Hematopoietic Growth Factor Expression and ATRA Sensitivity in Acute Promyelocytic Blast Cells

By Carolline Dubois, Marie Hélène Schlageret, Albane de Gentile, Fabien Guidée, Nicole Ballirand, Marie Elisabeth Toubert, Irène Krawice, Pierre Fenaux, Sylvie Castaigne, Yves Najean, Laurent Degos, and Christine Chomienne

Acute promyelocytic leukemia (APL) is a homogeneous subgroup of acute myeloid leukemias (AMLs) characterized by the presence of the t(15;17) translocation and the resulting promyelocytic myeloid leukemia/retinoic acid receptor α (PML/RARAα) fusion proteins. To date APL is the only AML that is sufficiently sensitive to all-trans retinoic acid’s (ATRA) differentiating effect. In vivo ATRA alone achieves complete remission in most APL patients. However, failure or partial responses are observed and the molecular basis of the absence of ATRA response in these patients has not been determined. To gain insights in the cell growth and differentiation of APL cells, expression of hematopoietic growth factors (HGF) shown to be produced by leukemic cells (interleukin-1β [IL-1β], IL-6, tumor necrosis factor α [TNFα], granulocyte colony-stimulating factor [G-CSF], granulocyte-macrophage colony-stimulating factor [GM-CSF], and IL-3) was studied in 16 APL samples. Twelve APL cases expressed IL-1β, IL-6, and TNFα, but not G-CSF, GM-CSF, and IL-3. These cases achieved complete remission with ATRA therapy. The four remaining patients (either TNFα negative or G-CSF, GM-CSF or IL-3 positive) did not achieve complete remission with ATRA. In all cases, in vivo response to ATRA therapy was correlated to the in vitro differentiation effect of all-trans retinoic acid. Thus, ATRA differentiation induction was strongly correlated to the HGF expression (P < .0001). These results suggest that the presence or absence of HGF’s expression by APL cells may contribute to the therapeutic effect of ATRA in this disease.

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MATERIALS AND METHODS

Patient samples and primary culture. BM or blood samples from 24 consenting AML patients were collected in heparinized tubes before any therapy. From these 24 patients, 16 were AML3 or APL, and 8 other AML subtypes (3 AML1, 3 AML2, 1 AML4, 1 AML5). The diagnosis was established by morphologic, according to the FAB criteria, and cytogenetic criteria. Hematologic characteristics of the APL patients are summarized in Table 1.

The leukemic cells were isolated by Ficoll-Hypaque gradient (Eurobio, France; density: 1.078 g/dL) centrifugation and enriched after monocyte adherence to plastic for 1 hour at 37°C. The AML blasts were then immediately prepared for in vitro suspension culture, the cells were incubated for up to 72 hours in RPMI 1640 medium supplemented with 15% fetal calf serum (FCS), L-glutamin (2 mmol/L), and antibiotics (GIBCO-BRL, France) at 37°C in humidified 5% CO2 atmosphere as previously described. The initial cell concentration was 10⁵ cells/mL and cytokine slides stained by May-Grünewald Giemsa technique and flow cytometry analysis after CD14 labeling showed the population to contain less than 1% of normal mononu-
HGF EXPRESSION AND ATRA SENSITIVITY IN APL

Table 1. Hematologic Characteristics of APL Patients at Presentation and Response to ATRA

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>FAB</th>
<th>Genetic Analysis</th>
<th>Status</th>
<th>Coagulopathy</th>
<th>% BM Blasts</th>
<th>WBC Count</th>
<th>In Vitro</th>
<th>In Vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AML3</td>
<td>t(15;17)</td>
<td>De novo</td>
<td>+</td>
<td>50</td>
<td>18,000</td>
<td>100</td>
<td>CR</td>
</tr>
<tr>
<td>2</td>
<td>AML3</td>
<td>t(15;17)</td>
<td>1st Relapse</td>
<td>+</td>
<td>90</td>
<td>87,000</td>
<td>49</td>
<td>F</td>
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<tr>
<td>3</td>
<td>AML3</td>
<td>t(15;17)</td>
<td>De novo</td>
<td>-</td>
<td>92</td>
<td>42,000</td>
<td>96</td>
<td>CR</td>
</tr>
<tr>
<td>4</td>
<td>AML3</td>
<td>t(15;17)</td>
<td>2nd Relapse</td>
<td>-</td>
<td>100</td>
<td>4,300</td>
<td>99</td>
<td>CR</td>
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<tr>
<td>5</td>
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<td>t(15;17)</td>
<td>De novo</td>
<td>+</td>
<td>95</td>
<td>80,000</td>
<td>98</td>
<td>CR</td>
</tr>
<tr>
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<td>AML3</td>
<td>t(15;17)</td>
<td>1st Relapse</td>
<td>+</td>
<td>90</td>
<td>2,000</td>
<td>34</td>
<td>F</td>
</tr>
<tr>
<td>7</td>
<td>AML3</td>
<td>t(15;17)</td>
<td>2nd Relapse</td>
<td>-</td>
<td>95</td>
<td>33,000</td>
<td>93</td>
<td>CR</td>
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<tr>
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<td>AML3</td>
<td>t(11;17)</td>
<td>1st Relapse</td>
<td>+</td>
<td>88</td>
<td>4,540</td>
<td>40</td>
<td>PR</td>
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<tr>
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<td>1st Relapse</td>
<td>+</td>
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<td>4,200</td>
<td>79</td>
<td>CR</td>
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<td>t(15;17)</td>
<td>1st Relapse</td>
<td>+</td>
<td>32</td>
<td>25,000</td>
<td>91</td>
<td>CR</td>
</tr>
<tr>
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<td>t(15;17)</td>
<td>1st Relapse</td>
<td>-</td>
<td>40</td>
<td>3,000</td>
<td>100</td>
<td>CR</td>
</tr>
<tr>
<td>12</td>
<td>AML3</td>
<td>t(15;17)</td>
<td>De novo</td>
<td>+</td>
<td>89</td>
<td>68,000</td>
<td>99</td>
<td>CR</td>
</tr>
<tr>
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<td>t(15;17)</td>
<td>2nd Relapse</td>
<td>+</td>
<td>80</td>
<td>3,000</td>
<td>0</td>
<td>F</td>
</tr>
<tr>
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<td>De novo</td>
<td>+</td>
<td>96</td>
<td>17,500</td>
<td>82</td>
<td>CR</td>
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<tr>
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<td>1st Relapse</td>
<td>+</td>
<td>90</td>
<td>10,400</td>
<td>92</td>
<td>CR</td>
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<tr>
<td>16</td>
<td>AML3</td>
<td>t(15;17)</td>
<td>De novo</td>
<td>+</td>
<td>65</td>
<td>1,300</td>
<td>90</td>
<td>CR</td>
</tr>
</tbody>
</table>

Abbreviations: FAB, French-American-British morphological classification; AML3 v, AML3 variant; t(15;17), translocation (15;17); t(11;17), translocation (11;17); WBC, white blood cell; ATRA Response In Vitro, percent of NBT positive cells after 5 days of incubation with all-trans retinoic acid 10^{-8} mol/L; ATRA Response In Vivo: CR, complete remission; PR, partial remission; F, failure after 90 days of therapy with all-trans retinoic acid 45 mg/m^2/d.

Cell viability. After incubation, cell aliquots were taken for cell viability, RNA extraction, and detection of CSF, and cytokine production was performed in the supernatants. For each sample, differentiation with ATRA was assessed at day 5 of culture by morphology, and the generation of superoxide anion O_2^- production was visualized by the nitroblue tetrazolium reduction assay as already described. Cell viability was assessed by the trypan blue exclusion dye test. Proliferation curves were established for each case and an index ratio (viable cell count after 72 hours versus cell count at hour 0) was calculated.

Culture of control hematopoietic cells and cell lines. Normal peripheral blood lymphomononuclear cells were isolated from heparinized normal blood samples after Ficoll-Hypaque gradient. Then, the purified cells were stimulated with phorbol 12-myristate 13-acetate 10^{-5} mol/L (PMA) and phytohemagglutinin 2 μg/mL (PHA) for 6 hours, in the same medium used for AML culture. The promyelocytic cell line NB4 (a gift from M. Lanotte, Paris, France) was grown in RPMI 1640 medium supplemented with 15% FCS and the 5637 human bladder carcinoma cell line was grown in RPMI 1640 with 5% FCS.

Biologic assay for G-CSF. G-CSF levels in supernatants collected from the leukemic cell culture after 72 hours were estimated by monitoring the proliferation of NFS-60 cells. The leukemic cell culture supernatants were concentrated (10- to 40-fold) by ultracentrifugation using a Centriprep concentrator 10 (Amicon, Danvers, MA) to increase the detectable level of G-CSF. NFS-60 cells (2 × 10^5) together with 25 μL of concentrated supernatants were cultured in each well of a microtiter plate at 37°C for 5 hours at 37°C. Dimethylsulfoxide (125 μL) was added and the optical density was measured at 550 nm. Thus, samples to be tested were titrated and related to an internal standard preparation. The sensitivity of this G-CSF assay was shown to be 10 pg/mL. The interassay coefficient of variation at level of 100 pg/mL was less than 10 (n = 6). As positive controls, cell culture media from lymphomononuclear cells stimulated by PMA and PHA and 5637 cell line were collected and tested.

GM-CSF, IL-3, IL-1β, TNFa and IL-6 enzyme-linked immunosorbent assay (ELISA). GM-CSF levels in the leukemic cell culture supernatants were estimated by an ELISA kit from Endogen (Boston, MA), and IL-3 levels by an ELISA kit from Amersham (Buckinghamshire, UK). For IL-1β, TNFa, and IL-6 levels, we have used ELISA kits from British Biotechnology (Oxford, England). GM-CSF, IL-3, IL-1β, and IL-6 ELISA were sensitive down to a concentration of 3 pg/mL and for TNFα ELISA, sensitivity was shown to be 12 pg/mL. For a secretion level of 100 pg/mL, the interassay coefficient of variation was less than 10 (n = 6). As positive controls, culture mediums from lymphomononuclear cells stimulated by PMA and PHA and 5637 cell line were collected and tested. Concentrated leukemic cell culture supernatants were used for GM-CSF and IL-3 detection. The results were expressed in pg/mL and in pg/10^6 cells, but no difference between these expressions were observed.

Northern blot analysis. Total cellular RNA from AML blasts was prepared by the guanidium isothiocyanate/cesium chloride centrifugation method as described by Davis et al. Briefly, 20 or 30 μg of RNA were size fractionated by agarose gel electrophoresis after denaturation in formamide loading buffer and transferred onto nitrocellulose membranes. These filters were prehybridized and hybridized at 65°C overnight in buffers containing 6× sodium chloride/sodium citrate (3C), 0.2% sodium dodecyl sulfate (SDS), and 10X Denhart’s solution. DNA probes were [32P]-labeled by the random priming method. After hybridization, the filters were washed under stringent conditions in 2× SSC, 0.1% SDS for 10 minutes twice at room temperature and then in 0.1× SSC, 0.1% SDS at 65°C for 10 minutes. The filters were autoradiographed at −80°C with intensifying screens for 2 days. As positive control for screening test, RNA from PMA- and PHA-stimulated lymphomononuclear cells and from the 5637 cell line were used.

DNA probes. cDNA probes for G-CSF and GM-CSF were kindly provided by Glaxo Laboratories (Lausanne, Switzerland). cDNA probes for IL-1β, TNFa, and IL-6 were obtained from British Biotechnology. A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to rehybridize the filters and normalize RNA expression.

Reverse transcription polymerase chain reaction (RT-PCR),
One microgram of total cellular RNA was reverse transcribed to cDNA by an incubation of 45 minutes at 37°C and 5 minutes at 99°C in a total volume of 20 μL containing 1X RT buffer (50 mmol/L TRIS HCl pH 8.3, 6 mmol/L MgCl₂, 40 mmol/L KCl, 10 mmol/L dithiothreitol), 1 mmol/L each deoxynucleotide triphosphate (Pharmacia, Piscataway, NJ), 12.5 U of Avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer, Indianapolis, IN) and 2.5 pmol of each specific PCR buffer (10 mmol/L TRIS HCl, 50 mmol/L KCl). Reaction times were 30 cycles (1 minute at 94°C, 2 minutes at 60°C or 55°C, 3 minutes at 72°C) and at the end 7 minutes at 72°C. Ten microliters of the amplified products was electrophoresed on 2% agarose gel stained with ethidium bromide.

The gels were transferred onto nylon filters and hybridized to 5' end-labeled oligonucleotides with 32P using polynucleotide kinase. The oligonucleotide probes were as follows: G-CSF, 5' GTGAGGAAGATCCACAGGGA 3'; GM-CSF, 5' AGAGACACTGCTGCTGAGA 3'; IL-1β, 5' CAAGACATTCTGATGGAAAA 3'; IL-6, 5' CCATATTGGAACCCTTGTTGTCG 3'; TNFα, 5' GTGAGGTGACGATGCAGAGGC 3'.

**RESULTS**

In vivo and in vitro characteristics of the APL cases studied. (Table 1). Sixteen APL cases were studied (patients 1 through 16). All patients were cytologically defined as APL leukemia; one case presented with a t(11;17) translocation.

Nine cases showed increased white blood cell (WBC) counts at presentation. All patients were treated with all-trans retinoic acid according to the previously published protocols. Only four cases (patients 2, 6, 8, 13) did not achieve complete remission with ATRA therapy alone. Leukemic cell suspension culture was performed in all cases at diagnosis. Four samples (patients 2, 6, 8, 13) showed poor to no differentiation (49%, 34%, 40%, 0% of nitroblue tetrazolium (NBT) positive cells respectively) in the presence of ATRA 10-8 mol/L, corroborating the absence of in vivo ATRA efficacy. Ten cases showed spontaneous increase of viable cells in vitro (index ratio >1.2) (Fig 1). Four of these cases (patients 1, 7, 9, 16) had an index ratio greater than 1.4. However, increased WBC at diagnosis was not correlated to the spontaneous increase of cell number in the suspension culture.

**Secretion of HGF (IL-1β, TNFα, IL-6, G-CSF, GM-CSF, and IL-3) by APL cells.** Secretion of HGF was studied in culture supernatants of highly enriched blast cell populations of seven APL patients (patients 1 through 4 and 14 through 16). All of these seven cases secreted IL-1β (median level, 1.1 pg/mL), TNFα (median level, 25 pg/mL), and IL-6 (me-
HGF EXPRESSION AND ATRA SENSITIVITY IN APL

To increase RNA sensitivity detection, RT-PCR was performed (Fig 4B). G-CSF mRNA was detected in only one case of the 13 samples studied (patient 6), GM-CSF mRNA in another case (patient 2) and IL-3 mRNA in 3 cases (patients 2, 6, and 13).

In summary, of the 16 APL cases tested for expression and/or secretion of HGF, 12 cases (80%) coexpressed or cosecreted IL-1β, TNFα, and IL-6, but not G-CSF, GM-CSF, and IL-3. Furthermore, when expression and secretion could be performed in the same sample, presence or absence of gene expression was always associated with the release or absence of the corresponding protein.

Expression and secretion of HGF by non-APL cells. In eight non-APL samples tested, CSF expression was detected in four cases (one case, G-CSF; one case, GM-CSF; one case, G-CSF + GM-CSF; and one case, G-CSF + GM-CSF + IL-3) (data not shown). All cases expressed IL-1β and IL-6 genes, but only four cases the TNFα gene (Fig 4). Higher levels of CSFs and cytokines were observed in these non-APL cases as compared with APL cases (Fig 2). Only one case (AML2) expressed IL-1β, TNFα, IL-6, but no CSFs. Leukemic cell infiltration and leukemic cell purification were the same in this non-APL population as for the APL samples, implying that random contamination by normal HGF-secreting cells cannot explain the difference between APL and non-APL samples.

HGF production by AML cells and in vitro characteristics. (Fig 1) In vitro cell growth and viability of AML cells was studied in a suspension culture system with standard condition medium by comparing viable cell counts at different time intervals with the number of seeded cells at day 0 of culture. Compared with non-APL cases, APL cells showed a better survival/growth in suspension culture. Only 3 APL cases had an index ratio less than 1 (patients 2, 8, 13). Among the 10 cases that showed in vitro leukemic cell growth (patients 1, 3 through 7, 9, 10, 12, 16), 9 cases expressed IL-1β, TNFα, and IL-6, but no CSFs. ATRA sensitivity was also analyzed. Induction of differentiation was assessed by morphologic and functional criteria (NBT test). Twelve of the 16 APL cases studied (patients 1, 3 through 5, 7, 9 through 12, 14 through 16) achieved differentiation in vitro in the presence of ATRA 10⁻⁶ mol/L (mean of 94 ± 6% NBT positive cells) and complete remission with ATRA therapy in vivo. These cases all coexpressed or coproduced IL-1β, TNFα, and IL-6, but not CSFs. The 4 APL cases that failed to respond significantly to ATRA therapy never exceeded more than 50% of differentiation and either expressed CSFs or did not produce TNFα. Non-APL cases did not show significant induction of differentiation by ATRA (varied from 0% to 20% with a mean of 5.5%) in vitro. Thus, ATRA differentiation induction was strongly correlated to the pattern of HGF expression (P = .0001 Fisher test).

DISCUSSION

This is the first study describing the coexpression of HGF in the APL subgroup of AML. We observed that of the 16 APL cases surveyed in this study, all but 4 cases were (IL-
1β, IL-6, TNFα) positive and (G-CSF, GM-CSF, IL-3) negative. These results are corroborated by the presence of TNFα in the APL cells by immunostaining, and by preliminary studies from our laboratory and other published data hinting at the absence of detectable G-CSF and GM-CSF levels and increased IL-6 levels in the serum of APL patients. This pattern was specifically correlated to ATRA sensitivity in vitro and in vivo. Interestingly, the four patients whose leukemic cells did not express TNFα or did express CSFs, did not achieve complete remission with ATRA therapy. Likewise non-APL cases that do not respond to ATRA, did not show the HGF expression pattern. Thus, part of the effect of ATRA’s efficacy may be the result of the growth factor pattern spontaneously expressed by APL cells. Differentiation of leukemic cells (cell lines and fresh blast in primary culture) can be induced by CSFs, TNFα, and interferons in association with all-trans retinoic acid (ATRA).18-20 The presence of these HGF expressions by APL cells may be involved in the in vitro sensitivity of APL cells to differentiate in the presence of ATRA. To evaluate the potential role of any of these growth factors, we have initiated in vitro assays using antibodies and antisense oligonucleotide probes to suppress each growth factor individually. In fact, the two patients whose cells did not express TNFα did not respond in vivo to ATRA therapy. A further question asked by the results presented here is whether the expression of HGF by APL cells has a relevance to cells’ proliferation or survival features in vitro. APL cells are noted to have a low doubling time, and absence of autonomous growth.22 These characteristics may be related to previously reported high levels of transforming growth factor-β and to TNFα production and absence of IL-3, GM-CSF, and G-CSF secretion shown in this study. Nevertheless, in the presence of added CSFs such as GM-CSF, APL cells are able to produce colony-forming unit-leukemia colonies and to show increased cell growth.24 This growth induction in the presence of GM-CSF is not surprising because we have shown APL cells to express high affinity GM-CSF receptors.25 In addition, the secretion of IL-1β and IL-6 by APL cells may, alone or in cooperation with the CSFs present in the culture medium, explain the good survival of APL cells in suspension culture.26

Clinically, APL is characterized by the presence of blood coagulation disorders and hyperleukocytosis at onset, mainly in the AML3 variant subtype.16 IL-1β has been reported to be linked to DIC in AML patients.27 The constant presence of IL-1β in APL cells may explain the predisposition of APL patients to present altered blood coagulation parameters. Contrary to what may have been expected, the cytokine/CSF production did not correlate with high WBC count at presentation. This suggests that other factors determinant for leukemic cell proliferation, such as egress from the BM, adhesion, and migration are probably involved. One of ATRA therapy’s main side effects is the induced ATRA syndrome (pulmonary infiltrates, respiratory distress)28 and the increase of peripheral blast cells. The fact that APL cells express HGFs (IL-1β, TNFα, and IL-6) that are involved in leukocyte activation29-31 may propose that an abnormal modulation of these factors by retinoic acid may provoke these side effects. The data presented here offer a model to study the mechanisms of induction of these side effects and identify potential preventive or curative therapies.

Thus, the secretion or absence of secretion of HGF by APL cells may be determinant for the survival, multiplication, and differentiation capacity of these cells. We suggest that the resulting expression of growth factors by APL cells may contribute to the clinical and biologic features of the disease and that the cause of this expression may be related to the molecular characteristics of APL. Answering these questions will be of utmost clinical and scientific relevance.

ACKNOWLEDGMENT

We thank the physicians participating in the study of all-trans retinoic acid therapy in M3 patients who sent patient samples to the
Fig 4. mRNA expression of HGF in APL and non-APL patients. (A) Northern blotting analysis is shown. The size of the transcripts are normal. Blots were rehybridized with a GAPDH probe for RNA expression normalization. (B) RT-PCR analysis is shown. For IL-10: ethidium bromide staining of the gel with IL-10-amplified cDNA. For TNFα, IL-6, IL-3, GM-CSF, and G-CSF Southern blot hybridization with a specific oligonucleotide probe. Patients 1 to 13, APL patients; patients 1' to 8', non-APL patients. Cases 2, 6, 8, and 13 showed partial or no response to all-trans retinoic acid.


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