Expression and Modulation of Specific, High Affinity Binding Sites for Erythropoietin on the Human Erythroleukemic Cell Line K562

By John K. Fraser, Fu-Kuen Lin, and Michael V. Berridge

**THE GLYCOPROTEIN** hormone erythropoietin (Epo) has long been acknowledged as a principal regulator of erythroid differentiation. More recently, however, other factors such as interleukin 3 (IL-3) in the mouse and burst-promoting activity (EPA) in humans have also been shown to contribute to erythroid differentiation. These observations suggest that erythroid differentiation is mediated by a complex interaction of factors which combine to "fine tune" circulating erythrocyte numbers in response to environmental requirements. The mechanism for these interactions remains unknown. Epo acts by stimulating committed erythrocyte precursor cells to proliferate and to differentiate toward mature erythrocytes. Its action appears to be mediated by binding to specific cell surface receptors. Difficulty in obtaining sufficient quantities of pure Epo and the observation that conventional 125I incorporation inactivates Epo have limited analysis of Epo receptors on normal erythroid precursor cells, however. This problem was overcome in one study by using tritiated Epo, but difficulties in obtaining Epo of sufficiently high specific activity have restricted application of this method.

In the present study, we used bioactive, 125I-labeled, recombinant human Epo (125I-rHuEpo) to examine Epo receptors on the human erythroleukemia cell line K562. This cell line expresses the erythrocyte surface marker glycoprotein A and can be induced to synthesize hemoglobin. Furthermore, the K562 cell line has been shown to be responsive to both EPA and Epo, suggesting that it might be a useful model for examining the human Epo receptor and its regulation. We demonstrate that K562 cells express low numbers of high-affinity surface receptors for Epo and that conditioned medium from the EPA-secreting cell line U937 stimulates and recombinant EPA increase expression of Epo receptors on K562. Induction of Epo receptors is associated with increased sensitivity of K562 to Epo.

**MATERIALS AND METHODS**

**Cells and cell culture.** K562 and the myelomonocytic cell line U937 were provided by Dr G. Findlay, Auckland Medical School (Auckland, New Zealand), and 5637 bladder carcinoma cells were provided by Dr M. Bodger, Cytogenetics Unit, Christchurch Hospital (Christchurch, New Zealand). These cell lines were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 1 mmol/L glutamine, 25 μg/mL penicillin, 25 μg/mL streptomycin, and 10% fetal calf serum (FCS) and were grown at 37°C in an atmosphere of 5% CO2 in air. U937-conditioned medium was prepared by seeding 1 to 2 × 106 cells into 50 mL culture medium and allowing the cells to expand and exhaust the medium at which time the medium was centrifuged, filtered through a 0.45 μm filter, and stored frozen at −20°C. Cell-conditioned medium (5637CM) was prepared by seeding 5 × 105 5637 cells into 25 mL Iscove's modified Dulbecco's Medium (IMDM, Gibco) supplemented with antibiotics and 10% FCS. The cells were allowed to expand for 8 to 10 days in an atmosphere of 5% CO2 in air, after which the supernatant was centrifuged, filtered through an 0.45 μm filter, and stored frozen at −20°C.

Epo receptor induction studies were performed by growing K562 cells in culture medium supplemented with 10% 5637CM or 20% U937CM, or with recombinant EPA (kindly supplied by Dr J. Gasson, Los Angeles) for 24 hours. Epo receptor reversibility was determined by culturing cells for 4 days (approximately 4 doubling times) in the presence of U937CM. Cells were then washed into IMDM medium plus antibiotics and 10% FCS and grown for 3 more days, at which time Epo binding was determined.

**Erythropoietin binding.** Pure recombinant human Epo (specific activity 104 U/μg; Amgen, Thousand Oaks, CA) was labeled to high specific radioactivity (6.6 to 9 × 103 cpm/μg) equivalent to 0.75 to

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RESULTS

Binding of Epo to K562 cells. All binding studies were carried out at 37°C with sodium azide to minimize internalization of Epo. Under these conditions >85% of cell-associated 125I-rHuEpo was released from the cell surface at pH 4. The time course of binding of 125I-rHuEpo to K562 cells is shown in Fig 1. Specific binding, determined by comparing total binding of 125I-rHuEpo at saturation with that in the presence of a 40-fold excess of unlabeled Epo, increased over the first 60 minutes and thereafter remained constant. Consequently, an incubation time of 75 to 90 minutes was used in subsequent experiments. Binding of 125I-rHuEpo to control K562 cells was saturable (Fig 2), corresponding to between 4 and 6 Epo receptors per cell (Fig 1 through 5, Table 1). Scatchard analysis of binding revealed a single class of high-affinity receptors having a KD of 270 pmol/L.

Fig 1. Time course of binding of 125I-rHuEpo to K562 cells. K562 cells (1 x 10⁵) were incubated in duplicate with near saturating concentrations of 125I-Epo (2.5 nmol/L) in the presence and absence of 40-fold excess unlabeled Epo for various times up to 90 minutes. Specific binding (1-5% to 10% of total binding) was calculated for each time point as described in the Materials and Methods section. Error bars are the sum of the SEM of total and nonspecific binding.

Fig 2. Saturation binding of 125I-rHuEpo to control K562 cells. K562 cells (1 x 10⁵) were incubated in duplicate with near saturating concentrations of 125I-Epo (2.5 nmol/L) in the presence and absence of 40-fold excess unlabeled Epo for various times up to 90 minutes. Specific binding (1-5% to 10% of total binding) was calculated for each time point as described in the Materials and Methods section. Error bars are the sum of the SEM of total and nonspecific binding.
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values after 12 to 16 hours of treatment with U937CM
doubling time was observed. That the effect of U937CM on
analysis were incubated with increasing concentrations
1251-Epo
incubated with 20% U937CM for 24 hours immediately prior to
U937CM-treated K562 cells. Control cells and cells that had been
Culture
treated K562.

Epo receptor expression on K562
cells. Cultures of K562 cells with 20% U937CM for 24 hours increased receptor expression by 3.5-fold (Table 1, Fig 3). Epo receptor expression on K562 cells reached maximum values after 12 to 16 hours of treatment with U937CM (results not shown) and remained at plateau values for at least 4 days (Table 1). During this period no effect on cell doubling time was observed. That the effect of U937CM on Epo receptor expression on K562 cells was reversible is also shown in Table 1 as subsequent culture of cells for 3 days in inducer-free medium resulted in Epo receptor expression returning to normal. In contrast to the effect of U937CM on Epo receptors, culture of K562 cells with 10% 5637CM had no effect on receptor expression. Scatchard analysis of Epo binding to U937CM-treated K562 cells showed a single class of high-affinity receptors having a K_D of 280 pmol/L (Fig 3). In three separate binding experiments, induced K562 cells expressed 13 to 23 receptors per cell with a K_D range of 245 to 300 pmol/L.

Effect of recombinant EPA on Epo receptor expression on K562. That the increased expression of Epo receptors on K562 cells following treatment with U937CM was due to EPA is indicated in Fig 4. At concentrations at which EPA is active in stimulating the growth of erythroid progenitor cells “in vitro,”3,21 EPA induced a 2.8 to 3.6-fold increase in Epo receptor expression on K562 cells.

Effect of K562 cell growth on Epo receptor expression. Recently, an EPA-mediated autocrine mechanism for growth stimulation of K562 cells was proposed based on the observation of EPA production by K562 and expression of specific high-affinity EPA receptors on K562 (J. Gasson, personal communication, July, 1986). All experiments described in the present study used K562 maintained at low to medium cell density (<1 x 10^6 cells/mL) growing in log phase. Thus, endogenous EPA in these cultures was maintained at low levels. To examine the effect of endogenous EPA on Epo receptors on K562 cells, cultures were allowed to expand to high cell density over a period of 8 days, and the responsiveness of Epo receptors to U937CM was determined. Figure 5 shows that Epo receptors increased with cell growth such that by the sixth day of culture, Epo receptor expression had increased to maximum levels previously observed on U937CM-induced or EPA-induced cells. Neither treatment with U937CM for 24 hours (Fig 5) nor recombinant EPA (results not shown) further increased this Epo binding. Treatment of control K562 cells (log phase, low density) for 18 hours in the presence of 50% supernatant from day 8 K562 cultures, resulted in a 2.5 to 3.5-fold increase in Epo binding to K562. Thus, K562 cells maintained at low cell density respond to EPA by increasing expression of Epo receptors 2.5 to 3.5-fold, whereas K562 cells grown to high cell density express maximum Epo receptor levels and show an associated loss of EPA responsiveness.

![Fig 3](image-url) Scatchard analysis of Epo binding to control and U937CM-treated K562 cells. Control cells and cells that had been incubated with 20% U937CM for 24 hours immediately prior to analysis were incubated with increasing concentrations of 1251-Epo (17 to 2,500 pmol/L) in the presence and absence of 40-fold excess unlabeled Epo. Specific binding data were plotted for Scatchard analysis; ■-■, control K562; ■-■, U937CM-treated K562.

![Fig 4](image-url) Concentration dependence of the effect of recombinant EPA on Epo receptors on K562. K562 cells that had been cultured for 24 hours in the presence of recombinant EPA concentrations ranging from 1 pmol/L to 5 nmol/L immediately prior to analysis were incubated with near saturating concentrations of 1251-Epo (2.5 nmol/L) in the presence and absence of 40-fold excess unlabeled Epo. Control K562 cells (no exogenously added EPA) were also included and were not significantly different from cells cultured in the presence of 1 pmol/L EPA. Error bars are the sum of the SEM of total and nonspecific binding.

![Fig 5](image-url) Effect of prolonged culture on Epo receptor expression and EPA sensitivity of K562 cells. K562 cells were allowed to expand in culture for up to 8 days without feeding. Every second day, cells were removed and Epo binding was determined in duplicate immediately or after culture for 24 hours in the presence of 20% U937CM in fresh medium. Epo binding to K562 cells, solid bars; Epo binding of K562 cells after treatment with U937CM, hatched bars. Error bars are the sum of the SEM of total and nonspecific binding.
counts, hatched areas; error bars, SEM.

tions of both conditioned media were 20%. CFU-E were scored using an inverted microscope on day 3 of culture. Control CFU-E and in the absence of additional Epo (B). The starting concentration of K562.

The effects of U937CM and Epo on K562 plating efficiency and hemoglobinization in semisolid culture are shown in Table 2. Epo alone had no effect on plating efficiency or hemoglobinization. In contrast, U937CM induced a 24% increase in the plating efficiency of K562 that was not significantly affected by coculture with 1 U Epo/mL. Although hemoglobinization remained unaffected in the presence of U937CM alone, when U937CM and Epo were added together an Epo-dependent increase in the number and percentage of colonies that expressed hemoglobin was observed. The percentage of hemoglobin-positive colonies increased by 33%, and the total number of hemoglobin-positive colonies increased by 70%.

**Effect of U937CM and U937CM on cord blood CFU-E differentiation.** To determine whether the effects of U937CM and Epo on erythroid differentiation of K562 cells were paralleled in normal erythropoiesis cord blood CFU-E were studied. Figure 6 shows that in the presence or absence of exogenously added Epo (1 U/mL), U937CM caused a concentration-dependent increase in cord blood CFU-E. In contrast, 5637CM inhibited cord blood CFU-E growth in the absence of added Epo but had no significant effect in the presence of Epo. When CFU-E were analyzed on the basis of size, the U937CM effect in the presence of Epo was largely confined to colonies of <32 cells presumably arising from more mature CFU-E progenitors (Table 3).

**DISCUSSION**

In the present study, we describe expression of a single class of saturable high-affinity receptors for Epo on the surface of cells of the human erythroleukemic cell line K562.

**Table 1. Effect of U937CM on Epo Binding to K562 Cells**

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>Total Epo (cpm)</th>
<th>Non-specific</th>
<th>Specific</th>
<th>Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14,796 ± 214</td>
<td>13,298 ± 107</td>
<td>1,498 ± 321 (P &lt; .025)</td>
<td>100 ± 21</td>
</tr>
<tr>
<td>20% U937CM</td>
<td>24 h</td>
<td>18,304 ± 227</td>
<td>13,086 ± 177</td>
<td>5,219 ± 404 (P &lt; .005)</td>
</tr>
<tr>
<td>96 h</td>
<td>18,992 ± 224</td>
<td>13,975 ± 160</td>
<td>5,017 ± 384 (P &lt; .005)</td>
<td>335 ± 26</td>
</tr>
<tr>
<td>96 + 72 h</td>
<td>14,691 ± 151</td>
<td>13,162 ± 46</td>
<td>1,529 ± 217 (P &lt; .01)</td>
<td>102 ± 14</td>
</tr>
<tr>
<td>Inducer-free</td>
<td>14,666 ± 199</td>
<td>13,189 ± 92</td>
<td>1,477 ± 291 (P &lt; .025)</td>
<td>99 ± 19</td>
</tr>
</tbody>
</table>

*Binding determined using 2.5 μmol/L 125I-Epo with or without a 40-fold excess of unlabeled Epo. SE associated with specific binding were obtained by adding the individual SE of total and nonspecific binding.

**Table 2. Effect of Epo and U937CM on K562 Plating Efficiency and Hemoglobinized Colony Formation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Colonies Per Plate</th>
<th>Control (%)</th>
<th>Hb* per Plate</th>
<th>Control (%)</th>
<th>Hb* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>437 ± 12</td>
<td>100 ± 3</td>
<td>122 ± 5</td>
<td>100 ± 4</td>
<td>28.3 ± 1.2</td>
</tr>
<tr>
<td>+ Epo</td>
<td>426 ± 11</td>
<td>99 ± 3</td>
<td>121 ± 7</td>
<td>100 ± 6</td>
<td>28.4 ± 1.1</td>
</tr>
<tr>
<td>+ U937CM</td>
<td>537 ± 15</td>
<td>124 ± 3</td>
<td>157 ± 6</td>
<td>129 ± 4</td>
<td>29.3 ± 1.1</td>
</tr>
<tr>
<td>+ Epo + U937CM</td>
<td>560 ± 20</td>
<td>130 ± 4</td>
<td>209 ± 8</td>
<td>171 ± 4</td>
<td>37.3 ± 1.3</td>
</tr>
</tbody>
</table>

Data are mean ± SE.

**Fig 6.** Effect of U937CM and 5637CM on Epo-dependent and Epo-independent growth of cord blood CFU-E. Cord blood mononuclear cells (1.6 x 10⁶) were cultured in methylcellulose in the presence of decreasing concentrations of U937CM (●●●●) and 5637CM (□□□□□) in the presence of saturating Epo (1 U/mL) (A), and in the absence of additional Epo (B). The starting concentrations of both conditioned media were 20%. CFU-E were scored using an inverted microscope on day 3 of culture. Control CFU-E counts, hatched areas; error bars, SEM.
These receptors are present at five sites per cell on the average. The $K_D$ of this binding is 260–290 pmol/L, a value similar to that which we observed for binding of radiolabeled recombinant human Epo to human bone marrow cells $K_D = 284$ pmol/L (J.K. Fraser and M.V. Berridge, manuscript in preparation) suggesting that the Epo receptor on K562 cells may be similar to that expressed on normal erythroid progenitor cells. A previous attempt to demonstrate Epo binding to K562 cells was not successful, but this study used $^{125}$I-Epo of much lower specific activity.

Several receptors for other human hematopoietic growth factors have now been characterized. For example, Dower et al. showed that the peripheral blood IL1 receptor is expressed at ~100 sites/mononuclear cell ($K_D = 17$ pmol/L), whereas Nicola et al. exploited the cross-species reactivity of murine G-CSF to show that the human neutrophil CSFβ receptor is expressed at 1,100 sites/cell ($K_D = 880$ pmol/L). The human receptor for GM-CSF has also been characterized and is expressed on the average at ~80 sites/neutrophil ($K_D = 17$ pmol/L). Thus, the receptor for Epo on K562 cells is within the range of affinities measured for other human hematopoietic growth factors although the level of expression is much lower. Whether this low level of Epo receptor expression is a reflection of low expression on erythroid precursor cells in bone marrow is not known, but the normal multipotential counterpart of K562 may also express low levels of Epo receptors.

In comparison with the present results, Friend virus-infected murine erythroblasts express 600 to 700 Epo receptors per cell ($K_D = 5.2$ nmol/L). More recent investigations have shown that these cells express two classes of receptors at 800 and 1,800 sites/cell ($K_D = 0.3$ and 1.8 nmol/L, respectively). Thus, the high-affinity receptor on these cells has a similar binding constant to the receptor on K562 cells and human marrow (see above).

In addition to demonstrating Epo receptor expression on K562 cells, we showed that recombinant EPA, and conditioned media containing EPA, increase Epo receptor expression of K562 cells without affecting receptor affinity. EPA exhibits erythroid lineage-specific stimulatory activity for both human and murine BFU-E and CFU-E (see Fig 4) and also stimulates the cloning efficiency of K562 (Table 2). Thus EPA may modulate cell growth by increasing Epo receptor expression. That K562 cells express autocrine secretion of Epo (J. Gasson, personal communication, July, 1986) which in turn up-regulates Epo receptors (Fig 5) may explain the sensitivity of U937CM-stimulated K562 cells to Epo (Table 2).

Modulation of expression of the GM-CSF receptor on the GM-CSF-responsive cell line HL-60 has also been described. In the unstimulated state, this receptor was shown to be present at an average of ~35 receptors/cell ($K_D = 14$ pmol/L) but when HL-60 was stimulated to differentiate with dimethylsulfoxide, receptor expression increased markedly to ~250 sites/cell ($K_D = 30$ pmol/L). This modulation of receptor expression has been described in a number of other systems. Transforming growth factor-β induces synthesis of epidermal growth factor receptors on fibroblasts, whereas in the murine hematopoietic system IL 3 and GM-CSF down-modulate expression of the receptors for G-CSF and M-CSF in short-term incubation and IL 3 upregulates M-CSF receptor expression in longer term. Generally, these effects are not associated with an alteration in $K_D$. Thus, hematopoiesis may be regulated by two interrelated systems, first by changes in the plasma concentration of the primary agonist and second by modification of the sensitivity of cells to the agonist by the action of a second factor on receptor expression. In acclimatization to high altitude, enhanced erythropoiesis is, at first, induced by increased plasma Epo, but with time Epo returns to normal while maintaining increased erythropoiesis. Consequently, the sensitivity of the erythropoietic system to Epo appears to be increased, possibly by alterations at the level of the Epo receptor.

The demonstration that EPA is identical to the tissue inhibitor of metalloproteinases (TIMP), which is present at relatively high concentration in normal plasma, is difficult to reconcile with a sensitive regulatory role in erythropoiesis. The concentration of EPA in plasma may not accurately reflect its concentration in the bone marrow microenvironment, however. Furthermore, it is difficult to explain the erythroid lineage-specific action of EPA in vivo and in vitro, the presence of specific high-affinity cell surface receptors for EPA (J. Gasson, personal communication, July, 1986), and the effect of EPA on Epo receptor expression in terms of a ubiquitous protease inhibitor. In addition, the concentration at which EPA is active on CFU-Es is similar to the concentration at which it is active in stimulating Epo receptor expression on K562 and K562 growth.

Overall, the similarity of the response of K562 and CFU-E to U937CM and to EPA suggests that the mechanisms of these responses might be similar, that is, that increased sensitivity of CFU-E to Epo might be a consequence of an EPA-induced increase in Epo receptor expression on CFU-E and their Epo-sensitive progeny.

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