Induced Megakaryocytic Maturation of the Human Leukemia Cell Line UT-7 Results in Down-Modulation of Erythropoietin Receptor Gene Expression

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ABSTRACT

Erythropoietin (Epo) affects not only erythocyte production but, in vitro, also promotes megakaryocyte maturation. However, the mechanism of action of Epo on megakaryocytic development remains to be determined. Recently, we reported the establishment of a human Epo-dependent megakaryoblastic leukemia cell line UT-7. Exposure of UT-7 to the tumor promoter, phorbol myristate acetate (PMA), resulted in the appearance of mature megakaryocytic properties, including the expression of platelet factor 4 and β-thromboglobulin. With exposure to PMA, however, UT-7 cells lost their responsiveness to Epo and Scatchard analysis showed an 85% decrease in the number of Epo receptors after 24 h. While the number of binding sites declined, the affinity of Epo binding was unchanged. Associated with the decrease in the number of Epo receptors was a profound decrease (>95%) in the level of Epo receptor (Epo-R) mRNA. To determine the level of regulation of the Epo-R gene, its rate of transcription was measured by nuclear run-off assay in untreated cells and in cells exposed to PMA for 6, 12, and 24 h. The rate of transcription was nearly identical at all time points in control and PMA-treated cells. Stability of Epo-R mRNA also was measured in the presence of actinomycin D, an inhibitor of transcription. The half-life of Epo-R mRNA in untreated and PMA-treated cells was 90 and 30 min, respectively. These results indicate that the down-modulation of the expression of the Epo-R gene is mainly caused by increased instability of mature mRNA of Epo-R. Post-transcriptional regulation may be an important mechanism in the regulation of hematopoietic growth factor receptor genes and one of the mechanisms by which lineage restriction is achieved.

INTRODUCTION

Epo² is the major regulator of erythroid proliferation and differentiation. However, both clinical and in vitro data suggest that red cell and platelet production are related and that the action of Epo may explain, at least in part, this relationship (1–5). In vitro, Epo promotes the differentiation of megakaryocytic progenitors and the maturation of isolated human or murine megakaryocytes under highly defined conditions (6–8). These findings imply that megakaryocytic progenitors, perhaps, and certainly megakaryocytes, themselves, have functional Epo receptors. This is supported by the observation that hematopoietic-restricted transcription factor GATA-1, which directly transactivates the Epo-R promotor (9) is also present in megakaryocytic cells (10, 11). Therefore, as suggested by other data which indicate a specific class of progenitor that has the capacity to give rise to both megakaryocytic and erythroid progeny (12), there would be a close relationship between megakaryopoesis and erythropoiesis. This idea raises the question as to the regulation of the two differentiation programs. However, studies of the interplay between two differentiation pathways are difficult since progenitors specific for both lineages cannot be identified precisely and thus isolated from normal human bone marrow.

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Recent studies suggest that the anti-convulsant drug valproic acid (VPA) may be a therapeutic agent for certain types of drug-resistant epilepsy. In this study, we investigated the effect of VPA on the expression of the Epo receptor gene in human blood cells. Bone marrow cells from patients with different types of leukemia were cultured in the presence of VPA and analyzed for Epo receptor expression by quantitative real-time PCR. The results showed that VPA increased the expression of the Epo receptor gene in all types of leukemia cells tested, with the exception of chronic myelogenous leukemia. Furthermore, the expression of other hematopoietic growth factor receptors, such as granulocyte colony-stimulating factor (G-CSF) and granulocyte/macrophage colony-stimulating factor (GM-CSF), was also increased by VPA treatment. These findings suggest that VPA may modulate hematopoiesis through the regulation of hematopoietic growth factor receptor gene expression.

MATERIALS AND METHODS

Cell Culture. UT-7 cell line was established from marrow cells obtained from a patient with acute megakaryoblastic leukemia (13). The cells were initially grown in the presence of GM-CSF (1 ng/ml). Over several weeks, the cells began to proliferate in the presence of GM-CSF and continued to increase in numbers in a liquid culture system with IMDM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FCS (HyClone Laboratories, Logan, UT). UT-7 was cloned from the original suspension cultures. Currently, the cloned cells have been cultured for more than 18 months in the presence of GM-CSF without morphological changes. UT-7 was subsequently shown to respond to recombinant human Epo and interleukin 3. In published studies, UT-7 also responded to PMA by expressing high levels of the megakaryocyte-specific proteins, platelet factor 4 and β-thromboglobulin (13).

Colony-forming Assay. The colony assay was carried out according to a modification of the method of Fauser and Messner (14). In brief, UT-7 cells were suspended in medium containing 0.9% methylcellulose (Dow Chemical Co., Midland, MI), 30% PCS, 5 × 10⁻⁵ M 2-mercaptoethanol (Eastman Organic Chemical, Rochester, NY), 1% bovine serum albumin (Sigma Chemical, St. Louis, MO), and 1 unit Epo/mI. One-ml aliquots of culture medium containing 250 or 500 UT-7 cells were cultured in 35-mm non-tissue culture dishes (Falcon, Oxnard, CA) at 37°C in a 5% air-5% CO₂ humidified tissue culture incubator. The plates were incubated for 10 days and colonies of >50 cells were counted with the use of an inverted microscope.

Reagents. Human recombinant Epo, with a specific activity of 1.7 × 10⁷ units/mg, was a gift of Snow Brand Milk Products Co. (Tochigi, Japan). Human recombinant GM-CSF, with a specific activity of 10 × 10⁵ units/mg, was kindly provided by Sumitomo Pharmaceutical Co. (Osaka, Japan). One mg PMA/ml was stored at −80°C in solution with dimethyl sulfoxide, was diluted in the IMDM culture medium just before use, and then was added to the liquid culture system.

Epo i.d. Inmation. Labeling of Epo with 125I was done by the solid phase method with the Iodo-Gen reagent (1,3,5,7-tetrachloro-3a,6a-diphenylglycouril; Pierce Chemical, Rockford, IL) as described previously (15). The specific activity of the labeled Epo obtained was 50–100 μCi/μg without loss of biological activity (data not shown).

Binding Studies with 125I-Epo. UT-7 cells grown in the presence of 1 ng GM-CSF/ml were washed with phosphate-buffered saline and suspended in 50 μl of IMDM supplemented with 10% FCS. For binding studies, a 50-μl cell suspension containing 2–5 × 10⁵ cells was mixed with 50 μl phosphate-buffered saline containing 60 mg 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2, 0.3% bovine serum albumin, 0.6% NaCl, and 125I-Epo with or without a 200-fold excess of unlabeled Epo. After 4 h 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 min in a microfuge (8000 × g). The tube was rapidly frozen at −80°C, and the tip containing the cell pellet was cut off. The radioactivity of the tip was counted on a gamma counter. Specific binding at a given concentration of radioactive Epo was defined as the difference in bound radioactivity between samples incubated in the absence of unlabeled Epo or in the presence of a 200-fold excess of unlabeled Epo. Scatchard analysis of Epo equilibrium binding data was performed as described (16). Epo data analysis and curve fitting were performed by using the computerized ENZFITTER program (Sigma).

**Results**

**Effect of Epo on Colony Formation by UT-7.** In the initial experiments, exposure of UT-7 to increasing concentrations of Epo resulted in increasing colony growth with the maximal response seen at 0.3–1.0 unit Epo/ml (Fig. 1A). Cloning efficiency was between 15 and 20% and no colonies formed in these conditions in the absence of added growth factor, in this case Epo. In the presence of 1.0 unit Epo/ml culture and increasing concentrations of PMA, however, UT-7 colony formation was progressively inhibited with 90% inhibition seen at a PMA concentration of 1 ng/ml (Fig. 1B).

**Effect of PMA on Expression of Epo-R in UT-7 Cells.** Fig. 2A shows the time course of changes induced by PMA in response to Epo of UT-7 cells. In these experiments, UT-7 cells were cultured in the presence of 10 ng PMA/ml for the times indicated. At each time point, the cells were recovered from culture and washed 3 times with IMDM. Cell viability remained at nearly 100% over 24 h of exposure to PMA (data not shown). The cells were then cultured in semisolid medium in the presence of 1.0 unit Epo/ml. As can be seen, there was a clear time-dependent effect of PMA on the subsequent ability of the cells to respond to Epo. These findings indicate that, in the PMA-induced process of megakaryocytic differentiation, the response to Epo is lost. In order to determine if this loss of Epo responsiveness was mediated by alterations in Epo-R expression, we performed Epo-binding assays on PMA-treated cells. As shown in Fig. 2B, exposure to PMA rapidly reduced the number of Epo-binding sites. This paralleled the kinetics of changes in the proliferative response to Epo. Scatchard analysis revealed that the number of Epo-binding sites per cell was approximately 13,000 in untreated UT-7 cells while, after 6 and 24 h of exposure to PMA, the numbers of Epo-binding sites were 6,000 and 2,100, respectively. The dissociation constants of Epo binding remained unchanged (Table 1; Fig. 3).

**Mechanism of Regulation of Epo-R Gene Expression during PMA-induced Megakaryocytic Differentiation.** The time course of expression of stable transcripts was determined by Northern blot analysis of UT-7 RNA isolated at different times after induction with PMA. The reduction of 2.2 kilobases Epo-R mRNA was found to be an early event in the differentiation process (Fig. 4). Within 3 h after PMA induction, stable Epo-R mRNA was slightly decreased, and there was a progressive decline in Epo-R mRNA with only a minute amount detectable after 12 h. Conversely, GM-CSF transcripts were clearly detected after exposure of UT-7 cells to PMA, as previously reported (22). This phenomenon was transient and, after 24 h, GM-CSF mRNA completely disappeared (22).
Fig. 2. (A) The effect of PMA on the response of UT-7 cells to Epo. UT-7 cells were cultured at an initial density of 10^5/ml with 10 ng PMA. At the indicated times, cells were harvested and washed with medium. These cells were then plated in a colony-forming assay in the presence of 1 unit Epo. Colonies were counted after 10 days of culture. The results are the mean ± SE from triplicate cultures containing 500 cells. (B) Time course of PMA-induced down-modulation of Epo receptors on UT-7 cells. UT-7 cells were cultured at an initial density of 10^6/ml with 10 ng PMA. At the indicated times, cells were harvested and prepared for binding assay as described in "Materials and Methods."

Table 1 Binding of 125I-Epo to UT-7 cells

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Experiment</th>
<th>No. of binding sites/cell</th>
<th>Dissociation constant (pm)</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>1</td>
<td>13,761 ± 318</td>
<td>83 ± 8</td>
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<tr>
<td></td>
<td>2</td>
<td>11,879 ± 217</td>
<td>112 ± 8</td>
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<td>12,820</td>
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<td>6,302 ± 86</td>
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<td></td>
<td>2,146</td>
<td>96</td>
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</table>

To determine whether the reduction of the Epo-R mRNA level was regulated at the transcriptional level, nuclear run-off transcription assays were performed by using nuclei isolated from untreated UT-7 cells or UT-7 cells treated with 10 ng PMA/ml for 6, 12, and 24 h (Fig. 5). Equal numbers of incorporated counts were hybridized to filters containing an excess of cDNAs for Epo-R and β-actin, as well as the plasmid DNA as a control for nonspecific hybridization. Moreover, in order to confirm that UT-7 cells had responded appropriately to PMA, we also checked the expression of GM-CSF as a positive control. Two independent experiments showed that the rate of Epo-R transcription did not change from its levels in uninduced cells. This observation suggested that, during the process of PMA-induced megakaryocytic differentiation, Epo-R expression is not regulated at the level of initiation plus elongation but, rather, by a posttranscriptional mechanism, possibly affecting the half-life of the transcripts in the cytoplasm. On the other hand, it was found that GM-CSF gene expression was regulated at the transcriptional level.

Consequently, we examined the effect of PMA on the half-life of cytoplasmic Epo-R mRNA in UT-7 cells with the use of the transcriptional inhibitor, actinomycin D. As shown in Fig. 6, since the level of Epo-R mRNA began to decrease after exposure to PMA for 2 h, cells were pretreated with PMA for 2 h and exposed to actinomycin D for various times. Total RNA was extracted at each time point and probed with human Epo-R cDNA. There was a big difference in the relative decrease in Epo-R mRNA concentration upon exposure of the cells to PMA. From the observed decline, we estimated the average half-life of the Epo-R mRNA in intact cells and cells treated with PMA to be 90 and 30 min, respectively. This suggests that PMA has affected the stability of Epo-R mRNA.

Fig. 3. The effect of PMA on the binding of 125I-Epo to UT-7 cells. After the exposure to PMA (10 ng/ml) for 0 (●), 6 (○), and 24 (△) h, UT-7 cells were harvested and incubated with increasing concentrations of 125I-Epo at 15°C for 4 h. (A) Specific binding curves. Specific binding was determined by subtracting the binding in the presence of a 200-fold excess of unlabeled Epo. Nonspecific binding was less than 10% of total binding. (B) Scatchard plot of the specific binding data. Each point shows the mean obtained from triplicate dishes in one representative of two separate experiments described in Table 1.
cells were harvested at the indicated times and total RNA was isolated. Thirty ug RNA (per on the right ordinate kb, kilobase.

Fig. 4. The effect of PMA on the expression of Epo-R mRNA and GM-CSF mRNA in UT-7 cells. UT-7 cells were cultured at an initial density of 10^5/ml with 10 ng PMA. The cells were harvested at the indicated times and total RNA was isolated. Thirty ug RNA (per sample) from each fraction were separated by electrophoresis, transferred to the membrane, and hybridized to the ^32P-labeled Epo-R cDNA, GM-CSF cDNA, or β-actin cDNA as described in “Materials and Methods.” The calculated sizes of the transcripts are shown on the right ordinate kb, kilobase.

Fig. 5. Nuclear run-off transcription analysis. Nuclei were isolated from untreated UT-7 cells and UT-7 cells treated with 10 ng PMA/ml for 0, 6, 12, and 24 h, and in vitro transcription was completed in the presence of [α-^32P]UTP as described in “Materials and Methods.” Radiolabeled RNA was isolated and equal numbers of counts were hybridized to filters containing the indicated probes.

Effect of Protein Synthesis Inhibition on PMA-induced Down-Modulation of Epo-R mRNA. In order to examine the role of labile proteins in the down-modulation of Epo-R mRNA, experiments were done using the protein synthesis inhibitor, cycloheximide. UT-7 cells were pretreated with 10 μg cycloheximide/ml for 2 h. After exposure of UT-7 cells to 10 ng PMA/ml for 0, 6, and 9 h, total RNA was harvested at each time point and probed with Epo-R cDNA. As shown in Fig. 7, cycloheximide diminished the PMA-induced down-modulation of Epo-R mRNA, suggesting that protein synthesis is required for any one of posttranscriptional processing of Epo-R transcript.

Discussion
Although the main action of Epo is on the proliferation and differentiation of erythroid cells, Epo modulates megakaryocytic proliferation and differentiation, at least in vitro. The mechanisms by which Epo affects megakaryocytic proliferation and the relationship of Epo responsiveness to terminal megakaryocytic maturation have not been detailed. To investigate these issues, we have examined the regulation of Epo-R in UT-7 cells which show Epo-dependent growth and which can differentiate into cells with properties of mature megakaryocytes in response to PMA (13).

In this study, we have shown that exposure of UT-7 cells to PMA for a period of time results in a progressive loss of the ability of the cells to respond to Epo. This alteration in response to Epo was marked by a parallel reduction in the number of functional Epo receptors on the surface of the cells. The reduced Epo receptor number was associated with a decrease in Epo-R mRNA levels. However, initiation of transcription of the Epo-R gene was not suppressed but, rather, gene expression was altered by posttranscriptional processing of mRNA within the cytoplasm. As a result of these effects, PMA-treated UT-7 cells lost their responsiveness to Epo. Gene regulation can occur at the transcriptional and/or posttranscriptional level. In some cases, gene expression is regulated posttranscriptionally by alterations in the stability of mRNA during differentiation (23–27). In a few cases, nuclear posttranscriptional regulation may be involved in transcript stability (26, 27). As a characteristic feature of eukaryotic cells, pre-mRNAs, the primary transcription products of their structural genes, undergo a variety of posttranscriptional modifications which result in the functionally mature mRNA. These processes include splicing, generation of a 5′-terminal cap structure, and polyadenylation. Since the decrease in Epo-R mRNA half-life may not completely account for the entire down-regulation of Epo-R mRNA induced by PMA, interference with any one of these processes could also contribute to a decrease in the stability of Epo-R mRNA.

The posttranscriptional mechanism responsible for regulating Epo-R gene expression appears to involve the synthesis of one or more labile proteins. This conclusion is based on the observation that cycloheximide partially reversed the decrease in Epo-R transcripts induced by PMA treatment. Alternatively, synthesis of a protein in-
Reduced by PMA could have resulted in the degradation of Epo-R transcripts. It is likely that protein-mediated transcript degradation is a relatively common mechanism for the regulation of gene expression in eukaryotic cells. As an example, the 3'-untranslated region of GM-CSF mRNA contains AUUUA sequences which are involved in message stability (28). Similar to the results presented here, treatment of GM-CSF-producing cells with cycloheximide resulted in reduced GM-CSF mRNA stability, suggesting that a labile protein must have been involved in the degradation of GM-CSF mRNA. There is no evidence to suggest that the AUUUA motif exists in the 3'-untranslated region of Epo-R mRNA (29, 30). However, it is possible that additional sequences other than the AUUUA motif are involved in the recognition and regulation of Epo-R mRNA stability. Identification of the labile protein(s) induced by PMA treatment would be helpful in understanding the regulation of Epo-R gene expression.

There are several other possible mechanisms to account for the down-modulation of Epo receptors. PMA-activated protein kinase C could have modified the binding activity of Epo receptors. For example, PMA-induced protein kinase C-mediated phosphorylation of the epidermal growth factor receptor and the insulin receptor results in a decrease in the activities of these receptors through internalization (31, 32). However, internalization of these receptors occurred within 30 min of PMA treatment, while the decrease in the number of Epo receptors on UT-7 cells was seen after 3 h of exposure to PMA. Therefore, the down-modulation of Epo receptor expression is not likely to be associated with receptor internalization. However, as with the epidermal growth factor receptor and insulin receptors, the activation of protein kinase C by PMA could modulate Epo receptor function since the Epo receptor has two candidate sites for protein kinase C-mediated phosphorylation within its intracytoplasmic domain (serines 273 and 335). Whether protein kinase C-mediated phosphorylation is involved in PMA-induced down-modulation of Epo receptor numbers and Epo-R mRNA in UT-7 cells remains to be determined.

We previously reported that PMA induced UT-7 cells to produce GM-CSF and this regulation occurred at the mRNA level (22). In this study, we also demonstrated that expression of GM-CSF mRNA induced by PMA was regulated at the transcriptional level. In order to rule out the possibility that the GM-CSF expression was involved in down-modulation of Epo-R mRNA induced by PMA, we checked Epo-R transcripts in the samples prepared from the cells treated with GM-CSF (1–100 ng/ml) for 12 h. No differences in Epo-R mRNA levels were found (data not shown). Therefore, we conclude that the PMA-induced down-modulation of Epo receptors could not be attributed to the effect of GM-CSF.

In our experiment, although short term exposure (<24 h) to PMA induced the down-modulation of Epo-R mRNA, after 5–7 days of exposure to PMA, Epo-R mRNA recovered to the basal level (data not shown). Therefore, the expression of Epo-R mRNA may be regulated at two levels in the process of megakaryocytic differentiation. This may account for the previous report that the number of Epo-binding sites increases with megakaryocyte maturation at a late stage (33). However, the biological significance of the increasing Epo-R expression in mature megakaryocytes remains unclear.

Finally, posttranscriptional processing of mRNA is an important mechanism for the regulation of gene expression. Our results provide evidence concerning the mechanism by which at least one hematopoietic growth factor receptor, that for Epo, may be regulated during differentiation, and provides a model by which lineage-restricted growth factor responsiveness is achieved.

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**REFERENCES**


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