Thrombopoietin levels in serum and liver tissue in patients with chronic viral hepatitis and hepatocellular carcinoma

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ABSTRACT
Thrombocytopenia in liver diseases is considered to be due to splenic platelet pooling and accelerated destruction. Since thrombopoietin (TPO), a regulator of thrombopoiesis, is produced mainly in the liver, decreased production of TPO may account for thrombocytopenia in liver diseases. To address this issue, we measured serum TPO, using a sensitive sandwich ELISA, in 108 patients with chronic viral hepatitis, which included chronic hepatitis (CH) and liver cirrhosis (LC), and hepatocellular carcinoma (HCC), and in 29 normal controls. TPO mRNA in 78 liver samples was examined by reverse transcription (RT)-PCR. Platelet counts in CH, LC, HCC and controls were 176 ± 15 x 10^9/l, 81 ± 8 x 10^9/l, 99 ± 7 x 10^9/l and 234 ± 9 x 10^9/l respectively. Serum TPO levels in CH, LC and HCC were 2.79 ± 0.4 fmol/ml, 1.49 ± 0.2 fmol/ml and 1.97 ± 0.2 fmol/ml, and were higher than those of controls. Serum TPO levels were positively correlated with prothrombin time and serum albumin (P < 0.05, in each case), and negatively correlated with Indocyanine Green test and Pugh score (P < 0.01 and P < 0.05 respectively). However, RT-PCR and immunohistochemistry showed that expression of TPO mRNA and protein were similar in the different liver diseases, suggesting that serum TPO is a reflection of the total mass of functional liver. Platelet counts were negatively correlated with spleen index, but not with serum TPO. These results suggest that thrombocytopenia in liver disease is not directly associated with serum TPO but is associated with hypersplenism.

INTRODUCTION
The platelet count in peripheral blood is controlled to maintain normal haemostasis. Thrombocytopenia is observed in various diseases, including chronic viral hepatitis and especially in liver cirrhosis (LC). Earlier studies of platelet dynamics in chronic liver disease accompanying thrombocytopenia showed that splenic platelet pooling and accelerated destruction are the main causes of thrombocytopenia [1–3]. However, consistent improvement of thrombocytopenia was not obtained by portal decompression procedure [4–7]. Indeed, thrombocytopenia can persist after splenectomy [8]. These findings suggest that other factors are involved in thrombocytopenia observed in liver disease.

Recently, thrombopoietin (TPO), the ligand for the c-mpl receptor, has been isolated by several groups [9–12]. TPO is the principal regulator of megakaryo-thrombopoiesis. It increases the number of megakaryocytes and platelets, and expands the number of megakaryocyte progenitor cells in vivo [13]. Serum TPO in patients with aplastic anaemia, acute lymphocytic

Key words: thrombocytopenia, thrombopoietin, chronic hepatitis, hepatocellular carcinoma, hypersplenism.
Abbreviations: TPO, thrombopoietin; CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; ICG, Indocyanine Green; HCV, hepatitis C virus; RT, reverse transcription.
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leukaemia and essential thrombocythaemia was increased in comparison with controls [14]. TPO is a physiological factor mediating the feedback loop between circulating platelets and bone marrow megakaryocytes. Although the regulatory mechanisms of TPO are not fully understood, the level of TPO has been reported to be determined by the ability of platelets to remove TPO from the circulation [15]. This hypothesis was confirmed by the reports that TPO mRNA is modulated by platelet count [16], and serum TPO levels are directly regulated by c-mpl-mediated binding to platelets [17].

TPO has been reported to be expressed in several organs, such as liver, kidney and muscle [9,10,18]. Of these, liver is the major organ that produces TPO. Hepatoma cells also express TPO mRNA [18,19]. Indeed, it is reported that serum TPO was low in liver cirrhosis but, however, it was increased after orthotopic liver transplantation, which was followed by an increase in platelet count [20–23]. These observations may support the hypothesis that impaired production of TPO in liver disease contributes to thrombocytopenia associated with chronic viral hepatitis. However, there is one report that serum TPO was unaltered in cirrhotic patients [24]. To examine the association of TPO with thrombocytopenia in liver disease, we have measured serum TPO and TPO mRNA, and immunoreactive TPO protein in liver tissue in patients with chronic viral hepatitis and hepatocellular carcinoma.

MATERIALS AND METHODS

Patients

The study was performed in 108 patients (73 men and 35 women) who were admitted to Tottori University Hospital (Yonago, Japan) between April 1996 and October 1997. The patients included 30 with chronic hepatitis (CH), 27 with LC and 51 with hepatocellular carcinoma (HCC). Serum TPO was measured in the 108 patients and 29 normal controls. Hepatic TPO mRNA was examined in liver tissues from 23 patients with CH, 29 with LC, 23 with HCC and three with fatty liver, which were used as controls. Immunohistochemical analysis was performed on 18 patients with liver disease, including three with fatty liver, five with CH, five with LC and five with HCC. All HCC patients had LC as underlying liver disease. Hepatitis B virus surface antigen and hepatitis C virus (HCV) antibody were measured by the reversed passive haemagglutination method (LCIA-200; Diatron Laboratories Inc., Tokyo, Japan), and an enzyme immunoassay (Immunocheck-HCVAb; International Reagent Corporation, Kobe, Japan) respectively. Spleen index, a parameter of splenomegaly [25], was measured by ultrasonography. Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

Measurement of serum TPO

Blood samples were collected early in the morning and were allowed to stand at room temperature for 30 min. Serum was separated from the clotted blood by centrifugation and stored at −30 °C until analysis of TPO. Serum TPO concentrations were measured by a sensitive sandwich ELISA, as described previously [14,24].

Liver tissues

Liver samples (78) were obtained from 23 CH patients, 29 patients with LC, 23 with HCC and three with fatty liver. The cirrhotic liver samples included four from LC patients and 25 from the non-cancerous tissues of HCC patients. All liver samples were obtained with a Majima needle [26] under ultrasonography or a Silvermann needle under laparoscopy.

cDNA synthesis, reverse transcription (RT)-PCR and Southern blotting

Total RNA was isolated from tissues by guanidium thiocyanate extraction using ISOGEN (Nippon Gene Inc., Toyama, Japan) according to the manufacturer’s instructions. Using the Ready-To-Go™ T-Prime First-Strand Kit (Pharmacia Biotech), the first-strand complementary DNA was prepared from 1 μg of total RNA with oligo(dT) as the primer and reverse transcriptase. Finally, 1 μg of total RNA was transcribed to 33 μl of cDNA solution. RT-PCR was performed using the Gene Amp PCR Reagent Kit (Perkin Elmer, Roche Molecular System Inc.). cDNA solution (5 μl) was added to 71.5 μl of water, heated at 95 °C for 5 min and then chilled on ice. Subsequently, 10 μl of PCR buffer [100 mM Tris/HCl (pH 8.3), 15 mM MgCl₂, 500 mM KCl and 0.1% (w/v) gelatin], 8 μl of 2.5 mM dNTP mix, 25 pmol of each primer and antisense primer, and 2.5 units of AmpliTaq DNA polymerase were added (final volume 100 μl). Amplification, using a thermocycler (Program Temp Control System PC-700, ASTEC, Japan), was by the following programme: denaturing, 94 °C for 1 min; annealing, 60 °C for 1 min, and elongation, 72 °C for 1 min; 30 cycles were used for TPO and 32 cycles for β-actin. Primers specific for TPO were: sense primer 5′-TGCGTTTCCTGATGCTTGTAG-3′ (nucleotides 396–415) and antisense primer 5′-AACCTTACCCTTGCGTTG-3′ (nucleotides 1071–1091). Primers specific for β-actin, used as an internal control, were: sense primer 5′-CACCCAGGGCGGTGATTG-3′ (nucleotides 504–524) and antisense primer 5′-AACCTTACCCTTGCGTTG-3′ (nucleotides 1071–1091). Primers specific for β-actin, used as an internal control, were: sense primer 5′-GGCGTTTCCTGATGCTTGTAG-3′ (nucleotides 504–524) and antisense primer 5′-AACCTTACCCTTGCGTTG-3′ (nucleotides 1071–1091). Aliquots of the PCR products (10 μl) were electro-
Table 1 Characteristics of the patients

<table>
<thead>
<tr>
<th>Aetiology: number of patients with hepatitis B virus (B)/hepatitis C virus (C)/or with neither (NBNC)</th>
<th>CH (n = 30)</th>
<th>LC (n = 27)</th>
<th>HCC (n = 51)</th>
<th>C (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>46 ± 2.2</td>
<td>57 ± 2.2*</td>
<td>64 ± 1.1**</td>
<td>25 ± 1.1</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>22/8</td>
<td>16/11</td>
<td>35/16</td>
<td>20/9</td>
</tr>
<tr>
<td>Aetiology (B/C/NBNC)</td>
<td>9/20/1</td>
<td>11/15/1</td>
<td>10/40/1</td>
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</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>1.0 ± 0.1</td>
<td>2.1 ± 0.4*</td>
<td>1.9 ± 0.3*</td>
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<tr>
<td>Albumin (g/dl)</td>
<td>4.0 ± 0.1</td>
<td>3.1 ± 0.1**</td>
<td>3.2 ± 0.1**</td>
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<tr>
<td>Glutamic oxaloacetic acid (units/l)</td>
<td>82 ± 17</td>
<td>94 ± 9</td>
<td>89 ± 8</td>
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<tr>
<td>Glutamic pyruvic transaminase (units/l)</td>
<td>121 ± 24</td>
<td>86 ± 10</td>
<td>73 ± 8*</td>
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<tr>
<td>Alkaline phosphatase (units/l)</td>
<td>249 ± 14</td>
<td>334 ± 28</td>
<td>387 ± 64</td>
<td></td>
</tr>
<tr>
<td>ICG rate at 15 min (%)</td>
<td>11.6 ± 1.0</td>
<td>36.3 ± 3.4**</td>
<td>33.6 ± 2.7**</td>
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<tr>
<td>Prothrombin time (%)</td>
<td>100 ± 4</td>
<td>62 ± 4**</td>
<td>73 ± 2**</td>
<td></td>
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<tr>
<td>Spleen index (cm²)</td>
<td>19.2 ± 1.4</td>
<td>25.0 ± 2.4*</td>
<td>26.1 ± 1.7*</td>
<td></td>
</tr>
</tbody>
</table>

*, P < 0.05 versus CH; **, P < 0.01 versus CH.
† Spleen index was defined as the product of the transverse diameter and half of the perpendicular diameter measured at the maximum cross-sectional image of the spleen using ultrasonography.

UV irradiation [27]. The membranes were hybridized with $1 \times 10^5$ c.p.m. of [$^{32}$P]dCTP-labelled TPO and [$^{32}$P]dCTP-labelled $\beta$-actin cDNA probes, prepared with the DNA Labelling Kit (Nippon Gene Inc., Toyama, Japan) by using cDNA of TPO and $\beta$-actin, respectively (kindly provided from Kirin Brewery Co. Ltd, Maebashi, Japan). The membranes were washed twice with 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate) containing 0.1% (w/v) SDS at room temperature for 15 min, and twice with 0.2× SSC containing 0.1% SDS at 65°C for 15 min. The blots were exposed to Kodak XAR-5 film (Eastman Kodak Company) at −80°C for 12 h. Standard size marker was HinClII-digested fragments of $\Phi X 174$ (Nippon Gene Inc.). The amount of expression was semi-quantitatively determined as the TPO/$\beta$-actin ratio using the NIH image version 1.58 computer software (Macintosh, Bethesda, MD, U.S.A.).

Immunohistochemistry

After fixation in 10% formalin, tissues were embedded in paraffin wax and 3 μm sections were prepared. The paraffin wax was removed and the sections were incubated in 0.3% (v/v) H$_2$O$_2$ for 30 min. After being washed three times in PBS, the sections were incubated in 1.5% (v/v) normal rabbit serum, diluted in PBS, for 30 min. The sections were then incubated with rabbit anti-human TPO polyclonal antibody (kindly provided by the Kirin Brewery Co. Ltd) at 4°C overnight. After three washes in PBS, sections were incubated with 0.5% (w/v) biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA, U.S.A.) for 30 min, avidin–biotin–peroxidase complex for 1 h and 200 μl diaminobenzene-H$_2$O$_2$ was added. Counterstaining was with haematoxylin.
Statistical analysis
The results were calculated as means ± S.E.M.. Statistical
significance was determined by the one-way analysis of
variance (‘ANOVA’). The significance level was set at \( P < 0.05 \). The relationships were examined by using
Spearman’s correlation coefficient.

RESULTS

Characteristics of the patients
Table 1 shows the clinical and laboratory findings of the
patients enrolled in the study. The aetiology of liver
diseases was 30 patients with hepatitis B virus, 75 patients
with HCV and three patients with non-hepatitis B virus,
non-HCV. All HCC patients had LC. The sizes of HCC
lesions in all cases were less than 5 cm in diameter. The
number of LC patients with Child score A, B or C was
38, 26 and 14 respectively. Spleen indices in CH,
LC and HCC were 19.2 ± 1.4 cm\(^2\), 25.0 ± 2.4 cm\(^2\) and
26.1 ± 1.7 cm\(^2\) respectively. Spleen indices in LC and

Platelet counts and serum TPO levels
Platelet counts in CH, LC, HCC patients and controls
were 176 ± 15 x 10\(^3\)/l, 81 ± 8 x 10\(^3\)/l, 99 ± 7 x 10\(^3\)/l and
234 ± 9 x 10\(^3\)/l respectively (Figure 1A). Platelet counts
in CH were lower than those in controls \((P < 0.01)\), and
counts in LC and HCC were lower than those in CH \((P
< 0.01\) in each case). Serum TPO levels in CH, LC and
HCC were 2.79 ± 0.4, 1.49 ± 0.2 and 1.97 ± 0.2 fmol/ml,
and were significantly increased compared with those of
controls \((P < 0.01\) in each case) (Figure 1B). In addition,
levels of serum TPO in LC and HCC were significantly
lower than those in CH \((P < 0.01\) in each case). Platelet
counts did not exhibit any correlation with serum TPO,
however, there was a negative correlation with spleen
index \((r = -0.294, P < 0.01)\) (results not shown).

Serum TPO levels were negatively correlated with
Indocyanine Green (ICG) retention rates at 15 min \((r =
-0.27, P < 0.05;\) Figure 2A), and were positively corre-
lated with prothrombin time \((r = 0.35, P < 0.01;\) Figure
2B). Serum TPO levels showed a positive correlation
with albumin \((r = 0.24, P < 0.05;\) Figure 3A), and a
negative correlation with Pugh score \((r = -0.24, P <
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Figure 4  PCR cycle number and exponential rise of PCR products
Exponential increase of PCR products in TPO (left panel) and β-actin (right panel). Southern blots of TPO and β-actin related to cycle number are shown in the bottom panels.

Figure 5  Hepatic expression of TPO and β-actin in liver tissue from fatty liver, CH, LC and HCC
The bands of TPO and β-actin are at 588 bp and 263 bp respectively. M, molecular marker; F, fatty liver; C, chronic hepatitis; L, liver cirrhosis; H, hepatocellular carcinoma.

0.05; Figure 3B). These results suggest that serum TPO is associated with liver function. In addition, no relationship was observed between spleen index and Pugh score (results not shown).

TPO mRNA expression in liver tissue
The exponential increase in PCR product formation for TPO was examined every 2 cycles from 26–36 cycles, and for β-actin from 28–38 cycles (Figure 4). The PCR cycle for each gene was determined by confirming that the cycle number exponentially fitted the linear relationship between the cycle number and the intensity of the bands. Figure 5 shows the PCR products of TPO and β-actin mRNA, which were 588 bp and 263 bp respectively. There was no difference in the ratio of TPO mRNA/β-actin mRNA among the diseases, suggesting that TPO mRNA per cell is almost equal among the liver diseases studied. Although the difference was not statistically significant, the intensity of TPO mRNA in patients with a Child C score tended to be lower than those with a Child A or Child B score (results not shown).

Immunohistochemical analysis of TPO in liver tissues
Staining for TPO was found in the cytoplasm of hepatocytes from fatty liver but a zonal distribution of TPO was not observed (Figure 6A). In liver tissues from CH, expression of TPO was heterogenous throughout the lobule (Figure 6B), in tissues from LC, TPO was expressed in hepatocytes in the pseudolobule (Figure 6C) and in HCC tissue, cancerous cells which formed trabecular structures expressed TPO in the cytoplasm (Figure 6D). Thus no difference in the intensity and distribution of TPO expression was found among the liver diseases investigated.
Figure 6  Immunohistochemical analysis of TPO in liver tissues
(A) Fatty liver, (B) CH, (C) LC, (D) HCC. Magnification × 100.
DISCUSSION

The development of an ELISA for TPO has revealed that serum TPO is increased in patients with aplastic anaemia and acute lymphocytic leukaemia, where platelet counts are decreased [14]. These results confirm the hypothesis of Kuter et al. [15] that TPO mediates the feedback loop between circulating platelets and bone marrow megakaryocytes, and that serum levels of TPO are directly determined by the ability of platelets to remove TPO from the circulation. Indeed, serum TPO was inversely correlated with platelet count in patients following chemotherapy [28] and in patients treated with interferon [29]. Thus TPO may increase in response to a reduction in platelet numbers in humans.

Thrombocytopenia secondary to liver disease is an independent prognostic factor in patients with LC [30], and can pose a problem when biopsies or other surgical interventions are undertaken. Although thrombocytopenia secondary to liver disease has been attributed to hypersplenism [1–3], cloning of TPO and identification of the liver as the main source could shed new light on the pathogenesis of thrombocytopenia in liver disease [9–12]. A recent study has indicated that TPO levels are high when thrombocytopenia is due to megakaryocyte deficiency, and that they are low when thrombocytopenia is due to increased platelet destruction [31]. Shimoda et al. [24] reported that serum TPO was not increased in patients with LC, and their data and that of Emmons et al. [31] suggest that thrombocytopenia associated with LC was not due to a defect in platelet production. However, it has been shown recently that serum TPO was increased after orthotopic liver transplantation, followed by an increase in platelet numbers to normal levels [20–23]. These reports suggest that inadequate production of TPO is a contributory factor in thrombocytopenia associated with LC. In the present study, there was no difference in hepatic TPO mRNA and immunoreactive TPO expression among the liver diseases, and the results confirmed a preliminary report by Kato et al. [32] who showed a 30–40% reduction in total liver TPO mRNA content in patients with LC and thrombocytopenia compared with patients without LC. Therefore, the lower levels of serum TPO in LC than in CH can be explained by lower content of total liver TPO mRNA. This speculation would accord with experimental results showing that knock-out mice had a 50% reduction in TPO mRNA, which resulted in a decrease in peripheral platelet counts to 67% of control values [33].

In the present study, the platelet count was inversely correlated with splenomegaly but not with serum TPO, suggesting that hypersplenism plays a major role in thrombocytopenia in CH, LC and HCC. On the other hand, serum TPO levels in CH were higher than those in LC and HCC. Serum TPO was associated with liver function tests such as ICG-retention rate, prothrombin time, albumin and Pugh score. These data suggest that concentration of serum TPO is, to some degree, dependent on the capacity of the liver to produce this protein. Since liver function in patients who underwent orthotopic liver transplantation seemed to be severely impaired [20–23], the discrepancy between the results of this study and ours may derive from difference in severity of liver dysfunction.

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Received 24 January 2000; 31 March 2000; accepted 9 May 2000