Molecular detection of c-mpl thrombopoietin receptor gene expression in chronic myeloproliferative disorders

S Duensing, A Duensing, J G Meran, A Kreft, G Büsche, A Ganser, A Georgii

Abstract

Background—Chronic myeloproliferative disorders (CMPD) originate from a pluripotent haematopoietic progenitor cell but show a marked degree of heterogeneity, especially between Philadelphia chromosome positive and negative disease entities. Abnormal megakaryopoiesis is a frequent finding in CMPD, often associated with thrombocytthaemic cell counts. Recent experimental data have suggested that the c-Mpl thrombopoietin receptor, together with its ligand thrombopoietin, are not only the major physiological regulators of megakaryopoiesis and platelet production, but also play a crucial role in chronic myeloproliferation.

Methods—A total of 18 peripheral blood mononuclear cell samples obtained from patients with CMPD (chronic myelocytic leukaemia (CML), n = 10; polycythaemia vera (PV), n = 6; and primary thrombocythaemia (PTH), n = 2) were analysed for c-mpl mRNA using the reverse transcriptase polymerase chain reaction (RT-PCR). In another 20 patients (CML, n = 10; chronic megakaryocytic granulocytic myelosis (CMGM), n = 3; PV, n = 3; PTH, n = 4), we compared the number of haematopoietic progenitors expressing c-Mpl, as characterised by coexpression with the CD34 antigen, in the bone marrow using double immunofluorescence staining.

Results—c-mpl mRNA was detected in all samples from patients with CML analysed, whereas only two of six PV and one of two PTH samples were positive (p < 0.008; χ² test). Expression of the c-mpl receptor gene was absent in healthy subjects used as controls. Similarly, an increase of c-Mpl expressing CD34 positive haematopoietic cells was detected in seven of 10 bone marrow aspirates obtained from patients with CML. Increased numbers of c-Mpl positive CD34 positive cells were found in only one of four patients with PTH, whereas in PV and CMGM the numbers of c-Mpl positive CD34 positive cells did not exceed normal values, despite thrombocythaemic cell counts.

Conclusions—These data confirm recent findings showing an impaired expression of the c-mpl thrombopoietin receptor gene in Philadelphia chromosome negative CMPD when compared with patients with Philadelphia chromosome positive CML. The relevance of this observation to the functional and morphological characteristics of abnormal megakaryopoiesis remains unclear. Thrombocytthaemic cell counts and a mature phenotype in megakaryocytes occur frequently in Philadelphia chromosome negative CMPD but require an intact c-Mpl receptor under physiological conditions. Therefore, further studies are warranted to elucidate the mechanisms contributing to megakaryopoiesis in CMPD disease entities with decreased c-mpl gene expression.

Keywords: c-Mpl thrombopoietin receptor; chronic myeloproliferative disorders; megakaryopoiesis

Thrombocytthaemic platelet counts in the peripheral blood are not restricted to essential or primary thrombocytthaemia (PTH) but may occur in patients with polycythaemia vera (PV), chronic idiopathic myelofibrosis (CIMF) or even in Philadelphia chromosome positive chronic myelocytic leukaemia (CML) in a substantial number of patients.

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ments showed that overexpression of the thrombopoietin gene in the animal model not only induced multilineage growth of blood cells but also extramedullary haematopoiesis and myelofibrosis similar to chronic myeloproliferation in humans.13–15 There is also evidence that c-mpl contributes to abnormal megakaryopoiesis. In patients with CMPD, Li et al found a decrease in spontaneous megakaryocytic colonies from peripheral blood using antisense strategies against the c-mpl receptor gene.16 With regard to these data, some interest arises in abnormalities of thrombopoietin mediated signalling through the c-Mpl receptor and the distribution of the receptor itself in CMPD. To evaluate potential differences between CMPD disease entities, we compared peripheral blood c-mpl mRNA expression between patients with Philadelphia chromosome positive CML and those with PV or PTH. The observed differences between CML and Philadelphia chromosome negative disorders were further investigated by studying the distribution of c-Mpl positive haematopoietic progenitors in the bone marrow of patients with CMPD.

### Material and methods

**PATIENTS AND HISTOPATHOLOGY**

Peripheral blood samples were collected from a total of 18 patients with CMPD (CML, n = 10; PV, n = 6; PTH, n = 2; table 1) and eight healthy subjects (not shown) after informed consent. The mean age of the patients was 48 years (range, 20–71 years). Because bone marrow aspirates were not collected from these patients, we analysed the distribution of c-Mpl expressing haematopoietic progenitors in a second group of 20 patients with CMPD (CML, n = 10; chronic megakaryocytic granulocytic myelosis (CMGM), n = 3; PV, n = 3; PTH, n = 4; table 2) and eight patients lacking histopathological alterations used as controls (data not shown). The mean age of patients with CMPD in the latter group was 59 years (range, 35–84 years).

Haematological diagnoses were confirmed by histopathology of trephine biopsies from the bone marrow. Histopathological classification was performed by two observers, according to the Hannover system.2 This may deviate from the new WHO nomenclature in early cases of CIMF, which are included among the descrip-
tive designation of CMGM, to distinguish early stages of the latter from essential thrombocythaemia (PTH). All patients with CML were proved to be Philadelphia chromosome positive by karyotyping; no substantial additional chromosomal abnormalities (including occurrence of 20q-) were observed by karyotyping in our laboratory.

**REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION (RT–PCR)**

Total cellular RNA was extracted from heparinised peripheral blood samples by the RNAzol A method (Cinna Biotecx, Houston, Texas, USA). Blood mRNA integrity was checked by reverse transcription with random hexamer primers and consecutive PCR with primers for pyruvate dehydrogenase 1 (PDH1), sense 5'-GGT ATG GAT GAG GAC CTG GA-3' and antisense 5'-CTT CCA CCC TGC ACT AA-3', giving rise to a 105 base pair (bp) fragment. Transcripts for c-mpl were detected using sense primer 5'-CTA GCT CCC AAG GCT TCT TC-3' (positions 927-946) and antisense primer 5'-GTC AGC ACC TTC CAG TCC-3' (positions 1298-1319), producing a 392 bp amplification product. Bone marrow mononuclear cells obtained from a patient with a normal bone marrow histopathology were used as a positive control. cDNA was synthesised using random hexamer primers (pdN3) and Moloney murine leukaemia virus reverse transcriptase. PCR was performed using 5 µl cDNA in a reaction containing a dNTP mix (2 mM dATP, 2 mM dCTP, 2 mM dGTP, and 2 mM dTTP), reaction buffer (at a final concentration of 50 mM KCl, 10 mM Tris/HCl, and 1.5 mM MgCl2), and 1.5 µl Taq polymerase (all reagents Boehringer Mannheim, Mannheim, Germany) in a final volume of 40 µl. Cycling conditions for PDH1 and c-mpl were 94°C for one minute, 68°C for one minute, and 72°C for one minute repeated 35 times, with an additional final extension at 72°C for five minutes. The PCR products (8 µl aliquots) were electrophoresed through a 2% agarose gel, stained with ethidium bromide, and visualised under UV light.

**IMMUNOFLUORESCENCE**

For double immunofluorescence staining of bone marrow aspirates, cytological preparations were fixed, washed in phosphate buffered saline (PBS), and blocked with normal rabbit serum. Slides were then incubated with a monoclonal antibody against c-Mpl (final concentration 0.1 µg/ml; Genzyme, Cambridge, USA) for 30 minutes at room temperature, followed by a fluorescence (Cy3) labelled antimouse antibody (Jackson Immunoresearch, West Grove, Pennsylvania, USA). Cells were then incubated with a fluorescein isothiocyanate (FITC) conjugated monoclonal anti-CD34 antibody (Pharmingen, Hamburg, Germany) overnight at 4°C, washed, and counterstained. Two hundred mononuclear bone marrow cells were evaluated under a fluorescence microscope.

**STATISTICAL ANALYSIS**

To assess significance, the χ² test was used wherever applicable.

**Results**

**PERIPHERAL BLOOD c-mpl mRNA EXPRESSION**

In patients with CMPD, a total of 13 of 18 peripheral blood samples were positive for c-mpl transcripts, showing an amplification product of the expected size of 392 bp (fig 1). By contrast, expression of c-mpl was not detected in any of the peripheral blood mononuclear cell samples obtained from healthy controls (eight, not shown).

All samples obtained from patients with Philadelphia chromosome positive CML were positive for c-mpl transcripts (10 of 10), regardless of white blood cell counts, platelet counts, or current treatment. By contrast, only two of six patients with PV and one of two patients with PTH had c-mpl transcripts. There was no correlation between peripheral blood c-mpl mRNA expression and platelet counts in the 18 patients studied. In addition, white blood cell counts did not correlate with c-mpl expression (table 1).

In summary, peripheral blood c-mpl mRNA expression occurred significantly more frequently in Philadelphia chromosome positive CML when compared with PV and PTH (p < 0.008; χ² test).

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**c-Mpl EXPRESSING HAEMATOPOIETIC PROGENITORS IN THE BONE MARROW**

In bone marrow aspirates obtained from patients lacking histopathological abnormalities, which were used as controls, the percentage of c-Mpl expressing mononuclear cells coexpressing CD34 did not exceed 1% (data not shown). In patients with CMPD, an increased number of c-Mpl positive haematopoietic progenitors (> 1%) was seen in eight of 20 samples (table 2).

Seven of 10 patients with Philadelphia chromosome positive CML had an increase of c-Mpl positive CD34 positive cells, as did one patient with PTH. None of the patients with CMGM or PV showed an increase in bone marrow progenitors coexpressing c-Mpl and CD34.

As in our findings in the peripheral blood, the number of c-Mpl expressing CD34 positive haematopoietic cells significantly increased in Philadelphia chromosome positive CML when compared with Philadelphia chromosome negative disease entities (p < 0.05; χ² test).

In all patients, trephine biopsies of the bone marrow were analysed simultaneously. All
increases of c-Mpl expressing bone marrow progenitors were found not to correlate with either an increased number of bone marrow megakaryocytes or with thrombocythaemic peripheral blood cell counts. By contrast, increased bone marrow megakaryocytes and thrombocythaemic blood cell counts were seen predominately in patients with CMGM, PV, and PTH, who mainly had physiological numbers of c-Mpl positive haematopoietic progenitors. There was a tendency towards raised peripheral blood platelet counts in patients with increased bone marrow megakaryocytes (p ≤ 0.055; χ²test). Bone marrow fibrosis was found in one patient with CML who did not have raised bone marrow megakaryocytes or c-Mpl positive CD34 positive mononuclear bone marrow cells.

Discussion

Chronic myeloproliferative disorders arise from the clonal expansion of a single pluripotent haematopoietic cell but show a large degree of heterogeneity in terms of blood counts, bone marrow histopathology, and the clinical course of disease. Abnormal megakaryopoiesis is found frequently in the bone marrow of patients with CMPD, often associated with thrombocythaemic cell counts. Characteristic histological differences in megakaryocytic morphology and distribution are recognised and permit the distinction of patients with and without the Philadelphia chromosome. The c-Mpl thrombopoietin receptor and its ligand (thrombopoietin) are major regulators of normal megakaryopoiesis and platelet production, and may also be involved in abnormal megakaryopoiesis in CMPD, as suggested by experimental data. However, little is known about the differential expression of the c-mpl gene in patients with CMPD and its potential role in the pathobiology of these disorders.

In our study, the rationale for analysing peripheral blood samples for c-mpl gene expression is based on numerous reports showing that circulating megakaryocyte progenitors may induce spontaneous in vitro megakaryocyte colony formation, which is frequently found in CMPD. Because megakaryocyte progenitors as well as multilineage precursors have been reported to express the c-mpl gene, peripheral blood mononuclear cells from patients with CMPD should express c-mpl transcripts, at least those from patients presenting with thrombocythaemic cell counts, as suggested by Kobayashi et al. In contrast to the expected results, our study revealed c-mpl mRNA in only a small proportion of patients with Philadelphia chromosome negative disorders and a significantly higher expression of c-mpl transcripts in Philadelphia chromosome positive CML. Although we did not evaluate circulating megakaryocytic precursors on a cellular level, we conclude that expression of the c-mpl receptor gene is impaired in PV and PTH, rather than increased in CML. Our data confirm recent results by Moliterno et al., who demonstrated reduced expression of the thrombopoietin receptor in megakaryocytes and platelets of patients with PV and idiopathic myelofibrosis, which is considered to be an advanced stage of CMGM. The authors also reported reduced thrombopoietin mediated tyrosine phosphorylation of platelet proteins in PV, which was absent in CML and haematological disorders unrelated to CMPD. Our data suggest reduced transcription of the c-mpl gene as a possible mechanism in the decrease of thrombopoietin receptor expression and function in PV. However, differences in tyrosine phosphorylation of platelet protein between PV and CML may not be based solely on sustained thrombopoietin receptor expression in CML, because tyrosine phosphorylation can also occur by Bcr-Abl kinase, which is constitutively activated in the malignant CML clone.

As in our findings in peripheral blood, the number of c-Mpl positive haematopoietic progenitors in the bone marrow, as characterised by coexpression of c-Mpl and CD34, was significantly lower in CMGM, PV, and PTH when compared with CML. Surprisingly, most patients with Philadelphia chromosome negative CMPD presented with thrombocythaemic cell counts and increased numbers of bone marrow megakaryocytes in the face of normal levels of c-Mpl positive haematopoietic progenitors. The incidence of increased bone marrow megakaryocytes in CML, in contrast to the high frequency of c-Mpl positive haematopoietic progenitors, may be related to shifts in the haematopoietic progenitor cell pool, as suggested recently.

With respect to morphological features, PV and other Philadelphia negative CMPD frequently present with predominantly large and hypersegmented bone marrow megakaryocytes showing an increased nuclear ploidy, which strongly suggests thrombopoietin mediated terminal maturation. It is not understood how impaired c-Mpl receptor expression can give rise to a mature appearing phenotype of bone marrow megakaryocytes and thrombocythaemic cell counts, respectively. Because thrombopoietin concentrations have been reported not to be increased in CMPD when compared with reactive thrombocytosis, other cytokines involved in the regulation of megakaryopoiesis might play a role. More speculatively, the underlying genetic aberration in Philadelphia chromosome negative disorders may give rise to regulatory pathways that allow megakaryocytic differentiation independent of the c-mpl/thrombopoietin system.

Further studies are warranted to elucidate the role of decreased c-mpl gene expression in the natural history of Philadelphia negative disorders and the relevance of c-Mpl/thrombopoietin in CMPD in general.

4 Kaloutsi V, Fritsch RS, Buhr T, et al. Megakaryocytes in chronic myeloproliferative disorders: numerical density...


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Mol Path 1999 52: 146-150
doi: 10.1136/mp.52.3.146

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