CD34⁺CD7⁺ Leukemic Progenitor Cells May Be Involved in Maintenance and Clonal Evolution of Chronic Myeloid Leukemia

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ABSTRACT

Purpose: We analyzed CD34⁺ cells coexpressing CD7 in chronic myeloid leukemia (CML) in chronic phase (CP) or accelerated phase (AP) to clarify their role in progression or regression of the disease during treatment.

Experimental Design: Enumeration of CD34⁺CD7⁺ cells was done on bone marrow nucleated cells from normal donors and CML patients. Fluorescence in situ hybridization analysis was done on sorted CD34⁺CD7⁺ and CD34⁺CD7⁺ cells to examine the occupancy rate of each fraction by BCR-ABL⁺ cells with or without additional cytogenetic abnormalities.

Results: The proportion of CD34⁺CD7⁺ cells was significantly affected by the treatment outcome and/or the disease status as follows: 20.5 ± 10.4% in normal donors (n = 22), 18.1 ± 10.2% in CP with major cytogenetic response (n = 14), 53.0 ± 12.9% in CP at diagnosis (n = 18), 55.0 ± 15.8% in CP with minor or no cytogenetic response (n = 28), and 70.2 ± 18.1% in AP (n = 6). The proportion of CD34⁺CD7⁺ cells decreased in parallel with cytogenetic improvement in individual patients. In six untreated CP patients, the ratio of BCR-ABL⁺ cells was comparable between each fraction. In three patients with major cytogenetic response, the ratio of BCR-ABL⁺ cells was remarkably lower in CD34⁺CD7⁺ cells than in CD34⁺CD7⁻ cells. In three AP patients with additional cytogenetic abnormalities, extra signals were detected at a much higher rate in CD34⁺CD7⁺ cells than in CD34⁺CD7⁻ cells.

Conclusions: Our results suggest that CD34⁺CD7⁺ cells may be involved in maintenance and clonal evolution of BCR-ABL⁺ cells in CML.

INTRODUCTION

Chronic myeloid leukemia (CML) results from a pluripotent stem cell acquiring the Philadelphia (Ph) chromosome, in which the ABL gene at 9q34 is juxtaposed to the 5.8-kb limited region of the BCR gene at 22q11, resulting in the generation of 210 kDa protein, p210BCR-ABL (1). This active tyrosine kinase is responsible for the clonal amplification of leukemia cells by inhibition of apoptosis and stimulation of cell cycling (1–3). Ph-positive stem cells are initially capable of producing almost all lineages of mature blood cells in CML (4–6), which evolves from chronic phase (CP) into blast crisis, sometimes via an accelerated phase (AP). During this evolution, a fraction of Ph-positive stem/progenitor cells may be susceptible to additional genetic changes and finally manifest maturation arrest at a given stage of the differentiation program. These genetic changes involve the inactivation of tumor suppressor genes (7, 8) as well as the formation of unique transforming genes (9). In other instances, the clonal evolution is often defined by the acquisition of additional chromosomal aberrations, including trisomy 8, i17q) and double Ph (10).

It is evidenced that normal stem/progenitor cells are still present in the bone marrow and peripheral blood of CML patients. Treatment with IFN-α or imatinib mesylate not only induces hematologic remission, but also restores benign polycythaemia. IFN-α induces complete cytogenetic remission in 10% to 20% of CP patients (11) and so does imatinib in about 40% of CP patients who failed to respond to IFN-α (12), although minimal residual disease usually persists (13). In addition, long-term bone marrow culture results in the dominant expansion of Ph-negative cells in CML-CP (14). There are some reports that benign primitive progenitors in CML-CP are enriched within the HLA-DRlow subfraction of CD34⁺ cells (15, 16). Van Den Berg et al. (17) reported the high incidence of CD7 and Thyl Lin⁻ cell fraction in IFN-resistant/intolerant CML. Nevertheless, normal and leukemic progenitors are not readily discriminated on the basis of phenotypic characteristics. Little is known concerning which fraction of Ph-positive CD34⁺ cells contributes to maintenance and progression of CML. Recently, we (18) and Martin-Henao et al. (19) reported the high incidence of CD7 and CD34 coexpression on blast cells in blast crisis, and the latter group also showed that CD7 was expressed to a higher degree on CD34⁺ cells from CML-CP patients than on those from normal donors (19). More recently, Sempowski et al. (20) reported that the relative number of CD34⁺CD7⁺ cells significantly increased in CML patients compared with normal donors. CD7 was initially recognized as an early differentiation marker of T/NK cells, but is known to be expressed on immature progenitor cells capable of producing T, B, and myeloid lineages (21). Thus, CD7 may be a candidate marker for studying the cellular origin of clonal evolution in CML.
In the present study, we found that the proportion of CD34+CD7+ cells were significantly higher in untreated and IFN-α-resistant CML than in normal donors and IFN-α-sensitive CML. Additional evidence suggests that CD34+CD7+ cells may be involved in maintenance and clonal evolution of Ph-positive cells.

MATERIALS AND METHODS

Patients and Samples. Bone marrow specimens were obtained with informed consent from normal donors and patients with CML in CP or AP. Patient characteristics are summarized in Table 1. The disease status was classified according to the criteria of the International Bone Marrow Transplant Registry, and the cytogenetic response criteria were defined as described previously (23). Bone marrow samples from CML patients were also subjected to standard G-banding analysis and fluorescence in situ hybridization (FISH) analysis.

Flow Cytometric Analysis. The following fluorescein isothiocyanate (FITC)–conjugated and phycoerythrin (PE)–conjugated monoclonal antibodies (MAb) and their respective isotype-matched controls were used: 8G12-FITC (anti-CD34) and mouse IgG1-FITC (Becton Dickinson, Sunnyvale, CA); 3A1E-12H7-PE (anti-CD7) and mouse IgG2b-PE (Coulter, Inc., Hialeah, FL). Enumeration of CD34+ cells was done using a FACS Calibur (Becton Dickinson) according to the modified Milan/Mulhouse protocol (23), in which CD34+ cells were separated with a MACS Cell Sorting. CD34+CD7+ or CD34+CD7− were sorted on a FACSVantage (Becton Dickinson) into defined unlabeled population. Cells in the low side light scatter fractions, which were shown to be more than 98% pure upon reanalysis.

Immunomagnetic Separation of CD34+ Cells. Non-phagocytic mononuclear cells were prepared by density gradient centrifugation over Lymphoprep (Axis-Shield, Oslo, Norway) after mixing bone marrow samples with silica suspension (IBL, Fujioka, Japan). CD34+ cells were separated with a MACS Cell Isolation Kit and the MiniMACS cell separator (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer’s instructions, and were stored in liquid nitrogen until use.

Cell Sorting. Frozen CD34+ cells were thawed and labeled with anti-CD34 and anti-CD7 MAb. Cells incubated with their respective isotype-matched controls were used to define the unlabeled population. Cells in the low side light scatter and low-intermediate forward light scatter windows were sorted on a FACSVantage (Becton Dickinson) into CD34+CD7+ or CD34+CD7− fractions, which were shown to be more than 98% pure upon reanalysis.

FISH Analysis of Sorted Cells. Aliquots of sorted CD34+ cell populations were spread onto glass slides, fixed, and then stained with May-Grunwald-Giemsa solution. The probes used were as follows: LSI bcr/abl ES dual color translocation probe (Vysis, Woodcreek, IL); chromosome 8 enumeration probe (CEP8; Vysis); and an AML1 probe, which was a mixture of overlapping P1 and cosmid clones (24). The labeled probes were hybridized to metaphase samples according to the standard procedures. Images of the hybridized signals were captured with a 4’,6-diamidino-2-phenylindole/green/orange triple-bandpass filter (Chromatechnology, Brattleboro, VT). For each slide, more than 100 interphase cells were analyzed. CD34+ cells from normal donors were used as a negative control and the frequency of false-positive cells was estimated to be 0.2% to 3.5%.

Clonal Methylcellulose Culture Assay. The recombinant human cytokines used were as follows: stem cell factor (Amgen Biologicals, Thousand Oaks, CA); interleukin-3 and erythropoietin (Kirin Brewery, Tokyo, Japan); granulocyte colony-stimulating factor (G-CSF; Chugai Pharmaceutical, Tokyo, Japan); and IFN-α2b (Schering-Plough Japan, Osaka, Japan). These were added as follows: stem cell factor, 100 ng/mL; interleukin-3, 200 units/mL; erythropoietin, 2 units/mL; and G-CSF, 10 ng/mL. IFN-α2b was added at 0, 102, and 103 IU/mL, respectively. The culture medium consisted of α modified Eagle’s medium (Invitrogen Corp, Grand Island, NY), 0.9% methylcellulose (Shimatsu Chemical Co., Tokyo, Japan), 30% fetal bovine serum (Hyclone, Logan, UT), 1% deionized bovine serum albumin (Sigma, St. Louis, MO), 5 × 10−5 mol/L 2-mercaptoethanol, and the cytokine cocktail. Aliquots of the mixture containing 300 sorted cells were plated in 35 mm dishes (Nunc, Roskilde, Denmark) and incubated at 37°C in 5% CO2. Cultures were done in triplicate and scored on day 14. Individual colonies were lifted with a micropipette, spread on glass slides, and analyzed by FISH with the BCR-ABL translocation probe, as described above.

Statistical Analysis. Results were expressed as means ± SD. Significance levels were determined by two-sided Student’s t test with P < 0.05 considered statistically significant.

RESULTS

CD7 Expression by CD34+ Cells From Normal Donors and CML Patients

The expression of CD7 on CD34+ cells occurred over a wide range of intensities (Fig. 1A, III). We compared CD7 expression by setting a common gating threshold for both normal donors and CML patients. A significant difference in the proportion of CD34+ cells coexpressing CD7 was noted between normal donors and untreated/IFN-α-resistant CML patients (Fig. 1B). CD34+CD7+ cells comprised only a minor population (20.5 ± 10.42%) of CD34+ cells in normal donors (n = 22), whereas CD7 was expressed on a major population of CD34+ cells from CML-AP patients at diagnosis (52.95 ± 12.89%) or in minor or no cytogenetic response (54.95 ± 15.78%). Interestingly, the relative number of CD34+CD7+ cells were significantly reduced to normal levels (18.14 ± 10.24%) in patients achieving partial or complete cytogenetic response, but increased to 70.19 ± 18.08% in AP patients (Fig. 1B).

<table>
<thead>
<tr>
<th>Table 1 Patient characteristics</th>
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<td>Clinical diagnosis</td>
</tr>
<tr>
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<tr>
<td>CML-CP therapy (−)</td>
</tr>
<tr>
<td>IFN-α (+)</td>
</tr>
<tr>
<td>partial/complete response</td>
</tr>
<tr>
<td>CML-AP</td>
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</tbody>
</table>
Correlation Between CD34⁺CD7⁺ Cell Population and Cytogenetic Response

In a prospective study of 15 newly diagnosed patients, we examined whether the relative number of CD34⁺CD7⁺ cells would be affected by the patients’ hematologic and/or cytogenetic responses to therapy. The results are shown in Fig. 1C. Twelve patients received IFN-α and the remaining three had imatinib from the start. All the patients achieved complete hematologic remission (data not shown). Except for two cases which failed to respond to IFN-α, the proportion of CD34⁺CD7⁺ cells decreased in parallel with cytogenetic improvement, although in the remaining two cases with no response, the proportion of CD34⁺CD7⁺ cells did not change (Fig. 1C), suggesting that there is a correlation between the proportion of CD34⁺CD7⁺ cells and the cytogenetic response in each patient. In addition, the proportion of CD34⁺CD7⁺...
cells at diagnosis was not likely to be a predictor of therapeutic response. Some of the IFN-α-resistant cases listed in Table 1 were moved to imatinib therapy and achieved complete cytogenetic remission with concomitant decrease of CD34+CD7− cell population (data not shown).

**FISH Analysis of Sorted CD34+CD7+ and CD34+CD7− Cells**

Both normal and leukemic progenitors can reside in the bone marrow of CML-CP patients, and IFN-α can preferentially eliminate leukemic progenitors in some patients but cannot prevent their clonal evolution in others. To gain insight into the significance of the particular behavior of CD34+CD7− cells in CML, we did FISH analysis of sorted CD34+ subpopulations using the BCR-ABL translocation probe to determine the occupancy rate of each fraction by Ph-positive cells (Table 2). Figure 2 shows a representative flow cytogram for sorting of CD34+CD7+ and CD34+CD7− cells from the enriched CD34+ cell population (patient 7 in Table 2). CD7hi and CD7− fractions were easily identified in such a population. At diagnosis of CML-CP, BCR-ABL fusion signals were found equally in both CD34+CD7+ and CD34+CD7− cells, ranging from 60% to 90% of total nuclei (patients 1 to 3, and 8 to 11). In three patients showing a major cytogenetic response to IFN-α treatment, CD34+CD7− cells exhibited a much lower rate of the fusion signals than CD34+CD7+ cells (13.0 versus 65.0% in patient 4, 8.1 versus 43.7% in patient 5, and 3.3 versus 50.0% in patient 8), indicating the preferential recovery of normal hematopoiesis in the CD34+CD7− cell fraction. On the other hand, in three patients who acquired additional chromosomal aberrations such as t(3;21) in patient 9 and trisomy 8 in patients 10 and 11, respectively, FISH analysis using the AML1 and CEP8 probes showed that CD34+CD7+ cells presented a much higher rate of extra signals than CD34+CD7− cells (48.1 versus 12.4% in patient 9, 58.0 versus 7.7% in patient 10, and 70.8 versus 15.1% in patient 11). In patient 9, who also showed a minor cytogenetic response to IFN-α, 81.7% of CD34+CD7− cells contained the BCR-ABL fusion signals, which was comparable to the pretreatment value (85.3%), whereas a smaller proportion of CD34+CD7− cells showed fusion signals than before treatment (34.2 versus 85.7%).

**Clonal Culture of Sorted CD34+CD7+ and CD34+CD7− Cells**

To examine whether the two CD34+ subpopulations possess distinct biological features, sorted CD34+CD7+ and CD34+CD7− cells were assayed for their capacity to form colonies in semisolid medium. In three separate experiments using samples from untreated patients (Table 3, patients 1-3), both CD34+CD7− and CD34+CD7+ cells produced various types of colonies, including erythroid bursts, granulocyte-macrophage (GM), and mixed colonies (Table 3). The cloning efficiency of CD34+CD7− and CD34+CD7+ cells were variable, but comparable in all three assays. However, we noted that CD34+CD7− cells contained more erythroid blast-forming units rather than CD34+CD7+ cells, and that CD34+CD7+ cells contained more granulocyte-macrophage colony-forming units than CD34+CD7− cells. Next, individual colonies were picked up and subjected to FISH analysis to dissect their clonality. A total of 45 colonies including erythroid, GM, and mixed colonies were all positive for the BCR-ABL fusion signals, indicating their leukemic origin (data not shown). Using the same samples, we examined the inhibitory effect of IFN-α on colony formation. IFN-α showed a weak inhibitory effect on erythroid bursts in a dose-dependent manner, but a negligible effect on GM progenitors. Although it seems that CD34+CD7− cells were more resistant to IFN-α than CD34+CD7+ cells, this did not reach statistical significance (Fig. 3).

**DISCUSSION**

Previous studies showed the presence of CD34+CD7− cells in the fetal liver (25) and thymus (25), as well as fetal (26) and adult bone marrow (27–29). In addition, CD7 is expressed not only on T/NK leukemia cells, but also on a subset of acute myeloid leukemia cells (30). These observations suggest that CD34+CD7− cells may include very primitive stem/progenitor cells capable of differentiating into T, B, and myeloid lineages, although in aggregate they are considered to be heterogeneous populations with respect to both differentiation stage and lineage commitment. In our clonal culture assays, both CD34+CD7+ and CD34+CD7− cells showed similar cloning efficiency and produced various types of colonies. The differentiation potentials of these two CD34+ subsets may be overlapping, but there is some evidence for loss of CD7 expression upon induction of differentiation of CD34+CD7− cells (19).

The present study was motivated by the recent finding that blast cells in nonlymphoid blast crisis preferentially coexpressed CD34 and CD7 (18). Two other groups reported that CD7 was expressed on a mean of 25.3% to 32.6% of CD34+ cells from CML-CP patients, and on a mean of 3.6% to 4.9% of CD34+ cells from normal donors (19, 20). There is somewhat of a discrepancy between these results, especially in the expression level of CD7 on normal CD34+ cells. The most likely reason may be the difference in fluorescent dye for labeling anti-CD7 MAb.

<table>
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<tr>
<th>Patient no.</th>
<th>IFN-α</th>
<th>Ph-positive metaphase</th>
<th>Additional abnormality</th>
<th>Total nucleated cells</th>
<th>Sorted CD34+ cells</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CD7−</td>
<td>CD7+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BCR-ABL (%)</td>
<td>Additional signal (%)</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>20/20</td>
<td></td>
<td>62.7</td>
<td>59.1</td>
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<tr>
<td>2</td>
<td>2</td>
<td>20/20</td>
<td></td>
<td>66.7</td>
<td>71.3</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
<td>20/20 (+Ph) 01/19</td>
<td></td>
<td>68.0</td>
<td>65.5</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
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<td></td>
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<td>65.0</td>
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<tr>
<td>5</td>
<td>08</td>
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<td>8.1</td>
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<tr>
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<td>01</td>
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<td>4.4</td>
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<td>88.0</td>
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<tr>
<td>9</td>
<td>08</td>
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<td>3.3</td>
<td>50.0</td>
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<td>10</td>
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<td>85.7</td>
<td>85.3</td>
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<tr>
<td>11</td>
<td>20</td>
<td>20/20 (+Ph) 08/20</td>
<td></td>
<td>68.9</td>
<td>67.8</td>
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</tbody>
</table>

**NOTE.** Total bone marrow nucleated cells were subjected to a standard G-banding analysis. CD34+CD7− and CD34+CD7+ cells sorted from purified CD34+ cells were grown onto glass slides and analyzed using the BCR-ABL translocation probe. At least 100 (114 on average) interphase cells were analyzed.

*Samples from patients 9 to 11 were also analyzed by AML1 probes.
*Samples from patients 9 to 11 were also analyzed by CEP8 probes.
We used the MAb labeled by PE, but others used FITC which is less fluorescent than PE.

These observations raise the question of why CD34+CD7+ cells expand in CML. In the present study, BCR-ABL fusion signals were detected in 60% to 90% of CD34+ cells in untreated patients. This score is comparable to the abnormal reference range (69-92%) reported by Dewald et al. (31) for bone marrow nuclei from most untreated patients. Takahashi et al. (32) reported similar results for sorted CD34+CD7+ cells from patients with 100% Ph-positive metaphases. Taken together, our results and those by other groups indicate that the majority of CD34+CD7+ cells are BCR-ABL+ in untreated CML patients. It is likely that p210 is involved either directly or indirectly in this phenomenon. For example, p210 may confer a growth advantage to a CD7+ subset of CD34+ cells by an unknown mechanism. Alternatively, p210 may activate the expression of certain genes encoding cytokines, etc., which may in turn up-regulate CD7 expression in leukemic as well as normal progenitors, by an autocrine or paracrine mechanism. In this respect, some attention should be given to the report by Jiang et al. (33) that both CD71CD45RA+ and CD71CD45RA- subsets of leukemic CD34+ cells constitutively produce interleukin-3 and G-CSF in CML.

Table 3 Colony formation by sorted CD34+ cells

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Cell type</th>
<th>Type of colony (%)</th>
<th>Cloning efficiency (%)</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>CFU-GM</td>
<td>BFU-E</td>
</tr>
<tr>
<td>1</td>
<td>CD34+CD7+</td>
<td>43.2</td>
<td>54.6</td>
</tr>
<tr>
<td>2</td>
<td>CD34+CD7+</td>
<td>52.0</td>
<td>44.1</td>
</tr>
<tr>
<td>3</td>
<td>CD34+CD7+</td>
<td>18.8</td>
<td>80.3</td>
</tr>
<tr>
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<td>CD34+CD7+</td>
<td>54.0</td>
<td>43.1</td>
</tr>
<tr>
<td>5</td>
<td>CD34+CD7+</td>
<td>23.1</td>
<td>75.0</td>
</tr>
<tr>
<td>6</td>
<td>CD34+CD7+</td>
<td>56.8</td>
<td>41.8</td>
</tr>
</tbody>
</table>

Abbreviations: CFU-GM, granulocyte-macrophage colony-forming unit; BFU-E, erythroid blast-forming unit.

Three hundred CD34+CD7+ and CD34+CD7- cells sorted from untreated CML-CP patients were cultured with stem cell factors, interleukin-3, G-CSF, and erythropoietin, respectively. Results are shown as the percentage of mean value in triplicate cultures.

FISH analysis of the sorted CD34+ subsets revealed the in vivo biological significance of CD34+CD7+ cells. CD34+CD7+ cells revealed quite different behavior from CD34+CD7- cells, depending on the IFN-α response in each patient. CD34+CD7+ cells seem more resistant to IFN-α than CD34+CD7- cells, and comprise the larger part of the AP clone with additional genetic changes. Assuming that CD7 is programmed to be transiently expressed in the stem/progenitor cell compartment and to be persistent only in the T/NK cell lineage, maintenance and clonal evolution of Ph-positive cells may originate from CD34+CD7+ stem cells. Normann et al. (20) also pointed out that all the CML patients with signs of disease progression showed expansion of CD34+CD7- cells, although the clinical risk scores did not differ at diagnosis irrespective of the proportion of CD34+CD7- cells. Very recently Jamieson et al. (34) reported that progression to blast crisis was associated with expansion of the myeloid progenitor fraction, which consists mainly of GM progenitors, rather than expansion of hematopoietic stem cells in CML. They showed that the β-catenin pathway was activated in leukemic GM progenitors (34). Although CD34+CD7- cell fractions include not only GM but also erythroid and common myeloid progenitors in untreated CML-CP, commitment of CD34+CD7+ cells might be restricted to the GM lineage in CML-AP. Furthermore, it is intriguing to explore the activation status of the β-catenin pathway in both CD34+CD7+ and CD34+CD7- cells because the human CD7 promoter contains putative TCF/LEF-1 binding sites (-ACAAAGT)-.4

CD7 is a 40-kDa membrane glycoprotein and a member of the immunoglobulin superfamily (20). The CD7 ligand has been recently identified, and this molecule, K12/SECTM1, was shown to be highly expressed by peripheral blood leukocytes (35). Cross-linkage of CD7, either by its ligand K12/SECTM1 or by MAb, leads to activation of a certain tyrosine kinase and recruitment of the phosphatidylinositol-3 kinase pathway (36), and contributes to protection from apoptosis through activation of the Akt kinase (37). Signaling through CD7 may confer a growth advantage to CD34+ stem/progenitor cells in vivo but not...
in vitro. Supporting this hypothesis, Hou et al. (38) showed that cross-linking of CD7 on myeloblasts by MAb results in production of GM-CSF. Then, in vivo repopulation assays using immunodeficient mice, or coculture studies with various types of stromal cells, is required to characterize the biological features of a primitive subset of CD34+/CD7− cells.

Our observations indicate that IFN-α is not a potent growth inhibitor of Ph-positive clonogenic cells. Similar to our results, Despres et al. (39) reported that IFN-α failed to inhibit GM-colony formation by CD34+ cells from patients with untreated CML-CP in the presence of stem cell factors with interleukin-3 or G-CSF. One possible explanation for the apparent discrepancy between the in vivo and in vitro inhibitory effects of IFN-α on hematopoiesis is that IFN-α may primarily stimulate accessory cells, including macrophages and stromal cells, to secrete inhibitors of the growth of CD34+ progenitors. The in vivo mechanism of IFN-α-mediated selective suppression of Ph-positive CD34+/CD7− cells cannot be explained by their inherent features, as the colony-forming assay indicated no evidence for different sensitivities to IFN-α between CD34+/CD7− and CD34+/CD7+ cells.

Imatinib is now the first choice in CML-CP. Although a few imatinib-treated cases were available in this study, the behavior of CD34+/CD7+ cells in these patients was similar to that in IFN-α-sensitive cases. The results of the present study suggest that CD34+/CD7+ cells play an important role in the maintenance and clonal evolution of Ph-positive cells, and that the selective antileukemic effects of IFN-α in CML may not be attributable to its direct antiproliferative activity on Ph-positive CD34+ cells.

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