Characterization of Native Human Thrombopoietin in the Blood of Normal Individuals and of Patients with Haematologic Disorders

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Summary

Thrombopoietin (TPO) isolated from thrombocytopenic plasma of various animal species has previously been shown to comprise only truncated forms of the molecule, presumably generated by proteolysis. Native TPO has now been partially purified from normal human plasma by immunoaffinity chromatography and was confirmed to be biologically active. Gel filtration in the presence of SDS revealed that TPO eluted in two peaks: a major peak corresponding to the elution position of fully glycosylated recombinant human TPO (rhTPO) consisting of 332 amino acid residues, and a minor peak corresponding to a smaller molecular size. Immunoblot analysis also revealed that most plasma-derived TPO migrated at the same position as fully glycosylated rhTPO, corresponding to a molecular size of ~80 to 100 kDa. Furthermore, the size distribution of circulating TPO in patients with various haematologic disorders did not differ markedly from that of plasma-derived TPO from healthy individuals. These results indicate that the truncation of circulating TPO is not related to disease pathophysiology, and that the predominant form of TPO in blood is a biologically active ~80- to 100-kDa species. The size distribution of TPO extracted from normal platelets was similar to that of TPO in plasma; the proportion of truncated TPO was decreased by prior incubation of platelets with hirudin, indicating that the endogenous truncated TPO, at least in platelet extract, was generated by thrombin-mediated cleavage.

Introduction

Native thrombopoietin (TPO), also known as c-Mpl ligand, has been isolated from thrombocytopenic plasma and the corresponding cDNAs have been cloned from various animal species (1-6). The purified TPO molecules were heterogeneous and appeared truncated at the COOH-terminal domain, exhibiting smaller molecular masses than those predicted from the cloned cDNAs (which encode polypeptides consisting of more than 300 amino acids). The predominant form of native TPO isolated from thrombocytopenic rat plasma exhibited a molecular mass of 19 kDa, but other forms ranging from 17 to 22 kDa were also detected (5, 6). TPO derived from canine plasma showed molecular sizes of 31 and 25 kDa, with the latter form predominating (3). TPO from sheep and porcine plasma exhibited sizes of 41.6, 35.7, and 27.8 kDa, with the 27.8-kDa form predominating (4), and 30, 28, and 18 kDa (1), respectively. However, the isolation of naturally occurring TPO from normal humans has not been described, although endogenous human TPO has been detected with an enzyme-linked immunosorbsent assay (ELISA) that recognizes a portion of the COOH-terminal domain of the molecule (7, 8). In addition, it is not clear whether the truncation or cleavage of TPO plays a role in the regulation of megakaryocytopoiesis and thrombopoiesis, although such posttranslational processing has been shown to modulate the biological activity of TPO (9, 10).

TPO is produced at all stages of development, from fetus to adult, predominantly by hepatocytes (11, 12), but it is also synthesized by other cell types including stromal cells in the bone marrow (13, 14). The molecular characteristics of native circulating TPO, the forms of the protein that exist between its secretion from production sites and its eventual degradation by target tissues, have not been fully elucidated. TPO secreted into the bloodstream subsequently binds to a specific receptor, c-Mpl, on platelets and megakaryocytes, and promotes megakaryocytopoiesis and thrombopoiesis (15). Because the production of TPO appears to be constitutive (16, 17), the binding of TPO to its receptor also contributes to the regulation of the blood concentration of this haematopoietic factor (18). In addition, platelets, whose TPO receptors remain functional (19, 20), contribute to the metabolism of TPO. Thus, 125I-labeled recombinant human TPO (rhTPO) bound to c-Mpl on the platelet surface is degraded to polypeptide fragments (21). In the presence of platelets, rhTPO is also cleaved by endogenous thrombin and thereby modulates TPO activity (9). The relative abundance and roles of full-length and truncated forms of human TPO in vivo thus remain to be determined.

To shed light on the life history of TPO, we have now partially purified TPO from human plasma and analyzed its biochemical characteristics, focusing on how the size of the molecule is affected by haematologic conditions such as thrombocytopenia and thrombocytosis. The present study demonstrates that the appearance of truncated TPO in the circulation is not related to platelet demand, and that the predominant form of TPO in both blood and platelets is a full-length (or almost full-length) molecule.

Materials and Methods

Preparation of blood samples, bone marrow, and platelet extract. Blood specimens were collected by venipuncture after obtaining informed consent from either healthy volunteers or individuals with haematologic disorders,
including aplastic anaemia (AA), essential thrombocythemia (ET), polycythaemia vera (PV), idiopathic thrombocytopenia purpura (ITP), and disseminated intravascular coagulation (DIC). These samples were then centrifuged at 850 × g, with or without the addition of 0.1 volume of 3.8% (w/v) trisodium citrate as an anticoagulant, to prepare plasma or serum. Bone marrow from a normal volunteer was similarly centrifuged, and the supernatant was subjected to analysis. To prepare platelet extract, we first isolated platelet-rich plasma from healthy volunteers by centrifuging whole blood at 200 × g for 20 min. After addition of prostaglandin E1 (Sigma) to a concentration of 1 μmol/l from a stock solution (1 mmol/l) in absolute ethanol, the platelet-rich plasma was then incubated for 30 min at 37°C in the presence of 1 mmol/l KH₂PO₄, 0.8 mmol/l MgCl₂, 10 mmol/l Hepes (pH 7.4) containing a mixture of protease inhibitors (Complete, Boehringer Mannheim), and was then incubated for 30 min at 37°C in the presence of 1 mmol/l CaCl₂, with or without 10 U/ml hirudin. Platelets were disrupted by sonication on ice, and, after removal of debris by centrifugation, the supernatant (platelet extract) was subjected to analysis.

**TPO assays.** The concentration of TPO was measured in triplicate or duplicate with a TPO-specific ELISA as previously described (22). The lower limit of detection of the assay was 0.045 fmol/ml, which corresponds to 1.6 pg of the polypeptide backbone of full-length TPO per milliliter. The biological activity of TPO was assayed in vitro with FDCP-hMpl5 cells, which have been genetically engineered to express human c-Mpl constitutively, as previously described (20). The cells were cultured for 72 h in microtiter wells (2.5 × 10⁵ cells per well) containing serial dilutions of plasma-derived TPO. Cell growth and survival were determined with an MTS colorimetric assay (CellTiter 96 AQ, Promega) on the basis of the dehydrogenase activity of metabolically active cells (23).

Analysis of the molecular size of TPO by gel filtration. Rabbit antisera to rhTPO, prepared as described previously (22, 24), was subjected to chromatography on a protein G-Sepharose column (Pharmacia) to obtain the immunoglobulin G (IgG) fraction, which was then applied to a column of NHS-activated Sepharose 4FF (Pharmacia) coupled with TPO in order to obtain antibodies to TPO (anti-TPO). The affinity-purified anti-TPO (27 mg of protein) was coupled to a 10 ml of preswollen NHS-activated Sepharose 4FF resin, which was then subjected to blocking and washing according to the manufacturer’s instructions (Anti-TPO resin). Plasma, serum, bone marrow samples (2 ml) or platelet extracts (1.2 ml) were incubated for 8 h at 4°C with 20 μl of anti-TPO resin. The resin was separated by centrifugation and washed first with Dulbecco’s phosphate-buffered saline (D-PBS) (Nissui Pharmaceuticals, Tokyo, Japan) containing 1 mmol/l EDTA, 0.1% (v/v) Tween 20, and 0.1% (w/v) NaN₃, and then with 10 mmol/l sodium phosphate buffer (pH 7.3) containing 0.5 mol/l NaCl. TPO was eluted from the resin with 100 μl of a solution containing 50 mmol/l Tris-Cl (pH 6.8), 1% (w/v) SDS, 2 mmol/l EDTA, and 10% (v/v) glycerol, and was then heated for 5 min at 95°C. The TPO sample was applied to a Superose 6 HR 10/30 column (Pharmacia) that had been equilibrated with D-PBS containing 0.1% SDS and 1 mmol/l EDTA at a flow rate of 0.2 ml/min; TPO was eluted with the same solution and fractions (0.5 ml) were collected. To eliminate excess SDS from the fractions, we added 20 μl of 500 mmol/l potassium phosphate (pH 6.8) to each in order to induce formation of a potassium-SDS complex, which was then removed by centrifugation (5). The resulting supernatants were concentrated and washed with D-PBS containing 1 mmol/l EDTA, 0.1% Tween 20, and 0.1% NaN₃, with the use of an Ultrafree-MC 10K filtration device (Millipore), and were then subjected to the TPO ELISA.

**Anti-TPO immunoaffinity column chromatography.** Anti-TPO resin was packed into a column (Anti-TPO column, 0.5 by 5.0 cm) and equilibrated with D-PBS containing 1 mmol/l EDTA, 0.1% Tween 20, and 0.1% NaN₃. Pooled human plasma, treated with protease inhibitors, was diluted with an equal volume of the column equilibration buffer and then applied to the anti-TPO column at 4°C and a flow rate of 30 ml/h. Nonspecific adsorption was reduced by first passing the diluted plasma sample through columns of plain Sepharose 4FF and of control rabbit IgG coupled to NHS-activated Sepharose 4FF that were connected in series with the anti-TPO column. After application of plasma, these first two columns were removed and the anti-TPO column was extensively washed first with 10 mmol/l sodium phosphate buffer (pH 7.4) containing 0.5 mol/l NaCl, and then with 0.15 mol/l NaCl. TPO was eluted with a solution containing 100 mmol/l glycine-HCl (pH 2.5), 150 mmol/l NaCl, and 5 mmol/l CHAPS detergent, and the pooled eluate (6 ml) was neutralized. For further purification, the pooled fraction was applied to the same anti-TPO column equilibrated with D-PBS containing 1 mmol/l EDTA, 0.1% Tween 20, and 0.1% NaN₃. The purification factor was increased by first passing the sample through an in-line column containing NHS-activated Sepharose 4FF coupled with anti-human plasma goat IgG that had been depleted of anti-TPO. TPO was eluted from the anti-TPO column as before.

**Estimation of the molecular mass of TPO by SDS-PAGE.** The eluate from the anti-TPO column was concentrated by ultracentrifugation in an Ultrafree-MC 10K filtration device and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (25) on a 10-to-20% gradient gel (Dai-iichi Chemicals, Tokyo, Japan). TPO was detected either by immunoblot analysis or by ELISA after extraction from the gel. Immunoblot analysis was performed as described (26), with the exception that immune complexes were visualized with the enhanced chemiluminescence (ECL) detection system (Amersham) (9). For recovery of TPO from the gel, gel slices were transferred to 0.3 ml of an extraction solution [20 mmol/l Tris-Cl (pH 8.0), 0.5 mol/l NaCl, 0.05% (w/v) bovine serum albumin] and then shaken for 6 h at 4°C. To remove SDS, we added potassium phosphate (pH 6.8) to a final concentration of 20 mmol/l and separated the resulting potassium-SDS complex by centrifugation. The supernatant was washed with D-PBS containing 1 mmol/l EDTA, 0.1% Tween 20, and 0.1% NaN₃, with the use of an Ultrafree-MC 10K filtration device.

**Results**

**Biological activity of partially purified native TPO.** We first examined whether TPO partially purified from human plasma by immunoaffinity column chromatography exhibited biological activity. The growth of TPO-dependent FDCP-hMpl5 cells was stimulated by the purified TPO fraction in a dose-dependent manner (Fig. 1), with the half-maximal biological activity apparent at 6 fmol/ml.

**Comparison of the molecular sizes of TPO isolated from plasma, serum, bone marrow, and platelets of healthy volunteers.** The size distributions of TPO derived from plasma (Fig. 2A), serum (Fig. 2B), and bone marrow (Fig. 2C) of healthy volunteers all revealed a major peak around fractions 14 or 15 [high molecular weight (MW) fraction] and a minor peak around fractions 19 or 20 (low-MW fraction) by gel-filtration chromatography. When a highly glycosylated full-length
rhTPO that exhibited a molecular size of ~80 to 100 kDa on SDS-PAGE (see Fig. 5) was subjected to identical gel-filtration chromatography, it eluted in the high-MW fraction (data not shown), suggesting that the endogenous TPO in the high-MW fraction is most likely a full-length form of the protein. The low-MW peak thus likely corresponded to truncated forms of TPO. Similar analysis of the size distribution of TPO in platelet extract of normal individuals also yielded two peaks, with the major peak corresponding to the high-MW fraction (Fig. 3).

Furthermore, prior incubation of platelets with hirudin, a specific inhibitor of thrombin (27-29), reduced the amount of TPO in the low-MW fraction, indicating that the generation of truncated forms of native TPO in platelets was mediated in part by thrombin cleavage.

**Molecular size distributions of TPO in the blood of individuals with haematologic disorders.** We also examined the size distributions of TPO derived from plasma of individuals with AA, ET, PV, ITP, or DIC. Such patients showed size distributions of TPO similar to that of normal individuals, with a major peak around fractions 14 or 15 and a minor peak around fractions 19 or 20 (Fig. 4). However, in individuals
Table 1  Partial purification of TPO from human plasma by immunoaffinity chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total protein (mg)</th>
<th>Total TPO (pg)</th>
<th>Purity (ng TPO/mg protein)</th>
<th>Purification factor</th>
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<tr>
<td>Plasma</td>
<td>32,500</td>
<td>16,900</td>
<td>5.21 x 10^{-6}</td>
<td></td>
</tr>
<tr>
<td>Anti-TPO column eluate</td>
<td>1.82</td>
<td>15,200</td>
<td>8.32 x 10^{-4}</td>
<td>1.6 x 10^{4}</td>
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<tr>
<td>Anti-TPO column eluate</td>
<td>0.0626</td>
<td>6,060</td>
<td>1.00 x 10^{-4}</td>
<td>1.9 x 10^{3}</td>
</tr>
</tbody>
</table>

![Absorbance at 492 nm](image)

**Fig. 5** Immunoblot and ELISA analysis of native TPO partially purified from human plasma by immunoaffinity chromatography. (Right panel) Partially purified human TPO (equivalent to ~23 ml of plasma) (lane 1) and 100 pg of rhTPO produced by CHO cells (lane 2) were subjected to SDS-PAGE under reducing conditions followed by immunoblot analysis with anti-TPO antibody. Lane M, biotinylated molecular size standards (200, 113, 97, 66, 40, 30, 20, and 14.4 kDa). (Left panel) The gel lane containing non-reduced human plasma TPO was sliced, and proteins extracted from each slice were subjected to the TPO ELISA.

with AA or DIC, whose plasma TPO concentrations were much higher than those of healthy subjects or of patients with ET, PV, or ITP, the proportion of TPO in the high-MW fraction appeared increased and that in the low-MW fraction decreased. Thus, most of the additional TPO in the blood of individuals with AA or DIC does not appear to be truncated.

**SDS-PAGE analysis of plasma-derived TPO.** For more precise characterization of the size distribution of native human TPO, the protein was partially purified from plasma of healthy volunteers by immunoaffinity chromatography (Table 1). This procedure resulted in a ~190,000-fold purification of TPO and provided ~6 ng of the protein (36% yield) from 450 ml plasma, as estimated by ELISA. The final purity was estimated to be ~1.0 x 10^{-4} mg of TPO per milligram of protein.

SDS-PAGE under reducing conditions followed by immunoblot analysis of the partially purified TPO preparation revealed a single immunoreactive band of ~80 to 100 kDa, which migrated at a position identical to that of the fully glycosylated, full-length rhTPO produced by CHO cells (Fig. 5). Thus, the polypeptide backbone of human plasma TPO would appear to terminate at or near amino acid 332, which is predicted to be the COOH-terminal residue of intact TPO on the basis of the corresponding cDNA sequence (30). The gel lane containing the partially purified human plasma TPO was sliced, and proteins extracted from each slice were subjected to the TPO ELISA. The peak of the ELISA profile corresponded to the position of the immunoreactive protein band detected by immunoblot analysis (Fig. 5). The ELISA also detected various truncated forms of TPO ranging in size from 35 to 20 kDa, consistent with the results of the gel-filtration analysis.

**Discussion**

As previous studies have shown (31), the concentration of TPO in blood is too low to allow purification to homogeneity for direct molecular analyses. We have therefore developed a method for the analysis of the size distribution of TPO in blood that relies on gel-filtration chromatography combined with a sensitive TPO ELISA. Because TPO is thought to be highly glycosylated, gel filtration was performed under denaturing conditions in the presence of SDS to allow more precise estimates of molecular mass. Importantly, the TPO ELISA used in the present study detects various TPO species uniformly, so that the molar TPO concentration values obtained directly reflect the number of TPO molecules in each sample. The availability of antibodies to TPO also enabled us to develop immunochemical methods for characterization of the molecule (24).

A sequence related to that of the COOH-terminal domain of TPO is not present in erythropoietin or in any other known haematopoietic growth factor. The function of this domain remains unknown, although the N-glycans attached to this region play an important role in TPO secretion (32). This latter observation suggested that TPO enters the bloodstream predominantly as full-length, rather than truncated, molecules. On the other hand, TPO purified from thrombocytopenic plasma of various species comprised truncated forms, suggesting that, after its secretion, TPO is cleaved by proteolytic enzymes present in blood. We investigated the possibility that such posttranslational processing of circulating TPO might serve to modulate TPO activity in response to changes in the demand for thrombopoiesis by examining the size profiles of circulating TPO in individuals with various haematologic disorders. However, most circulating TPO from patients with AA, ET, PV, ITP, or DIC, like that from healthy individuals, eluted from the gel-filtration column in a high-MW fraction corresponding to a molecular size of ~80 to 100 kDa. A small amount of truncated forms of TPO was detected in most plasma samples analyzed, but no marked difference in the proportions of the high-MW and low-MW forms was apparent among healthy subjects and patients with the various disorders. Thus, regardless of pathological state, the concentration of TPO, or platelet number in blood (22), the truncation of circulating TPO remained almost constant.

As far as we are aware, the presence of a full-length molecule among native TPO species in plasma from normal volunteers has not previously been analyzed. We partially purified TPO from normal human plasma by immunoaffinity column chromatography. Immunoblot analysis revealed that the purified protein migrated at the same position, corresponding to a molecular mass of ~80 to 100 kDa, as did fully glycosylated, full-length rhTPO produced by CHO cells. A subtle difference in the intensity of staining on the immunoblot was apparent between plasma TPO (which stained most intensely around 80 kDa) and rhTPO (which stained uniformly from 80 to 100 kDa). To examine whether this difference was due to a difference in sugar content or in the polypeptide backbone, we performed immunoblot analysis of the plasma-derived TPO with a monoclonal antibody (24) that reacts with the terminal (erythropoietin-like) domain with the use of antibodies specifi-
ic for the COOH-terminal region (7, 8). In this study, the native protein showed a marked reactivity with this monoclonal antibody (data not shown), indicating that the predominant form of plasma TPO contains more than 312 amino acid residues and is likely a full-length molecule. The diffuse nature of the native immunoreactive band likely reflects a difference in the extent or structure of glycosylation compared with that of rhTPO.

The mechanism and physiological relevance of the generation of truncated TPO by deletion of the COOH-terminal region appear to differ from those for the processing of other cytokines, such as transforming growth factor-β and interleukin-1, that undergo proteolytic conversion from a latent to an active form (33-36). We have previously shown that thrombin-mediated cleavage generates truncated forms of TPO that exhibit increased activity (9). Given that thrombin is activated during blood clotting, it might have been expected that in normal serum or in the plasma from patients with DIC, the proportion of low-MW TPO would be increased relative to that in normal plasma. This was not the case. The high-MW form of TPO was also predominant in platelet extract from normal individuals. However, prior incubation of platelets with hirudin reduced the amount of TPO in the low-MW fraction of platelet extract, indicating that TPO underwent proteolysis by platelet-associated thrombin. The proportion of native truncated TPO in platelet extract was much less than that of labeled truncated molecules obtained after incubation of human platelets with 125I-labeled rhTPO (21). This discrepancy may be due to a difference in examined TPO, the exogenous 125I-labeled rhTPO and native TPO. It remains to be determined whether the full-length TPO detected in platelet extract in the present study was internalized into the platelets or retained on the platelet membrane.

TPO is secreted into the bloodstream from liver, kidney, and other tissues. We have now shown that most secreted TPO circulates as apparently full-length molecules. Some TPO molecules interact with target cells expressing c-Mpl, such as megakaryocyte progenitors in bone marrow, and thereby exert biological actions, such as promotion of megakaryopoiesis. Interaction of TPO with c-Mpl on platelets can serve to regulate the blood concentration of TPO as well as to clear TPO from the circulation by platelet-mediated catabolism. Further studies are required to determine whether the biological activity of TPO is modified by posttranslational processing, including proteolysis in microenvironments such as bone marrow, in which TPO production has been shown to be induced in patients with thrombocytopenia (14).

References


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