Erythropoietin Receptor Characteristics on Primary Human Erythroid Cells

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Erythropoietin (EP) exerts its effects on erythropoiesis by binding to a cell surface receptor. We examined EP receptor expression during normal human erythroid differentiation and maturation from the burst-forming unit-erythroid (BFU-E) to the reticulocyte level. In contrast to previous studies, we assessed EP receptor number and affinity in erythroid precursors immunologically purified from fresh bone marrow aspirates or fetal liver samples and in reticulocytes purified from peripheral blood. EP receptors were quantitated by equilibrium binding experiments with $^{125}$I-EP. We found that purified primary erythroblasts from both adult and fetal sources exhibited a single high-affinity (kd 100 pmol/L) binding site for EP under our experimental conditions, and 135 or 250 receptors per cell, respectively. Reticulocytes were devoid of EP receptors. We compared these data to in vitro-derived BFU-E progeny at both early and late stages of maturation. Cultured BFU-E progeny also displayed a single class of receptors of slightly lower affinity (210 to 220 pmol/L). Preparations enriched in colony-forming unit-erythroid (CFU-E) and proerythroblasts (day 9 BFU-E progeny) displayed approximately 1,100 receptors per cell, whereas populations containing mature erythroblasts (day 14 BFU-E progeny) exhibited approximately 300 receptors per cell. Furthermore, information from binding experiments was complemented by autoradiography in both enriched BFU-E preparations, cultured BFU-E progeny (days 9 and 14), and marrow mononuclear cells. These studies are consistent with a peak in EP receptor expression at the BFU-E/proerythroblast stage and a decrease with further maturation to undetectable levels at the reticulocyte stage. These data examining EP receptor characteristics on freshly isolated erythroid precursor cells complement previous data on EP receptor biology using culture-derived erythroblasts.

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Erythropoietin (EP) plays a central role for the survival, proliferation, and maturation of erythroid cells. The biologic effects of EP are mediated through binding to a specific cell surface receptor. Understanding the cellular distribution and binding characteristics of EP receptors during normal erythroid differentiation may provide insights into the mechanisms of EP action, and the pathophysiology of familial or acquired erythrocytosis.

EP receptor expression in human cells has been examined using human erythroid cell lines or culture-derived erythroid precursors. Although these populations have advantages in terms of numbers of cells available and purity, they are either generated under high EP stimulus (culture-derived erythroblasts) or they are nonresponsive to EP (many erythroblastic lines) and therefore may not reflect the receptor characteristics present in bone marrow erythroblasts. The binding parameters of the EP receptor on normal human or murine cells remains controversial, with reports of one or two classes of binding sites.

We purified fresh erythroid precursor cells from bone marrow aspirates or fetal liver by immunologic means and quantitated EP receptors on these cells. We also performed parallel experiments with culture-derived erythroblasts. Our results show that primary erythroblasts purified from adult marrow exhibit a single class of high-affinity EP receptors (kd 100 pmol/L) with approximately 135 receptors per cell. Cultured cells (burst-forming unit-erythroid [BFU-E] progeny) display a single class of binding sites of slightly lower affinity (kd 220 pmol/L), possibly due to exposure to high concentrations of EP in vitro. Preparations enriched in colony-forming unit-erythroid (CFU-E)/proerythroblasts exhibit approximately 1,100 receptors per cell, whereas preparations containing mainly late erythroblasts had approximately 300 receptors per cell, demonstrating a decrease in receptor expression with terminal erythroid maturation.

MATERIALS AND METHODS

Isolation of erythroid precursor cells from bone marrow or fetal liver. Normal human bone marrow cells were aspirated from the posterior iliac crest of consenting healthy adult volunteers. The marrow samples were obtained in the Clinical Research Center at the University of Washington (Seattle). Human fetal liver cells (gestational age 55 to 80 days) were obtained from therapeutic abortions. Consent was obtained for the use of these tissues, and these studies were approved by the Institutional Review Board at the University of Washington. Purified populations of normal human erythroid precursor cells were isolated from adult marrow buffy coat cells or from fetal liver cells by direct immune adherence with monoclonal antibody (MoAb) EP-1. The antigen recognized by EP-1 is preferentially expressed on late erythroid progenitor cells and erythroid precursor cells. The entire immune adherence procedure was complete within 2 hours after obtaining the marrow aspirate or fetal liver sample.

Isolation of reticulocytes. Reticulocytes were isolated from the peripheral blood of a normal adult and a patient with sickle cell disease by Percoll density centrifugation (Sigma, St Louis, MO). Brilliant cresyl blue staining demonstrated that the cell preparations varied between 20% (normal donor) and 90% (sickle cell patient) reticulocytes.

Isolation of BFU-E progeny. Peripheral blood (50 to 100 mL) was obtained from healthy adult volunteers, and mononuclear cells were isolated by Ficoll-Hypaque density centrifugation (Organon Teknika Corp, Durham, NC). The mononuclear cells were cultured at a density of $5 \times 10^7$/mL in Iscove's modified Dulbecco's medium (IMDM) supplemented with 30% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT), 1% deionized bovine serum albumin (BSA; Reheis, Phoenix, AZ), 1.5 U/mL recombinant human EP, and 2 U/mL purified recombinant human interleukin-6

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kin-3 (IL-3; Genetics Institute, Cambridge MA), and made semi-solid with 1.4% methylcellulose (Dow, Midland, MI). The EP used to culture cells was partially purified (6,000 U/mg) from the supernatants of BHK cells transfected with the human EP gene. The plates were incubated in a humidified atmosphere containing 5% CO₂. Erythroid colonies were identified by characteristic morphology under the inverted microscope. Under these conditions the plates contained less than 10% myeloid colonies. To obtain erythroid populations comprised mainly of proerythroblasts, individual colonies were plucked early (day 8 or 9 of culture). Mature, well-hemoglobinized bursts cultured in parallel from the same normal donor were plucked later (day 13 or 14 of culture). In some experiments, the mature bursts were plucked on day 12 and placed in suspension culture for 1 to 2 days to permit further maturation to occur. The suspension culture medium consisted of IMDM supplemented with 10% FCS, 1% BSA, 1.5 U/mL EP, and 1 U/mL IL-3. This approach yielded up to 15 × 10⁶ cells from the day 9 colonies, and 30 × 10⁶ cells from the mature bursts. For each experiment, cytopreparations of cells were stained with benzidine and were evaluated in conjunction with binding data. To determine whether CFU-E were present, cells plucked on day 8 were cultured in plasma clots. The cells (10⁹/mL) were cultured in IMDM supplemented with 30% FCS, 1% BSA, 10% beef embryo extract (GIBCO, Grand Island, NY), 1 U/mL EP, 1 U/mL IL-3, and 10% bovine citrated plasma. After a 5-day incubation at 37°C, the plasma clots were harvested, stained with benzidine, and erythroid colonies of ≥ 8 cells were counted.

Identification of equilibrium binding conditions. We examined the time course of [³²P]EP binding to normal marrow cells at 15°C. Because the rate of binding may be slower with lower concentrations of ligand, these experiments were performed with 60 pmol/L [³²P]EP. Plateau binding was achieved by a 6-hour incubation at 15°C; continuing the incubation up to 24 hours did not significantly increase the amount of cell-surface-associated EP. Internalization of EP at 15°C was evaluated by using glycine-HCl to strip the cell surface. The results of these experiments showed that 85% of the cell-associated EP was surface bound after a 6-hour incubation at 15°C.

Quantitation of EP receptors. EP receptors were measured by incubating cells with [³²P]EP (300 to 900 Ci/mmol; Amersham) for 1 hour at 37°C in the presence of 0.2% azide. The final concentration of [³²P]EP was 1 nmol/L. At the conclusion of the incubation, the cell suspension was layered onto Percoll (density 1.02 g/mL) and centrifuged for 1 minute at 4°C in a Beckman Microfuge 11 to separate cell-associated [³²P]EP from free [³²P]EP. Cytopreparations were made in a Shandon Southern cytospin. The slides were processed as described and exposed for 3 to 4 weeks. Specific binding was determined by performing grain counts on marrow mononuclear cells incubated with [³²P]EP with or without a 100-fold molar excess of unlabelled EP; the results of both measurements are presented. Specific binding for the enriched BFU-E and for the day 9 and day 14 BFU-E progeny was determined by counting grains overlying the cells and in an area immediately adjacent to each cell; the grain count in the adjacent area was then subtracted.

RESULTS

EP receptors on purified primary erythroid cells. Normal human erythroblasts were isolated from adult marrow or fetal liver by direct immune adherence with EP-1. Up to 40 × 10⁶ erythroid precursor cells would be isolated with EP-1 within 2 hours after the fetal liver or adult marrow specimen was obtained. These populations of cells were greater than 95% erythroid and consisted mainly of late erythroblasts, less than 1% of the cells were CFU-E, and no BFU-E were identified by colony assay, suggesting that the EP-1 antigen must be expressed at low density on BFU-E. Equilibrium binding analysis of EP receptors on purified adult marrow or fetal liver erythroblasts showed a single class of high-affinity sites (Figs 1 and 2). In four experiments, fetal erythroblasts were found to have 250 ± 35 receptors per cell with a KD of 100 ± 35 pmol/L (mean ± SEM). Primary adult marrow erythroblasts exhibited 135 ±
Fig 1. EP binding to primary marrow erythroblasts. (A) Adult marrow buffy coat cells. (B) Erythroblasts isolated from marrow buffy coat cells by direct immune adherence with EP-1. (A and B) Benzidine stain with Wright-Giemsa counterstain, original magnification x40. (C) Primary marrow erythroblasts, $1.2 \times 10^8$, were incubated with $^{125}$I EP (2.3 pmol/L to 2.6 nmol/L) with or without a 100-fold excess of unlabeled EP for 6 hours at 15°C. The average of duplicate points is shown.
20 receptors per cell, $kd$ 108 ± 40 pmol/L (mean ± SEM of three experiments). Reticulocytes did not have detectable binding of $^{125}$I EP (two experiments).

**EP receptors on in vitro BFU-E progeny.** Peripheral blood mononuclear cells were cultured in semisolid medium supplemented with EP and IL-3, and erythroid colonies were plucked on day 9 or day 14. Day 9 cells were large pale early erythroblasts (Fig 3). Immunofluorescent staining showed that 100% of the day 9 cells were positive with EP-1.15 Plasma clot assay showed that 9.2% of day 8 cells were CFU-E. By day 14, the population included smaller well-hemoglobinized late erythroblasts (Fig 3). Both populations of cells exhibited a single class of EP receptors (Fig 3). In this experiment, the early erythroblasts displayed 1,740 receptors per cell with a $kd$ of 180 pmol/L; the late erythroblasts had 580 receptors per cell with a $kd$ of 215 pmol/L. In two additional experiments, day 9 cells exhibited an average of 800 receptors per cell with a $kd$ of 230 pmol/L. The day 14 cells displayed 235 ± 50 receptors per cell, $kd$ 230 ± 5 pmol/L (mean ± SEM of three additional experiments). A summary of EP receptor numbers and binding affinity in normal human cells is presented in Tables 1 and 2.
ERYTHROPOIETIN RECEPTORS ON HUMAN CELLS

Cellular distribution of EP receptors. Autoradiographic analysis of $^{125}$I EP binding to bone marrow mononuclear cells showed that only erythroblasts and megakaryocytes display EP receptors. No specific binding of $^{125}$I EP to myeloid or lymphoid cells was detected. Grain counts showed a progressive decrease in the binding of EP in conjunction with erythroid maturation (Fig 4). Proerythroblasts displayed 15- to 20-fold more EP binding than orthochromatric erythroblasts.

Peripheral blood preparations enriched for BFU-E also bound $^{125}$I EP (Fig 5). In this experiment, 45% of the cells (88 of 194 cells analyzed) were noted to have specific binding of $^{125}$I EP. When these cells were replated in plasma clots, there were 31 BFU-E per 100 cells cultured (average of duplicate clots). No CFU-E were observed. The slightly higher fraction of cells binding EP than percentage of BFU-E detected by colony assay could be due to a plating efficiency less than 100%.

Culture-derived BFU-E progeny displayed specific binding of $^{125}$I EP (Fig 6). The day 9 cells exhibited an average of 3.5 grains per cell (grain count performed on 86 cells), whereas the day 14 cells displayed an average of 0.8 grains per cell (grain count performed on 156 cells).

DISCUSSION

Prior studies of EP receptors on normal human cells have used in vitro generated erythroid cells. Because these cells were generated under nonphysiologic high levels of EP, they may not reflect the true number or affinity of EP receptors on native erythroid precursors. Therefore, we examined EP receptor expression in erythroid cells freshly purified from both adult and fetal sources.

A single class of high-affinity binding sites was identified on all of the normal human erythroid precursor cells studied under the conditions we used (equilibrium binding at 15°C). This finding held true whether the data were analyzed by the ligand method or by the method of Lipkin et al. Although a two-site model could be fitted to the data using the ligand program in approximately 30% of the experiments, the one site model was preferred whether the experiments were analyzed individually or pooled (F-test). We interpret these data to show that the high-affinity site predominates on normal human erythroid precursors.

The binding affinity of the EP receptor was similar in adult and fetal erythroid cells (108 ± 40 pmol/L and 100 ± 35 pmol/L, respectively), despite the observation that EP may play a unique role in fetal erythropoiesis. Fetal erythroid progenitor cells (BFU-E) can differentiate into CFU-E in the presence of EP alone in single-cell cultures under serum-free conditions. In contrast, BFU-E from adults require both EP and IL-3 to undergo the same differentiation steps. However, erythroblasts obtained by culturing BFU-E displayed a lower affinity EP receptor (kd 210 to 220 pmol/L). EP levels in normal adults are 20 to 40 mU/mL, whereas levels of EP used for cell culture are usually 1 to 2 U/mL, 50-fold higher. Exposure to a high EP level or a change in membrane composition during culture may result in the expression of a lower affinity EP receptor. Local alterations in membrane proteins may affect EP receptor-binding affinity. Bousios et al. showed that receptor affinity may change with the developmental stage during yolk sac erythropoiesis. However, human embryonic cells have not been available for study, and this phenomenon has not yet been found during the late stages of fetal or adult erythroid maturation.

EP receptor display is not a static property of the cell, but changes with cellular differentiation and maturation. The present report provides several pieces of evidence to support this concept. Fetal liver erythroblasts exhibited a higher number of receptors per cell than did adult marrow erythroblasts, reflecting the greater number of early erythroblasts among the fetal cells. Our data show that the final stages of normal erythroid maturation are accompanied by a decrease in EP receptor expression: equilibrium binding experiments showed that day 9 erythroblasts expressed threefold more receptors per cell than day 14 erythroblasts, and reticulocytes were devoid of EP receptors. Autoradiographic analysis of $^{125}$I EP binding to cultured BFU-E progeny showed that day 9 erythroblasts had fourfold more grains per cell than day 14 erythroblasts, confirming and strengthening the Scatchard analysis results. Autoradiography of $^{125}$I EP binding to marrow mononuclear cells verified the decrease in EP binding with terminal erythroid maturation, in accord with prior reports. We demonstrate herein that a preparation of cells containing BFU-E without CFU-E displays specific bind-

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<td>Adult marrow erythroblasts</td>
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<tr>
<td>Fetal liver erythroblasts</td>
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<th>Cells</th>
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<td>Day 9 erythroblasts</td>
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<td>Day 14 erythroblasts</td>
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Fig 4. Autoradiographic analysis of $^{125}$I EP binding to human marrow cells. (square), Binding in the presence of 1 nmol/L $^{125}$I EP; (diamond), binding in the presence of 1 nmol/L $^{125}$I EP plus 100 nmol/L unlabeled EP. Grain counts were performed on 28 proerythroblasts, 35 basophilic erythroblasts, 62 polychromatophilic erythroblasts, and 104 orthochromatric erythroblasts.
Fig 5. Autoradiography of $^{131}I$ EP binding to enriched peripheral blood BFU-E. Original magnification $\times 90$.

Fig 6. Autoradiography of $^{131}I$ EP binding to BFU-E progeny. (A) Day 9 erythroblasts. (B) Day 14 erythroblasts. Original magnification $\times 90$ in (A) and (B).
ing of EP, and a previous report showed that binding of EP continuously increases as BFU-E mature to CFU-E in vitro.\textsuperscript{26} Taken together, these studies show that EP receptor expression peaks at the CFU-E/proerythroblast stage and decreases with terminal erythroid maturation.

Autoradiography shows that megakaryocytes are rich in EP receptors, and quantitative studies indicate that megakaryocytes display 6,500 receptors per cell.\textsuperscript{28} Although megakaryocytes clearly express EP receptors, the biologic significance of these receptors remains unclear.\textsuperscript{35} The use of EP in humans has not been associated with clinically significant thrombocytosis.

Prior reports describe high-affinity and low-affinity sites on erythroid precursor cells isolated from mice infected with the anemia strain of the Friend virus, enriched populations of murine CFU-E, and normal human erythroid cells.\textsuperscript{4,10,16,17,37} However, other investigators have found one class of sites on normal murine or human erythroid cells.\textsuperscript{41-158} The conditions used to achieve equilibrium binding (prolonged incubation at 4°C v higher temperatures) may partially explain the discrepancy in results.

We find predominantly the high-affinity EP receptor which, from a physiologic point of view, is probably the most relevant one. The function of the lower-affinity receptor in normal human erythropoiesis remains unclear at present.

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