Asynchronous expression of myeloid antigens in leukemic cells in a PML/RARα transgenic mouse model


Abstract

Acute promyelocytic leukemia (APL) is characterized by the expansion of blasts that resemble morphologically promyelocytes and harbor a chromosomal translocation involving the retinoic acid receptor α (RARα) and the promyelocytic leukemia (PML) genes on chromosomes 17 and 15, respectively. The expression of the PML/RARα fusion gene is essential for APL genesis. In fact, transgenic mice (TM) expressing PML/RARα develop a form of leukemia that mimics the hematological findings of human APL. Leukemia is diagnosed after a long latency (approximately 12 months) during which no hematological abnormality is detected in peripheral blood (pre-leukemic phase). In humans, immunophenotypic analysis of APL blasts revealed distinct features; however, the precise immunophenotype of leukemic cells in the TM model has not been established. Our aim was to characterize the expression of myeloid antigens by leukemic cells from hCG-PML/RARα TM. In this study, TM (N = 12) developed leukemia at the mean age of 13.1 months. Morphological analysis of bone marrow revealed an increase in the percentage of immature myeloid cells in leukemic TM compared to pre-leukemic TM and wild-type controls (48.63 ± 16.68, 10.83 ± 8.11, 7.4 ± 5.46%, respectively; P < 0.05). Flow cytometry analysis of bone marrow and spleen from leukemic TM identified the asynchronous co-expression of CD34, CD117, and CD11b. This abnormal phenotype was rarely detected prior to the diagnosis of leukemia and was present at similar frequencies in hematologically normal TM and wild-type controls of different ages. The present results demonstrate that, similarly to human APL, leukemic cells from hCG-PML/RARα TM present a specific immunophenotype.

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Key words
• Acute promyelocytic leukemia
• Acute myelogenous leukemia
• Transgenic mice
• Flow cytometry
• Leukemogenesis

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Acute promyelocytic leukemia (APL) is a distinct subtype of acute myelogenous leukemia invariably associated with recurrent chromosomal translocations involving the retinoic acid receptor α (RARα) locus on chromosome 17 (1). In about 98% of APL cases, t(15;17) is detected and causes the fusion of RARα to the promyelocytic leukemia locus (PML) on chromosome 15. The resulting hybrid gene PML/RARα encodes a
PML/RARα fusion protein, which retains the majority of the functional domains of the parental PML and RARα proteins (1). Another hallmark of APL is the block of differentiation at the promyelocytic stage presented by the leukemic cells. Pharmacological doses of retinoic acid induce the degradation of the PML/RARα fusion protein and disease remission (2). APL has a specific immunophenotypic profile, characterized by the co-expression of the pan-myeloid markers CD13 (with a heterogeneous intensity of expression) and CD33 (homogeneous intensity of expression), and absence or low expression of HLA-DR, CD11a and CD18 (3-5). APL is mostly CD34 negative and the low expression of this marker has been associated with the microgranular variant and with the expression of the bcr3 PML/RARα isoform (6,7). In addition, a singular CD34 and CD15 pattern of expression has been reported, in which leukemic cells lose CD34 before they acquire CD15 expression, with the latter being never expressed at high levels (3). Finally, CD117 is frequently expressed by APL cells, but with variable intensity (5). The study of the immunophenotypic profile of APL has prompted some groups to develop flow cytometry methods for APL diagnosis and minimal residual disease detection (3,4).

The generation of transgenic mouse (TM) models harboring the PML/RARα fusion gene in their genome became a very useful tool for the study of APL pathogenesis. Several groups have used the human cathepsin G (hCG) minigene to drive the expression of PML/RARα to the promyelocytic stage of myeloid differentiation, and these TM developed a lethal form of leukemia that closely resembles human APL (8,9). Overt acute leukemia occurred after a long pre-leukemic phase (12-15 months) and affected only 10 to 15% of the TM, suggesting that PML/ RARα is necessary but not sufficient for full-blown leukemogenesis. In fact, Kogan et al. (10) demonstrated that double TM for MRP8-PML/RARα and MRP8-BCL-2 developed leukemia earlier than single MRP8-PML/RARα TM, suggesting that in this model BCL-2 cooperated with PML/RARα to accelerate leukemogenesis and to block myeloid differentiation.

Like human APL, leukemia in the hCG-PML/RARα TM responded to retinoic acid treatment (8). Although leukemic cells of hCG-PML/RARα TM are morphologically similar to human promyelocytes, their immunophenotypic features have not been fully characterized. This characterization is an important pre-requisite for studies aiming to analyze the gene and/or protein expression profiles, additional cytogenetic abnormalities, and proliferative and survival pathway deregulation in APL cells.

Thus, the objective of the present study was to determine if, similarly to human APL, leukemic cells from hCG-PML/RARα TM also present a distinct immunophenotypic profile. If so, it was important to analyze TM prior to the development of leukemia and to establish if there is a progressive accumulation of immature myeloid cells during the pre-leukemic phase of the TM model or if the block of maturation is a late event in leukemogenesis.

hCG-PML/RARα TM were provided by Prof. Pier Paolo Pandolfi (Memorial Sloan-Kettering Cancer Center, New York, NY, USA) and their generation has been described elsewhere (8). The mice were bred and maintained under pathogen-free condition at the Animal Facility of the Fundação Hemocentro, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo. Genotyping was performed by PCR analysis of tail genomic DNA (11) using the pair of primers C1 and D, and the reaction conditions described by van Dongen et al. (12). For monitoring peripheral blood (PB) counts, hCG-PML/RARα TM and their wild-type (WT) littermates were bled from the tail monthly starting from the third month of life, and automated counts were performed.
using a Coulter T-890 counter (Coulter Corporation, Hialeah, FL, USA). PB differential counts were performed on Wright-Giemsa-stained smears. The diagnosis of leukemia was based on the following criteria: a) presence of blasts/promyelocytes in the PB; b) leukocytosis (leukocyte counts >30 x 10^3/µL), and c) anemia (hemoglobin <10 g/dL) or thrombocytopenia (platelet counts <500 x 10^3/µL). In the present study, hCG-PML/RARα TM developed leukemia at the mean age of 13.1 months. For brevity, hCG-PML/RARα TM aged between 6 to 12 months presenting PB counts within the normal range were named ‘pre-leukemic’.

Twelve leukemic hCG-PML/RARα TM, 6 pre-leukemic hCG-PML/RARα and 12 sex- and age-matched WT controls aged 6 to 12 months were sacrificed by CO2 asphyxiation at the time of diagnosis (leukemic), or at 4, 8, and 12 months of age (2 pre-leukemic and 4 WT controls at each time point). Bone marrow (BM) cells were obtained by flushing the bone cavity with RPMI 1640 (Gibco, Grand Island, NY, USA) and splenic cell suspensions by mechanical disruption of the spleen. The cells were washed once and the pellet was resuspended in PBS at a concentration of 10^6/mL. Cytospin slides were prepared in a Cytospin 3 Cell Preparation System (Shandon, Pittsburgh, PA, USA), air-dried, and Wright-Giemsa stained. A minimum of 200 cells were counted and myeloid cells were classified as immature, intermediate or mature according to the Bethesda Proposals for Classification of non-lymphoid hematopoietic neoplasms in mice (13). The leukemic phase of the transgenic model was characterized by a marked increase of the number of white blood cells with a shift to the left and intense thrombocytopenia accompanied by mild to moderate anemia (Table 1). The percentage of blasts in PB was only moderately elevated, in contrast to what was observed in BM and spleen (Table 1). The leukemic cells morphologically resembled human promyelocytes. Invariably, leukemic mice presented splenomegaly with a spleen weight (mean ± SD) 1.25 ± 0.9% of body weight. Along with the lack of abnormalities in PB, a discrete, but nonsignificant, increase in spleen size was detected in pre-leukemic TM (0.27 ± 0.2 vs 0.1 ± 0.1% body weight in pre-leukemic and WT mice, re-

Table 1. Peripheral blood, bone marrow, and spleenic cell counts of leukemic (N = 12), pre-leukemic (N = 6), and wild-type (N = 12) mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hemoglobin (g/dL)</th>
<th>White blood cells (x 10^3)</th>
<th>Platelets (x 10^3)</th>
<th>Immature cells (%)</th>
<th>Intermediate cells (%)</th>
<th>Differentiated cells (%)</th>
<th>Lymphocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Peripheral blood</strong></td>
<td></td>
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</tr>
<tr>
<td>LEU</td>
<td>9.90 ± 3.53*</td>
<td>107.78 ± 122.96*</td>
<td>250.32 ± 180.29*</td>
<td>17.90 ± 12.04*</td>
<td>15.65 ± 10.18*</td>
<td>36.00 ± 16.39</td>
<td>30.05 ± 20.27*</td>
</tr>
<tr>
<td>PRE</td>
<td>15.02 ± 3.65</td>
<td>9.26 ± 4.51</td>
<td>1100.00 ± 514.87</td>
<td>0.00 ± 0.00</td>
<td>0.67 ± 1.03</td>
<td>24.33 ± 11.83</td>
<td>73.33 ± 12.69</td>
</tr>
<tr>
<td>WT</td>
<td>14.08 ± 3.23</td>
<td>7.41 ± 3.47</td>
<td>1055.13 ± 443.22</td>
<td>0.00 ± 0.00</td>
<td>0.70 ± 1.34</td>
<td>31.30 ± 17.18</td>
<td>66.80 ± 15.55</td>
</tr>
<tr>
<td><strong>Bone marrow</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LEU</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>48.63 ± 16.68*</td>
<td>32.32 ± 10.36*</td>
<td>12.00 ± 9.97*</td>
<td>6.84 ± 6.26*</td>
</tr>
<tr>
<td>PRE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>10.83 ± 8.11</td>
<td>21.33 ± 4.93</td>
<td>46.17 ± 21.48</td>
<td>17.50 ± 9.89</td>
</tr>
<tr>
<td>WT</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>7.40 ± 5.46</td>
<td>20.70 ± 4.79</td>
<td>41.10 ± 14.43</td>
<td>27.50 ± 9.96</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEU</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>48.00 ± 20.88*</td>
<td>27.90 ± 18.25*</td>
<td>9.70 ± 6.38</td>
<td>20.67 ± 19.61*</td>
</tr>
<tr>
<td>PRE</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.17 ± 0.41</td>
<td>0.50 ± 0.84</td>
<td>11.50 ± 7.77</td>
<td>87.83 ± 6.91</td>
</tr>
<tr>
<td>WT</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>0.44 ± 0.53</td>
<td>1.00 ± 0.71</td>
<td>2.56 ± 1.33*</td>
<td>91.78 ± 11.21</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SD. LEU = leukemic mice; PRE = pre-leukemic mice; WT = wild-type mice; NA = not applicable.

*P < 0.05 for LEU vs PRE and LEU vs WT comparison. +P < 0.05 for WT vs LEU and WT vs PRE comparison (one-way ANOVA followed by Bonferroni’s multiple comparison test)
spectively). The only significant difference between pre-leukemic and WT mice detected by cytomorphological analysis of PB, BM and spleen was a higher percentage of terminally mature myeloid cells in the spleen of the former.

For immunophenotypic analysis BM and splenic single cell suspensions were prepared as described above and 100 µL of the suspension were incubated with 5 µL of each monoclonal antibody (mAb) for 20 min at 4°C protected from light. The following combinations of mAbs directly conjugated with fluorochromes [fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridin chlorophyll protein (Per-CP)] were employed: CD117-PE/CD34-FITC/CD45-Per-CP, CD117-PE/CD11b-FITC/CD45-Per-CP, Gr-1-PE/CD11b-FITC/CD45-Per-CP and CD3-FITC/CD19-PE/CD45-Per-CP. Fluorochrome-conjugated isotypic antibodies of irrelevant specificity were used as negative controls. The anti-CD117 and anti-CD19 mAbs were obtained from Southern Biotechnology Associates (Birmingham, AL, USA); anti-CD3-ε from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the remaining mAbs were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Red blood cells were lysed immediately after labeling by incubation with 2 mL of FACS lysing solution (Becton Dickinson, San Jose, CA, USA) as recommended by the manufacturer. A minimum of 10,000 events/tube were acquired with a FACSScan flow cytometer. Viable CD45-positive cells were gated and the percentage of each cell subset was determined using the CellQuest software (Becton Dickinson).

The spleen and BM of leukemic mice were infiltrated by CD117+CD34+ and CD117+CD11b+ cells, the former being the most frequent subset detected (Table 2). In contrast, the same phenotype was expressed by less than 3% of cells in spleen samples from WT and in pre-leukemic TM (Table 2). No differences were observed between pre-leukemic mice aged 4, 8 or 12 months. Importantly, a significant increase in the more mature phenotype CD11b+Gr-1+ (19.42 ± 25.04 vs 4.29 ± 2.57%, P < 0.05) but not of the immature subset CD34+CD117+CD11b+, was detected in the spleen of pre-leukemic TM compared to WT.

In the present study, the immunophenotypic analyses demonstrated that BM and spleen of leukemic TM were infiltrated by CD117+CD34+ and CD117+CD11b+ cells, a phenotype that was seldom detected in the WT spleen. The CD117 antigen is rare in normal BM in both humans and mice, and is characteristic of the early stages of myeloid differentiation, decreasing after the colony-forming unit-granulocyte-monocyte stage.

### Table 2. Immunophenotypic profile of leukemic (N = 12), pre-leukemic (N = 6), and wild-type (N = 12) mice.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>CD117+CD34+ (%)</th>
<th>CD11b+CD117+ (%)</th>
<th>CD11b+Gr-1+ (%)</th>
<th>CD3+CD19+ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bone marrow</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEU</td>
<td>35.29 ± 16.39*</td>
<td>27.80 ± 7.89*</td>
<td>60.43 ± 21.92</td>
<td>6.18 ± 4.41</td>
</tr>
<tr>
<td>PRE</td>
<td>8.67 ± 1.74</td>
<td>8.79 ± 1.89</td>
<td>53.45 ± 24.15</td>
<td>2.77 ± 0.93</td>
</tr>
<tr>
<td>WT</td>
<td>6.67 ± 3.41</td>
<td>7.38 ± 3.35</td>
<td>48.45 ± 15.68</td>
<td>3.03 ± 3.80</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEU</td>
<td>43.43 ± 24.93*</td>
<td>26.95 ± 20.53*</td>
<td>35.04 ± 18.26*</td>
<td>3.92 ± 6.04</td>
</tr>
<tr>
<td>PRE</td>
<td>2.35 ± 1.61</td>
<td>3.58 ± 1.01</td>
<td>19.42 ± 25.04*</td>
<td>3.80 ± 2.83</td>
</tr>
<tr>
<td>WT</td>
<td>2.45 ± 1.96</td>
<td>2.98 ± 2.35</td>
<td>4.29 ± 2.57</td>
<td>8.41 ± 4.32</td>
</tr>
</tbody>
</table>

*Data are reported as mean ± SD. LEU = leukemic mice; PRE = pre-leukemic mice; WT = wild-type mice. *P < 0.05 for LEU vs PRE and LEU vs WT comparison. +P < 0.05 for PRE vs LEU and PRE vs WT comparison. One-way ANOVA followed by Bonferroni’s multiple comparison test.
(4,14). Similarly, CD34 is an important marker of human hematopoietic stem cells and is lost before the decline in CD117 expression (15). On the other hand, in adult mice, only 20% of the hematopoietic stem cells are CD34+ (16). Ishida et al. (17) have demonstrated the co-expression of different CD11b levels (an antigen expressed late during granulocytic differentiation) in a small fraction of CD34+ hematopoietic stem cells of normal adult mice. Therefore, in view of the rarity of the immunophenotypic profile the co-expression of CD34, CD11b and CD117 should be considered asynchronous. In fact, in human patients the expression of CD11b in association with immaturity markers like CD34 and CD117 has been reported as an example of asynchronous antigen expression characteristically observed in acute myelogenous leukemia (18).

Westervelt et al. (19), analyzing a knock-in model in which the PML/RARα cDNA was expressed under the control of the murine cathepsin G locus, reported that 90% of these mice developed APL between 6 and 16 months of age. Interestingly, the leukemic cells presented an abnormal immunophenotype co-expressing CD34 and Gr-1. Similarly, Pollock et al. (20) described the same immunophenotype in leukemic cells from double transgenic mice co-expressing the bcr3 isoform PML/RARα and the reciprocal product of the t(15;17), RARα/PML. Therefore, the asynchronous myeloid antigen expression is not dependent on the promoter region controlling PML/RARα expression, on the PML/RARα isoform, or on the presence of the reciprocal.

In pre-leukemic TM, we did not detect an increase of the percentage of cells with the CD117+CD34+ and CD117+CD11b+ phenotype compared to WT controls. In contrast, there was an infiltration by the more mature myeloid subset identified morphologically and immunophenotypically (CD11b+ Gr-1+) in the spleen of pre-leukemic and leukemic TM when compared to WT controls. Likewise, the weight of the spleen increased in the same fashion. The follow-up of hCG-PML/RARα TM using the more sensitive and specific immunophenotypic method, associated with morphological analysis, allowed us to demonstrate that the block of differentiation is a late event during the pre-leukemic phase, thus suggesting that it may depend on additional mutagenic events.

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References

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