Establishment of a Leukemia Cell Line with i(12p) from a Patient with a Mediastinal Germ Cell Tumor and Acute Lymphoblastic Leukemia

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ABSTRACT

We report the establishment of a leukemia cell line (UoC-B10) from a patient who developed leukemia several months after the diagnosis of a mediastinal yolk sac tumor. The patient’s yolk sac tumor responded to combination chemotherapy, and a mature teratoma with focal areas of hematopoiesis was subsequently resected. However, 5 months after the initial diagnosis, the patient developed an acute lymphoblastic leukemia with a precursor B-cell phenotype. Cytogenetic analysis showed an i(12p) abnormality in the patient’s leukemia cells and in the UoC-B10 cell line. The i(12p) was also identified retrospectively in the mediastinal tumor cells by fluorescent in situ hybridization analysis. The UoC-B10 cell line, which has been growing continuously for >24 months in culture, was Epstein-Barr virus negative and was generally concordant with the patient’s leukemia cells by analysis of immunophenotype, karyotype, and genotype. The UoC-B10 cell line possesses receptors for granulocyte-colony-stimulating factor, a cytokine which the patient received as part of his treatment protocol. This cell line may be useful in studying the relationship between i(12p) and hematological differentiation of human mediastinal germ cell tumors.

INTRODUCTION

Approximately 700 mediastinal GCTs occur each year in the United States (1). These tumors typically have yolk sac histology, are locally extensive or metastatic at the time of diagnosis, respond well to chemotherapy, but are cured in less than 50% of the cases (2). Recently, an association between mediastinal GCTs and malignant hematological disorders has been recognized. Over 30 cases have been reported and, while most of the second malignancies are AML, there has been a disproportionate number of uncommon subtypes reported, such as erythroleukemia and acute megakaryoblastic leukemia (3–26). In some patients, the leukemia was present at the time of (or even prior to) the diagnosis of the mediastinal GCT and, in most patients, the hematological dysfunction was detected within 12 months of the initial diagnosis (3–26). Thus, this syndrome differs from therapy-related AML that follows prior exposure to alkylating agents, topoisomerase II inhibitors, or radiation therapy.

In a review of 722 patients treated with non-etoposide-based regimens for germ cell tumors at Memorial Sloan-Kettering Cancer Center over a 30-year interval, Redman et al. (11) observed an increased incidence of leukemia. Patients who developed leukemia tended to have been treated with radiation or alkylating agents, and the median interval from diagnosis of the primary tumor to development of leukemia was 45 months. Recently, the antineoplastic agent etoposide has been associated with the development of acute myeloid leukemia, usually with specific chromosomal translocations involving 11q23 (27–32). Thus, patients with a mediastinal GCT, who are now frequently treated with etoposide-based regimens as well as radiation and alkylating agents, are at risk to develop a hematopoietic malignancy induced by one of several possible mechanisms.

We report on a patient who developed an overt hematological malignancy with features characteristic of acute lymphoblastic leukemia 5 months after the diagnosis and treatment of a mediastinal yolk sac tumor. Cytogenetic analysis showed an i(12p) abnormality in the patient’s leukemia cells and in a cell line (UoC-B10) established from the leukemia cells. The i(12p) was retrospectively identified in the mediastinal tumor cells by FISH analysis. The UoC-B10 cells possessed receptors for G-CSF, a cytokine which the patient received as part of his treatment protocol.

MATERIALS AND METHODS

Case Report. A previously healthy 26-year-old male presented in September, 1991, with acute onset of back pain. Radiographic studies revealed an epidural mass with bony erosion and an anterior mediastinal mass. The complete blood count was normal, AFP was 3210 ng/ml (normal, <5 ng/ml) and the βHCG was 18 milli-international units/ml (normal, <5 milli-international units/ml). The patient underwent a decompression laminectomy, and pathological review of the specimen revealed a yolk sac tumor (Fig. 1A). In the BM present within this specimen, the myeloid and erythroid lines appeared normal, micromegakaryocytes were observed, and focal involvement with tumor cells was noted. The patient was treated with cisplatin, etoposide, and bleomycin chemotherapy, followed by daily G-CSF on Intergroup protocol 3887. He received 21-day cycles of cisplatin 20 mg/m²/day × 5, etoposide 100 mg/m²/day × 5, bleomycin 30 units i.v. on days 2, 9, and 16, and G-CSF (5 µg/kg s.c.) from day 7 to day 18 with G-CSF doses omitted on days 9 and 16.

During chemotherapy, the patient’s hemoglobin gradually decreased from 14.3 to 8.2 g/dl, but this was felt to be related to cisplatin. After 4 cycles of chemotherapy, the mediastinal mass had decreased markedly in size, and the AFP and βHCG levels had returned to normal. A bone marrow examination was done to assess cellularity and document the absence of bone marrow involvement by tumor prior to a bone marrow harvest for cryopreservation. The peripheral blood smear was notable for extreme red cell anisopoikilocytosis and marked polychromatophilic as well as a large number of nucleated RBC. The platelets were moderately decreased, and the WBC appeared normal. Erythroid hyperplasia was present, and the myeloid line showed a shift towards immaturity. There was no evidence of metastatic tumor or reticulum fibrosis. The overall impression of this bone marrow specimen was exuberant regenerating marrow in a patient receiving a hematopoietic growth factor following intensive chemotherapy.

A bone marrow harvest and resection of the residual mediastinal mass was performed. On histological review, the mediastinal mass showed extensive necrosis and elements of mature teratoma which contained hematopoietic tissue (Fig. 1, B and C). Prior to the resection and bone marrow harvest, mild thrombocytopenia was noted (70,000/µl). Postoperatively, the patient received

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3 The abbreviations used are: GCT, germ cell tumor; AML, acute myeloid leukemia; UoC-, cell line established at the University of Chicago; FISH, fluorescence in situ hybridization; G-CSF, granulocyte-colony-stimulating factor; AFF, serum α fetoprotein; βHCG, serum β-subunit of human chorionic gonadotropin; BM, bone marrow; MDS, myelodysplastic syndrome; BCP-ALL, B-cell precursor acute lymphoblastic leukemia; TdT, terminal deoxynucleotidyl transferase; ADA, adenosine deaminase; cDNA, complementary DNA; EBV, Epstein Barr virus; PCR, polymerase chain reaction.
Source of Malignant Cells. Leukemia cells were obtained from peripheral blood and BM samples at the time of diagnosis of leukemia, anticoagulated with preservative-free heparin, and separated into aliquots for cell culture experiments, immunophenotyping, genotyping, and cytogenetic analyses. The protocol procedures were approved by the Institutional Review Board and informed consent was obtained.

Establishment and Maintenance of the Cell Line. The technique for culturing leukemia cells was a modification of our previously reported method (33). Briefly, Ficoll-Hypaque gradient-separated cells were washed twice, plated (1 × 10^6 cells/ml; 0.3 ml) onto 24-well Petri dishes, and cultured in an incubator gassed with 5% O_2/6% CO_2/89% N_2. Each well contained a feeder layer consisting of a mixture of media, agar (0.5%), and human serum (10%).

Characterization of Cellular Antigens. Cell surface antigens were evaluated on BM cells and the UoC-B10 cell line by indirect immunofluorescence using fluorescein isothiocyanate-conjugated antibodies and analyzed by flow cytometry.

Tumor Markers and Enzyme Evaluation. AFP and βHCG were measured using the IMx AFP and IMx total βHCG assays, respectively (both from Abbott Laboratories, Abbott Park IL). TdT activity was assayed by an immunofluorescence kit (Supratechs, Bethesda, MD), while ADA and nucleoside phosphorylase activity were measured as described previously (34).

Gene Rearrangement Studies. DNA was extracted from BM and the UoC-B10 cell line and analyzed by Southern blot hybridization (35). DNA was digested with two restriction enzymes (EcoRI and HindIII), electrophoresed on 0.8% agarose gels, transferred to nylon membranes, and hybridized with cDNAs from the human immunoglobulin heavy chain gene (λ) and the Epstein-Barr virus.

Cytogenetic Analyses. Cytogenetic analyses using a trypsin-Giemsa banding technique were performed on the “MDS” BM aspirate, the “ALL” BM aspirate, and the UoC-B10 cell line. Metaphase cells were prepared directly or following short-term (24 or 48 h) culture without mitogens. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (1991).

FISH Analysis. FISH analysis was performed using a biotinylated probe specific for the α-satellite repeat sequences in the centromeric region of chromosome 12 (Oncor, Gaithersburg, MD). FISH analysis was performed on the MDS specimen, on fresh leukemia cells, and tissue sections of the mediastinal germ cell tumor using methods described previously (36–38). The cell nuclei in the tissue sections were counterstained with propidium iodide.

G-CSF Studies. The expression of G-CSF receptors was evaluated on the UoC-B10, U937 (positive control), and UoC-M1 (negative control) cell lines by PCR and binding studies. Reverse transcription was performed on mRNA isolated from the cell lines. The resulting cDNA was amplified (35 cycles) by PCR using primers specific for the G-CSF message: 5’ primer, 5’-CACCTGCCCTCTGGAACGTG-3’; 3’ primer, 5’-CAGTGTCTGTGACCGTCTATG-3’ (positions 2086 to 2105 and 2232 to 2303, respectively; Ref. 39).

The presence of cell surface G-CSF receptors was evaluated using a Fluorokine G-CSF flow cytometry kit (R&D Systems, Minneapolis, MN). Cells were processed according to kit specifications and incubated with: (a) unlabeled G-CSF; (b) streptavidin-phycoerythrin; (c) phycoerythrin-conjugated G-CSF (G-CSF-PE); or (d) G-CSF-PE after the cells had been preincubated (60 min) with 100-fold molar excess of rG-CSF (Amgen, Thousand Oaks, CA). Cells were analyzed by flow cytometry using a 488-nm wavelength laser excitation.

The effect of rG-CSF on leukemic cell growth was determined. Test cells with >95% viability were evaluated while in log phase growth and while growing in McCoy 5A media supplemented with either 10% fetal calf serum or serum substitutes (40). Cells (1–10 × 10^6/well) were cultured with and without supplemental rG-CSF (1–200 U/ml) for 48 h, pulsed with 1 μCi [3H]thymidine (2 Ci/mmol; Amersham, Arlington Heights, IL) for 4 h, and thymidine incorporation was determined by a liquid scintillation analyzer (TRI-CARB; Packard Instrument Co., Downers Grove, IL).

RESULTS

Establishment of UoC-B10 Cell Line. In the cultures of the peripheral blood sample, leukemia cell viability gradually fell to less than 1% during the first 14 days of culture. However, during the third day, the platelet and RBC transfusions for a mediastinal hemorrhage and persistent thrombocytopenia.

A bone marrow examination was then performed 3 weeks postoperatively and, in comparison to the preoperative BM biopsy, showed a hypercellular marrow with decreased megakaryocytes, increased blasts (5%), and dysplastic features in all cell lineages, findings consistent with MDS. Two weeks later, blasts were noted in the blood, the serum lactate dehydrogenase rose dramatically, and a second bone marrow sample showed a marked hypercellular marrow with 65% blasts. The blasts were myeloperoxidase negative and generally had L-3 morphology, but some resembled erythroblasts while others had L-3 morphology (Fig. 2A). The immunophenotype was consistent with BCP-ALL (Table 1). Remission induction chemotherapy was started but the patient suffered an intracranial hemorrhage and died 10 days after the start of therapy. Permission for an autopsy was denied.
week, cell proliferation was observed which continued after the cells had been passed to suspension culture. The UoC-B10 cell line has sustained growth for more than 200 passages and has proliferated for more than 24 months in suspension culture.

**Immunophenotype.** The leukemia cells from the patient and the cell line expressed the immunophenotype of a B-cell leukemia: CD45+, HLA-DR+, CD10+, CD19+, CD38+ (Table 1). In contrast to the patient's leukemic cells, the UoC-B10 cell line expressed IgD/lambda and CD4 but none of the 6 other T-lineage antigens tested. While the patient's marrow expressed 30% positive cells for glycophorin, it was not directly determined whether the patient's blasts expressed glycophorin. It is of interest that the cell line had weak, but definite and reproducible, expression of glycophorin (12%).

**Cytochemical Stains, Tumor Markers, and Enzymes.** Both the patient's leukemic blasts and the UoC-B10 cells were nonreactive when stained with the myeloperoxidase, α-naphthyl acetate esterase, and PAS stains, and both lacked TdT activity. The UoC-B10 cells had low ADA activity (4.6 EU/mg) compared to typical BCP-ALL cells and BCP-ALL cell lines (41, 42). Also, AFP and carcinoembryonic antigen were not detected in either concentrated (10-fold) or unconcentrated UoC-B10 cell line culture media (the sensitivity of the assays was 1 ng/ml and 5 milli-international units/ml, respectively). These results are consistent with the results of Paiva et al. (43) who demonstrated that somatic components within GCTs lack AFP.

**Karyotype Analysis.** Cytogenetic analysis on the MDS BM aspirate revealed a male karyotype with four chromosomally abnormal clones (Table 2). The primary clone contained a Robertsonian translocation involving the chromosome 13 homologues [45,XY,der(13;13) (q10;q10), 21%]. A gain of an isochromosome for the short arm of chromosome 12, i(12p), was observed in one clone (clone 4; 6%) and in eight of the nine nonclonal abnormal cells. In addition, there were two additional unrelated clones (2 and 3) observed.

Analysis of the leukemic BM aspirate revealed three of the four abnormal clones observed initially, as well as two new clones, and eight nonclonal abnormal cells. The i(12p) was noted in 80% of cells examined. Karyotype analysis of the UoC-B10 cell line revealed many of the cytogenetic changes present in the predominate clone of the leukemic specimen, and an i(12p) was present in all of the cells. The Robertsonian translocation involving chromosomes 13 homologues (45,XY,t(13q;13q) was also present in all of the UoC-B10 cells.

**Gene Rearrangement.** Southern blot analysis of the patient's leukemia cells and the UoC-B10 cell line was done using the immunoglobulin heavy chain (JH) and EBV probes (Fig. 3). The rearranged nongermline band present in the cell line co-migrated with the rearranged band present in the patient's leukemia cells. The UoC-B10 cell line (and the patient's leukemia cells) lacked the EBV genome because the cells did not hybridize to the EBV cDNA probe (data not shown).

**FISH Analysis.** Using the chromosome 12 probe, the leukemia cells showed three hybridization signals (Fig. 2B); two were of normal size and one was small, likely due to the centromeric structure of the i(12p), as previously reported (36). The MDS BM aspirate was studied retrospectively and occasional cells (<5%) were found with the same three-signal pattern (Fig. 2C). Deparaffinized sections of the germ cell tumor were studied according to a protocol of Hopman (38) and, although tissue sectioning made the analysis difficult, some cells with two bright signals and one weak signal were observed (Fig. 2D).
Table 1 Immunophenotype of the patient's leukemia cells and the UoC-B10 cell line

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Cluster designation (antigen)</th>
<th>Bone marrow cells (%)</th>
<th>UOC-B10 cell line (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-lymphoid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>B22</td>
<td>49%</td>
<td>100%</td>
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<tr>
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<td>B22</td>
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<td>100%</td>
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<td>CD19</td>
<td>B22</td>
<td>50%</td>
<td>100%</td>
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<tr>
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<td>ND</td>
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<tr>
<td>IgD</td>
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<td>68%</td>
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<td>Progenitor</td>
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<td>1%</td>
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<td>NK Associated</td>
<td>CD56 (NKH-1)</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>Macrophage/Myeloid</td>
<td>CD13 (My7)</td>
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<td>1%</td>
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<tr>
<td></td>
<td>CD14 (MO2)</td>
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<td>0%</td>
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<tr>
<td></td>
<td>CD15 (Leu M1)</td>
<td>8%</td>
<td>1%</td>
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<tr>
<td></td>
<td>CD33 (My9)</td>
<td>5%</td>
<td>0%</td>
</tr>
<tr>
<td>Other</td>
<td>CD45 (KCS6)</td>
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<td>100%</td>
</tr>
<tr>
<td></td>
<td>CD38 (Leu 17)</td>
<td>39%</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>HLA-DR (I2)</td>
<td>53%</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>CD61 (GPIll)</td>
<td>3%</td>
<td>1%</td>
</tr>
<tr>
<td>Glycophorin</td>
<td></td>
<td>30%</td>
<td>12%</td>
</tr>
</tbody>
</table>

* Not done.

This signal pattern was not observed in normal control cells, and three signals were found in only 0.5% of nuclei (37).

G-CSF Studies. Using a reverse transcription-PCR protocol, mRNA for the G-CSF receptor was detected as a single 237-base pair band in the U937 cell line (positive control) and the UO-B10 cell line. The UOC-M1 cell line served as a negative control for both the reverse transcription-PCR and binding studies. Using a Fluorokine G-CSF kit, cell surface binding of G-CSF to the UO-B10 cell line was detected (Fig. 4). G-CSF binding to the UO-B10 cells was partially blocked (approximately 60%) when the UO-B10 cells were preincubated with 100-fold molar excess of G-CSF (data not shown). Supplemental rG-CSF, while it was associated with an increase in [3H]thymidine uptake in the control cells, had no effect on the UO-B10 cells cultured in either serum-free media or media with 10% fetal calf serum.

DISCUSSION

We report the establishment of an i(12p) positive leukemia cell line cultured from a patient with a metastatic mediastinal GCT (yolk sac) who had progressive bone marrow dysfunction and i(12p) positive leukemia. Of note, at initial diagnosis, micromegakaryocytes were observed in the bone marrow, suggesting that abnormal hematopoietic differentiation was already occurring. After the completion of chemotherapy, the patient developed thrombocytopenia and multilineage...
The relationship between the progressive hematological changes and streptavidin-phycoerythrin (SA-PE), and phycoerythrin-conjugated G-CSF (GCSF-PE) were resistant to cisplatin, etoposide, and bleomycin chemotherapy. Chromosome abnormality, which is a specific marker of GCT (21, 36, 44, 45), was detected by FISH and/or cytogenetic analysis in the bone marrow specimens, it seems likely that the protean nature of the bone marrow changes was mediated by evolving GCT metastasis which were resistant to cisplatin, etoposide, and bleomycin chemotherapy. Patients with similar hematological changes, which were consistent with GCT metastasis undergoing hematological differentiation and clonal evolution, have been reported previously (14, 17, 24, 26, 32). For example, among four patients with GCT and AML reported by Bajorin et al. (32), one was not karyotyped, but two of the remaining three leukemias had complex karyotypes which included i(12p) and abnormalities of chromosome 13. The i(12p) chromosome abnormality found in the germ cell tumor may have been directly involved in the genesis of this patient’s leukemia.

This patient received 36 doses of G-CSF (5 µg/kg/dose) to ameliorate the expected myelosuppression of chemotherapy. The B-lymphoid cell line established from the patient’s leukemia cells possessed G-CSF receptors. While G-CSF receptors are generally restricted to hematopoietic cells of the myeloid lineage (46, 47), Drach et al. recently reported G-CSF receptors on the leukemia cells from each of the 21 patients with BCP-ALL. Also, in four of their eight patients studied, BCP-ALL colony growth was enhanced when leukemia cells were cultured with G-CSF (50 ng/ml). Others have reported a rise in the number of leukemic blasts in the blood of patients treated with G-CSF (48, 49), and in 2 patients with severe aplastic anemia, treatment with G-CSF was followed by the development of leukemia (50). However, exogenous G-CSF did not change the growth rate or induced differentiation of the UoC-B10 cell line.

Recently, Orazi et al. (26) reported data on six patients with secondary leukemia who initially presented with mediastinal GCT. They observed morphologically identifiable hematological cells (which were CD34+) within the yolk sac tumor component of the GCT and hypothesized that the leukemias were derived from pluripotent stem cells found within the yolk sac tumor (26, 51). Leukemias with i(12p) may represent a malignant counterpart of embryonal hematopoiesis because there is a higher frequency of histiocytic and megakaryoblastic differentiation observed in mediastinal GCT patients when compared to the more mature myeloid subtypes observed in patients without mediastinal GCT. This parallels hematopoietic development in the embryo where the development of macrophages occurs at 4 weeks gestation and thrombopoiesis at 8 weeks gestation (52, 53). Many features of the case presented here support this hypothesis, including the rapid evolution of the patient’s marrow disease from a MDS involving myeloid, erythroid, and megakaryocyte lineages to a BCP-ALL.

There was good general concordance between the UoC-B10 cell line and the patient’s leukemia cells as determined by a comparison of the morphology, immunophenotype, genotype, and karyotype results. Both had features of mature B-lymphocytes with the expression of B-cell antigens (CD19, CD10, and CD20), the rearrangement of the immunoglobulin heavy chain gene (54, 55) and enzyme activities (TdT and ADA) commonly observed in mature B-cell malignancies (41). In the cell line, the discordant expression of IgD/lambda may represent B-lymphoid differentiation in culture, whereas the aberrant expression of CD4 (along with the expression of glycoporphin) suggests that the cell line may have the capacity to differentiate along more than one lineage.

Previously, a leukemia cell line with megakaryocytic features (EST-IU) was established from a patient with a mediastinal GCT who developed AML 4 months after completing chemotherapy (56). Since this cell line failed to proliferate beyond 6 months in culture, UoC-B10 appears to be the first leukemia cell line available to investigators with an i(12p) karyotypic marker. UoC-B10 cells, and the 1 liter of the patient’s cryopreserved BM which contains i(12p) blasts, will be useful in: (a) studies of molecular consequences induced by allele loss on chromosome 12; (b) determining the relationship between i(12p) and hematological differentiation of GCT; and (c) studies of acquired cisplatin-resistance in human GCT (57). Finally, the accumulating evidence suggests that patients with mediastinal GCTs should be carefully evaluated for a hematological disorder. Allogeneic bone marrow transplantation performed early in the course of the disease may alter its devastating natural history.

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