Establishment and Erythroid Differentiation of a Cytokine-Dependent Human Leukemic Cell Line F-36: A Parental Line Requiring Granulocyte-Macrophage Colony-Stimulating Factor or Interleukin-3, and a Subline Requiring Erythropoietin

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We have established a new nonlymphoid leukemic cell line from a patient with myelodysplastic syndrome (MDS), which progressed to overt leukemia. The parental cell line and a subline derived from this line have absolute dependency on several cytokines for their long-term survival and growth. The parental line designated F-36P requires granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) for continuous growth, while a subline designated F-36E can be maintained in the presence of erythropoietin (Epo) alone. When these cytokines are depleted, both the parental and the subline cells die within several days, even in medium supplemented with fetal calf serum (FCS). F-36E, maintained in the presence of Epo, constitutively synthesizes hemoglobin at a significant level. F-36P, which is usually maintained in the presence of GM-CSF or IL-3, can be induced to synthesize hemoglobin when GM-CSF or IL-3 is substituted by Epo. The surface marker profile shows that the F-36P cells are positive for the leukocyte common antigen (CD45) and some common multilineage markers such as CD13, CD33, and CD34, and negative for T- and B-cell antigens and mature myelomonocytic antigens. However, some monoclonal antibodies recognizing erythroid and platelet glycoproteins react with these cells. Thus, this cell line has a multilineage phenotype, suggesting that the transformation event occurred in a multipotent stem cell. It is also evident that the F-36 cells can be induced to differentiate into the erythroid lineage in the presence of Epo. This, to our knowledge, is the first description of a human leukemic cell line that can be stimulated to synthesize hemoglobin by Epo.

Establishment of the cell line.

MATERIALS AND METHODS

Cytokines. Recombinant human Epo was a generous gift from Kirin-Amgen (Thousand Oaks, CA). Recombinant human GM-CSF was kindly provided by Schering Plough (Osaka, Japan). Recombinant human IL-3 was supplied by Kirin Brewery (Tokyo, Japan).

Establishment of the cell line. We observed a male patient diagnosed with refractory anemia with excess blasts (RAEB), a subtype of myelodysplastic syndrome (MDS). On October 12, 1989, approximately 5 months after the initial diagnosis, the disease progressed to overt leukemia, at which time the cells used to establish the line were obtained from the patient’s pleural fluid. At the intermediate stage of RAEB in transformation (RAEB-T), the bone marrow aspirate morphology showed some characteristics of erythroleukemia (~50% of the nucleated cells were erythroblasts, many of which were multinucleated or abnormally large in size), but did not meet the French-American-British (FAB) criteria for M6 subtype of acute myeloid leukemia. These cells were cultivated in flat-bottom 24-well plates (Becton Dickinson Labware, Lincoln Park, NJ) in α-modified Dulbecco’s medium supplemented with 20% FCS with or without G-CSF, GM-CSF, or IL-3.

Morphology and cytochemistry. Light microscopy examination was performed on Wright-Giemsa–stained cytopsin preparations. Cytochemical staining of myeloperoxidase (MPO), double esterase by α-naphthyl acetate and α-naphthyl butyrate, and Fe and periodic acid–Schiff (PAS) staining were performed by a standard protocol.

Electron microscopy. For the standard procedure, the pellet was fixed in a 2.5% glutaraldehyde, sliced, and postfixed in 1% osmium tetroxide. Specimens were dehydrated in ethanol, embedded in Epon, and stained with uranyl acetate and lead citrate. The

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platelet peroxidase (PPO) was performed by the method of Breton-Gorous et al. Cytogenetic studies. Three months before obtaining established cells, 18 mitotic figures of freshly aspirated bone marrow cells were analyzed by the trypan-Giemsa G-banding method. Sixteen mitotic figures of the cultured cells were analyzed by the same method 3 months after the culture was started.

Surface marker study. Surface markers were detected by an immunofluorescence assay using commercial monoclonal antibodies listed in Table 1.

Assay for cell proliferation. Short-term cell proliferation was examined by a colorimetric assay according to Mosmann. Cells were incubated at a density of 1 x 10^6 cells/100 μL in 96-well plates for various periods in RPMI 1640 medium supplemented with 10% FCS in the presence or absence of 10 ng/mL GM-CSF or IL-3, or 20 U/mL of Epo at 37°C. Next, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added at a final concentration to 0.5 mg/mL. Following a 4-hour incubation at 37°C, the insoluble product was dissolved in isopropanol containing 0.04N HCl. The optical density (OD) was measured at 630 nm. For dose-response plots, the assay was performed in a similar manner as described above, except that 2 x 10^6 cells were initially plated and various concentrations of GM-CSF, IL-3, or Epo were added, respectively. MTT was added after 48 to 72 hours of incubation.

Results

Establishment of F-36, a parental line F-36P and a subline F-36E. Cells cultured in the presence of GM-CSF or IL-3 gradually started active proliferation, following a lag time of approximately 1 month, when most of the cells cultured in the absence of these cytokines had stopped proliferation. During the subsequent 2 months, the proliferative rate of the active cells increased, but the cells cultured in the presence of IL-3 alone stopped proliferation at the end of the third month and died thereafter. At this time, therefore, the still actively proliferating cells were all maintained in the presence of GM-CSF. We found that these cells would die within several days when transferred to the medium without any exogenously added cytokines, even if supplemented with FCS, but that they would continue to proliferate in a similar manner when GM-CSF was substituted by IL-3. This parental line was designated F-36P. When GM-CSF was substituted by Epo, the cells survived for 1 to 2 weeks without active proliferation, followed by the death of most of the cells. However, after a lag time of an additional 1 month, dividing cells were apparent in one of these Epo-substituted flasks. These cells started active proliferation in the presence of Epo, but would die with the depletion of Epo. This subline was designated F-36E.

The established parental and subline of the F-36 cells have been maintained in RPMI 1640 medium supplemented with 10% FCS and GM-CSF or IL-3 (for F-36P), or Epo (for F-36E), respectively. They had been cultured continuously for 18 months at the time of submission of this report.

Morphology and cytochemistry. F-36P proliferates as adherent cells and as single cells in suspension. F-36E has much less tendency to adhere to the flask. Both of the F-36 cell lines have an irregular, often nonspheric shape, with spurs or blebs in suspension. Morphology of F-36P and F-36E resembled that of bizzare nonlymphoid leukemia blast (Fig 1A) or immature erythroblast (Fig 1B), respectively. Cyttoplasmic blebs were outstanding in all cytospin preparations. Dark nucleoli were often present. Peripheral splittings of cytoplasmic fragments were observed, some of which contained amorphous eosinophilic zones continuing from a perinuclear region (Fig 1A). Mitotic figures were observed at a frequency of up to 3% to 4%. Multinucleated cells were present at up to 1%.

Cytological stains of MPO, or α-naphthyl and double esterase, were negative. Fe was negative in the established cell line, although it was positive on some occasions in some of the patient’s freshly aspirated bone marrow cells. Some of the established cells, as well as the patient’s aspirated bone marrow cells, showed large granular staining with PAS (Fig 1C).

Electron microscopic studies showed that the F-36P cells exhibited a small number of microvilli, ruffles, and elongated cytoplasmic processes of the cell surface. The cells showed a round but occasionally multilobulated nucleus without marginal chromatin and with a few large nucleoli, and a prominent cytoplasm that contained a moderate number of rough endoplasmic reticulum, mitochondria, and occasionally scattered open vesicles, and Golgi appara-
GM-CSF/IL-3- and Epo-dependent sublines

tus of varying sizes (Fig 1D). The cells did not exhibit MPO. However, only a small population (1% to 2%) of cells exhibited apparent PPO (data not shown), which may correspond to the positivity of IbIIa antigen as described below, and thus may suggest some megakaryocytic properties of the cells.

Karyotype. Chromosomal study of the freshly obtained bone marrow cells indicated the presence of normal and abnormal clones. Thirteen of 18 mitotic figures showed normal karyotype (43,XY). Four showed essentially the same karyotype: -5, -7, -9, -17, -18, 19p+, and some markers, although some additional random loss was observed in each mitotic figure. The majority of the established cells had a homogeneous karyotype (Fig 2); 43,Y (Xp+, -5, -7, -13, -16, -17, -19, -21, 2q-, 9p+, 10q+, +4mar).

Surface marker profiles. The surface marker profile of F-36P is summarized in Table 1. The results were essentially the same in F-36E subline. The cells were positive for the leukocyte common antigen (CD45) and some common multilineage markers such as CD13, CD33, and CD34, and negative for T- and B-cell antigens and mature myelomonocytic antigens. However, monoclonal antibodies recognizing glycoporphin A (erythrocyte glycoprotein) or platelet glycoprotein IbIIa reacted with the cells.

Cytokine requirements. The survival and growth of the F-36 cells are shown in Fig 3. Neither the parental or the subline cells proliferated in the absence of any cytokines, and both types of cells started to die within 36 hours despite sufficient supplementation with FCS. IL-5, IL-6, and G-CSF had no effect on the survival or growth of the cells, either alone or in combination with IL-3 or GM-CSF (data not shown). Both the parental and the subline cells proliferated dramatically with the addition of GM-CSF or IL-3 at 10 ng/mL.

In F-36P, Epo (20 U/mL) prolonged the survival of the cells for up to 2 weeks, but these Epo-added cells died thereafter unless either GM-CSF or IL-3 was added. In contrast, in the F-36E subline, the proliferation of the cells could be sustained by Epo, as well as by GM-CSF or IL-3. Furthermore, observing F-36E cells for a longer period showed that Epo was the most potent cytokine among the three, because some populations of F-36E cells did die in the presence of GM-CSF or IL-3 alone (data not shown). Dependence of concentrations of GM-CSF, IL-3, or Epo is shown in Fig 4. Cytokine requirements for the growth of the parental line and the subline correlated well with the survival time course shown in Fig 3. Specifically, the maximal stimulation by Epo was approximately half that by GM-CSF or IL-3 for F-36P, but maximal stimulation by any of the three cytokines was almost equal to the F-36E subline in short-term assays. A colony formation study showed almost the same tendency as a characterization by the colorimetric assay; F-36P formed colonies only in the presence of GM-CSF or IL-3, but not Epo alone, and F-36E formed colonies in the presence of any of these three cytokines, although it did not in the absence of any of these (data not shown).

Epo-induced hemoglobin synthesis. The F-36E cells (maintained by Epo) constitutively synthesized hemoglobin at a significant level by estimation with benzidine staining (data not shown), by peak absorbance of cytosolic protein at 414 nm (Fig 5B), and by the reddish color of the cell pellet (data not shown). The level of hemoglobin synthesis by F-36P cells (maintained by GM-CSF or IL-3) was undetectable grossly (as judged from color of the cell pellet) or colorimetrically (Fig 5A). However, after GM-CSF or IL-3 was removed and replaced by Epo, F-36P started to synthesize hemoglobin as shown by the estimation with benzidine staining, by the reddish color of the cell pellet (data not shown), and by colorimetry at 414 nm (Fig 5A).

The reverse experiment, the replacement of Epo by GM-CSF in F-36E subline cells, seemed to decrease cellular hemoglobin (Fig 5B), although a significant change was not observed. 8-ALA also induced hemoglobin synthesis in F-36P in the presence of GM-CSF or IL-3 (Fig 5A), and increased hemoglobin synthesis in F-36E maintained in Epo (Fig 5B). However, F-36P was not induced to synthesize hemoglobin by sufficient concentrations of Epo when GM-CSF or IL-3 coexisted even at a low concentrations (0.01 ng/mL).

GM-CSF receptor on F-36 cells. GM-CSF receptor was identified by affinity labeling using 125I-GM-CSF. The result was similar to our previous experiments using other leukemic cell lines: two major bands composed of the a-chain and the b-chain, respectively, were labeled. However, the ratio of the intensities of the two bands were found to be different from the cells we studied previously. As shown in Fig 6, in the U-937 cells, the intensity of the lower molecular weight band originating from the a-chain was approximately 10-fold greater than the higher molecular weight band originating from the b-chain (lane a) when a high concentration (2 to 4 nmol/L) of 125I-GM-CSF was incubated, as described by us previously. In contrast, the lower molecular weight band identified in either the parental or the subline of F-36 was much fainter in comparison to the U-937 cells (lanes e and c). Comparison of the GM-CSF receptors on F-36P and F-36E by the ligand affinity labeling showed no significant difference between these two sublines.

DISCUSSION

A small number of cytokine-dependent or cytokine-requiring human leukemic cell lines have been reported recently. However, with some exceptions, most of them can be maintained even in the absence of any exogenous cytokines if enough FCS is supplemented, although various hematopoietic growth factors and other cytokines enhance their growth. It is apparent that the human hematopoietic cells are much more difficult to establish as cytokine-dependent cell lines compared with the murine cells, which are easily established with dependence on various cytokines, especially IL-3. This difference has been attributed to frequent intrinsic viral integration in mouse cells or may represent the different physiologic roles of cytokines between mouse and human. The previously reported erythroleukemia cell line TF-1 is one example of such a truly cytokine-dependent human leukemic cell line.
Fig 1. Morphology of F-36 cells (A through D). Wright-Giemsa stains of (A) F-36P and (B) F-36E. (C) PAS staining of F-36P, and (D) a transmission electron microscopic photograph of F-36P (original magnification ×9,800; bar, 1 µm).
As judged from Wright-Giemsa and PAS stains, the F-36 cells seem to have characteristics of immature erythroid cells. Although the host patient from whom F-36 was derived was initially diagnosed with MDS by FAB classification, his disease had many aspects of erythroleukemia as described in Materials and Methods. The morphological similarity with immature erythroid cells was not unexpected.

F-36 has complex chromosomal abnormalities. Among the karyotypic abnormalities of F-36, -5 and -7 are both well documented as an abnormality in MDS. The deletion of chromosome 17 has been infrequently described in MDS.

Fig 1. (Cont’d).

Fig 2. Karyotype of F-36 cells. A major karyotype was 43,Y (Xp+, -5, -7, -13, -16, -17, -19, -21, 2q-, 9p+, 10q+, +4mar).
but may be of interest since the malfunction of the p53 antioncogene located on chromosome 17q has recently been demonstrated in a number of tumors, including some leukemias.²⁸ The difference of karyotype between the primary cells and the cell line could be explained as further divergence, such as additional chromosomal loss (2q-, 13, 16, 19, and -21) or duplication of chromosomes (9p+ and 10q+).

F-36 was found to have some features of a megakaryocytic lineage. Platelet glycoprotein IIbIIIa was detected and PPO was positive, although at a low level. However, the platelet glycoprotein Ib was negative, and we did not obtain any other evidence that the cells are megakaryocytic or differentiate into more mature megakaryocytic cells. Further studies are necessary for detailed characterization of the lineage and differentiating potency of these cells.

Successful establishment of F-36E, a subline of F-36P, makes the F-36 cell line unique. We did not find significant biochemical differences between the two lines; GM-CSF receptors identified by affinity labeling were indistinguishable in F-36P and F-36E. However, F-36E differs from F-36P in that it can grow continuously in the presence of Epo alone. Thus, F-36E must have some mechanism that allows it to proliferate in the presence of Epo alone, an issue that can be clarified by future studies. This may be due to an alteration of Epo receptor, as was recently reported.²⁹

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**Fig 3.** Survival and proliferation of F-36P and F-36E in the presence or absence of hematopoietic growth factors. The results shown are a mean of triplicate data. The experiments were performed 3, 6, and 14 months after the primary culture with similar results.

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**Fig 4.** Responsiveness of F-36 sublines to hematopoietic growth factors. The results are shown as a mean of triplicate data.
or to convergence of a cellular mechanism downstream from the Epo receptor and the receptors for GM-CSF or IL-3 in F-36E. In any case, the establishment of an Epo-dependent continuous human cell line described for the first time here will be an important tool for the study of growth through Epo and its receptor. The two F-36 cell lines may also be useful in the analysis of the relationship of growth signals mediated by GM-CSF, IL-3, and Epo.

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Establishment and erythroid differentiation of a cytokine-dependent human leukemic cell line F-36: a parental line requiring granulocyte-macrophage colony-stimulating factor or interleukin-3, and a subline requiring erythropoietin

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