together with at least two reference samples. Since rhTpo appeared to be unstable, i.e. the amount of rhTpo decreased over time, a pool of EDTA-anticoagulated plasma of six patients with a high Tpo level was used as a standard. The standard curve consisted of eight two-fold dilutions, of which the first dilution was arbitrarily set at 100 Arbitrary Units (A.U.). One Arbitrary Unit equals 1 pg of fresh rhTpo from Genentech Inc. and 9 pg when compared with rhTpo from Research Diagnostics Inc. All samples were measured in triplicate. If their concentration exceeded the linear part of the calibration curve, the samples were diluted in HPE.

**Collection and preparation of blood samples**

**Control values:** EDTA-anticoagulated blood was obtained from 198 healthy individuals by venepuncture. After collection, platelet counts were determined by standard electronic particle counting and the blood was spun at 850 g for 15 minutes. The platelet-poor plasma was again spun for another 10 minutes at 850 g to remove any residual platelets. Plasma was stored until use at -20°C. Platelet counts were all in the normal range, between 150 and 450 x10⁹/L, except for five individuals (platelet counts 146, 484, 503, 510 and 600 x10⁹/L, respectively) who were excluded from the study. The mean age was 38 yrs with a range of 18-69; 53% were male, 47% female.

**Comparison of Tpo levels in plasma and serum:** Serum and EDTA-anticoagulated plasma was obtained during one donation from 137 volunteers. The Tpo concentration in serum versus that in EDTA-anticoagulated blood was measured by means of the ELISA. For five EDTA-anticoagulated samples, Tpo concentration was also measured after recalcification with 1.68 mM CaCl₂ and subsequent clotting.

**Transfusion data:** EDTA-anticoagulated blood was obtained from five patients, directly before and within 10 minutes after they received a platelet transfusion of 5 units, administered within 30 minutes up to one hour. Patients were hemato-oncologic patients with persistent thrombocytopenia after chemotherapy and/or stem cell transplantation.
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**Figure 1: Standard Tpo dose-response curve.** The calibration curve was calculated according to logit regression. For each concentration a duplicate measurement was done. All measurements are represented with a letter. The detection limit of this particular test, defined as the concentration Tpo reflecting twice the background extinction multiplied by the sample dilution, was 1.1 A.U. A pool of EDTA-anticoagulated plasma from six thrombocytopenic patients with high Tpo levels was used as a standard.

**Chemotherapy data:** EDTA anticoagulated blood was drawn at different time points during myeloablative treatment of patients (n=7) with ovarian cancer who had relapsed from a previous remission. At the Netherlands Cancer Institute, patients were treated with Carboplatin (AUC 10) and Endoxan (500 mg/m²) at a 21-day interval, starting at day 0. In addition to platelet counts, Tpo levels were measured.

**Data analysis**
Statistical analysis was performed with the software package SPSS for windows, release 6.1.3 (SPSS Inc.). Differences between groups were assessed with the Mann-Whitney U-Wilcoxon rank sum W test and the Student’s t test. The correlation between two variables was calculated with Pearson’s correlation coefficient (r) and the Spearman correlation coefficient (r_s).

**RESULTS**

**Selection of MoAbs recognizing Tpo for the ELISA**
Out of seven MoAbs directed against rhTpo, five high affinity MoAbs (αTpo5, αTpo12, αTpo13, αTpo14 and αTpo15) were selected by means of a RIA in which a fixed amount of ^125^I-labeled rhTpo was added to serial dilutions of the different MoAbs. Denaturation of ^125^I-Tpo by addition of sodium dodecyl sulphate (0.075% SDS, w/v) in the RIA completely inhibited the binding (data not shown), indicating that the MoAbs are not directed against an epitope that is exposed upon denaturation. MoAbs were tested in an ELISA in various combinations for capture and detection, to assess epitope specificity and to select an optimal combination of MoAbs for Tpo detection. Only αTpo12 and αTpo13 showed cross inhibition, suggesting that they either recognize the same or an overlapping epitope.
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Figure 2: Comparison of the titration curves of Tpo from different sources. The mean of duplicate samples from one test are shown. Line a-c represent the titration of Tpo present in EDTA-anticoagulated plasma from three patients with thrombocytopenia. Line d represents the titration of rhTpo from Genentech Inc. Not shown in this figure are the titration curves of the standard, of the Tpo-containing concentrated culture supernatant of the HepG2 human liver cell line and of rhTpo from Zymogenetics.

The most sensitive assay was obtained with a combination of equal amounts of two MoAbs, αTpo5 and αTpo14, to capture Tpo and with biotinylated αTpo12 to detect the bound Tpo. All three MoAbs had the IgG1 isotype. Fig. 1 shows the standard dose-response curve of the ELISA in this format. The use of either one of the two capture MoAbs alone, markedly diminished the sensitivity of the assay (data not shown). The limit of detection of the assay is 2±0.8 A.U. (mean±SD).

Evaluation of the ELISA: specificity and reproducibility

To check for test specificity, titration curves were made with Tpo from different sources (Fig. 2). Line a, b and c represent the (native) Tpo concentration in plasma from three different thrombocytopenic patients. Line d was obtained after titration of the rhTpo from Genentech Inc. (South San Francisco). Truncated Tpo (174 AA) comprising the N-terminal domain of Tpo, was not recognized in the assay when tested in concentrations ranging from 453 pg/ml up to 500 ng/ml. The titration curves of the rhTpo from Zymogenetics (Seattle), the Tpo containing concentrated supernatant from the human liver cell line HepG2 and the standard that was used in the ELISA showed identical curves to the curve of the Genentech Inc. rhTpo (data not shown). As is shown in Fig. 2, all lines are parallel, which implies that native Tpo is recognized equally well as rhTpo in our ELISA and that plasma components did not influence the measurements. The latter was confirmed by recovery experiments in which rhTpo was added to plasma samples and measured. Table I shows the recovery of Tpo from plasma. The data shown are representative data from one out of two experiments. rhTpo was added in different concentrations to different plasma samples and to elisa buffer. The latter was used to establish the spiking concentration in Arbitrary Units. By comparison of the measurements in elisa buffer, in plasma without rhTpo and in plasma with rhTpo, the recovery could be calculated. From table I it can be seen that rhTpo was completely recovered. Recovery experiments in serum samples gave similar results.
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Table I: Recovery of rhTpo

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Plasma samples with different endogenous concentrations of Tpo were spiked with rhTpo. Shown are the representative data from one out of two experiments. Given are the mean values of triplicate measurements. Recovery was assessed by comparison of the expected and the observed Tpo concentration.

No cross-reactivity with Epo was found, which is highly homologous to Tpo in the N-terminal domain, nor was there any cross-reactivity with IL-3, IL-6, GM-CSF, SCF or G-CSF (data not shown). All cytokines were tested in a concentration from 50 pg/ml to 5 ng/ml. Epo was tested at concentrations ranging from 0.035 to 3.5 Units/ml.

To estimate the reproducibility of the ELISA, the intra- and inter-assay variation were assessed. Five samples containing various concentrations of Tpo were assayed at seven different positions on a microtiter plate in one run, yielding an intra-assay variation from 5-7%. The inter-assay variation ranged from 7-13% and was determined by testing five samples with various Tpo concentrations at five different days.

Reference values

Tpo was measured in EDTA plasma of 193 healthy adult donors. In Fig. 3, the Tpo levels are shown as a function of platelet count. The mean Tpo level was 11 ± 8 AU/ml (mean ± SD), with the 2.5th and 97.5th percentile of 4 and 32 AU/ml, respectively. No correlation between platelet count and Tpo level was demonstrated in this population (r_s=-0.12, p>0.05). However, the Tpo concentration did vary slightly with age in that with increasing age, Tpo levels tended to decrease (r_s=-0.18, p<0.05). No difference in Tpo concentration between the sexes was found (Mann Whitney U-Wilcoxon Rank Sum W test, p>0.05). Age was not correlated with platelet count (r=-0.7, p>0.05), nor was there a difference in age between males and females (Student's t test, p>0.05).

In addition to Tpo measurement in EDTA plasma, serum Tpo levels were measured in a population of 137 healthy donors (Fig. 4). Tpo plasma and serum levels were positively correlated (r_s=0.8, p<0.001). Serum levels were on average 3.4 times higher than plasma levels. An artificial difference due to the presence of fibrinogen or other coagulant substances in plasma was excluded, since Tpo levels in EDTA plasma did not change after coagulation by recalcification (n=5).
Stability of plasma Tpo
Repeated freezing and thawing of EDTA plasma up to nine times did not affect the Tpo measurements. The time interval between blood collection and plasma isolation up to 25 hrs did not have an effect on the plasma Tpo concentration either (data not shown).

Platelet transfusion and Tpo level
It is now known from transfusion studies in rabbit and mouse models [10,19], that platelets are able to bind Tpo which leads to a decrease of Tpo in the circulation. Thus, similar results would be expected in humans. In Fig. 5, it is shown that plasma Tpo levels indeed decreased as a result of a platelet transfusion in thrombocytopenic patients.

Figure 3: Platelet counts vs Tpo levels in healthy controls

Figure 4: Plasma vs serum Tpo levels in healthy controls

Figure 5: Plasma Tpo concentrations in patients with thrombocytopenia before and after platelet transfusion. Patients were transfused with 5 units of platelets, and EDTA anticoagulated blood was drawn just before and within 10 minutes after transfusion. The percentages of Tpo decrement from top to bottom are 32%, 30%, 21%, 20% and 2%. Only the last patient had a normal plasma Tpo concentration.
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**DISCUSSION**

In this report a sensitive, reproducible and specific sandwich ELISA for the detection of Tpo is described. In contrast to previously reported Tpo ELISAs [12-14], this assay is based solely on MoAbs. The five MoAbs that were selected for potential use in the ELISA, bound to at least four different epitopes. The use of a combination of Tpo5 and Tpo14 as capture Abs and biotinylated Tpo12 as a detecting Ab, yielded an assay with high sensitivity. Truncated rhTpo was not recognized in the assay. When this truncated form was coated on an elisa plate, only Tpo5 was bound. Therefore, Tpo5 recognizes an epitope on the N-terminal domain, whereas Tpo14 and Tpo12 probably recognize an epitope on the C-terminal domain of Tpo.

Levels in plasma from all healthy individuals could easily be measured, since the limit of detection of the assay, 2±0.8 A.U. (mean±SD), is lower than the concentration Tpo present in normal plasma (11±8 A.U., mean±SD). A pool of plasma from patients with elevated Tpo levels was chosen as a standard, because rhTpo appeared to be unstable, in contrast to native Tpo in pooled plasma. The start of the standard curve was arbitrarily set at 100 A.U. When compared with fresh recombinant preparations, 1 unit equals 1 pg of rhTpo from Genentech Inc. and 9 pg of rhTpo from Research Diagnostics Inc. This marked difference is unexplained but could, for instance, be due to differences in the expression system used to prepare full-length rhTpo. It indicates that standardization is needed.
The specificity of the Tpo assay was demonstrated in several experiments. The Tpo ELISA showed no cross-reactivity with other cytokines. In addition, from the titration experiment it can be concluded that the affinity of the MoAbs for native Tpo was equal to their affinity for rhTpo. This test also showed that plasma components did not interfere with the detection of Tpo. The recovery experiments supported this hypothesis. However, the presence of interfering substances can never be fully excluded. For instance, autoantibodies against cytokines such as IL-6, IL-1α and TNFα, interfering with the assays for these cytokines, have been described both in healthy individuals and in patients [20-22]. The presence of autoantibodies against Tpo could give a false (low) measurement if these antibodies interfere with the binding of the MoAbs to Tpo. The same may hold true when a soluble form of the Tpo receptor binds to Tpo. The existence of such a receptor (sMPL) has been suggested by several groups [23,24]. Hornkohl et al. [15] even measured high sMPL concentrations in healthy individuals. However, our data show no evidence that sMPL interferes with the Tpo measurements, because rhTpo added to plasma was completely recovered.

In order to assess reference values for Tpo, plasma samples of 194 healthy volunteers were tested in the ELISA system. Although several groups have reported ELISAs for the detection of Tpo [12-14], not all tests were sensitive enough to measure Tpo levels in healthy individuals [12]. With the assay that is described here, Tpo levels in all healthy controls could be determined. The Tpo level was independent of sex but was slightly negatively correlated with age. No correlation with platelet count was found in this group of healthy controls, which is in concordance with previously published results [14]. Many reports support the hypothesis that the Tpo level is inversely related to the platelet count or mass outside the normal range [5,10,16,19,25]. Indeed, when we measured plasma Tpo concentration in thrombocytopenic patients before and after platelet transfusion, a definite decline in the Tpo level was seen. Similar results were reported by Kuter et al. [10], who did a transfusion study in a rabbit model and monitored the Tpo level by means of a bioassay. Fielder et al. [19] also found a decrease in Tpo level after injection of normal platelets into c-mpl" mice. Moreover, they showed that this decrease is a result of Tpo binding and internalization by platelets. A correlation between platelet count and Tpo level was also present in patients treated with chemotherapy. When platelet levels declined, a concomitant rise in Tpo could be seen. Nichol et al. [26] described similar results in patients with lymphoid malignancies who received chemotherapy.

Thus, a relationship between platelet count and Tpo level does seem to exist, but this becomes clear only in case of platelet counts below normal. The lack of correlation between platelet counts and Tpo levels in the control population might be explained by normal inter-individual variation in, for example, the rate of Tpo production, Tpo clearance, and/or the number of c-mpl receptors that are expressed by the megakaryocyte/platelet lineage.

In addition to EDTA-plasma samples, the level of Tpo was also measured in simultaneously isolated serum samples from healthy individuals. The levels in serum were found to be on average 3.4 times higher than the Tpo level in EDTA plasma. Since the recovery of rhTpo in plasma and serum was equally well and recalciification of plasma samples did not change Tpo measurements, the difference seems not to be caused by an artifact. An explanation for the
plasma-serum difference might be that during blood clotting, Tpo is released from the platelets, which leads to an elevated Tpo concentration in serum. About the origin of this Tpo can be speculated. Release can occur from Tpo bound to the receptor, but it is also possible that intact Tpo is stored in the platelet or is present in the surface-connected canalicular system. In addition to intact Tpo, Tpo degradation products are present in platelets [19], and release of these fragments might be detected with the Tpo ELISA. If the released Tpo is biologically active, it might indicate the existence of another feed-back loop in platelet homeostasis. Platelet activation in vivo (such as upon coagulation, during sepsis, etc.), with subsequent platelet destruction, may lead to increased Tpo levels in the body fluids, which in turn stimulates new platelet production. We have recently initiated new studies to test this hypothesis and to investigate Tpo storage and destruction mechanisms.

In summary, the monoclonal antibody-based Tpo ELISA we describe is easy to perform, sensitive, reproducible and specific. It provides a valuable tool for the measurement of Tpo in research and clinical settings.

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REFERENCES

12. Emmons RVB, Reid DM, Cohen RL, Meng G et al. Human thrombopoietin levels are high when thrombocytopenia is due to megakaryocyte deficiency and low when due to increased platelet destruction. Blood 1996; 87: 4068-71.


