Constitutive Expression of Mpl Ligand Transcripts During Thrombocytopenia or Thrombocytosis

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Mpl ligand (thrombopoietin [TPO]) is the physiological regulator of platelet production. In mice, mRNA encoding the Mpl ligand (Mpl-L) is predominantly found by Northern blot analysis in the liver and kidney. To investigate the mode of regulation of the Mpl-L gene, we have developed several experimental models of severe thrombocytopenia differing in their kinetics and an opposite model of chronic thrombocytosis. Northern analysis performed at various times after induction of a thrombocytopenic state demonstrates that, whatever the number of circulating platelets, no change in Mpl-L mRNA level occurs in liver and kidney. By ribonuclease protection assays, we analyzed the ratios between mRNAs coding for the wild-type Mpl-L form and various splice variants encoding inactive or nonsecreted Mpl-L proteins. No modification in levels of these various isoforms was detected confirming the data of a previous report. Because the highest level of Mpl-L bioactivity in sera was observed only in mice with drastically reduced numbers of both platelets and megakaryocytes, these results further suggest that not only platelets, but also megakaryocytes, must be involved in the regulation of the level of circulating Mpl-L. In addition, we show that no downregulation of wild-type Mpl-L mRNA and no change in the ratio of Mpl-L mRNA isoforms were detected in mice in which a chronic thrombocytosis was induced. Together, these different models extend and further confirm that the regulation of Mpl-L does not occur at a transcriptional level or by a modulation in the ratios of Mpl-L mRNA isoforms.

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Four mutually exclusive theories may account for the regulation of Mpl-L activity. Modification of Mpl-L level with the platelet demand might result from a transcriptional regulation with variations in Mpl-L mRNA levels in the two major organs responsible for its synthesis, ie, liver and kidney, as for the erythropoietin regulation.12 Synthesis of Mpl-L might be constant; its plasma level would depend on its specific uptake by platelets that express Mpl-R,13 as it was proposed for the macrophage colony-stimulating factor (M-CSF)15,16 and the granulocyte colony-stimulating factor (G-CSF)17 regulation. This could explain the inverse correlation between Mpl-L plasma level and circulating platelet numbers.18 However, this hypothesis does not exclude the possibility of a modulation of Mpl-L synthesis by a feedback regulation either at transcriptional or posttranscriptional levels. Indeed, splice variants encoding inactive or nonsecreted forms of Mpl-L proteins have been described and these variants appear to be as abundant as the wild-type form.19,20 Finally, Mpl-L synthesis might be regulated in hematopoietic organs as it was suggested by a study showing that Mpl-L-specific transcripts levels appeared to be upregulated in marrow of mice rendered thrombocytopenic.21

To date, only a few reports have investigated the regulatory mechanisms controlling Mpl-L production. A recent study has provided evidence that no upregulation and no change in the profile of Mpl-L mRNA isoforms could be detected in liver and kidney from thrombocytopenic mice.22 The goal of the present study was to further examine whether severely decreased or highly increased platelet and megakaryocyte numbers would modify the expression of the Mpl-L gene. To this aim, we have studied three experimental models of thrombocytopenia differing by their kinetics: (1) a slow thrombocytopenic state was induced by a lethal irradiation (9.5 Gy) that led to the destruction of progenitor and precursor cells; (2) a combination of 5-fluorouracil (5-FU) and RAMPS treatment caused an acute destruction of circulating platelets without rapid reconstitution of the platelet mass; and (3) a combination of 5-fluorouracil (5-FU) and RAMPS treatment caused an acute thrombocytopenia that resumed within a few days. We also created a model of chronic thrombocytosis to examine whether high platelet numbers would...
result in quantitative or qualitative changes of Mpl-L transcripts. In these four different models, no change of Mpl-L mRNA levels or in the profiles of Mpl-L mRNA isoforms was detected in liver or kidney. Comparison of the serum levels of biologically active Mpl-L in the different models of thrombocytopenia supports the hypothesis that not only platelets, but also megakaryocytes, are involved in Mpl-L clearance through Mpl-R uptake and catabolism.

MATERIALS AND METHODS

Experimental murine models. Female C57Bl/6×DBA/2 (B6D2) F1 mice of 8 to 10 weeks of age were purchased from Janvier (Lyon, France). Mice were totally irradiated with a 40 Gy source delivering 0.4 Gy/min. For RAMPs-treatment (kindly provided by Dr T. McDonald, Knoxville, TN), mice were intraperitoneally injected with 0.05 mL of serum (RAMPs #135) 12 hours after irradiation. 5-FU (Roche, Neuilly-sur-Seine, France) was intravenously injected via the retroorbital sinus at a single dose of 150 mg/kg (body weight). Five animals were killed for each determination. Peripheral blood platelets isolated from individual mice after red blood cell lysis (Unopet; Becton Dickinson, Rutherford, NJ) were counted using a hemocytometer at a 400× magnification after a 4-hour sedimentation in a humidified chamber. Mice were either anesthetized and bled by cardiac puncture. Sera were pooled for each determination of Mpl-L activity. Organs were rapidly frozen in liquid nitrogen for RNA extraction.

To induce a thrombocytosis by a chronic exposure to Mpl-L, mice were subcutaneously grafted with factor-dependent-cell-P1 (FDC-P1) cells engineered to secrete murine Mpl-L. Briefly, FDC-P1 cells were infected with a retrovirus containing the full-length cDNA encoding the murine Mpl-R. Infected clones were isolated from colonies formed in agar culture containing 10 ng/mL r-mu-Mpl-L and amplified in liquid culture. A clone was then infected with a retrovirus carrying the full-length murine Mpl-L cDNA and autonomously proliferating colonies were isolated from nonstimulated agar cultures. A high Mpl-L-producer clone was selected and used to inoculate B6D2 mice (1×10^6 cells/mouse). As previously shown with GM-CSF, autocrine FDC-P1 cells produce tumor masses at the site of injection. Moreover, grafted FDC-P1 continued to secrete Mpl-L as evidenced by the dramatic increase (5- to 10-fold) in platelet numbers occurring 10 days after inoculation.

Serum Mpl-L activity determination. Exponentially growing Bal/F3-mpl cells were starved of interleukin-3 (IL-3) for 4 hours before being seeded (2×10^4 cells per well) in 96-well plates, in the presence of decreasing concentrations of sera to be tested. After 48 hours in culture, 6,3H thymidine (0.5 μCi) was added for 6 hours. Cultures were collected onto glass fiber filters, and radioactivity incorporated into cellular DNA was determined by liquid scintillation counting.

Northern blot analysis. Total RNAs were extracted and 20 μg were electrophoresed through a 1% agarose gel, in the presence of 3H formaldehyde and transferred to GeneScreen hybridization transfer membranes (NEN Dupont, Boston, MA) in 0.025 M phosphate buffer (pH 6.5). Hybridization was performed for 16 hours at 42°C with a 32P randomly labeled (Rediprime: Amersham, Bucks, UK) PsI UNco I fragment of a murine Mpl-L cDNA (nucleotides 377 to 948 of the coding sequence). The membrane was washed twice with 2X saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at room temperature and twice with 0.1× SSC, 0.1% SDS at 56°C. To quantify Mpl-L mRNA, membranes were stripped and rehybridized with a murine α-actin probe. Blots were exposed to hyperfilm MP (Amersham).

Ribonuclease protection assay. A 32P Pridine triphosphate (UTP)-labeled antisense probe was transcribed from the T7 promoter of an Xho I linearized plasmid (PBT TPO 550) containing a murine Mpl-L cDNA. To construct PBT TPO 550, a Xho I-PstI fragment containing nucleotides 1 to 542 of the Mpl-L coding sequence was inserted into the polylinker site of the Bluescript M13 vector (Stratagene, La Jolla, CA). In one experiment, a 32P-labeled antisense murine actin cDNA probe transcribed from the T3 promoter of the BstNI digested pBACT5 plasmid was used as an internal control for each reaction sample. The plasmid (pBACT5), containing the 5′ half of actin cDNA, was constructed by inserting a PsI I-Tag I actin fragment into the polylinker site of the Bluescript M13 vector (kindly provided by F. Dautry, IGR, Villejuif, France). Total RNAs (20 μg) from liver and kidney of normal, thrombocytopenic or thrombocyto- tomic mice were hybridized with radioactive probes at 55°C overnight. Nonhybridizing RNAs were digested with RNase A (10 μg/mL) and RNase T1 (1,000 U/mL) for 1 hour at 37°C. To stop RNases action, SDS (0.6%) and protease K (145 μg/mL) were added for 15 minutes at 37°C. Protected fragments were extracted in the presence of 15 μg of carrier transfer RNA with phenol/chloroform/isoo- amyl alcohol and precipitated with absolute ethanol at −20°C. Fragments were resolved on a 4% polyacrylamide, 7 mol/L urea gel, and autoradiographed on hyperfilm MP (Amersham). Size of fragments was determined by labeled Msp I–digested pBR322 (New England Biolabs, Beverly, MA). Quantification of protected fragments was performed with a phosphorimager system (MACBAS program).

RESULTS

Detection of Mpl-L mRNA in total RNA from multiple tissues by Northern blot. We first examined whether Mpl-L transcripts could be detected in B6D2 control mice by Northern analysis of total RNA. Figure 1A shows a major 1.8-kb Mpl-L transcript predominantly expressed in the liver and kidney, and more faintly in skeletal muscles. As previously reported, a long Mpl-L–specific transcript of about 5 kb was seen in the brain. Upon longer exposure periods, no additional bands appeared. As no major differences were detected between total and poly (A)+ RNA, subsequent experiments were performed using total RNA.

Mpl-L transcripts are essentially localized in hepatocytes. To further characterize in which liver cell population the Mpl-L gene was transcribed, hepatocyte and nonhepatocyte fractions were separated from normal rat livers by the collagenase perfusion method. The level of Mpl-L transcripts in the two fractions was compared with that of total rat liver. A strong signal was detected in the hepatocyte fraction, while the signal obtained with the nonhepatocyte fraction was much lower (Fig 1B). This signal may result from a 5% to 10% contamination of this fraction by hepatocytes. These results indicate that hepatocytes could be the major cell type in normal liver that produces Mpl-L. However, they do not totally exclude the possibility that a minor population contained in the nonhepatocyte population also expresses the Mpl-L gene. Further in situ hybridization studies are required to precisely determine which cell type(s) produces Mpl-L.

Experimental models of thrombocytopenia. Three experimental models of thrombocytopenia differing in their kinetics were studied. In the first model, mice were lethally irradiated at a dose of 9.5 Gy. As shown in Fig 2A, platelet numbers began to decrease on day 2 and reached a nadir 8 days after irradiation. Serum Mpl-L activity was significantly augmented on day 6 postirradiation and continued to increase as platelet numbers decreased. In the second model, mice
were irradiated at a sublethal dose of 6 Gy and injected with RAMPS. Mice were severely thrombocytopenic, but on day 6 platelet numbers had returned to control values (Fig 4A). The Mpl-L activity in sera from these mice remained much lower than that detected in the two precedent models. To ascertain that the proliferative activity determined on Ba/F3-
LIGAND REGULATION

in thrombocytopenic mice with high levels of plasmatic Mpl-L. Northern blot analyses of liver and kidney mRNAs were performed on different times after treatments. Whatever the times chosen for examinations or the methods used to induce thrombocytopenia, Mpl-L mRNA levels remained similar to those seen in control mice (Figs 2B, 3B, and 4B). A more accurate quantification was obtained by densitometric analysis of bands corresponding to Mpl-L transcripts on the autoradiograms. No significant changes in the level of Mpl-L mRNA was observed in both organs (Table 1). These data show that the increase in Mpl-L activity in sera from thrombocytopenic mice does not result from an upregulation of Mpl-L mRNA in these organs. Previous results performed with a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) technique have suggested that Mpl-L transcription could be modulated by platelet counts in the

mpl bioassay was really due to Mpl-L, proliferation assays were performed in parallel with sera preincubated with a soluble form of Mpl-R. In all cases, preincubation of sera with soluble Mpl-R resulted in a total loss of the biological activity (data not shown).

Expression of Mpl-L transcripts in thrombocytopenic mice. To determine if Mpl-L mRNA level was increased

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**Fig 3.** Analysis of Mpl-L activity and Mpl-L mRNA in mice rendered acutely thrombocytopenic by a sublethal irradiation and an injection of RAMPS. (A) Relationship between platelet number (■) and Mpl-L activity in serum (○) at various times after treatment. Each point represents the mean ± SD (5 mice per determination). (B) Northern blot analysis of 20 µg total RNAs from livers, kidneys or spleens pooled at the times indicated. (C) Ribonuclease protection assay of 20 µg of total RNA. Arrows on the right indicate the position of the protected fragments.

**Fig 4.** Analysis of Mpl-L activity and Mpl-L mRNA in mice treated with 5-FU and RAMPS. (A) Relationship between platelet number (■) and Mpl-L activity in serum (○) at various times after treatment. Each point represents the mean ± SD (5 mice per point). (B) Northern blot analysis of 20 µg total RNAs from livers or kidneys pooled at the times indicated.
Table 1. Mpl-L mRNA Level Ratios in Liver and Kidney From Thrombocytopenic Mice Compared With Control Mice

<table>
<thead>
<tr>
<th>Time Intervals</th>
<th>Irradiated Mice (9.5 Gy)</th>
<th>Irradiated (6 Gy) + RAMPS</th>
<th>5-FU + RAMPS</th>
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<tr>
<td>Liver</td>
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<tr>
<td>Mpl-L mRNA (treated)</td>
<td>1.3 1.8 0.9 ND 1.5 1.1 1.1 0.9</td>
<td>1 0.75 0.6 0.4</td>
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<tr>
<td>Mpl-L mRNA (control)</td>
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<tr>
<td>Kidney</td>
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</tr>
<tr>
<td>Mpl-L mRNA (treated)</td>
<td>1 1.2 0.9 0.8 1.5 1.3 1.3 1.5</td>
<td>1.8 2.2 1.5 1.1</td>
<td></td>
</tr>
<tr>
<td>Mpl-L mRNA (control)</td>
<td></td>
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The ratios of Mpl-L mRNA were calculated from the intensity of the bands measured by scanning densitometry of Northern blots from liver or kidney.

Abbreviation: ND, not done.

spleen or marrow. Mpl-L mRNA levels in spleens of mice with a sustained thrombocytopenic state were analyzed by Northern blot analysis. Even after a long exposure, bands corresponding to Mpl-L transcripts were not shown in the experimental group (Fig 3B). However, when these RNAs were analyzed by RT-PCR, Mpl-L-specific bands were detected, but their intensity did not vary more than twofold when compared with control mice (data not shown).

Profiles of Mpl-L mRNA splice variants in thrombocytopenic mice. The existence of various Mpl-L isoforms has been reported. They encode either nonsecreted or inactive proteins. We studied whether any modulation of mRNAs corresponding to these isoforms could be detected by RNase protection assay using an antisense riboprobe containing the 12 last nucleotides of exon 3, all nucleotides of exons 4, 5, and 6, and 147 nucleotides of exon 7. From the previous reports, this probe should yield at least four different protected fragments. When mRNAs from livers or kidneys of control mice were examined, two predominant fragments were protected corresponding to the wild-type (550 nts) and probably the LPLQ- (400 nts) forms, respectively (Figs 2C and 3C). The LPLQ- isoform appeared to be as abundant as the wild-type form. As best illustrated in Fig 2C, two other protected fragments were also detected that could correspond to murine homologues of the exon 7 variant and the 112 nucleotides-deleted variant. Analyses of the band intensity ratios of wild-type and LPLQ- forms in kidney or liver from thrombocytopenic mice showed no significant changes when compared with normal organs (Figs 2C and 3C). Thus, the results indicate that the increase of Mpl-L activity detected in sera from these mice is not due to increased relative expression of wild-type Mpl-L transcript encoding for active protein vis-à-vis Mpl-L isoforms encoding inactive or nonsecreted proteins.

Levels of Mpl-L mRNA in thrombocytotic mice. To analyze whether high platelet counts would downregulate Mpl-L mRNA or change the profile of Mpl-L isoforms, we developed a model of chronic thrombocytosis. The murine FDC-P1 cell line was engineered to secrete Mpl-L and grow autonomously. Mpl-L production was calculated to vary from 30 to 120 U/1 x 10^6 cells/mL in 4-day conditioned media (1 U of Mpl-L activity is equal to 300 pg/mL of purified recombinant mu-Mpl-L protein as defined on the BaF3-mpl bioassay). Autocrine FDC-P1 cells injected subcutaneously produced tumor nodules at the site of injection. On day 10 after the graft, platelet numbers were increased 10-fold above normal values (10 x 10^6 platelets/µL of blood v 8.6 x 10^7/µL in control mice). Mice were examined on day 15. RNase protection assays were performed with an internal antisense actin riboprobe to quantify Mpl-L mRNA levels and ratios of Mpl-L wild-type transcript to Mpl-L isoforms. The data (Fig 5) show comparable levels of Mpl-L mRNA and no modification in the ratios between wild-
type transcript and isoforms in liver or kidney from control and thrombocytotic mice.

DISCUSSION

In the present study, we have investigated by quantitative and qualitative Northern blot and RNase protection analyses whether a modulation of Mpl-L transcripts could be detected in liver and kidney of mice rendered either severely thrombocytopenic or thrombocytotic. We show, that in mice with low level or excess of platelets, no change in Mpl-L mRNA levels or in the profile of Mpl-L mRNA isoforms were detected suggesting the absence of a transcriptional regulation.

To study whether Mpl-L activity was regulated at a transcriptional level, we developed several models of thrombocytopenia and a model of thrombocytosis. Kinetic analyses were performed with thrombocytopenic mice to determine whether an increase of Mpl-L mRNA would parallel the augmentation in Mpl-L activity in serum. Our results confirmed the inverse relationship between low platelet numbers and high Mpl-L activity in serum. However, no significant change in Mpl-L mRNA levels was detected in the liver and kidney, irrespective of the time interval following treatment or the method used to induce low platelet counts and/or to decrease the megakaryocyte mass. One possible mechanism of Mpl-L regulation relies on the existence of various Mpl-L splice variants encoding either nonsecreted or inactive proteins.19,20,28 By RNase protection experiments, we show that no change in the ratios of Mpl-L wild-type form to isoforms occurred in thrombocytopenic mice. These data confirm the conclusions drawn from a similar study performed with two models of thrombocytopenic mice.22

We have extended these results by an analysis of an opposite model of thrombocytosis. We have investigated whether a 10-fold increase in circulating platelets would induce a downregulation of Mpl-L transcripts or a modulation in the ratio between Mpl-L mRNA isoforms. We observed no decrease of Mpl-L mRNA levels in mice with an excess of platelets. Furthermore, no modulation in the ratio between Mpl-L wild-type form and its isoforms was detected in the thrombocytotic mice.

Therefore, from our study we can conclude that neither Mpl-L mRNA levels, nor the ratio between isoforms are changed in response to decrease or increase platelet numbers. These results show that Mpl-L mRNAs levels in liver and kidney are not modified by feedback mechanisms. This conclusion is further supported by the results recently obtained with genetically modified Mpl-L knock-out mice.20 The investigators have shown that circulating platelet numbers in heterozygous mice were about 50% decreased, while a 90% reduction in platelet numbers was seen in homozygous animals. The gene dosage effect observed in heterozygous mice strongly suggests that the Mpl-L gene is not directly regulated by the platelet mass.

Several models have suggested that platelets could regulate circulating Mpl-L produced at a constant rate by receptor mediated uptake and destruction. Indeed, recent reports have demonstrated that platelets expressed high affinity Mpl-R that bind, internalize, and degrade Mpl-L.22,30

Our different models of thrombocytopenia further suggest that the megakaryocyte mass could also be involved in Mpl-L clearance. Indeed, comparison of Mpl-L bioactivity in the three models of thrombocytopenia showed large differences. Mice with low platelet numbers and drastically reduced number of megakaryocytes in their marrow and spleen (9.5 Gy or 6 Gy irradiated mice) have high Mpl-L levels in the serum. In contrast, in 5-FU and RAMPS-treated mice with low platelet numbers, but active megakaryopoiesis, serum Mpl-L activity remains low. Two reports are in favor of the involvement of the megakaryocytes in Mpl-L clearance. First, we have previously demonstrated that late colony-forming unit megakaryocyte, promegakaryoblasts and megakaryocytes expressed functional Mpl-R.31 Since the time period of megakaryocyte maturation and platelet life-span are almost identical (about 5 days in the mouse), the platelet mass and megakaryocyte mass must be equivalent in normal animals. Therefore, it can be hypothesized that the numbers of Mpl-R expressed on megakaryocytes and platelets might be in the same order of magnitude. Secondly, the hypothesis that megakaryocytes may play an active role in the Mpl-L clearance is also suggested by the model of the NF-E2 knockout mice. Mice with disrupted NF-E2 alleles died at birth of hemorrhage due to a total absence of platelets. Unexpectedly, no elevation of Mpl-L in plasma from these mice could be demonstrated.31 However, these mice have increased numbers of megakaryocytes with disordered and expanded demarcation membranes. Although not formally demonstrated, it is tempting to speculate that Mpl-L is actively cleared from plasma by binding to its receptors expressed most probably at high number on the abnormal megakaryocytes. Other levels of Mpl-L regulation might occur within the bone marrow and spleen microenvironment. As already suggested, these organs might be sites of Mpl-L production.31 In support of this hypothesis is the demonstration that endothelial and stromal cells support megakaryocytogenesis in vitro32,33 and produce Mpl-L.33

In physiological states, Mpl-L appears to be constitutively produced and cleared from the plasma by receptor uptake and catabolism. However, several other possible important mechanisms must be further investigated to fully understand the mode of Mpl-L regulation. Mpl-L might be also captured by circulating soluble Mpl receptors32 or locally concentrated in the extracellular matrix of the marrow microenvironment, as already demonstrated for other cytokines.33

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