Differentiation and Erythropoietin Receptor Gene Expression in Human Erythroid Progenitor Cells

Amittha Wickrema, Sanford B. Krantz, John C. Winkelmann, and Maurice C. Bondurant

Proliferation and differentiation of mammalian erythroid progenitors into red blood cells are controlled by the hormone erythropoietin (EPO). In recent years, both murine and human model systems have been developed using purified erythroid progenitors to study molecular events occurring during cellular differentiation mediated by EPO. In one murine model, Friend virus-infected spleen cells (FVA) have been used to obtain a highly pure population of erythroid progenitors that are at the colony-forming unit-erythroid (CFU-E) and cluster-forming unit-erythroid stages of development. In a human model, peripheral blood from normal adult donors has been used to obtain partially purified erythroid progenitors that are at a much earlier stage of development, and these cells, termed burst-forming units-erythroid (BFU-E), have been cultured for 7 days and further purified to obtain relatively pure CFU-E. Using this system, Sawada et al have defined the effects of various hormones and growth factors on human CFU-E colony formation when grown in a semisolid medium.

Specific receptors for EPO have been shown to be present on both murine and human cells of hematopoietic origin. Both high- and low-affinity receptors have been shown to be present in FVA cells, CFU-E derived from fetal mouse liver, human blood BFU-E-derived CFU-E, and several hematopoietic cell lines, whereas other murine cell lines, such as the Friend murine erythroleukemia cells (MEL), B6SutA cells, and DA-1 cells, have a single class of receptors. In addition, a separate study using purified human primary erythroblasts from adult and fetal sources have shown the presence of only the high-affinity receptors on the cells. The binding studies performed on human CFU-E showed a steady decline in the amount of EPO binding with increased maturation of the cells. Recently, both mouse and human EPO receptor cDNAs have been isolated and analyzed. The mouse and human EPO receptors have a great similarity (82%) in their deduced amino acid sequences. The deduced polypeptide for the human EPO receptor has 508 amino acid residues and contains the Trp-Ser-X-Trp-Ser motif and the four cysteine residues in the extracellular domain of the primary structure. This polypeptide, along with its mouse counterpart, belongs to an expanding family of cytokine receptors that include several interleukin receptors as well as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-CSF (G-CSF), growth hormone, and prolactin receptors.

The abundance and stability of EPO receptor messenger RNA (mRNA) in mouse FVA cells have been determined and the relatively low number of 25 copies per cell that was observed is in agreement with the low receptor numbers (800 to 1,000) detected in the same cells. In this study, we have used a human EPO receptor cDNA clone as a specific probe to detect the levels of EPO receptor mRNA, and its half-life in differentiating human CFU-E derived from peripheral blood BFU-E. Although an absolute requirement of EPO for human CFU-E has been reported, the timing of various macromolecular events in these cells has not been studied extensively in vitro due to the difficulty in performing experiments on semisolid culture medium. In this study, we have developed an in vitro liquid culture system instead of the traditional semisolid medium to

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analyze the disappearance of EPO requirement in human erythroblasts in addition to determining the period of heme synthesis. Also, in the liquid medium we have been able to correlate the changing morphology of the differentiating erythroid progenitors with the expression of β-globin and the EPO receptor gene in addition to the stabilities of their mRNAs. Finally, the liquid culture system supports the cells well throughout the enucleation process, which we have examined in terms of its timing and extent.

**MATERIALS AND METHODS**

**Materials.** Human recombinant EPO (rEPO) was a gift from Ortho Pharmaceutical (Raritan, NJ). 59Fe, ferric chloride in 0.5 HCl, was purchased from Dupont de Nemours & Co (Wilmington, DE). Human transferrin was purchased from Calbiochem (La Jolla, CA). Nytran blotting membranes were purchased from Schleicher & Schuell (Keene, NH). Mouse α-actin clone PAM91 was a gift from Michael J. Getz (Mayo Foundation, Rochester, MN). CD45/MY-11 and CD16/MY23 monoclonal antibodies (MoAbs) were a gift of Curt Civin (Johns Hopkins School of Medicine, Baltimore, MD). The plasmid containing the mouse β-major globin gene was provided by James Darnell (Rockefeller University, New York, NY). 125I-EPO was a gift from Stephen Sawyer (Vanderbilt University, Nashville, TN).

**CFU-E purification and cell culture.** Peripheral blood was collected from normal adult volunteers after informed consent approved by the Vanderbilt University and Veterans Affairs Medical Center institutional review boards. Purification and culture of CFU-E were performed as previously described with some modifications. Briefly, 400 mL of whole blood was collected in sodium heparin (20 U/mL). Light-density mononuclear peripheral blood cells were separated by centrifugation over Ficoll-Hypaque (1.077 g/mL). The interface mononuclear cells were collected and washed twice with a minimum essential medium (α-MEM) and resuspended in 50% Iscove’s modified Dulbecco’s medium (IMDM)/50% α-MEM. These cells were then incubated with sheep erythrocyte cytoskeleton antibodies and nonsensitized cells were separated from rosette-forming cells by centrifugation over Ficoll-Hypaque. Cells were washed twice with α-MEM followed by an overnight incubation in polystyrene flasks at 37°C in a 5% CO2 incubator with 20% fetal calf serum (FCS) and 10% giant cell tumor-conditioned medium (GIBCO, Grand Island, NY) to deplete monocytes. Nonadherent cells were incubated at 4°C for 1 hour in a mixture of four MoAbs: CD11b/OKM1 plus CD2/OKT11 (Ortho) and CD45/MY11 plus CD16/MY23 to coat granulocytes, monocytes, CPU-granulocyte-macrophage, T and B lymphocytes, and natural killer cells. Cells were washed twice with cold α-MEM and incubated in polystyrene culture dishes that had been coated with affinity-purified goat anti-murine IgG at 4°C for 90 minutes (Boehringer Mannheim Biochemicals, Indianapolis, IN). Nonadherent cells were centrifuged, and resuspended in 20% FCS and pipetted into a polystyrene tissue culture flask for incubation at 37°C for 30 to 60 minutes. These cells were cultured at a concentration of 2 x 10^6 cells/mL in 30% FCS, 10% bovine serum albumin (BSA), 2 U/mL rEPO, 50 U/mL interleukin-3 (IL-3), 10 μg/mL insulin, 5 x 10^-5 mol/L β-mercaptoethanol, 500 U/mL penicillin, 40 μg/mL streptomycin, and 0.9% methylcellulose. On day 6 or 8 (day 0 being the day blood was collected from donors), cells were collected and further purified by adherence to plastic tissue culture flasks and Ficoll-Hypaque density centrifugation as previously reported. Purified CFU-E (the day 6 or day 8 cells described above) were grown in liquid cultures at 37°C in humidified air plus 5% CO2. The liquid culture medium contained 15% FCS, 15% human AB serum, 1% human serum albumin (HSA), IMDM, 1 U/mL rEPO, 10 U/mL insulin, 500 U/mL penicillin, and 40 μg/mL streptomycin. In the experiments in which cells were in liquid culture for more than 3 days, cells were recovered by centrifugation and recultured with fresh medium on day 3 of incubation. All the experiments were performed using the cells plated in liquid medium at a concentration of 0.5 to 1.0 x 10^6 cells/mL. Initial cell purity was assessed by cytocentrifugation of cells onto microscope slides, followed by staining with 3,3'-dimethoxybenzidine and hematoxylin. Greater than 80% of the cells had morphology characteristic of erythroid precursors. In the experiments in which the half-lives of mRNAs were determined, 5 μg/mL of actinomycin D was added to the cell cultures. The lag period between addition of actinomycin D to the culture and the initiation of transcriptional inhibition was determined by incubating cells with and without actinomycin D in the presence of 2.5 μCi of [3H]-uridine (37.5 Ci/mmol). The cells were tested for inhibition of [3H]-uridine incorporation into RNA by scintillation counting the trichloroacetic acid precipitable aliquots taken at 5-minute intervals.

**Cell morphology and enucleation.** Day 6 cells that were cultured in liquid suspension media were removed between days 7 and 14 and cytospin onto glass slides before staining with 3,3'-dimethoxybenzidine and hematoxylin for viewing by light microscopy. Likewise, in experiments performed to determine the extent of enucleation in the differentiating CFU-E, 2 x 10^6 cells were cytospin onto glass slides and stained with 3,3'-dimethoxybenzidine and hematoxylin before viewing by light microscopy. The percentage of enucleated cells on each day was assessed by determining the number of bare nuclei and reticulocytes present when a total of 300 nuclei (cellular nuclei plus bare nuclei) were counted in the same microscope fields using an oil immersion lens.

**59Fe incorporation into heme and cell proliferation.** Kinetics of heme synthesis during the day 6 through 14 time period was monitored by measuring the rate of 59Fe incorporation into protoporphyrin to yield heme, which is predominantly from hemo-globin (Hb), as previously described by Koury et al. Briefly, day 6 cells cultured in 0.5-mL aliquots with and without EPO were pulsed with 50 μL of a 2.4 mg/mL solution of human transferrin containing 0.5 μCi of 59FeCl3 (specific activity, 44 Ci/mg). After a 3-hour pulse, cells were collected, washed with IMDM, and centrifuged at 600g. The cell pellets were lysed in Drabkin’s solution and were acidified with HCl, followed by extraction with diethyl ether. The heme fraction was extracted with ether and this was measured by determining the 59Fe counts in a gamma counter. A similar experiment was performed to determine the effect of EPO concentration on erythroblasts proliferation in liquid media by plating day 6 cells with rEPO ranging from 0 to 1 U/mL and incubating with 59Fe-transferrin for 3 hours on day 10 of culture, followed by heme extraction and measurement of 59Fe in a gamma counter. The number of viable cells were determined by counting the cells that excluded trypan blue in a hemocytometer every 24 hours during the day 6 through 14 culture period.

**Disappearance of the EPO requirement in CFU-E descendants.** In the experiments performed to determine the length of time EPO is required, day 8 cells were cultured in the presence of 1 U/mL of EPO and 0.05 μCi/mL 59Fe-transferrin. On days 8 through 12, the cells were removed at 24-hour intervals and washed twice at 4°C with 50 mL of IMDM per wash. These cells were recultured in 0.5-mL aliquots with fresh media containing 0.5 μCi/mL of 59Fe-transferrin. During reculture, only one set of these 0.5-mL aliquots received EPO. The other set was recultured without added EPO. On day 14, all the samples were collected, washed, and concentrated as described above. The 59Fe incorporated into heme was determined by measuring the radioactive 59Fe in a gamma counter.
DNA probes. The EPO receptor probe used in Northern blot hybridization was a 1.1-kb EcoRI/BamHI DNA fragment obtained from the human EPO receptor clone ER2.20 This probe lacks the sequence of poly A poly T representative of the poly A portion of the mRNA. The actin probe was a 5′/3′ fragment containing the entire α-actin coding region obtained from mouse actin clone PAM91.21 The β-globin probe was a 5′/3′ fragment containing the first two exons from the β-major globin gene of mouse.22

RNA isolation and Northern analysis. Total RNA was isolated from human CFU-E by hot phenol extraction.23 Briefly, cells were washed in IMDM and the cell pellets resuspended in a solution of 10 mmol/L NaAc (pH 5.2)/0.5% sodium dodecyl sulfate (SDS). Immediately after resuspension, an equal volume of phenol that had been equilibrated with 10 mmol/L NaAc (pH 5.2) was added and extracted for 5 minutes at 56°C. The solution was rapidly cooled to 4°C in an ice bath followed by centrifugation for 5 minutes at 12,000g. The aqueous phase was extracted a second time and the RNA was precipitated twice with 0.3 mol/L NaAc (pH 6.3) plus 2.5 vol of ethanol. The total cellular RNA extracted from CFU-E were quantitated by determining absorbance at 260 nm.

For Northern analysis experiments, RNA was electrophoresed in 1.0% agarose gels containing 0.66 mol/L formaldehyde according to the method described by Davis et al.24 Immediately before electrophoresis, 10 μg of ethidium bromide was added to each sample. After electrophoresis, the gels were photographed and blotted onto nylon membranes. Prehybridizations and hybridizations were performed in 50% formamide, 6× SSC (1× SSC = 0.15 mol/L NaCl, 0.015 mol/L sodium citrate), 1× Denhardt’s solution (0.02% Ficol, 0.02% polyvinylpyrrolidone, 0.02% BSA), and 0.5% SDS. DNA probes were labeled by the random primer labeling method25 using [α-32P]dCTP (800 Ci/mmol). Hybridizations were performed at 42°C for 16 to 20 hours using 1.0× 106 cpm of the labeled probes per milliliter of hybridization fluid. Blots were washed with 2× SSC, 0.5% SDS at room temperature for 20 minutes, followed by 0.1× SSC, 0.1% SDS at 55°C for 1.5 hours. Quantitation of the autoradiographic signal was performed with a laser scanning densitometer (BioRad video densitometer model 620; BioRad, Richmond, CA). The relative amounts of total RNA present in each lane were estimated by scanning a photographic negative of the ethidium bromide-stained gel over the 18S ribosomal RNA band. For each lane, the autoradiograms and the photographic negatives of the ethidium bromide stained gels were within the linear range of the densitometer. Control experiments also showed that the estimates of RNA amounts based on photography and scanning of stained gels were linear with respect to actual amounts of RNA loaded onto the gels.

Binding of 125I-EPO. CFU-E grown in liquid culture were washed twice with 50 mL of IMDM/1% FCS per wash to eliminate EPO. Washed cells (1×106) were resuspended in 100 μL of binding media (1% FCS, 1% HSA, IMDM) at 37°C in a 5% CO2 incubator for 1 hour. 125I-EPO (4 U/mL) was added and the incubation was continued for an additional 1 hour at 37°C. Cells were washed with excess IMDM/1% FCS at 4°C and centrifuged through 0.5 mL of n-butyl phthalate in microfuge tubes. The tubes were frozen and the bottom of the tubes containing the pellets were cut and counted. Nonspecific binding was determined by incubating 1×106 cells in the presence of 100 U/mL of rEPO in addition to 125I-EPO. Specific binding was determined by subtracting nonspecific binding from the total binding.

RESULTS
Human CFU-E, derived from peripheral blood BFU-E and grown in liquid suspension culture, mature and differentiate into reticulocytes during 14 days of incubation (Fig 1). During their terminal differentiation, these erythroid progenitors undergo a significant decrease in size. Figure 1A shows the morphology of the stained CFU-E that were further purified from BFU-E cultures on day 7 and started in liquid suspension cultures. At this time, the majority of the cells have a basophilic erythroblast. When the cells are stained after 8 and 10 days of culture, most have synthesized large amounts of globin and are in the polychromatophilic erythoblast stage of their development (Fig 1B through D). By day 12 of incubation, the cell population is composed of both orthochromatic erythroblasts and reticulocytes (Fig 1E). Enucleating cells can be detected easily throughout the culture during this time period. By 14 days of culture, both bare nuclei and reticulocytes are widespread, and accumulated Hb can be seen as a deep brown color (stained with benzidine) within the irregular shaped reticulocytes (Fig 1F). Under the culture conditions used in the present study, 14 days of incubation yielded approximately 54% bare nuclei and 40% reticulocytes (Fig 2).

Measurements of heme synthesis during proliferation and differentiation showed that the cells cultured with EPO exhibit an increase in the rate beginning on day 7 and reaching a maximum rate by day 10 of culture, whereas cultures without EPO did not have an increase in heme synthesis (Fig 3A). After day 10, there is a decrease in the rate through day 14. Figure 3B shows the extent of cellular proliferation of erythroblasts from purified CFU-E cultured with EPO in liquid suspension culture beginning on day 6. Between day 11 and 12, these cells reached their maximum number (approximately fivefold the initial number plated) and then a steady level in the cell number was observed through day 14. Figure 4 shows the relationship between EPO concentration and the rate of heme synthesis on day 10 in cells that were initially cultured at a concentration of 0.5×106/mL in liquid medium. Because day 10 is the day of maximum heme synthesis in these cells, a measurement of heme synthesis rate at this time reflects the magnitude of EPO effect on erythropoiesis. The dose response curve shows that a plateau concentration is approached at approximately 0.5 U/mL.

We next determined whether there is a time period during erythroblast maturation beyond which the cells do not require EPO for development (Fig 5). Early withdrawal of EPO from these cells at various times showed that between day 10 and 11 of culture the erythroblasts became independent of EPO for further development (Fig 5). During this time period, the majority of the cells arc in the polychromatophilic erythoblast stage and the proliferation of erythroblasts has greatly declined.

Table 1 shows the abundance of total cellular RNA in human erythroblast during development. Quantitation of the cellular RNA per 1×106 cells between 8 and 14 days of culture showed a decrease throughout the culture period.
Fig 1. Photomicrograph of differentiating human CFU-E stained with 3,3′-dimethoxybenzidine and hematoxylin. (A) Day 7, (B) day 8, (C) day 9, (D) day 10, (E) day 12, and (F) day 14 of culture. Bar = 10 μm.

The ethidium bromide-stained lanes total RNA shown in Fig 6A were from erythroblasts isolated at progressively later stages of development (days 8 to 14). These cells expressed EPO receptor mRNA levels that are detectable by Northern hybridization using approximately 10 μg of total cellular RNA (Fig 6B). The major band detected using a human cDNA probe was 2.2 kb in size. In addition, two minor bands could be seen at 4.4 and 6.4 kb, respectively. This pattern has been reproduced in multiple analyses of human erythroblasts. Total RNA extracted from two non-erythroid human cell lines (HL60 and U937) and analyzed by electrophoresis and Northern hybridization along with the erythroblast RNA did not show a band corresponding to 2.2 kb, although a minor band corresponding to 4.4 kb could be seen on a highly exposed autoradiogram. The second minor band seen in erythroid cells (corresponding to 6.4 kb in size) could not be visualized, even on a highly exposed x-ray film in nonerythroid cells.
Figure 7A and B shows an analysis of the quantity of EPO receptor mRNA in cultured erythroblasts between days 8 and 14 of culture. During this time period, a steady decline in the EPO mRNA per cell is observed. To quantify the EPO receptor mRNA, we first normalized the autoradiographic band to the amount of 18S RNA present in each lane by scanning a photographic negative of the ethidium bromide-stained gel. After normalizing to rRNA, we took into account the decline in the total cellular RNA (Table 1) during terminal differentiation to arrive at the relative EPO receptor mRNA per cell. Figure 7B shows the results of the analysis of such timed RNA samples for β-globin message in addition to EPO receptor mRNA on the same blot. The experiment in Fig 7B showing the decline of the EPO receptor mRNA was repeated four times for days 8, 10, and 12 and twice for days 13 and 14. Results of the changes in the level of globin mRNA shown in Fig 7B are from triplicate determinations. After normalization of the autoradiographic band, for both equal loading and for the decline of the ribosomal RNA content during development, a 12% reduction in the EPO receptor mRNA is seen during the time period between days 8 and 10 of culture. By day 12 of culture, only 30% of the initial amount is present per cell and by day 14 of culture, when the majority of the cells are in the reticulocyte stage, only 5% of the initial amount was present in these cells. Figure 7C shows the results of 125I-EPO binding to determine the level of EPO receptor protein present on the cell surface of the differentiating erythroblasts. Almost 50% of the surface binding was lost in these cells between days 7 and 9 of culture; after 13 days in liquid culture, the binding was down to less than 5% of the amount present on day 7. With respect to the level of globin mRNA during the day 8 to day 14 culture period, an approximate threefold increase was evident between days 8 and 10 of culture. After reaching a maximum level of globin mRNA expression on day 10, there is a slow decline between days 10 and 12, followed by a faster rate of decline in the mRNA level between days 12 and 14 of culture.
determined on day 9 of culture by the addition of the transcriptional inhibitor actinomycin D followed by RNA extraction and Northern analysis. Figure 8 shows the autoradiogram of the Northern blot hybridized with EPO receptor, α-actin, and β-globin cDNAs along with the ethidium bromide-stained gel and the decay curve for the EPO receptor mRNA. There is approximately a 30-minute lag period during which actinomycin D enters the cell and begins to take effect, as determined by [3H]uridine incorporation into RNA (data not shown). The half-life of the EPO receptor message was approximately 90 minutes after taking into account this initial lag period. During the 4-hour time period of the experiment, there was no significant decay of the actin and β-globin mRNAs that could be measured by Northern analysis.

DISCUSSION

In the mouse models for erythroid differentiation, to obtain a highly enriched population of erythroid precursors (CFU-E) the animals are made anemic by treating with thiamphenicol or they are injected with an anemia-inducing strain of Friend virus. Human erythroid progenitors used in the present study were isolated and purified from primary cultures of peripheral blood cells derived from normal adult donors using modifications of the system.
EPO receptor gene expression and of β-globin mRNA during maturation and terminal differentiation of human erythroid progenitors. (A) Ethidium bromide-stained gel of approximately 7 µg of total RNA and autoradiograms of a Northern blot showing the levels of EPO receptor and globin mRNAs during days 8 to 14 of culture. A strong signal seen in day 13 lane is due to the overloading of total RNA for this lane. (B) Changes in the levels of EPO receptor (○) and globin (●) mRNAs per cell during days 8 to 14 of culture after accounting for the loading variations. The data are mean ± SD of quadruplicate determinations for days 8 to 12 and duplicate determinations for days 13 and 14. (C) Specific binding of ¹²⁵I-EPO to cell surface receptors on human erythroid progenitors (●). The data are mean ± SD of triplicate determinations.

devised by Sawada et al. Starting with 400 mL of whole blood and culturing partially purified BFU-E for 6 or 8 days in vitro, we are able to purify between 10 and 30 × 10⁶ cells that are CFU-E. These cells, when cultured in liquid media with EPO, will mature and differentiate into reticulocytes in vitro. The ability to sustain human CFU-E in liquid suspension cultures has allowed us to characterize both biochemical and morphologic events in a temporal fashion.

Results of this study show several interesting differences between human and mouse erythroid precursors with respect to the timing of key events in the differentiation program. In human cells, a maximum rate of Hb synthesis is observed on day 10 of culture, 2 days before enucleation (Figs 1 and 3). Very little Hb synthesis occurs in human cells by day 14, when more than 50% of the cells are reticulocytes. In a mouse model (FVA cells), maximum Hb synthesis occurs at a time when a majority of the cells have enucleated. Also, in human cells the globin mRNA level is at its maximum (Fig 7B) at day 10 (when the majority of the cells are in the orthochromatic erythroblast stage), whereas in mouse FVA cells, the maximum level of globin mRNA is
present before the maximum rate of Hb synthesis is observed. Thus, it appears that human cells complete Hb synthesis earlier relative to the enucleation process than do mouse cells. In addition, the decline in the total cellular RNA content (which primarily reflects ribosomal RNAs) seen during the day 8 to day 14 culture period (Table 1) in human erythroblasts is quite different from the observations that have been made with the FVA cells. In FVA cells, total RNA levels stay constant at least through the first 24 hours of culture, when the majority of the cells are in the basophilic and polychromatophilic erythroblast stages, whereas in human erythroblasts, total RNA content is reduced by greater than twofold during the same stage of development (between 8 and 10 days of culture). Between day 10 and day 14, human cells continue to lose their cellular RNA while going through the orthochromatic erythroblast stage to become reticulocytes. The studies on the differentiating primary mouse erythroblast cells have been performed using cells derived from spleens of mice infected 2 weeks previously with the anemia strain of Friend virus. All available evidence suggests that the FVA cells are analogous in differentiation stage to mouse CFU-E or slightly more mature cluster-forming proerythroblasts, and that the characteristics of their in vitro differentiation recapitulate the events of late-stage differentiation of normal mouse CFU-E in terms of the relative timing of events. However, mouse CFU-E that are not infected with virus have not been evaluated as fully with respect to many of the molecular processes of late differentiation as have FVA cells. Thus, it is possible that the preceding differences between human and mouse (FVA) cell differentiation could reflect an effect of the virus infection of the mouse cells.

Withdrawal of EPO from human CFU-E between days 8 and 12 of culture showed that all of the cells in the population become independent of EPO by day 11, as measured by 59Fe incorporation into heme (Fig 5). In addition, quantitation (on day 14) of the number of enucleated cells in cultures that were deprived of EPO after day 11 showed that there was no difference with respect to enucleation between these and the control cultures containing EPO throughout the culture period (data not shown). An interesting finding of these experiments is that the timing of the disappearance of the EPO requirement in the human cells coincides with the marked decline of proliferation (Figs 3 and 5). Such a coincidence was not obvious in previous studies with mouse (FVA) cells, perhaps because in mouse cells the total differentiation period is about 48 to 60 hours as opposed to 6 to 7 days in the case of human CFU-E. Therefore, the time resolution of biochemical events occurring during differentiation can be clearly determined in the case of the human system.

The extent of enucleation (54% bare nuclei) observed in these cells is similar to that seen in FVA cells. The results showing lower reticulocyte counts compared with the number of bare nuclei throughout the time course (Fig 2) are probably attributable to the fact that reticulocyte membranes are much more fragile and, as a result, may rupture during the cytospining of these cells.

Human EPO receptor mRNA is 2.2 kb, almost 400 bases longer than its mouse counterpart based on the migration pattern in formaldehyde/agarose gels. The 6.4-kb band, which was seen only in human erythroid cell RNA (Fig 6), is a specific hybridization signal and is likely to be an unprocessed form of the EPO receptor gene transcript because the molecular size of this band falls within the size of the complete human EPO receptor genomic clone. The 4.4-kb band may be due to a slight, nonspecific interaction between the probe and 28S ribosomal RNA. Comparable minor species of RNA were not observed in our analysis of the mouse EPO receptor mRNA. A decrease in the level of EPO receptor mRNA (Fig 7A and B) observed during maturation and terminal differentiation of the human cells is similar to the decrease seen in mouse FVA cells. There is an initial 12% decline in the receptor mRNA level between days 8 and 10, followed by an accelerated decrease between days 10 and 14 of culture. In fact, by day 14, less than 5% of the EPO receptor mRNA is left in these cells. EPO binding experiments performed on these cells showed a steady decline in the surface binding during maturation and terminal differentiation of these cells (Fig 7C). These results are in agreement with the binding studies performed by Sawada et al and Broudy et al with human CFU-E cultured in semisolid medium, by Sawyer and Kouny with FVA cells, and by Landschultz et al with mouse spleen cells. In the case of mouse erythroblasts, the decrease in the level of EPO receptor mRNA occurs against a background of a constant content of ribosomal RNA per cell, whereas in human cells the ribosomal RNA content itself is diminishing throughout the culture period. Therefore, in human cells, before rRNA can be used as a standard for equal loading, the amounts must be adjusted to reflect the changes in total cell RNA content.

The half-life of human EPO receptor mRNA is approximately 90 minutes, as measured by the addition of the transcriptional inhibitor actinomycin D (Fig 8). Also, there was no change in the half-life of this message measured at different times during development (results not shown). This compares well with the stability of the mouse EPO receptor mRNA (75 minutes) determined by the same method. Because the half-life of the EPO receptor mRNA stays unchanged through day 11, the decrease in the EPO receptor mRNA level observed during the course of cell culture probably reflects a decrease in the transcription rate over time. The relatively short half-life of EPO receptor mRNA indicates that for this message to persist, continuous transcription must take place. Both the actin and globin mRNAs do not show an appreciable amount of decay during the 4-hour time period with actinomycin D. This is consistent with the fact that, at least in mouse, globin and actin have half-lives approximately 50 hours and 16 to 26 hours, respectively.

Adapting the original system devised by Sawada et al to liquid suspension cultures has enabled us to follow biochemical and molecular events of human erythroid cells in a temporal fashion. By measuring the EPO receptor mRNA...
levels, the globin gene expression, the timing of the EPO requirement, the kinetics of Hb synthesis, and the changes in morphology, we have been able to relate the changes during erythroid differentiation of human cells and those changes described in mouse erythroid progenitors. Because the cell population is quite homogeneous and undergoes the differentiation process in a relatively synchronous manner, this cellular system should be useful in the future to study particular aspects of human erythroid development, including a variety of aberrant conditions of erythropoiesis. Also, it might be useful to study developmental stages of the malaria parasite within the infected erythroblast, including the receptors that are involved.

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REFERENCES


Differentiation and erythropoietin receptor gene expression in human erythroid progenitor cells [see comments]

A Wickrema, SB Krantz, JC Winkelmann and MC Bondurant