Expression of the c-mpl Proto-oncogene in Human Hematologic Malignancies


Similar to two other hematopoietic growth factor receptors, the c-fms (macrophage colony-stimulating factor receptor) and the c-kit genes, c-mpl has been discovered through the study of oncogenic retroviruses. Unlike c-fms and c-kit, which both belong to a subgroup of tyrosine kinase receptors, the c-mpl proto-oncogene encodes a new member of the cytokine receptor superfamily. We have studied the expression of c-mpl in a series of 105 patients with hematologic malignancies using Northern blot analysis. The levels of c-mpl transcripts in lymphoid malignancies and in chronic myeloproliferative disorders were not significantly different from those found in normal bone marrow cells, in which c-mpl was barely detectable. In contrast, c-mpl expression was increased in 26 of 51 patients with acute myeloblastic leukemia (AML) and in 5 of 16 patients with myelodysplastic syndromes. Amplification of the c-mpl gene was detected in genomic DNA of one M4 AML patient. There was no significant correlation between c-mpl expression and the French-American-British classification of AML. Patients with high c-mpl expression appeared to belong to a subgroup of AML with a low rate of complete remission and a poor prognosis, including secondary leukemia and AML with unfavorable cytogenetic abnormalities.

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MATERIALS AND METHODS

Patients and cell line. Peripheral blood (PB) or bone marrow (BM) samples were obtained from 105 patients (104 adults and 1 child) who had been referred to the Hematology Department at the Hôpital Cochin between 1988 and 1992. All specimens were collected before the initiation of therapy and were obtained from 51 patients with AML, 11 with acute lymphoblastic leukemia (ALL), 19 with myeloproliferative disorders (MPD) in chronic phase, 8 with non-Hodgkin's lymphoma (NHL), and 16 with myelodysplastic syndromes (MDS) (Table 1). The diagnosis of each AML was established on the basis of morphologic and cytochemical staining, and patients were classified according to the criteria of the French-American-British (FAB) committee.

Although c-mpl expression was detected only in malignancies with immature blast cell proliferation, its expression did not uniformly correlate with the presence of immature blast cells. Our results suggest that c-mpl expression in AML patients could be of prognostic significance in predicting response to induction therapy.

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leukemia (CML), essential thrombocytemia (ET). As a control, PB and normal BM samples were frozen at -80°C until isolation after Ficoll-Hypaque density gradient centrifugation. The 4 untreated patients included 2 elderly patients (unique patient numbers UPN 23 and UPN 49). The cytogenetic study was performed on 37 patients with AML at diagnosis on BM samples. Cells were cultured for 24 or 48 hours before chromosome preparation. When possible, 20 metaphases were photographed and analyzed after reverse heat G (RHG) banding of the chromosomes.56 Karyotypes were described according to the International System for Human Cytogenetic Nomenclature.26

**Cytogenetic analyses.** The cytogenetic study was performed on 37 patients with AML at diagnosis on BM samples. Cells were cultured for 24 or 48 hours before chromosome preparation. When possible, 20 metaphases were photographed and analyzed after reverse heat G (RHG) banding of the chromosomes. Karyotypes were described according to the International System for Human Cytogenetic Nomenclature. Statistical analysis. The χ² test was used to determine significance of qualitative variable differences. Probabilities of <.05 were considered statistically significant. The log-rank test was used to compare the groups of patients with respect to achieving CR and disease-free survival.

**RESULTS**

c-mpl is expressed in patients with AML or RAEB. RNAs isolated from PB or BM from 105 patients with hematologic malignancies described in Materials and Methods and Table 1 were studied for c-mpl expression by Northern blot analysis. We previously reported that c-mpl was detected as a major 3.7-kb and a minor 2.7-kb mRNA species in the human HEL cell line. Both transcripts have been cloned, and they potentially code for polypeptides that share common extracellular and transmembrane domains but differ in their cytoplasmic regions. These two mRNA species probably derive from a unique c-mpl gene by alternative splicing. To study c-mpl expression, we used as a probe a fragment located in the mpl extracellular domain that detects the two c-mpl transcripts in HEL cells (Fig 1, lane a).

As shown in Fig 1, no signal was detected in mRNA isolated from normal PB cells (Fig 1, lane b), and c-mpl was barely detectable as a faint 3.7-kb transcript in BM samples from healthy individuals (Fig 1, lane c). Very low c-mpl expression was detected in the 19 patients with lymphoproliferative disorders and in the 19 patients with MPD in chronic phase. In contrast, c-mpl mRNA levels appeared to be elevated in 26 of 51 (51%) AML patients and in 5 of 16 (31%) MDS patients. Typical results are shown in Fig 1. Variability in intensity was seen among patients despite equal loading (as verified by β-actin hybridization), but c-mpl expression in patient samples was usually lower than in HEL cells, except for 1 patient (UPN 23 in Table 2) who presented with a diagnosis of AML, subtype M4 (Fig 1, lane g). Although the 3.7-kb c-mpl transcript was consistently expressed more than the 2.7-kb c-mpl mRNA species, as

**Table 1. c-mpl Expression in Human Hematologic Malignancies**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Increase Over Normal c-mpl Expression</th>
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<tr>
<td>AML (51 patients)</td>
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<td>M0</td>
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<td>M1</td>
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<td>MDS (16 patients)</td>
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<td>MPD (19 patients)</td>
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<td>NHL (6 patients)</td>
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RNA loading was verified by hybridization of each filter with a murine β-actin probe. The level of c-mpl expression was compared with that observed in HEL cells and in normal BM cells. The presence of megakaryocytes or platelets in BM and PB samples was detected with a glycoprotein IIb (GPIIb) cDNA probe given by Dr G. Uzan (U 217-Grenoble, Grenoble, France).

**DNA preparation and Southern blot analysis.** High molecular weight DNA was extracted by conventional methods. DNAs (10 μg) were digested with restriction enzymes under reaction conditions recommended by the manufacturers. Agarose gel electrophoresis, Southern blot transfer, and hybridization were performed as previously described.17 Probes used as markers of the 1p32-34 chromosomal region were tal-1, L-myc, c-jun (provided by Dr D. Mathieu-Mahul, U 301, Paris, France), lck (provided by Dr R. Benarous, U 332, Paris, France), Rhesus (from Dr J.P. Cartron, CNTS, Paris, France), and a G-CSF receptor probe (given by Dr S. Gillis, Immunex, Seattle, WA).

**mRNA preparation and Northern blot analysis.** PB or BM cells were isolated after Ficoll-Hypaque density gradient centrifugation and washed three times in Hanks' balanced salt solution. RNA was extracted by the guanidine thiocyanate procedure.29 After precipitation in ethanol at −20°C, RNA pellets were resuspended in water. RNA concentrations were determined by optical density, and samples were frozen at −80°C until use.

RNA samples (10 μg of total RNA) were denatured in glyoxal, dimethyl sulfoxide, and phosphate buffer at 65°C for 10 minutes, size-fractionated by electrophoresis through 1% agarose gel, and transferred to nylon membrane. Membranes were hybridized with random 32P-labeled probes and washed under stringent conditions as previously described.17 The c-mpl probe used in this study was a 280-bp-long fragment located in the human c-mpl extracellular domain.

*Vigon et al.*
observed in normal BM and in HEL cells (in BM, the level of mpl expression is not high enough to detect the 2.7-kb transcript), the 2.7-kb c-mpl transcript was found to be quite abundant in the two cases shown in Fig 1, lanes g and i. In AML patients, it appeared that blast cell populations were heterogeneous with respect to the expression of c-mpl. No significant correlation was found between c-mpl expression and the FAB classification because c-mpl transcripts

<table>
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<th>Table 2. AML Patient Characteristics</th>
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Abbreviations: FAB, French-American-British classification; BM, bone marrow; PB, peripheral blood; CR, complete remission; F, failure; NT, not treated; R, relapse; D, dead; A, alive.

* Secondary leukemia.
were found in patients with M2, M3, M4, M5, and M6 AML (Table 1). However, it is noticeable that, although the percentage of blast cells were lower in M6 AML samples than in other AML subtypes, all patients with M6 leukemias were c-mpl positive.

Of the 16 MDS patients, none of the 5 patients with RSA had increased c-mpl expression, whereas 5 of the 11 patients with a diagnosis of RAEB showed increased c-mpl transcripts (Table 1).

The c-mpl gene is amplified in one patient with an M4 AML. To determine whether genomic alterations might be responsible for high expression, genomic DNAs of leukemic cells from patients expressing the highest levels of c-mpl mRNA were subjected to Southern blot analysis. No gross alteration of the c-mpl gene was detected in these DNA samples, except in DNA from UPN 23, in which a c-mpl gene amplification was found (Fig 2A, lanes a and d). Although cytogenetic analysis was not available for this patient, these results could be explained by either an amplification encompassing the c-mpl locus or by chromosome duplication. The c-mpl gene was previously located on human chromosome 1p34. Therefore, probes for 6 genes located in the small arm of chromosome 1 (tal-1, L-myc, c-jun, lck; Rhesus, and G-CSF receptor) were hybridized to DNA from UPN 23. No amplification was shown with any other probe tested (see, for example, hybridization with a L-myc probe Fig 2B), suggesting that the c-mpl locus was most likely amplified in the leukemic cells from this patient. Dot blot analysis performed on DNA dilutions indicated that the c-mpl gene was eightfold more abundant in DNA from UPN 23 than in control DNA (data not shown).

Correlation between c-mpl expression and clinical and biologic features in AML. The characteristics of the AML population we studied are listed in Table 2. We found no significant correlation between c-mpl expression and characteristics such as sex, age, white blood cells, and platelets counts or the origin of sampling (PB or BM) (Table 2). The HEL cell line which has megakaryocytic and erythroid characteristics is the only human hematopoietic cell line we tested in which we detected c-mpl transcripts by Northern blot analysis. Consequently, we investigated the possibility of a correlation between GPIIb, glycophorin, and c-mpl expression in AML. By immunophenotyping, only M6 AML expressed the early erythroid glycophorin marker. Northern blots from AML patients were rehybridized with a GPIIb probe. No correlation was found between GPIIb and c-mpl expression (data not shown).

Of the 51 AML cases we studied, 16 were presumably secondary leukemias; 12 were blast crises arising during the progression of either chronic MDS (UPN 7, 14, 15, 30, 50, and 51) or MPD (UPN 8, 12, 17, 24, 28, and 35); 1 patient (UPN 49) had been professionally exposed to irradiation; and 3 patients (UPN 3, 25, and 32) had received chemotherapy after diagnosis of a solid tumor. Sixty-eight percent (11 of 16) of these 16 secondary leukemias were c-mpl positive versus 43% (15 of 35) of the 35 AML patients presenting with a diagnosis of de novo AML (Table 3).

Prognostic value of c-mpl expression in AML. Thirty seven patients with AML could be classified into prognostic groups based on the results of cytogenetic studies according to the prognostic cytogenetic criteria of Keating et al.
c-mpl EXPRESSION IN LEUKEMIAS

Among the 18 c-mpl-negative AML, 14 had a poor prognosis karyotype. The presence of a poor prognosis karyotype was highly significant among patients with c-mpl-positive AML (35%) achieving CR, whereas 16 of 24 AML with a high-risk karyotype were classified as c-mpl negative. AML with a good prognostic karyotype (13 of 17) was significantly lower rate of CR in comparison to the c-mpl-negative AML. Therefore, we wondered whether c-mpl expression in AML is responsible for the impaired differentiation of AML blasts; but, because c-mpl is a component of a growth factor signal transduction pathway, expression of the c-mpl product could play a role in the proliferation of these cells. c-mpl expression in AML may be caused by an alteration of the c-mpl gene or may reflect the expansion of a cell population that normally expresses the c-mpl gene and represents only a small proportion of normal BM cells.

Amplification of the c-mpl gene evidenced in AML DNA was probably responsible for high c-mpl expression in 1 M4 AML (UPN 23). Two AML had translocations involving the lp36 region, but 1 (UPN 38) did not express c-mpl. However, UPN 32 expressed a normal-sized c-mpl 3.7-kb transcript at relatively low levels (not shown). No gross alteration or amplification of the c-mpl gene was detected in the DNA of other c-mpl-positive AML.

c-mpl expression in AML was associated with poor prognosis because, compared with c-mpl-negative AML, the c-mpl-positive AML had a significantly lower rate of CR in response to chemotherapy. Chemotherapy resistance disease was the main cause of induction failure in c-mpl-positive AML. Therefore, we wondered whether c-mpl expression would correlate with other factors known to be of prognostic significance in AML. c-mpl expression did not significantly correlate with age, leucocyte count, or platelet count. Characteristics of the 26 patients with c-mpl-positive AML and of the 25 patients with c-mpl-negative AML were compared and showed that c-mpl was more frequently expressed in secondary than in de novo AML. Moreover, our results showed that c-mpl expression correlated with the presence of cytogenetic abnormalities known to be of major prognostic value in the outcome of AML in terms of both CR rate and long-term disease-free survival. We noted that 11 of the 12 AML patients with abnormalities leading to loss of the whole chromosome or part of chromosome 5 and/or chromosome 7 were c-mpl positive. AML with deletion of chromosomes 5 and/or 7 represent a subset of AML characterized by poor prognosis usually because of chemotherapy-resistant disease.

Although c-mpl did not correlate with the FAB classification, the fact that 5 erythroleukemias (FAB M6) were all

| Table 3. Relationship of c-mpl Expression to AML Patient Characteristics |
|-----------------------------|-----------------------------|
| Increase Over Normal Expression |
| Median | Range | Median | Range |
| Age (yr) | 50.9 | 20-90 | 51.7 | 11-87 |
| Sex ratio (M/F) | 1.07 | 1.1-6 |
| White blood cell count (<10⁹/L) | 37.7 | 1.5-200 | 42.3 | 3.5-180 |
| Platelet count (<10⁹/L) | 95.8 | 16-344 | 91.2 | 15-600 |
| De novo AML (35 patients) | 20 (57%) | 15 (43%) |
| Secondary AML (18 patients) | 5 (31%) | 11 (68%) |
| Good prognostic karyotype (20 patients) | 14 (70%) | 6 (30%) |
| Poor prognostic karyotype (17 patients) | 4 (23%) | 13 (76%) |

Schouten et al20 [good prognostic: normal karyotype, t(8;21) (q22q22), t(15;17)(q22q11), or inv(16)(q22); poor prognostic: all others]. Among the 18 c-mpl-negative AML, 14 had a good prognostic karyotype [11 had a normal karyotype, 3 had a translocation t(15;17)(q22q11)], and 4 had a poor risk karyotype. In contrast, among the 19 c-mpl-positive AML, only 6 had good prognostic characteristics: 3 had no cytogenetic abnormalities, 2 had a translocation t(15;17)(q22q11), and 1 had a t(8;21)(q22q22). However, 13 patients had chromosomal abnormalities affecting chromosome 5 and/or 7 or chromosome deletions or additions. As summarized in Table 3, 70% (14 of 20) of AML with a good prognostic karyotype were c-mpl negative, whereas 76% (13 of 17) of AML with a high-risk karyotype were classified as c-mpl positive. The correlation between high c-mpl expression and the presence of a poor prognosis karyotype was highly significant (P < .001).

Of 51 AML patients, 47 were treated with doxorubicin and cytosine arabinoside as described in Materials and Methods. Of the 4 untreated AML patients, 1 was c-mpl negative (UPN 42) and 3 were c-mpl positive (UPN 49, 50, and 51), presenting with a diagnosis of M6 leukemia that evolved from a myelodysplastic disorder (UPN 49 and 50) or secondary exposure to irradiation (UPN 51). CR was obtained in 24 of 47 patients treated (51%). Eight of 23 c-mpl-positive AML (35%) reached CR, whereas 16 of 24 c-mpl-negative AML (67%) reached CR (P = .01). Resistance disease was the main cause of induction failure in c-mpl-positive AML (13 of 15) and was also observed in 3 of the 8 c-mpl-negative AML who did not achieve CR. Of the 13 c-mpl-positive patients who failed to enter CR, 5 patients with a good performance status were treated with a second course of chemotherapy, as indicated in Materials and Methods. None of these 5 patients reached CR. However, in our experience, this intensive therapy leads to CR in 45% of refractory AML, suggesting a primary resistance in c-mpl-positive AML.22 Because too few patients in the c-mpl-positive group achieved CR, a meaningful comparison of the duration of remission could not be performed.

DISCUSSION

In this report, the expression of the c-mpl gene that encodes a new member of the cytokine receptor superfamily was examined by Northern blot analysis in cells from patients with various hematologic neoplasms. RNA blot analysis showed that c-mpl transcripts were not detectable in normal PB and were expressed at very low levels in normal BM cells. We found c-mpl mRNA to be expressed above normal BM cell levels in 51% of AML and in 5 of 11 RAEB samples. No increase in expression was observed in acute leukemias of lymphoid origin, in lymphoma cells, or in samples of myelodysplastic or myeloproliferative disorders in chronic phase. This result contrasts with the biologic properties of MPLV, a murine retrovirus that has transduced a constitutively activated form of this receptor chain and induces the overproliferation of differentiating hematopoietic progenitors. Therefore, it seems very unlikely that c-mpl expression in AML is responsible for the impaired differentiation of AML blasts; but, because c-mpl is a component of a growth factor signal transduction pathway, expression of the c-mpl product could play a role in the proliferation of these cells. c-mpl expression in AML may be caused by an alteration of the c-mpl gene or may reflect the expansion of a cell population that normally expresses the c-mpl gene and represents only a small proportion of normal BM cells.
c-mpl/positive raised questions concerning the possible influence of the phenotype of these leukemias on c-mpl expression. Alternatively, because 3 M6 AML patients had secondary AML and 4 of 5 had abnormalities of chromosomes 5 and/or 7 frequently associated with c-mpl-positive AML, these M6 leukemias could represent a subset of AML in which c-mpl is frequently expressed.

Another significant prognostic indicator of response to therapy is the expression of the glycoprotein CD34 antigen (My10). High incidence of secondary AML versus de novo AML and chromosomal abnormalities covariated with CD34 expression in a large multivariate analysis. Our preliminary results indicate that, although CD34 and c-mpl expression both correlate with the same poor prognostic factors, their expression does not completely overlap because some CD34-negative AML did not overexpress c-mpl, and, conversely, some c-mpl-positive AML were CD34-negative (data not shown). Analysis of a larger series of patients would indicate to what extent these two markers covariate.

Interestingly, our study on MDS patients shows that, similar to AML, elevated c-mpl expression could also be associated with a poor prognosis. Indeed, 5 of 11 RAEB patients had increased c-mpl expression versus none of the 5 RSA patients. A survey of c-mpl expression in a larger panel of MDS patients is currently being performed in an attempt to define the precise relationship between c-mpl expression and the emergence of a blastic cell population.

Further studies on the c-mpl protein will show whether c-mpl is a ligand binding chain or a part of a multimeric complex. Because c-mpl has the overall structure of cytokine receptors, one could suppose that expression of the c-mpl gene product may promote the proliferation of myeloid blast cells in vivo.

ACKNOWLEDGMENT

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Expression of the c-mpl proto-oncogene in human hematologic malignancies

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