Establishment of an Interleukin-3-Dependent Leukemic Cell Line From a Patient With Chronic Lymphocytic Leukemia in the Acute Phase

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A novel human leukemic cell line, TMD2, which proliferates dependently on interleukin-3 (IL-3), was established from the peripheral blood (PB) of a patient with chronic lymphocytic leukemia (CLL) in the acute phase. After 8 years of the chronic phase of CLL, lymphoblastoid cells appeared and became dominant in the PB. After repeated subcultures of the patient's PB cells in the acute phase, lymphoblastoid cells have proliferated actively in the presence of recombinant human IL-3, and the TMD2 cell line has been established. The lymphoblastoid cells in acute phase and TMD2 cells proliferated dependently on IL-3, whereas growth of the small lymphocytes in chronic phase was not supported by IL-3. Other ILs (IL-1, 2, and 4 through 6) or CSF did not support the growth or survival of TMD2 cells. The existence of high-affinity receptors for IL-3 was shown on TMD2 cells (binding sites 88 per cell, $K_d = 76.9 \text{ pmol/L}$). DNA extracted from the small lymphocytes in the chronic phase, the lymphoblastoid cells in the acute phase, and TMD2 cells showed the same rearrangement pattern of the immunoglobulin heavy-chain gene. Therefore, these cells were considered to have originated from the same clone. These results imply that a genetic event that caused the responsiveness to IL-3 in the cell of the chronic phase caused the acute transformation of CLL in this patient. We consider that TMD2 cell line is valuable as a model of cells of CLL in the acute phase and as a tool for studying the signal transduction system of IL-3.

Materials and Methods

Patient. A 67-year-old man was hospitalized at Tokyo Metropolitan Komagome Hospital in June 1990 because of marked splenomegaly. He had had CLL since 1982. On his admission, his WBC count was 303,600/$\mu$L with 76% large lymphoblastoid cells, 21% small lymphocytes, and 3% neutrophils. The lymphoblastoid cells were larger than CLL cells and had a relatively abundant cytoplasm, an irregular nuclear outline, fine chromatid condensation, and several nucleoli. He was diagnosed as having CLL in the acute phase or lymphoblastic transformation and received cyclophosphamide, vincristine, adriamycin, and prednisolone. After the chemotherapy, most of the lymphoblastoid cells disappeared from the peripheral blood (PB) and the disease progressed to the chronic phase. Soon, however, the lymphoblastoid cells again became dominant and repeated chemotherapy was required (Y. Maruyama, manuscript in preparation).

Cell culture. Mononuclear cells were separated by Ficoll-Hypaque density gradient (1.077 g/mL) from the patient's PB in the acute phase, with his informed consent. The cells were plated in a 24-well plate (Falcon, Oxford, CA) at 1 $\times 10^6$ cells/mL in $\alpha$-minimal essential medium (GIBCO, Grand Island, NY) with 20% fetal calf serum (GIBCO) and 5 ng/mL recombinant human IL-3 (rhIL-3, Genzyme, Boston, MA). rhIL-3 was produced using a cDNA obtained from activated T cells and expressed in yeast. The purity of rhIL-3 is 95%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in gels developed with silver staining before addition of carrier protein. Approximate specific activity is 5.0 $\times 10^7$ proliferation units per milligram, as determined by the proliferation assay measuring human bone marrow (BM) precursor cell incorporation of $^{3}H$-TdR in a 96-hour microassay (Genzyme). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO$_2$ in air. Every 4 days, half the medium was exchanged with fresh medium. The cells have proliferated exponentially, and a new cell line established after repeated subcultures has been designated TMD2.

Cellular morphology, cytochemistry, immunophenotype, and karyotype. The cells were placed on a glass slide using Cytospin 2 (Shandon, London) and stained with Wright's stain, peroxidase reaction with 3,3'-diaminobenzidine tetrahydrochloride, and methylene blue. The cells were also stained with anti-CD19, anti-CD20, and anti-CD3 monoclonal antibodies using the peroxidase-antiperoxidase method. The surface antigens of TMD2 cells, the small lymphocytes obtained from the patient in the chronic phase, and the large lymphoblastoid cells from the patient in the acute phase were analyzed with FACStar (Becton-Dickinson, Mountain View, CA) with commer-

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cational monoclonal antibodies (MoAbs). Chromosomal analysis of TMD2 cells was made by the trypsin-Giemsa G banding method.8

Southern blot analysis. DNA was extracted from TMD2 cells and mononuclear cells separated from the PB (PBMCN) of the patient in chronic and acute phases. Human placenta DNA was used as a control. The samples of DNA were digested with HindIII, EcoRI, or BamHI, electrophoresed, transferred by Southern blotting technique, hybridized to 32P-labeled probe, and visualized on autoradiograms.23 The probes used were the IgH gene joining region JH probe [3.4-kilobase (kb) EcoRI-HindIII fragment], Cβ probe, bcl-2 probe (Oncor) and c-myc third exon probe (all from Oncor, Gaithersburg, MD).

Effects of cytokines on the proliferation of TMD2 cells. To determine the growth factor essential for optimal proliferation of TMD2 cells, the proliferation of TMD2 cells stimulated with various cytokines was examined by colorimetric assay as described by Mosmann23 and by the clonogenic assay as described by Nara et al.12 The cytokines studied were rhIL-1α and rhIL-β (gifts from Dr Y. Hirai, Ohtsuka Pharmaceutical, Tokushima, Japan), rhIL-2, rhIL-3, rhIL-4, rhIL-6 (Genzyme), rhIL-5 (Amer- sham, Buckinghamshire, England), rhGM-CSF (Hoechst Japan, Tokyo), rhG-CSF, rh-erythropoietin (rhEPO, Chugai Pharmaceutical, Tokyo), and rhM-CSF (Ohtsuka Pharmaceutical).

In the colorimetric assay (MTT assay), 104 TMD2 cells were incubated in a 96-microwell microplate (Sumitomo Bakelite, Osaka, Japan) in 0.1 mL RPMI 1640 medium (GIBCO) with 10% FCS (GIBCO) with some cytokine at 37°C in a humidified atmosphere of 5% CO2 in air. After 5-day incubation, MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide, Sigma Chemical, St. Louis, MO) was added and incubated for 4 more hours. Isopropanol containing 0.04 N HCl was then added, and optical density (OD) was measured with an enzyme-linked immunosorbent assay (ELISA) reader using test and reference wavelengths of 570 and 620 nm, respectively.

When 104 TMD2 cells were plated in a 96-microwell plate in 0.1 mL α-MEM with 20% FCS, 0.8% methylcellulose (Dow Chemicals, Midland, MI) with 1 to 10 ng/mL rhIL-3, colonies of more than 20 cells formed after 7-day incubation at 37°C, 5% CO2 in air. The effect of other cytokines on colony formation of TMD2 was tested. Furthermore, the effect of anti-IL-3 antibody on colony formation stimulated by 5 ng/mL rhIL-3 was examined. Mouse anti-human IL-3 MoAb was purchased from Genzyme. This anti-IL-3 antibody has been shown by ELISA and dot-blot analysis to bind rhIL-3, and 0.5 mg of this antibody neutralizes approximately 2,500 U rhIL-3. No detectable cross-reactivity of this antibody with rhIL-2, IL-4, or GM-CSF has been observed. Because this antibody class was IgG1, mouse IgG1, (ICN Biomedicals, Lisle, IL) was used as a control.

Binding of 125I-IL-3 to cells. To detect the specific IL-3 receptors on TMD2 cells, binding studies using 125I-IL-3 were performed by a previously described method15; 7.0 × 107 cells in 200 μL binding buffer (RPMI 1640 medium supplemented with 0.1% bovine serum albumin (Sigma) and 10 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (Sigma), pH 7.4) containing 125I-IL-3 at various concentrations with or without a 100-fold excess of unlabeled IL-3 were incubated for 2 hours at 18°C in Micro Labtubes (Ieda Trading, Tokyo) with gentle continuous mixing.15 125I-IL-3 was purchased from Amersham Japan. 125I-Labeled rhIL-3 was prepared by the method based on that of Bolton and Hunter14 and was purified by gel-filtration high-performance liquid chromatography. The specific activity was 18.5 TBq/μmol. After the cells were saturated with 125I-IL-3, bound and free 125I-IL-3 were separated by removing duplicate 80-μL aliquots from the incubation mixture, layering those on 100 μL di-n -butyl phthalate (Wako Pure Chemical Industries, Osaka, Japan) in tapered 400-μL Micro Labtubes, and centrifuging for 2 minutes at 10,000g. The oil allowed cells to sediment but was more dense than binding buffer. After aspiration of the supernatants, the lower layer containing the cell pellets was cut off by scissors, and the radioactivity was counted in a γ-counter. Radioactivity bound to cells in the presence of 100-fold excess of unlabeled IL-3 was considered nonspecific binding and was subtracted from the total binding.

Viral study. To rule out contamination of TMD2 cells by Epstein-Barr virus (EBV), EBV nuclear antigen (EBNA) was tested by the method of Reedman and Klein.55

RESULTS

Establishment and characterization of the cell line. The PBMCN obtained from the patient in the acute phase proliferated actively when cultured in a 24-well plate in α-MEM with 20% FCS and 5 ng/mL rhIL-3. After several passages, the cells increased in number and were then cultured in culture flasks (25 cm2, Falcon) in the same conditions as the primary culture. The cells have been proliferating for 8 months with no detectable change in morphology and proliferative activity. Thus, the cell line has been considered to be established and is designated TMD2. When PBMCN were cultured without IL-3, the cells did not proliferate in culture and most of the cells died in about 10 days.

TMD2 cells have a round or slightly irregular nucleus with three to six nucleoli and have relatively abundant cytoplasm by Wright's stain (Fig 1). They were negative for POX, PAS, or dual esterase reaction. On the basis of morphology and cytochemistry, TMD2 cells are similar to lymphoblastoid cells in the PB of the patient in the acute phase.

The immunophenotypes of TMD2 cells, lymphocytes in the patient in the chronic phase and lymphoblastoid cells in the acute phase are summarized in Table 1. TMD2 cells were positive for CD10, CD19, CD20, HLA-DR, surface IgM, and surface k. This finding was identical to that of lymphoblastoid cells in the acute phase. In contrast, lymphocytes in the chronic phase were negative for CD10 but positive for CD11b and CD13, both of which were markers of myeloid cells. This finding indicated that lymphocytes in the chronic phase were of the mixed lineage of lymphoid and myeloid cells. The details of the characteristics of CLL cells in the chronic phase will be discussed later (Y. Maruyama, manuscript in preparation). EBNA was not detected in TMD2 cells; TMD2 cells were not contaminated by EBV.

The karyotype of TMD2 cells was determined as 46,X,−Y,5q−,6p−,−8,9p−,17q+,19p+,20q+,−4 markers (1 cell), 46,XY,−11,−13,−17,−19,−20,5q−,8q+,9p−,−4 markers (1 cell), 46,XY,−3,−11,−17,−19,20,5q−,8q+,9p−,−4 markers (1 cell), and undetermined (2 cells). Chromosomal analysis of CLL cells in the chronic phase was not performed. Furthermore, the karyotype of BM cells in the acute phase varied greatly. Therefore, determining the clonal origin of TMD2 cells on the basis of cellular karyotype was difficult.

To determine the clonal origin of TMD2 cells, the DNA

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Fig 1. Morphologic appearance of original leukemic cells and TMD2 cells (Wright's stain). (A) PBMNC in acute phase. They were composed of lymphoblastoid cells and small lymphocytes. (B) TMD2 cells were similar to the lymphoblastoid cells in the acute phase.

analysis was then performed. Southern blot analysis showed the same rearrangement pattern of immunoglobulin heavy-chain gene in DNA among TMD2 cells, PBMNC in the chronic phase, and PBMNC in the acute phase. Figure 2 shows the result of DNA digested by HindIII. Results showed that these three cellular populations originated from the same clone. These three DNA samples did not have the rearranged band for T-cell receptor gene β-chain, bcl-2 gene, or c-myc gene (data not shown).

Growth requirement of TMD2 cells. Proliferation in culture of TMD2 cells was studied by colorimetric assay and clonogenic assay to determine the growth factor(s) necessary for optimal proliferation of TMD2 cells. Figure 3 shows the IL-3 dose-response curves for proliferation of TMD2 cells, PBMNC in chronic phase and PBMNC in acute phase, as determined by MTT assay. When these cells were incubated for 5 days with various concentrations of rhIL-3, rhIL-3 stimulated proliferation of TMD2 cells and PBMNC in the acute phase in a dose-dependent manner.

Table 1. Immunophenotype of TMD2 Cells and Original Cells

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Abbreviations: CP, chronic phase; AP, acute phase; s, surface.

Fig 2. Southern blot analysis using Jκ probe. Each lane contains HindIII-digested DNA from TMD2 cells (A), PBMNC in chronic phase (B), PBMNC in acute phase (C), and placenta as a control (D). G, germline; R, rearranged band. Lanes A through C showed the same rearrangement pattern.
but 0.02 to 5.0 ng/mL IL-3 did not stimulate proliferation of PBMNC in the chronic phase. The proliferative activity of TMD2 cells and PBMNC in the acute phase appeared to reach plateau by IL-3 at a concentration greater than 2.5 ng/mL. When TMD2 cells were plated in methylcellulose media with IL-3 added, colonies of more than 20 cells formed. Colonies were picked using a fine micropipette, placed on a glass slide, stained by Wright's stain, and examined morphologically. The colonies were thus confirmed to be composed of cells with the morphologic appearance of TMD2 cells. rhIL-3 stimulated colony formation in a dose-responsive manner, as shown by MTT assay; 2 ng/mL IL-3 showed almost maximal effect on colony formation of TMD2 cells (data not shown).

Figure 4 shows the time-course of TMD2 cell proliferation. Cultured with rhIL-3, TMD2 cells proliferated exponentially. Without IL-3, although viable cell number increased slightly until day 7, most cells ceased to proliferate after the day 8.

Next, we attempted to clarify whether other cytokines also were effective on proliferation of TMD2 cells. First, proliferation of TMD2 cells was assessed by MTT assay with some cytokine added. The cytokines tested were rhIL-1α (100 U/mL), rhIL-1β (100 U/mL), rhIL-2 (1,000 U/mL), rhIL-4 (100 U/mL), rhIL-5 (5 ng/mL), rhIL-6 (100 ng/mL), rhGM-CSF (10 ng/mL), rhG-CSF (10 ng/mL), rhM-CSF (1,000 U/mL), and rh-EPO (10 U/mL). The concentration of each cytokine was chosen because results of our previous published and unpublished studies showed that each cytokine at each concentration displayed some effect on leukemia cells. These cytokines did not stimulate or suppress proliferation of TMD2 cells in MTT assay. Neither were PBMNC in the chronic and acute phases affected by these cytokines. Second, we examined the effect of 1 to 100 ng/mL rhGM-CSF or G-CSF on the growth of TMD2 cells in the clonogenic assay. Neither GM-CSF nor G-CSF stimulated colony formation of TMD2 cells at concentrations of 1 to 100 ng/mL.

The above data suggest that TMD2 cells require IL-3 exclusively for their growth. To confirm this hypothesis, we tested whether anti-IL-3 antibody released the stimulatory effect of IL-3 on TMD2 cells. TMD2 cells were plated in methylcellulose media in the presence of 5 ng/mL rhIL-3; 0.05 to 100 μg/mL anti-human IL-3 MoAbs were added to the culture of TMD2 cells. After 7-day incubation, colonies of more than 20 cells were counted. Figure 5 shows the effect of anti-IL-3 antibody on colony formation of TMD2 cells. Anti-IL-3 antibody reduced colony formation stimulated by 5 ng/mL IL-3, and 50 μg/mL anti-IL-3 antibody completely abolished the stimulatory effect of IL-3 on TMD2 cells. Mouse IgG1, used as a control, did not significantly affect colony formation of TMD2 cells.

Receptors for IL-3 on TMD2 cells. To confirm further the role of IL-3 on proliferation of TMD2 cells, we investigated the expression of IL-3 receptors on TMD2 cells. Figure 6A shows the result of the binding experiment in which increasing concentrations of 125I-IL-3 were incubated with TMD2 cells. Nonspecific binding increased
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A plot of specific binding as a function of radioligand concentration indicates that binding was dose responsive and saturable. Scatchard plot analysis of the binding data yielded an apparent kd of 76.9 pmol/L with 88 binding sites per cell (Fig 6B). Fewer IL-3 receptors were expressed on TMD2 than were expressed on some strains of human myeloid leukemia cell line KG-1, which had a single class of high-affinity receptors (240 to 310 receptors per cell) with a kd of approximately 100 pmol/L.22

DISCUSSION

We have established a novel IL-3-dependent cell line, TMD2, from a patient with CLL in the acute phase. To our knowledge, a cell line established from CLL in the acute phase has not yet been reported. Therefore, the TMD2 cell line may be useful as model of CLL cells in acute transformation just as the K562 cell line has proved to play important roles in studies of CML in blast transformation.7

The morphology and phenotypes of TMD2 cells were similar to those of lymphoblastoid cells of the patient in the acute phase (Fig 1). The responsiveness to IL-3 of TMD2 cells was also similar to that of the lymphoblastoid cells in the acute phase (Fig 3). These findings support the hypothesis that the TMD2 cell line is a clonal expansion from the original leukemic cells of the patient. In contrast, TMD2 cells were different from the CLL cells of the patient in the chronic phase in terms of the morphology, phenotypes, and responsiveness to IL-3, but DNA showed the same rearrangement pattern of the immunoglobulin heavy-chain gene among TMD2 cells, lymphoblastoid cells in the acute phase, and lymphocytes in the chronic phase (Fig 2). Therefore, TMD2 cells, lymphoblastoid cells in the acute phase, and lymphocytes in the chronic phase were considered to have originated from the same clone. During the long-term clinical course, CLL cells are believed to have made a transformation, resulting in lymphoblastoid cells. Although CLL cells in the chronic phase did not respond to IL-3, lymphoblastoid cells in the acute phase proliferated actively by stimulation of IL-3. Some CLL cell(s) in the chronic phase may have acquired responsiveness to IL-3, responded to IL-3, transformed, and obtained high proliferative activity. Alternatively, in the process of transformation, CLL cells may have acquired responsiveness to IL-3.

IL-3 stimulated proliferation of TMD2 cells in a dose-responsive manner. The maximal proliferation of TMD2 was induced by 2.5 ng/mL rhIL-3 assessed by MTT assay, and half-maximal proliferation was obtained by approximately 0.3 ng/mL rhIL-3 (Fig 3). This dose-response curve was similar to that observed in another IL-3-dependent cell line, TF-1 established from erythroleukemia; 1 ng/mL and 0.03 to 0.1 ng/mL rhIL-3 induced maximal and half-maximal proliferation of TF-1 cells, respectively.7 Although the responsiveness to IL-3 of TMD2 cells was comparable to that of TF-1, the growth requirement of these cell lines was different. TMD2 responded only to IL-3, whereas TF-1 responded not only to IL-3 but also to GM-CSF and EPO.

To demonstrate further the specificity of the IL-3 effect on TMD2 cells, we investigated whether TMD2 expressed receptors for IL-3 and whether an anti-IL-3 antibody blocked the effect of IL-3 on proliferation of TMD2 cells. TMD2 cells possessed high-affinity receptors for IL-3 (Fig 3), although their number was quite small as compared with those of IL-3 receptors expressed on some strains of the KG-1 cell line.22 Furthermore, anti-IL-3 MoAb completely released the stimulatory effect of IL-3 on TMD2 cells (Fig 5). On the basis of these findings, the TMD2 cell line is a quite novel IL-3-dependent lymphoid cell line that expresses surface immunoglobulin Mk and contains the rearrangement of immunoglobulin heavy chain. The TMD2 cell line, therefore, will be a valuable tool for studying signal transduction system of hIL-3. So far, some murine IL-3-dependent cell lines have played an important role in studying signal transduction of IL-3,20 but the signals of
murine IL-3 are not necessarily the same as those of hIL-3. Therefore, an hIL-3-dependent cell line is required to explore the signal transduction system of IL-3 in human cells. Human cell lines that respond to both IL-3 and GM-CSF have been reported. With these cell lines, signal transduction of human IL-3 has been studied. Because IL-3 and GM-CSF have been believed to induce a nearly identical tyrosine phosphorylation, however, the possibility that IL-3 stimulates not only signal transduction system of IL-3 but also that of GM-CSF cannot be excluded. Therefore, the TMD2 cell line, which responds only to IL-3 but not to GM-CSF, can be expected to serve as a suitable model for studying the signal transduction system of IL-3 in human cells.

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