Dynamics of Erythropoietin Receptor Expression on Erythropoietin-Responsive Murine Cell Lines

By Virginia C. Broudy, Betty Nakamoto, Nancy Lin, and Thalia Papayannopoulou

We examined erythropoietin receptor expression in two murine cell lines, B6SUtA and DA-1, that respond to erythropoietin in different ways. While B6SUtA cells undergo erythroid differentiation with limited proliferation after addition of erythropoietin, DA-1 cells show only a proliferative response. Equilibrium binding experiments with $^{125}$I-erythropoietin revealed that both B6SUtA and DA-1 cells express a single class of erythropoietin receptors. In the absence of erythropoietin, B6SUtA cells exhibited 145 receptors per cell with a dissociation constant (kd) of 380 pmol/L. Six days after induction with erythropoietin, the B6SUtA cells displayed 310 receptors per cell without a change in binding affinity; exposure to erythropoietin also increased cellular hemoglobin content. DA-1 cells adapted to erythropoietin-dependent growth over a period of months and exhibited a progressive increase in erythropoietin receptor expression, from 85 per cell (kd = 540) to 550 per cell (kd = 530), although the cells remained uniformly benzidine-negative. We interpret the data with B6SUtA cells to indicate that early erythroid differentiation stages are attended by an increase in erythropoietin receptor display, coordinate with the initiation of expression of erythroid-specific genes. In contrast, the results with DA-1 cells are most compatible with clonal selection as the mechanism underlying enhanced receptor expression. Thus, display of the erythropoietin receptor is dynamic and can be modulated during the course of erythropoietin-induced differentiation.

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**METHODS**

**Erythropoietin and hematopoietic growth factors.** The $^{125}$I-erythropoietin used for these studies had a specific radioactivity of 300 to 900 Ci/mmol. Recombinant human erythropoietin expressed in baby hamster kidney cells and partially purified by chromatography on CM Affi-Gel Blue (Bio-Rad, Richmond, CA) was used for cell culture.$^{21,22}$ The biologic activity of this preparation of erythropoietin was approximately 6,000 U/mg of glycoprotein.

Recombinant murine interleukin-3 (IL-3) and murine granulocyte-macrophage colony-stimulating factor (GM-CSF) were expressed in COS cells and were provided by Dr K. Kaushansky (University of Washington, Seattle, WA). The concentration of IL-3 or GM-CSF in COS cell conditioned medium was determined by colony-forming assays.$^{23}$ For experiments with the B6SUtA cell line, the source of growth factors was medium conditioned by the WEHI-3 cell line.$^{24}$

**Cell culture.** The DA-1 murine lymphoblastoid cell line was obtained from Dr L. Guilbert (University of Alberta, Edmonton, Alberta)$^{25}$ and was cultured in Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 5% heat-inactivated fetal calf serum (FCS; Armour Pharmaceutical Co, Terreton, NY) and 100 U/mL of GM-CSF or 20 U/mL of IL-3. To select a subclone capable of growing in erythropoietin alone, the DA-1 cells were cultured for 1 to 5 months in IMDM supplemented with 5% FCS and 2.5 U/mL erythropoietin. To determine whether continuous presence of erythropoietin is required for receptor expression, DA-1 cells were cultured in IMDM supplemented with 5% FCS, 20 U/mL of IL-3, and polyclonal anti-erythropoietin antibodies$^{26}$ sufficient to neutralize the quantity of erythropoietin potentially present in FCS. The neutralizing ability of the antibody preparation was tested in murine erythroid colony-forming assays.$^{27}$ The effect of serum on erythropoietin receptor numbers and binding affinity was examined by culturing DA-1 cells in serum-free medium$^{28}$ supplemented with 20 U/mL IL-3. DA-1 cells were maintained in

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parallel in serum-replete medium as described above. Receptors were quantitated after 7 days of culture.

The B6SUtA cell line, obtained from Dr. J. Greenberger (University of Massachusetts Medical Center, Worcester, MA), was maintained in Kincaide's medium with 15% FCS and WEHI-3 conditioned medium as a source of IL-3. To determine the effect of differentiation on erythropoietin receptor expression, the B6SUtA cells were cultured in IMDM supplemented with 30% FCS, 10% human AB plasma, 1% bovine serum albumin (Reheis, Phoenix, AZ), 100 mmol/L β-mercaptoethanol, and WEHI-3 conditioned medium (inducing medium), with or without 2.5 U/mL erythropoietin for periods of time varying from 6 hours to 6 days. To achieve a higher proportion of benzidine-positive cells in some experiments, the B6SUtA cells were cultured in inducing medium with erythropoietin but without WEHI-3-conditioned medium. The fraction of cells stained with benzidine was counted just before measurement of erythropoietin receptor numbers. The effect of serum on erythroid differentiation of B6SUtA cells was examined by culturing the cells under serum-deprived conditions supplemented with 2.5 U/mL erythropoietin for 7 days as described, omitting the methycellulose. FBL-3 and EL-4 nonerythroid murine cells were used as a control for the erythropoietin binding experiments (obtained from Dr. J. Klarner, University of Washington).

In a parallel in serum-replete medium as described above. Receptors were quantitated after 7 days of culture.

**Results**

B6SUtA and DA-1 demonstrated specific binding of 125I-erythropoietin, while EL-4 and FBL-3 did not. Experiments presented here showed that erythropoietin promoted the growth and erythroid differentiation of B6SUtA cells (Table 1) in agreement with previous data. This also held true under serum-free conditions (Fig 1). Over the course of 6 days, an increased number of receptors per cell was evident concomitant with an increased fraction of hemoglobinized cells (Fig 2). B6SUtA cells maintained in WEHI-3-conditioned medium displayed 145 receptors per cell with a kd of 380 pmol/L; these cells were benzidine negative. When cultured with WEHI-3-conditioned medium plus erythropoietin (2.5 U/mL) for 3 days, the B6SUtA exhibited 190 receptors per cell; 10% of these cells stained with benzidine. After a 6-day incubation in WEHI-3-conditioned medium plus erythropoietin, the B6SUtA cells expressed 310 receptors per cell, and 40% of the cells were benzidine-positive (Fig 2). Similar results were obtained in three additional experiments (Table 2). The highest fraction of benzidine positive cells (up to 83%) was observed when B6SUtA cells were cultured with erythropoietin in the absence of WEHI-3-conditioned medium (Table 2). Both erythropoietin-induced and uninduced cells displayed a single class of erythropoietin receptors, and the kd did not change with erythroid differentiation (Fig 2).

To determine how rapidly increased erythropoietin receptor expression occurs, erythropoietin (2.5 U/mL) was added to the B6SUtA culture medium and receptors were quantitated at various times from 6 to 24 hours later. The B6SUtA cells displayed 140 receptors per cell before the addition of erythropoietin. No change in receptor expression was seen at 6 or 12 hours after adding erythropoietin. Increased receptor expression (260 receptors per cell) was first evident 24 hours after the addition of erythropoietin.

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Benzanidine (+)</th>
<th>Benzanidine (−)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>WEHI-3 CM</td>
<td>1.9</td>
<td>56.1</td>
<td>58.0</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>36.8</td>
<td>7.5</td>
<td>44.3</td>
</tr>
</tbody>
</table>

B6SUtA cells (10 × 10⁶) were cultured in inducing medium containing WEHI-3 conditioned medium (CM) or in inducing medium plus 2.5 U/mL of erythropoietin, without WEHI-3 conditioned medium. After 5 days, the total number of cells and the fraction of cells staining with benzidine were counted. Results are expressed as cell number x 10⁶.
kd of 540 ± 160 pmol/L (mean ± SEM of three experiments). In the presence of IL-3 (20 U/mL), the cells exhibited 144 ± 41 erythropoietin receptors per cell, with a kd of 500 ± 100 pmol/L (mean ± SEM of four experiments).

 Addition of neutralizing anti-erythropoietin antibodies to the DA-1 culture medium did not alter erythropoietin receptor expression. DA-1 cells cultured in IMDM plus 5% FCS and 20 U/mL IL-3 with or without anti-erythropoietin antibodies for 7 days expressed a similar number of receptors per cell (two experiments, data not shown). These results indicate that display of the erythropoietin receptor does not require the continuous presence of erythropoietin.

 To select a subclone of DA-1 cells capable of growing in erythropoietin alone, erythropoietin (2.5 U/mL) was substituted for IL-3 in the culture medium. Surviving cells were maintained in erythropoietin, without any other source of hematopoietic growth factors, for up to 6 months. Erythroid differentiation of the cells (assessed by benzidine staining) did not occur during culture with erythropoietin. The number and affinity of erythropoietin receptors was determined at various times after the DA-1 cells were established in erythropoietin (Table 3, Fig 3). There was a progressive increase in receptor expression over the first 3 months without a change in the affinity of the receptor for erythropoietin. Receptor expression appeared to reach a plateau at approximately 500 receptors per cell between 3 and 5 months (Table 3). A single class of binding sites was evident throughout the culture period (Fig 3). DA-1 cells cultured in erythropoietin for 3 months retained their ability to respond to IL-3; substitution of IL-3 (20 U/mL) for erythropoietin as the source of growth factor sustained cell growth for more than 2 months. To examine the effect of serum on erythropoietin receptor expression, DA-1 cells that had been maintained in erythropoietin for more than 1 year were cultured under serum-deprived or serum-supplemented conditions for 7 days, and receptors were quantitated. The serum-deprived DA-1 cells displayed 590 receptors per cell (kd = 760 pmol/L), while the DA-1 cells cultivated in serum-containing medium exhibited 500 receptors per cell (kd = 680 pmol/L).

**DISCUSSION**

B6SuTA and DA-1 are multipotent cell lines arrested in differentiation at an early developmental stage, a stage at
which their normal counterparts are sparse and difficult to purify. These cell lines respond to a number of hematopoietic growth factors. Thus, the B6SUtA and DA-1 cell lines offer a convenient model in which to examine the dynamics of receptor expression during continuous proliferation or differentiation to hemoglobinized cells.

Data presented here suggest that erythropoietin can increase the expression of its receptor in a population of cells undergoing erythroid differentiation/maturation. Increased display of the erythropoietin receptor occurred over a period of 1 to 6 days concomitant with an increase in cellular hemoglobin content in B6SUtA cells. In contrast to these results, other investigators have found that erythropoietin receptor numbers decrease as cell lines or erythroid precursor cells are cultured with erythropoietin. Fukamachi et al found a decrease in the number of erythropoietin receptors on TSA8 cells cultured in erythropoietin for 48 hours, although a very high concentration of erythropoietin (20 U/mL) was used. Fraser et al observed that supraphysiologic levels of IL-3 (2500 U/mL) or erythropoietin (50 U/mL) downmodulate erythropoietin receptors on murine erythroblasts within 1 to 2 hours. A decrease in erythropoietin receptor expression was seen when erythroid precursor cells derived from fetal mouse liver were cultured with erythropoietin. However, a majority of the fetal mouse liver cells were hemoglobinized before exposure to erythropoietin, and erythropoietin receptor expression was thus examined during late erythroid maturation. A similar decline in erythropoietin receptor expression occurred during suspension culture of the fetal liver cells, even in the absence of erythropoietin. The differences between this report and our data probably reflect differences in the target cells selected for study. The B6SUtA cell line represents cells early in hematopoietic differentiation (at least bipotent), since they can form mixed colonies containing neutrophils and basophils in semisolid medium; by contrast, the murine erythroblasts and fetal liver cells used in previous studies are a more mature population of cells. There is an increase in erythropoietin receptor expression as B6SUtA cells differentiate to early erythroblasts; however, these cells do not progress to the terminal level of erythroid maturation and enucleation achieved by fetal liver cells or normal marrow cells.

interest, display of the IL-3 receptor decreases dramatically as the B6SUtA cells undergo erythroid differentiation.

Enhanced erythropoietin receptor expression seen with erythropoietin-induced differentiation of B6SUtA cells could be due to an increase in either the number of receptors on individual B6SUtA cells or the fraction of receptor-bearing cells. Selective survival of erythropoietin receptor-bearing cells is certainly a factor in the present experiments with the DA-1 cells. However, the magnitude of the expansion in hemoglobinized B6SUtA cells could only be accounted for if benzidine-positive cells had multiplied at an unacceptably high and much faster rate than benzidine-negative cells (Fig 1). These data suggest that erythropoietin recruited some initially benzidine-negative B6SUtA cells to become benzidine-positive. Since benzidine-negative B6SUtA cells display fewer receptors than benzidine-positive cells (Table 2, Fig 2), erythropoietin-induced benzidine positivity is accompanied by a higher receptor number per cell. Alternatively, some benzidine negative B6SUtA cells may lack erythropoietin receptors. High levels of receptor expression could be present on other benzidine negative cells, and erythropoietin may selectively amplify this subset of cells. We cannot currently discriminate between these two possibilities. However, either interpretation suggests that during early stages of erythroid differentiation, the average number of receptors per cell increases. This is in agreement with autoradiographic analysis demonstrating that binding of erythropoietin increases as early BFU-E mature to CFU-E in the presence of erythropoietin and IL-3, although the latter experiments do not indicate whether enhanced binding is due to an increase in receptor numbers or in affinity.

Both B6SUtA and DA-1 cells express a single class of low affinity erythropoietin receptors, yet are erythropoietin responsive. Failure to detect a low number of high affinity receptors, already occupied by erythropoietin present in serum used to culture the cells, is unlikely in that experiments with serum-free media did not reveal an occult high affinity site. Thus, our results document that erythropoietin can stimulate proliferation or differentiation by binding to a low affinity site. JK-1 cells exhibit a high affinity site, yet respond to erythropoietin with proliferation alone. These observations indicate that expression of a high or low affinity erythropoietin receptor does not predict the cellular response to erythropoietin (proliferation and/or differentiation). Murine erythropoietin-responsive cell lines have been reported to display one (Rauscher, SKT6) or two (TSA8) classes of receptors. The number of classes of erythropoietin receptors on normal erythroid precursor cells remains controversial.

ACKNOWLEDGMENT

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