Establishment and Characterization of an Erythropoietin-Dependent Subline, UT-7/Epo, Derived From Human Leukemia Cell Line, UT-7

By Norio Komatsu, Masayuki Yamamoto, Hiroyoshi Fujita, Akiyoshi Miwa, Kiyohiko Hatake, Toshiyasu Endo, Hiromitsu Okano, Takanori Katsube, Yasuyuki Fukumaki, Shigeru Sassa, and Yasusada Miura

UT-7 is a human leukemic cell line capable of growing in interleukin-3 (IL-3), granulocyte/macrophage colony-stimulating factor (GM-CSF), or erythropoietin (Epo) (Komatsu et al. Cancer Res 51:341, 1991). To study the effect of Epo on proliferation and differentiation of UT-7, we maintained the UT-7 cell culture for more than 6 months in the presence of Epo. As a result, a subline, UT-7/Epo, was established. The growth of UT-7/Epo could be supported by Epo but not by GM-CSF or IL-3. UT-7/Epo showed a greater level of heme content and ratio of benzidine-positive staining cells than did UT-7. Butyric acid promoted the synthesis of hemoglobin in UT-7/Epo, but not UT-7. Further, the mRNA concentrations of the c-myc oncogene and GM-CSF receptor β-subunit were decreased substantially in UT-7/Epo cells. These findings showed that UT-7/Epo cells had progressed further in erythroid development than UT-7 cells, and suggested that long-term culture in Epo had promoted this differentiation. Whereas availability of the Epo receptor (Epo-R) for binding of Epo was reduced in UT-7/Epo cells compared with UT-7 cells, the Epo-R showed a similar affinity for Epo. This observation suggested that change(s) in postreceptor signaling step might be involved in the establishment and maintenance of the UT-7/Epo phenotype.

© 1993 by The American Society of Hematology.

MATERIALS AND METHODS

Reagents. Recombinant human Epo, with a specific activity of 1.7 × 10^11 U/mg, was a gift of the Life Science Research Institute of Snow Brand Milk Products Company (Tochigi, Japan). Recombinant human GM-CSF, with a specific activity of 10^9 U/mg, was provided by Sumitomo Pharmaceutical Company (Osaka, Japan). Recombinant human IL-3, with a specific activity of 10^9 U/mg, was a gift of Genetics Institute (Cambridge, MA). Recombinant human IL-6 was purchased from Genzyme Co (Boston, MA). Butyric acid (BA), hexamethylene-bis-acetamide (HMBA), N’-methylnciccinamide (NMN), and phenol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co (St Louis, MO). Plasmid DNAs for human c-myc cDNA and human ribosomal genomic DNA (5’ portion) were provided by the Japanese Cancer Research Resources Bank. A human Epo-R cDNA was newly cloned from UT-7. Human GATA-1 and GATA-2 cDNAs were kindly pro-
vided by Dr S.H. Orkin.12,13 cDNAs encoding α- and β-subunit of human GM-CSF-R were kindly provided by Dr K. Hayashida. Cell culture. The UT-7 cell line was established from marrow cells obtained from a patient with acute megakaryoblastic leukemia.3 The original UT-7 cells were maintained in liquid culture with Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT) and 1 ng GM-CSF/mL, by replacement of one half of the medium with fresh medium every 3 or 4 days. UT-7/Epo was maintained with Epo 1 U/mL and 10% FCS by replacement of three fourths of the medium every 3 or 4 days.

Colorimetric MTT assay. Cell growth was estimated by a modified colorimetric MTT assay of Mosmann.14 Briefly, 20 μL of a sterilized 5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] solution were added to each culture well containing a final volume of 0.1 mL of cell suspension. After 5 hours of incubation, 100 μL of 10% sodium dodecyl sulfate (SDS) solution was added to the wells and mixed thoroughly. Each culture well of the plate was examined using a microplate reader (model 3550; Bio-Rad Laboratories, Richmond, CA) at a wavelength of 595 nm.

Analysis of cell surface markers by immunofluorescence. Cell surface antigens were detected by immunofluorescence staining with the monoclonal antibodies (MoAbs) P2 (CD41a, specific for platelet glycoprotein [GP] IIb/IIIa) and S22 (CD42b, specific for platelet GP Ib). These MoAbs were purchased from Immunotech (Marseilles, France). Surface marker studies were carried out as follows: UT-7 cells were incubated for 30 minutes at 4°C with the appropriately diluted MoAbs. After washing, the cells were reincubated with fluorescein-labeled goat antimouse IgG for 30 minutes at 4°C. After a second washing, fluorescence analysis was performed using a Becton Dickinson Flow Cytometer (Facstar; Becton Dickinson, Mountain View, CA), using 10,000 cells for each antibody. The proportion of positive cells was determined by comparison with cells that were reacted with the fluorescein isothiocyanate (FITC)-conjugated secondary antibody alone.

Quantitative analysis of platelet factor 4 (PF4) and β-thromboglobulin (β-TG). Enzyme-linked immunosorbent assay (ELISA) kits (Asserachrom PF4 and Asserachrom β-TG; Diagnostica Stago, Marnes-la-Coquette, France) were used for quantitative determinations of PF4 and β-TG concentrations in UT-7 and UT-7/Epo cells as described.3 Briefly, cell lysate and international standards of PF4 and β-TG were incubated in a multiewell ELISA plate coated with anti-human PF4 or anti-human β-TG rabbit antibodies; second antibody conjugated with horseradish peroxidase was added to each well. After completion of the reaction, the substrate solution (0.4 mg/mL orthophenylenediamine and 0.015% H₂O₂) was added. The peroxidase reaction was stopped with sulfuric acid and the absorbency at 490 nm was measured using an immunoplate reader (Immunno-Reader NJ-2,000; InterMed, Tokyo, Japan). The concentrations of PF4 and β-TG in the cell lysates were determined by comparison of results with those obtained with the standards.

RNA preparation and RNA blot hybridization analysis. UT-7 cells (1 × 10⁶ cells/mL) in IMDM supplemented with 10% FCS in the presence or absence of 10 ng PMA/mL were incubated at 37°C for the indicated period. Total cellular RNA was prepared from cells using a guanidinium isothiocyanate procedure described by Chomczynski et al.15 RNA was electrophoresed on an agarose formaldehyde gel, transferred to nylon membranes in 10× standard sodium citrate (SSC), and hybridized to human Epo-R cDNA fragment, c-myb cDNA fragment, bovine β-actin cDNA fragment, human GATA-1 and GATA-2 cDNA fragments, GM-CSF-R α- and β-subunit cDNA fragments, and human ribosomal genomic DNA fragment. Those cDNA fragments were labeled with 32P-αCTP by a random priming method. Filters were autoradiographed using Kodak XAR-5 film (Eastman Kodak, Rochester, NY) with intensifying screens at −70°C.

Fluorometric assay of heme. Suspensions containing 1 × 10⁵ cells were transferred to 6 × 50 mm disposable glass tubes. The tubes were centrifuged at 680g for 5 minutes. Cell pellets were washed once with 500 μL of Eagle’s buffer devoid of Ca²⁺ and Mg²⁺ (pH 7.4). Five hundred microliters of 2 mol/L oxalic acid were then added to the cell pellets. The suspension was mixed, and the mixture was immediately heated for 30 minutes at 100°C. Fluorometric analysis of heme was performed as described previously.16

Characterization of hemoglobin. Hemoglobin fractions were analyzed by isoelectric focusing (IEF) according to the method previously described.17 In brief, samples were prepared for IEF using 0.05% KCN and LKB ampholines with a pH 6 to 9 gradient, and separated on an acrylamide slab gel. After electrophoresis, benzidine staining was performed to detect the peroxidase activity of hemoglobin. To characterize the hemoglobins, adult red blood cells (HbA) and cord blood cells (HbF) were used as controls.

Epo iodination. Labeling of Epo with 125I was done by the solid phase method with the IODO-GEN reagent (1,2,4,6-tetrachloro-3-α,6-diphenylglycouril; Pierce Chemical Co, Rockford, IL) as described previously.18 The specific activity of the labeled Epo was 50 to 100 μCi/ng and there was no loss of biologic activity.

Binding studies with 125I-labeled Epo. UT-7 cells grown in the presence of 1 ng GM-CSF/mL were washed thoroughly with phosphate-buffered saline (PBS) and suspended in 50 μL of IMDM supplemented with 10% FCS. For binding studies, 50 μL of a suspension containing 2 × 10⁵ cells was mixed with 50 μL PBS containing 60 mmol/L HEPES (pH 7.2), 0.3% bovine serum albumin (BSA), 0.6% NaCl, and 125I-Epo with or without a 200-fold excess of unlabeled Epo. After 4 hours of incubation at 15°C, the cells were transferred to a microtube and binding was terminated by sedimenting the cells through a mixture of dibutyl phthalate oil (60 μL; Sigma) and bis(3,5,5-trimethylhexyl) phthalate oil (40 μL; Fluka Chemical, Ronkonkoma, NY) for 1.5 minutes in a microfuge (8,000g). The tube was rapidly frozen at −80°C, and the tip containing the cell pellet was cut off. The radioactive tip was counted on a gamma counter. Specific binding at a given concentration of radioactive Epo was defined as the difference in bound Epo iodide determined in the absence of or with 200-fold excess of unlabeled Epo. Epo iodination was analyzed by isoelectric focusing (IEF) according to the method previously described. Epo data analysis and curve fitting were performed using the computerized “ENZ-FITTER” program (Sigma).

RESULTS

Establishment of UT-7/Epo cell line. To establish growth factor–dependent sublines of UT-7, we cultured UT-7 cells in the presence of IL-3 (10 U/mL), GM-CSF (1 ng/mL), or Epo (1 U/mL) for more than 6 months. As a result, UT-7 cells proliferated continuously in all these culture conditions. We tentatively designated these cells UT-7/IL-3, UT-7/GM-CSF, and UT-7/Epo, respectively. Figure 1 shows the result of the MTT assay of these UT-7 cells in response to these growth factors. Whereas UT-7/IL-3 and UT-7/GM-CSF showed almost the same pattern of response to the growth factors as the original UT-7, UT-7/Epo completely lost its response to both IL-3 and GM-CSF. Therefore, we concluded that UT-7/Epo was a subline of UT-7 whose growth was totally dependent on the presence of Epo. Supporting this conclusion, the growth profile of
UT-7/Epo in liquid culture was significantly different from that of the original UT-7 (Fig 2). UT-7 had a saturation density of 3 to 5 × 10⁶/mL and a doubling time of 36 to 48 hours in log growth phase, whereas UT-7/Epo showed a saturation density of 1 × 10⁶/mL and a doubling time of 18 to 24 hours.

UT-7/Epo has committed more to erythroid lineage but less to megakaryocytic lineage compared with original UT-7. The ratio of the benzidine-positive to benzidine-negative cells was routinely monitored during the course of UT-7/Epo culture to follow erythroid differentiation. The frequency of the benzidine-positive cells increased to 3% to 4% in UT-7/Epo compared with less than 1% in the original UT-7. We previously observed that original UT-7 grew as dispersed cells in suspension, but UT-7/Epo formed aggregates of 10 to 20 cells (Fig 3A). The cytoplasm of the original UT-7 cells was relatively basophilic and the nuclear chromatin was not condensed. In contrast, cytoplasm of UT-7/Epo was stained relatively eosinophilic and chromatin condensation was observed (Fig 3B). These observations suggest that UT-7/Epo cells attained a more mature stage of erythroid differentiation than the original UT-7.

We also examined UT-7/Epo for changes in the expression of megakaryocyte-specific antigens, and also for its response to PMA. As a result, we found that UT-7/Epo expressed lower levels of GPIIIa/IIb and GPIb than original UT-7 (Fig 4). This showed marked contrast to UT-7/IL-3 and UT-7/GM-CSF, in which abundant megakaryocyte-specific antigens were expressed in similar concentration to that of original UT-7 (data not shown). PMA promoted the production of β-TG and PF4 in original UT-7, but not in UT-7/Epo (Table 1). PMA-treated UT-7 cells adhered to plastic dishes and extended pseudopods. Conversely, UT-7/Epo did not show morphologic changes by PMA treatment (Fig 5). These observations indicated that the megakaryocyte differentiation program was repressed in the UT-7/Epo cell line.

BA induced erythroid differentiation of UT-7/Epo but not UT-7. Cell pellets of UT-7/Epo showed slightly red in color upon centrifugation, suggesting the existence of hemoglobin. To confirm this, we quantitated the heme content in UT-7/Epo, as shown in Table 2. The basal level of heme in UT-7/Epo was three times higher than that in original UT-7 cells.

We chose several chemicals known to induce erythroid differentiation of cultured erythroleukemia cell lines and tested whether they could induce hemoglobin synthesis in UT-7/Epo cells. We added BA, dimethylsulfoxide (DMSO), HMBA, and NMN to the culture medium. Of these chemicals, only BA induced an increase in heme content of UT-7/Epo cells. None of the chemicals could induce an increase in the heme level of original UT-7 cells (Table 2).

We next analyzed the types of hemoglobin expressed in UT-7/Epo cells. IEF analysis of the cell lysates showed the synthesis of HbF (α₂γ₂), HbA (α₂β₂), HbX (ε₂α₂), and Hb Bart's (γ₄) in UT-7/Epo cells, but not in UT-7. The BA-treatment of UT-7/Epo cells markedly induced hemoglobin synthesis in the cells without changing the expression pattern of hemoglobin subtypes (Fig 6). UT-7/Epo cells seemed to have acquired an ability to produce large quantities of hemoglobin, an essential character of erythroid lineage.
cells. This observation supported the theory that UT-7/Epo had progressed further in erythroid development than had UT-7.

Expression of Epo-R, c-myb, and GATA-1 in UT-7/Epo. We also examined the expression of Epo-R, c-myb oncogene, and GATA-1 transcription factor in UT-7/Epo cells by using RNA blot hybridization analysis. As shown in Fig 7, the level of Epo-R transcript was decreased in UT-7/Epo cells compared with that in the original UT-7 cells. BA treatment of the UT-7/Epo cells strongly decreased the level of Epo-R mRNA initially; the mRNA level partially recovered by 5 days of treatment (Fig 7) and recovered to almost the basal level by 7 days of the treatment (data not shown). This was a somewhat surprising result because the growth of the UT-7/Epo cell line was totally dependent on the presence of Epo. Therefore, we analyzed Epo-R expression in UT-7/Epo in detail, as will be seen in the next section.

The GATA-1 transcription factor had been reported to be important for transcriptional activation of erythroid, megakaryocytic, and mast cell–specific genes. The original UT-7 cells expressed GATA-1 mRNA as abundantly as UT-7/Epo (Fig 7). This suggested that UT-7 cells had already committed to erythroid and/or megakaryocytic lineages. Steady-state level of the GATA-1 mRNA was markedly decreased by BA treatment of the UT-7/Epo cells, but the level partially recovered by 3 to 5 days of the treatment. This pattern of response was similar to that of the Epo-R mRNA level to BA treatment. In contrast, mRNA level of GATA-2, another member of the GATA transcription factor family, did not show recovery by 3 to 5 days of the treatment. Because a number of GATA consensus sequences (binding sites for GATA transcription factors) have already been found in the regulatory region of the Epo-R gene, this result suggested that Epo-R expression might be...
Table 1. Comparison of PF4 and β-TG Production Between UT-7 and UT-7/Epo

<table>
<thead>
<tr>
<th></th>
<th>PF4 (ng/10⁶ cells)</th>
<th>β-TG (ng/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 d</td>
<td>1.4 ± 0.1</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>5 d</td>
<td>431.4 ± 9.9</td>
<td>465.6 ± 17.2</td>
</tr>
<tr>
<td>10 d</td>
<td>612.8 ± 13.3</td>
<td>637.5 ± 25.2</td>
</tr>
<tr>
<td>UT-7/Epo</td>
<td>1.0 ± 0.4</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>0 d</td>
<td>1.5 ± 0.5</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>10 d</td>
<td>0.7 ± 0.4</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

UT-7 or UT-7/Epo cells were cultured in the presence of PMA (10 ng/mL) for 5 or 10 days, harvested, and prepared for the quantitation of PF4 and β-TG as described in Materials and Methods. Cell viability was greater than 80% over 10 days of exposure to PMA.

regulated by the GATA-1 factor. However, it is not clear from this analysis whether GATA-1 alone can direct cell type-specific expression of the Epo-R gene (see Discussion).

The c-myb mRNA level in UT-7/Epo was far lower than that in original UT-7 cells (Fig 7). BA treatment of UT-7 cells further decreased the expression of c-myb mRNA. This pattern, an initial decrease followed by a gradual increase in the mRNA level in response to BA, was similar to that observed for Epo-R and GATA-1. This observation agreed with the previous report that downregulation of c-myb oncogene expression is an essential event for Epo-induced erythroid differentiation and, therefore, provided another line of evidence supporting the conclusion that UT-7/Epo represents a later stage of erythroid development.

Change in postreceptor signaling events may be involved in the establishment and maintenance of the UT-7/Epo phenotype. We examined UT-7 and UT-7 Epo cells to determine the number of cell-surface Epo receptors, and their affinity for Epo (Table 3). UT-7/Epo had 3,000 Epo-binding sites per cell, whereas UT-7 had 13,000 binding sites per cell. Dissociation constants of Epo binding were 68 pmol/L and 100 pmol/L for UT-7/Epo and UT-7, respectively. This decrease in the concentration of Epo-R in UT-7/Epo cells correlated well with the observed decrease in the level of Epo-R mRNA (Fig 7). These results clearly showed that proliferation of UT-7/Epo was not maintained by any stimuli provided by either an increased number of Epo-R or an increased affinity of the Epo-R to Epo. Therefore, it was suggested that there might be some modifications of postreceptor signaling pathways in UT-7/Epo cells that led the cell to acquire the ability to proliferate in medium containing Epo as the sole growth factor.

UT-7/Epo cells lose their response to GM-CSF and IL-3 because of a decrease of the GM-CSF-β-subunit expression. UT-7/Epo cells lose their response to both GM-CSF and IL-3. Receptors for these growth factors share a common β-subunit and the signals seem to be transduced through a common intracellular pathway. The β-subunit generates high-affinity binding sites through combining α-subunit (low-affinity binding chain). In our preliminary binding analysis, UT-7/Epo was found to have GM-CSF binding sites on the surface of the cells, but number of high-affinity binding sites on UT-7/Epo cells was clearly decreased compared with that on UT-7 cells (data not shown). This suggested that UT-7/Epo might have a defect in a common component of GM-CSF and IL-3 signal...
transduction pathway, perhaps the receptor β-subunit. Therefore, we examined the expression of α- and β-subunits of GM-CSF-R by RNA blot hybridization analysis using cDNA clones encoding receptor α- and β-subunits. The result is shown in Fig 8. Whereas the level of α-subunit in UT-7/Epo cells was almost the same as that in the UT-7 cells, the level of β-subunit in UT-7/Epo was markedly reduced compared with that in UT-7. This suggested that the downregulation of the common β-subunit made UT-7/Epo refractory to both GM-CSF and IL-3 stimulation.

DISCUSSION

To study the effect of Epo on the proliferation and differentiation of UT-7 in long-term culture, we maintained UT-7 cells in the presence of Epo and, after 6 months, a subline designated UT-7/Epo was established. The UT-7/Epo phenotype differed from that of the parent cells (UT-7) in the following respects: (1) UT-7/Epo lost the response to IL-3 and GM-CSF, resulting in complete dependence on Epo for maintenance and growth; (2) it lost the ability to express the megakaryocytic program in response to PMA; and (3) it acquired the ability to respond to BA, resulting in increased hemoglobin synthesis. Among the several human and murine Epo-dependent cell lines, UT-7/Epo cells are unique in that growth can be maintained solely by Epo and in that the cells have a large number of Epo binding sites on the surface. Therefore, taking advantage of this fact, the cell line may serve as a useful system for the bioassay of Epo and modified Epo molecules because MTT reduction paralleled the concentration of Epo in the range of 0.01 to 1 U/mL of Epo (Fig 1).

There are two possible explanations for the derivation of UT-7/Epo from UT-7. One is that a subline that responds exclusively to Epo has spontaneously replaced the parent UT-7 cells as a result of long-term exposure to Epo. The other explanation is that Epo has promoted the erythroid differentiation of UT-7. We note that UT-7 cultured with Epo for 3 months responds to BA and begins to lose its response to GM-CSF and IL-3 (data not shown). A concomitant decrease in the number of Epo-R starts at this stage (5,500/cell, Table 2). These observations suggest that the latter possibility is likely. Epo seems to promote the ery-

Table 2. Heme Content in Chemical-Induced UT-7 and UT-7/Epo

<table>
<thead>
<tr>
<th></th>
<th>Control (medium)</th>
<th>DMSO (1.5%)</th>
<th>HMBA (5 mmol/L)</th>
<th>BA (1.3 mmol/L)</th>
<th>NMN (8 mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT-7</td>
<td>15.0 ± 0.4</td>
<td>11.5 ± 1.5</td>
<td>11.2 ± 1.7</td>
<td>19.9 ± 1.3</td>
<td>14.2 ± 0.2</td>
</tr>
<tr>
<td>UT-7/Epo</td>
<td>40.0 ± 3.4</td>
<td>36.5 ± 1.0</td>
<td>33.2 ± 0.8</td>
<td>121.5 ± 1.1</td>
<td>48.5 ± 0.8</td>
</tr>
</tbody>
</table>

UT-7 or UT-7/Epo cells were incubated with any one of the chemicals for 5 days and prepared for heme content analysis. Values are the heme content (pmol/10^6 cells).
Fig 7. RNA blot hybridization analysis of Epo-R, GATA-1, GATA-2, and c-myb mRNAs in UT-7, UT-7/Epo, and BA-treated UT-7/Epo cells. UT-7/Epo cells were treated with 1.3 mmol/L BA for 1, 3, or 5 days. Cells were harvested and RNA was extracted from each cell sample. Total cellular RNA (20 μg) isolated from UT-7 (lane 1), UT-7/Epo (lane 2), or BA-treated UT-7/Epo (lane 3, 1 day; lane 4, 3 days; lane 5, 5 days) was subjected to agarose/formaldehyde gel electrophoresis and then was hybridized to 32P-labeled Epo-R, GATA-1, GATA-2, or c-myb cDNA probes. 18S ribosomal RNA was hybridized to 32P-labeled human ribosomal DNA probe as described in Materials and Methods.

It is of interest that UT-7/Epo loses its response to both IL-3 and GM-CSF because receptors for these growth factors share a common β subunit. In the present analysis, we found that UT-7/Epo cells contained a much lower level of the β-subunit mRNA than did UT-7 cells (Fig 8). This suggests that UT-7/Epo has lost its ability to respond to GM-CSF and IL-3 because the cells cannot generate high-affinity binding sites for GM-CSF and IL-3. However, further analysis using specific antibody is necessary to clarify the molecular mechanism underlying this phenomenon. Liboi et al recently reported that Epo-induced differentiation of murine erythroid cell lines was accompanied by downregulation of AIC2B, a murine counterpart of the β-subunit of human GM-CSF–R. This observation suggests that the downregulation of the common β-subunit in UT-7/Epo cells is the result of Epo-mediated erythroid development of the UT-7/Epo cells. An alternative explanation for the downregulation of the β-subunit mRNA level is that a point mutation that generates a termination codon makes the mRNA unstable and reduces the steady-state level of the β-subunit mRNA.

UT-7 cells have been cultured for 6 months in the presence of GM-CSF or Epo and tentatively named UT-7/GM-CSF or UT-7/Epo, respectively. In this study, UT-7 sublines were cultured in the absence of hematopoietic growth factors for 12 to 16 hours. Then, each subline was prepared for Epo binding assays and Scatchard analyses were performed. The data shown for each experiment represent the mean value ± SE from duplicate cultures.

* Original UT-7 cells were newly thawed and prepared for Epo binding assays.
† The original UT-7 cells were continuously cultured with Epo (1 U/mL) for 3 months and prepared for Epo binding assays.

mRNA unstable and reduces the steady-state level of the β-subunit mRNA.

It is noteworthy that UT-7/Epo loses its response to PMA and acquires erythroid properties on long-term exposure to

Table 3. Binding of 125I-Epo to UT-7 Sublines

<table>
<thead>
<tr>
<th>UT-7</th>
<th>Experiment No.</th>
<th>No. of Binding Sites/Cell</th>
<th>Dissociation Constant (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original*</td>
<td>1</td>
<td>11,879 ± 217</td>
<td>112 ± 8</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>13,761 ± 318</td>
<td>83 ± 8</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>12,820</td>
<td>97</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>1</td>
<td>10,764 ± 263</td>
<td>130 ± 1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10,757 ± 156</td>
<td>97 ± 6</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>10,761</td>
<td>113</td>
</tr>
<tr>
<td>Original Epo</td>
<td>1</td>
<td>4,795 ± 56</td>
<td>122 ± 54</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6,184 ± 86</td>
<td>94 ± 9</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>5,490</td>
<td>108</td>
</tr>
<tr>
<td>Epo</td>
<td>1</td>
<td>2,392 ± 26</td>
<td>66 ± 3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4,240 ± 92</td>
<td>65 ± 6</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>3,316</td>
<td>66</td>
</tr>
</tbody>
</table>

Fig 8. RNA blot hybridization analysis of α- and β-subunit mRNAs of GM-CSF-R in UT-7 and UT-7/Epo cells. RNA blot hybridization analysis was performed as described in Fig 7. As a standard, 18S ribosomal RNA was probed with 32P-labeled human ribosomal DNA.
Epo. Because the importance of protein kinase C (PKC) activity in the Epo signal transduction pathway has been reported,30 Epo-induced erythroid differentiation of UT-7 should be associated with the activation of PKC. On the other hand, PMA, an activator of PKC, can induce UT-7 but not UT-7/Epo to differentiate into mature megakaryocytes. Therefore, the activation of PKC may also be essential for the megakaryocytic differentiation of UT-7. Because PKC is reported to have isoforms that have distinct tissue distribution, localization, and requirements for activation,31 the PKC isoform activated by Epo may be different from that activated by PMA. Although we do not have experimental evidence, we speculate that different isoforms of PKC may be involved in erythroid versus megakaryocytic differentiation.

We find that expression of GATA-1 and Epo-R mRNAs is closely coordinated during BA-induced erythroid differentiation of UT-7/Epo. This result suggests that GATA-1 may have an important role in the regulation of Epo-R gene expression. GATA sequences have been found in the regulatory region of both murine and human Epo-R gene, and Epo has been reported to induce GATA-1 mRNA level in murine erythroid cell lines.32,33 However, changes in Epo-R mRNA expression are not fully explained by the changes in GATA-1 expression, because the levels of GATA-1 expression in UT-7 are almost the same as UT-7/Epo, although Epo-R mRNA is expressed at a much higher level in UT-7 than in UT-7/Epo. Heberlein et al.34 recently reported that a correlation between Epo-R and GATA-1 gene expression could not be found in embryonal stem cells. We find that Epo downregulates Epo-R gene transcription without changes in GATA-1 mRNA concentration (unpublished observation). These results imply an additional regulatory mechanism(s) for Epo-R transcription. The binding of GATA-1 to the GATA consensus sequence of the Epo-R gene may be affected by other transcription factors including myb, ets, myc, Sp1, and GATA-2. These oncogenes and transcription factors are suggested to be important for erythroleukemia cell differentiation,35-37 and homologies to the consensus binding motifs for these factors are found in the Epo-R gene region.35,36 It is of interest to study the relationship between Epo-R and these transcription factors using UT-7 and UT-7/Epo.

The present study suggests that there might be a close relationship between megakaryopoiesis and erythropoiesis. This is consistent with previous observations that a specific class of progenitor has the capacity to give rise to both megakaryocytic and erythroid progeny.38 This idea raises a question as to the regulation of expression of these two differentiation programs. At least two transcription factors, GATA-1 and GATA-2, are candidates for the lineage determinant transcription factors, because these factors exist in both megakaryocytic and erythroidic cells. GATA binding motifs are found in the promoter regions of megakaryocytic-specific genes such as GP IIB and platelet factor-4, as well as in erythroblast-specific genes, such as Epo-R and globin. GATA factors are probably necessary for the activation of both erythrocyte- and megakaryocyte-specific genes. However, the Epo-R gene is downregulated, and the PF-4 and β-TG genes are upregulated during megakaryocytic maturation.32,33 Thus, the on/off switching mechanism of gene expression during hematopoietic cell differentiation remains to be clarified. The UT-7 and UT-7/Epo cell lines, which mimic the differentiation patterns of erythroid and megakaryocyte progenitors, could serve as model systems for resolving these problems.

ACKNOWLEDGMENT

The helpful discussions and critical comments of Dr John W. Adamson (The New York Blood Center) are gratefully acknowledged. We thank Dr. Stuart H. Orkin for his generous gift of the human GATA-1 and GATA-2 cDNAs, Dr Kazuhiro Hayashida for cDNAs encoding α- and β-subunits of human GM-CSF-R, and the Japanese Cancer Resources Center for c-myb and ribosomal gene. We also thank Dr. Seiji Okada and Yukie Urabe for their technical assistance, Dr. Susan J. Stamler for advice, and Motoko Yoshida for the manuscript preparation.

REFERENCES

12. Zon Li, Tsai S-F, Burgess S, Matsuda I, Bruns GAP, Orkin SH: The major human erythroid DNA-binding protein (GF-1);
Primary sequence and localization of the gene to the X chromosome. Proc Natl Acad Sci USA 87:668, 1990
Establishment and characterization of an erythropoietin-dependent subline, UT-7/Epo, derived from human leukemia cell line, UT-7

N Komatsu, M Yamamoto, H Fujita, A Miwa, K Hatake, T Endo, H Okano, T Katsube, Y Fukumaki and S Sassa