Long-Term Growth of Malignant Thymocytes In Vitro

By Stephen D. Smith, Pam McFall, Rodman Morgan, Michael Link, Fredrick Hecht, Michael Cleary, and Jeffrey Sklar

We report a new methodology for the long-term growth of malignant T-lymphoblasts from patients with T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LL). When malignant cells were cultured in the presence of insulin-like growth factor I under hypoxic conditions, cellular proliferation occurred that resulted in the establishment of immortal cell lines from ten of 12 patient tumors. Authenticity of each cell line was verified by a direct comparison of the immunophenotype, karyotype, and immunogenotype with the patient’s tumor cells. This improved method of cell culture permits frequent establishment of cell lines from patients with T-ALL/T-LL, thereby aiding in analysis of thymocyte transformation and neoplasia.

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MATERIALS AND METHODS

IGF-I. Previously, we screened our initial T-cell lines (K-T1, SUP-T1 through -T4) for the presence of receptors to peptide growth hormones. Using iodinated peptides, the malignant thymocyte cell lines were found to express receptors for IGF-I but lacked receptors for insulin, growth hormone, and insulin-like growth factor II (IGF-II).14 K-T1 possessed 1,047 high-affinity IGF-I binding sites per cell. K-T1 cells doubled every 36 hours when grown in nutrient media (McCoy 5A media [Irving Scientifics, Santa Ana, CA] with 10% fetal calf serum [FCS]) and cultured in an incubator gassed with 6% CO2, 5% O2, and 89% N2. In experiments designed to determine the biologic effect of IGF-I on malignant lymphoblasts that possessed IGF-I receptors, K-T1 cells were washed free of nutrient media, resuspended in serum-free media that contained or lacked IGF-I (AMGen Biologicals, Thousand Oaks, CA) and cultured in an incubator gassed with 6% CO2, 5% O2, and 89% N2 (Fig 1).

In order to evaluate the importance of oxygen concentration on the IGF-I mitogenic effect, a K-T1 subline (K-T1a) was developed by culturing K-T1 cells in nutrient media for 2 months in an incubator gassed with 6% CO2 in air. K-T1a cells had the same peptide receptor profile as K-T1 cells but had a slower doubling time (56 hours) than the parent cells. In experiments evaluating the difference between cell lines K-T1 and K-T1a, cells were washed free of nutrient media, resuspended in serum-free media with IGF-I (5 ng/mL) and replaced in the incubator of origin.

Patient samples. Tumor samples were collected from 12 patients with T-ALL or T-LL. The patients ranged in age from 3 to 19-years-old, except for one 74-year-old man (patient no. 4). Fresh tissue from eight patients had been cultured previously using our standard methodology but complete cell death had occurred by 1 to 3 months in culture.18 Each sample was immunophenotyped and karyotype analysis was successful on six samples (Tables 1 and 2). Tumor samples had been collected at the time of diagnosis in six patients and at the time of relapse in six patients. Approval was obtained from the Institutional Review Board for these studies. Patients were informed that blood and bone marrow samples were obtained for research purposes, and that their privacy would be protected.

In vitro culture of tumor samples. The patient’s tumor cells had been previously separated by a Ficoll-Hypaque gradient and frozen in 5% DMSO with 15% FCS at —70°C. Twelve samples, each frozen for at least 6 months but no longer than 42 months, were thawed, suspended in nutrient media (McCoy 5A media with 15% FCS), and divided into two equal aliquots. One aliquot was plated into 24-well tissue culture plates that contained a feeder layer consisting of a gel of nutrient media, agar (0.5%), and human serum (10%). The cells
were washed free of DMSO (final calculated concentration 0.01%), fed with 0.3 mL nutrient media with or without supplemental IGF-I (10 ng/mL), and incubated in a Hereaus incubator (Hereaus Inc., Pleasant Hills, CA) gassed with a mixture of 6% CO₂, 5% O₂, 89% N₂ or a standard incubator gassed with 6% CO₂ in air. The other aliquot of cells was incubated in a glass bottle overnight (37°C, Hereaus incubator), and the nonadherent cells were aspirated, centrifuged through FCS (to remove dead cells), and loaded into wells containing a feeder layer. Cells were fed with 0.3 mL nutrient media that contained or lacked 10 ng/mL IGF-I, and placed in a Hereaus or standard incubator.

**Characterization of cell antigens.** Monoclonal antibodies (MoAbs) to CD-5 (Leu-1, Pan-T), CD-8 (Leu-2a, T-cytotoxic/suppressor), CD-4 (Leu-3a, T-helper), CD-3 (Leu-4, Pan-T), CD-2 (Leu-5, sheep erythrocyte receptor), CD-1 (Leu-6, thymocyte), CD-7 (Leu-9, Pan-T), CD-10 (common ALL antigen, CALLA), CD-13 (My7, granulocytes and monocytes), CD-24 (My9, granulocytes and monocytes), and HLA-DR were generously provided by Becton-Dickinson (Mountain View, CA). Cell surface antigens were identified by the binding of MoAb as determined by indirect immunofluorescence. The cells were analyzed for fluorescence staining with a fluorescence-activated cell sorter (FACS-IV; Becton-Dickinson Electronics Laboratory, Mountain View).

Malignant cells were evaluated for CD-25 (Tac antigen) expression by the immunohistochemical staining (IHCS) technique to add supplemental data to the FACS analysis since IHCS detects cytoplasmic as well as cell surface antigens.15-17

**TdT evaluation.** Terminal deoxynucleotidyl transferase (TdT) activity was assayed by a testing system from Supertechs (Bethesda, MD).

**Chromosome preparation and analysis.** Chromosomes were prepared for analysis from cell lines and from peripheral blood and bone marrow cells. The cell lines were in log phase growth at 3 x 10⁵ cells/cc at 37°C. Peripheral blood and bone marrow cells were divided into two aliquots. One aliquot was untreated while the other was exposed to methotrexate (10⁻⁴ mol/L) for 18 hours and released with thymidine (10⁻³ mol/L) for six hours to produce cell synchronization and high-resolution chromosome banding. Both aliquots were incubated for 24 hours at 37°C in 6% CO₂ in air. One drop of Colcemid (GIBCO, Grand Island, NY) (10 μg/mL) was added for 30 minutes to arrest cells in mitosis. The cells were pelleted, resuspended in 0.075 mol/L KCl hypotonic solution and fixed three times in fresh methanol/glacial acetic acid (3:1). The chromosomes were trypsin-Giemsa banded. From each sample, 20 or more cells were analyzed and karyotypes were prepared.

**Molecular genetics.** Southern blot analysis of the β and γ T-cell receptor gene rearrangements were performed on the patient's tumor cells and the corresponding cell lines. Analyses were performed as previously described using hybridization probes specific for the human beta T-cell receptor (TCR) gene (combined Jβ1 and Jβ2) probes on DNA digested with BglII restriction enzymes (case nos. 2, 6, 9, and 10); Jβ2 alone on DNA digested with EcoRI (case 8); Cβ probe on DNA digested with EcoRI (case 7); or TCR gene (Jγ probe) on DNA digested with BamHI restriction enzyme.18-20

**RESULTS**

**IGF-I studies.** When K-T1 cells were plated at 10⁴ cells/cc in serum-free media and incubated in 5% O₂, 6% CO₂, and 89% N₂, cell viability remained relatively constant, but cellular proliferation did not occur (Fig 1). However, supplemental IGF-I supported cellular proliferation at ng/mL concentrations in a dose-dependent manner (Fig 1). Moreover, in identical experiments in which K-T1 cells (10⁴ cells/cc) were cultured in serum-free media supplemented with IGF-I (10 ng/mL) and incubated in an environment of 6% CO₂ in air, rapid cell death was observed.
of supplemental IGF-I to these culture plates resulted in glass adherence. In experiments in which cells were cultured, cellular proliferation did not occur (Fig 2).

After adherent cell removal, lymphoid cell groups (ten to 50 samples were cultured in hypoxia with supplemental IGF-I in hypoxic conditions, malignant lymphoblast proliferation was abundant monocyte proliferation with cell survival up to 3 weeks. The addition of 6% CO2. 5% O2, and 89% N2 resulted in the proliferation of normal monocytes that survived up to 3 weeks. The addition of 6% CO2 in air, cellular proliferation was not observed and all cells were dead within 20 days. However, when tumor samples were cultured directly after thaw in an incubator gassed with 6% CO2 in air, proliferation of cells cultured from patient samples. The viability of thawed cells was routinely grown in a gaseous environment of 5% O2, 6% CO2, and 89% N2, lack of cell growth in 6% CO2 in air could have been secondary to the stress of switching the cells into a different gaseous environment. Thus, a subline of K-Tl (K-Tla) was developed that maintained for more than 30 days. However, when tumor cultures could not be supported both the initial and subsequent proliferation of each T-cell line.

Cellular antigens. Each patient tumor sample (except case no. 6) expressed pan-T cell markers (CD-5, CD-7) and at least one other T-cell-associated antigen (Table 1). Each sample was also nonreactive with MoAbs against Tac (CD-25), CALLA (CD-10), and HLA-DR, and failed to express surface immunoglobulin (sIg). In addition, each cell line was nonreactive with MoAbs against granulocytes and monocytes (CD-13, CD-33) except for the SUP-T8 cell line, which was weakly reactive (25%) with CD-33.

The malignant tumor cells (and corresponding cell lines) were present in each well. After 2 weeks of culture, cell groups were passaged to fresh plates to reduce the potential for overgrowth of fibroblasts and lymphoblastoid cells. The volume of the overlayer was kept at or below 0.3 mL to allow full diffusion of gases. Continued cell growth occurred in ten of 12 thawed samples and ten new cell lines were established. Subsequent experiments documented that IGF-I was not necessary for continued cell proliferation when the cell concentration approached 10^6 cells/mL. Different batches of FCS supported both the initial and subsequent proliferation of each T-cell line.

Cultures of patient samples. The viability of thawed samples was >90% before glass adherence, and >50% after glass adherence. In experiments in which cells in air, cellular proliferation was not observed and all cells were dead within 20 days. Cells cultured directly after thaw in an incubator gassed with 6% CO2, 5% O2, and 89% N2 resulted in the proliferation of normal monocytes that survived up to 3 weeks. The addition of supplemental IGF-I to these culture plates resulted in abundant monocyte proliferation with cell survival up to 3 months. In experiments in which nonadherent cells were cultured in hypoxic conditions, malignant lymphoblast proliferation was observed briefly but cell cultures could not be maintained for more than 30 days. However, when tumor samples were cultured in hypoxia with supplemental IGF-I after adherent cell removal, lymphoid cell groups (ten to 50 cells) were present in each well. After 2 weeks of culture, cell groups were passaged to fresh plates to reduce the potential for overgrowth of fibroblasts and lymphoblastoid cells. The volume of the overlayer was kept at or below 0.3 mL to allow full diffusion of gases. Continued cell growth occurred in ten of 12 thawed samples and ten new cell lines were established. Subsequent experiments documented that IGF-I was not necessary for continued cell proliferation when the cell concentration approached 10^6 cells/mL. Different batches of FCS supported both the initial and subsequent proliferation of each T-cell line.

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The malignant tumor cells (and corresponding cell line) from patient no. 6 were classified as an early thymocyte because cells were CD-7-positive but lacked other T-cell–associated antigens. Tumors from four patients (no. 1, 2, 5, 8) had cell surface antigen profiles characteristic of the

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Diagnosis</th>
<th>Cell Source</th>
<th>Cell Line</th>
<th>Chromosome Abnormality</th>
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<tr>
<td>1</td>
<td>T-LL</td>
<td>BM</td>
<td>SUP-T5</td>
<td>46,XY, der(7;9)(q34;q34), -9,11q+, ,12p+, +mar</td>
</tr>
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<td>SUP-T6</td>
<td>46,XY, t(7;9)(q34;q34), del(6)(q21)</td>
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<td>BM</td>
<td>SUP-T8</td>
<td>44,XX, del(1)(p32p35), +1p, +1p, +4,14(19;13),7p+, +8, +9, 10p+, del(11)(q21q25), -12, -16, -17, der(17)(12;17)(p13p13), -22, +mar</td>
</tr>
<tr>
<td>4</td>
<td>T-ALL</td>
<td>BM</td>
<td>SUP-T9</td>
<td>46,XY, t(6;14;21)(q23;q11.2;q22), del(11)(q23q25), t(15;21)(q15; q22)</td>
</tr>
<tr>
<td>5</td>
<td>T-ALL</td>
<td>BM</td>
<td>SUP-T10</td>
<td>47,XY, del(5;9)(q31), t(7;11)(p13;p13), t(8;12)(q13;p13), t(9;16)(p22; p13), t(17;18)(q11.2;q23), +mar</td>
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<td>6</td>
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<td>BM</td>
<td>SUP-T12</td>
<td>46,XY, t(1;7)(p34;q34)</td>
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<td>PE</td>
<td>SUP-T13</td>
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<td>BM</td>
<td>SUP-T14</td>
<td>46,XY, del(6)(q15), del(9)(p22)</td>
</tr>
</tbody>
</table>

Underlined chromosomes in karyotype denotes discordance between tumor cells and cell lines.

Table 2. Chromosome Results
midthymocyte maturation stage and expressed CD-4, CD-8, and CD-1, and had low levels or lacked CD-3. Patient nos. 7 and 9 (CD-1 -, CD-3 +, CD-4 +, CD-8 +, CD-25 -) and patient no. 4 (CD-1 -, CD-3 +, CD-4 -, CD-8 -, CD-25 -) had a more mature (late thymocyte stage) but uncommitted immune profile. Malignant cells from patient 10 (CD-1 -, CD-3 -, CD-4 +, CD-8 -) appeared to possess an immune profile consistent with a T-helper cell.

While patient no. 3 was originally classified as T-ALL (CD4 +, CD-5 +, CD-7 +), the malignant cells might be better classified as immature monocytes because the patient’s tumor cells (and the SUP-T8 cell line) lacked terminal deoxyribonucleotide transferase, stained positively with esterase, had a normal adenosine deaminase level and possessed chromosome abnormalities involving 4q and 11q (Tables 1 and 2).22,24

The immunophenotype on each cell line showed good general agreement with the patient’s tumor cells (Table 1). As previously reported for the SUP-T1 through -T4 cell lines, the rare discordance between the surface antigen profile of the patient’s tumor and the corresponding cell line tended to occur in cases where a low level of surface antigen was expressed on the patient’s tumor cells while the cell line lacked the specific antigen.12

TdT analysis. Activity for TdT was present in >90% of the malignant cells from each cell line except SUP-T8, which was nonreactive with TdT.

Karyotype analysis. Each cell line was karyotyped and showed clonal cytogenetic changes in every cell (Table 2). In each case, except case no. 1, the cell line was established from an aliquot of the clinical sample that was karyotyped. In case no. 1, the SUP-T5 cell line was established from the patient’s first clinical relapse (pleural effusion) while the tumor karyotype was obtained from the patient’s second clinical relapse (bone marrow).

In five cases, a direct comparison between the cell line karyotype and the patient’s tumor karyotype was possible (Table 2). Complete karyotype concordance was present in two cases (patient no. 2, SUP-T6; patient no. 4, SUP-T11) while cell lines from patient nos. 1, 3, and 8 showed karyotypic changes compatible with clonal evolution. However, specific translocation breakpoints were highly conserved in each cell line. Moreover, the breakpoint on chromosome 7 band q34, at or near the loci of the β chain gene of the TCR, appears to be at the same site in each of the five cell lines and four bone marrow karyotypes in which it was observed.

Molecular genetics. For the majority of tumor specimens (case nos. 2, 4, 5, 7-10), Southern blot analyses using probes specific for the β and γ TCR genes showed nongermline fragments together with germline fragments in DNA obtained from the patient’s leukemia cells. In each of the cases, the tumor specific nongermline fragments co-migrated with the rearranged bands present in the corresponding cell line (Fig 3). Case nos. 3 and 6 showed only germline fragments in analyses for both the β and γ TCR genes. However, the corresponding cell lines established from these cases also failed to show nongermline fragments. The cell
line from case no. 1 (SUP-T5) was not analyzed because it was lost in a laboratory accident. These data demonstrate that the cell lines were derived from the predominant malignant cells in the patient’s tumor and that the cell lines represent a clonal population of cells in each case.

DISCUSSION

Our data indicate that malignant thymocyte cell lines can be established frequently when patient samples are cultured in a low oxygen environment with nutrient media supplemented with IGF-I. The oxygen concentration of normal bone marrow approaches 5% and oxygen concentrations of 1% to 10% have been shown to enhance the in vitro growth of both normal and malignant hematopoietic cells. IGFI is a 70 amino acid polypeptide that has similar structural and functional properties as insulin. IGFI will induce cellular proliferation of competent mesenchymal cells that possess specific IGF-I receptors. On the test cells used here (malignant thymocytes), the IGF-I–induced cell proliferation was restricted to a low oxygen environment and was blocked at a higher oxygen concentration. Previously, investigators have shown the response of epiphyseal chondrocytes to IGF-I was enhanced in 5% oxygen and blocked by 95% oxygen.

Clinically, T-ALL and T-LL are aggressive malignancies that are associated with an uncontrolled proliferation of malignant thymocytes. Patients with these neoplasms, which represent up to 20% of cases of ALL and lymphoma, often possess an anterior mediastinal mass, are in the first or second decade of life and, despite aggressive therapy, have a relatively poor prognosis. In the past, we (and others) established cell lines from 5% to 10% of T-ALL/T-LL samples and cells usually grew in vitro only from patients with a rapidly proliferating neoplasm. The new methodology reported here resulted in cell line establishment from ten of 12 frozen samples that were obtained from patients at presentation or relapse. In addition, using this technique, cell lines have been established recently from fresh tissue from five of six patients with T-ALL and T-LL at diagnosis.

Characteristically, T-ALL and T-LL cells possess an immature immunophenotype similar to cortical thymocytes developing in the normal thymus. The immaturity of these cells is characterized by the presence of reactivity with MoAbs against the CD-1 antigen and a lack of reactivity with antibodies against CD-3 and CD-25 (TAC, the receptor for interleukin-2 [IL-2]). Each of the new cell lines reported here lacked reactivity with the CD-25 antibody and each had an immunophenotype consistent with a stage I to III thymocyte. Since lack of expression of the IL-2 receptor restricts the ability of nonmalignant T lymphocytes to proliferate in response to IL-2, CD-25 nonreactive T-ALL/T-LL blasts will proliferate in response to IL-2 as measured by 3H-thymidine uptake and colony formation. This IL-2 response may be mediated by an IL-2 receptor that is not detected by CD-25. Since the SUP-T1 → T14 cell lines do not require exogenous IL-2 for establishment and maintenance (and do not produce IL-2; data not shown), and since the IL-2 induced proliferation of T-ALL cells is short-lived, IL-2 may induce terminal differentiation in vitro. Alternate-ly, since cell selection in cell line establishment is so great, specific subpopulations of T-ALL cells may respond differently to IL-2.

Karyotype analysis on each cell line (SUP-T5 through T14) showed clonal karyotype changes consistent with a lymphoid malignancy. In the five instances where a comparison to the tumor karyotype was possible, the cell line karyotypes showed either complete concordance or minor cytogenetic changes consistent with clonal evolution. Most of the karyotype abnormalities observed in the cell lines have been reported in ALL previously. Recurring sites of chromosomal translocations occurred at 7q34, 12p13, 14q11.2, 19p13, and 21q22. Five cell lines showed a chromosome translocation involving 7q34 at or near the site of the gene for the β chain of the TCR. Molecular analysis of the t(7;9) (q34;q32) in SUP-T5 and t(7;19) (q34;p13.1) in SUP-T7 has shown that the chromosome 7 breakpoint is located within the TCR gene. The SUP-T8 cell line had a t(4;19)(q21;p13) and del(11)(q21q25) with chromosomal breakpoints similar to the t(4;11)(q21;q23) seen in ALL with mixed lineage features.

Molecular analyses of the tumor cells showed rearrangements of the β and/or γ TCR genes in seven of the nine cases analyzed. Rearrangement of the TCR gene(s) documents the lineage and clonality of these neoplasms. Co-migration of the rearranged bands by the tumor samples and cell lines demonstrates that the cell lines represent the major malignant population in the patient's tumor. The germ line patterns of two cell lines support the monocytoid features of the SUP-T8 cell line and the uncommitted (or primitive T-cell) nature of the SUP-T10 cells.

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