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Specific binding of erythropoietin to its receptor on responsive mouse erythroleukemia cells

(erythroid differentiation/affinity labeling/Scatchard analysis/receptor molecular weight/Friend cells)

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ABSTRACT **Erythropoietin (Epo) is a glycoprotein factor that specifically regulates the proliferation and differentiation of erythroid progenitor cells. Here we describe the isolation of Epo-responsive mouse erythroleukemia cell line SKT6, the characterization of the specific binding of biologically active ^{125}I-labeled human Epo (^{125}I-Epo) to its membrane receptor, and, finally, report information concerning the molecular structure of the receptor. About 75% of erythroid colony-forming precursor cell-like colonies derived from SKT6 cells were hemoglobin-positive after 3- to 4-day exposure to Epo in methylcellulose culture. Radioiodinated Epo bound specifically to SKT6 cells, and Scatchard analysis of the data showed a high affinity for ^{125}I-Epo (K_d = 0.15 nM) but displayed only a small number of specific receptors (≈470 per cell). Membrane components that specifically interact with ^{125}I-Epo were identified by covalent crosslinking with disuccinimidyl suberate, and three receptor species with apparent M_r 63,000, 94,000, and 119,000 were found in membrane from SKT6 cells, suggesting the complex structure of the receptor molecules. Specific bindings were also detected in all of the Epo-unresponsive Friend erythroleukemia cells examined, and crosslinking study revealed the presence of only the 63,000 species as a binding site.**

In vitro hemopoiesis is regulated by a set of specific glycoproteins that affect the proliferation and differentiation of the progenitor cells (1). Among these humoral factors, two glycoproteins specific for erythropoietic lineage have been identified by recombinant DNA technology, and their physiological functions have been clarified (2–5). Burst-promoting activity, which was identified as one of the interleukin 3 (IL-3) activities (2), enhances proliferation of immature erythroid cells (BFU-E). Erythropoietin (Epo) acts on relatively mature erythroid progenitor cells (CFU-E) and induces differentiation of the cells to mature erythrocytes (3, 4).

Epo has been purified to homogeneity from the urine of patients with aplastic anemia (6). Clones of complementary and/or genomic DNA encoding the human and mouse Epo were recently isolated, and recombinant human Epo is produced in mammalian cells (3, 4, 7). However, very little is known about the molecular mechanism of the erythroid cell differentiation induced by this hormone, owing mainly to the difficulty of obtaining sufficient quantities of pure target cells and purified hormone.

To study the nature of the cells responsive to Epo and to gain insight into the mechanism by which the Epo receptor mediates the response to Epo, we established an Epo-responsive mouse erythroleukemia cell line SKT6 and characterized the specific binding of recombinant Epo to its membrane receptor; from these results we briefly discuss the molecular structure of the Epo receptor.

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MATERIALS AND METHODS

Proteins and Virus. Purified human erythropoietin (70,000 units/mg; M_r 34,000) produced by genetically engineered Chinese hamster ovary cells was supplied by T. Kaneko (Kirin Brewery, Tokyo). Recombinant mouse IL-3 from COS-7 cells was the gift of K. Arai (DNAX Institute, Palo Alto, CA). Anemia-inducing Friend leukemia virus complex (FVA) was obtained from C. Friend (City University of New York).

Isolation of Epo-Responsive Cell Line SKT6. Female 6-week-old DBA/2J mice (Charles River Breeding Laboratories) were injected intravenously with ≈10^4 spleen focus-forming units of FVA. Eight to ten weeks after infection the mice were sacrificed, and a single cell suspension of spleen cells was maintained in Ham's F-12 medium supplemented with 10% fetal calf serum [HyClone (Logan, UT) lot 100411]; fetal calf serum that does not induce differentiation of SKT6 cells was used. Clonal cell lines were obtained by limiting dilution. A cell line, SKT6, highly responsive to Epo was established after several reclonings and was maintained in suspension for over 1.5 years.

Erythroid Colony Assay. Logarithmically growing SKT6 cells (1×10^5) were plated in 1 ml of 0.9% methylcellulose (Dow A4M) in Ham's F-12 medium supplemented with 10^{-4} M 2-mercaptoethanol, 1% pure bovine serum albumin (Calbiochem), and 30% fetal calf serum, with or without pure recombinant human Epo (2 units/ml unless indicated), recombinant mouse IL-3 (30 units/ml), and pure human holotransferrin (10 ng/ml). The hemoglobin-positive cells in the culture were stained by the modified procedure of Ikawa *et al.* (8). A lower concentration of benzidine (0.2%) was used to avoid crystallization.

Radioiodination of Epo. Recombinant human Epo was labeled using Iodo-Gen (Pierce) (9) and carrier-free ^{125}I in NaOH solution (pH 8.5, 100 mCi/ml; 1 Ci = 37 GBq), and was purified using Sephadex G-25 gel filtration in phosphate-buffered saline containing 0.02% Tween-20. The determined specific radioactivity of ^{125}I-labeled Epo (^{125}I-Epo) ranged from 8.0×10^5 Bq/μg to 1.4×10^6 Bq/μg for different preparations (0.44–0.79 ^{125}I atom per Epo molecule). ^{125}I-Epo prepared this way retained full biological activity when assayed by SKT6 cell colony formation.

^{125}I-Epo Equilibrium Binding to Cells. To detect binding of ^{125}I-Epo, cells were grown to the logarithmic phase, harvested by centrifugation, and washed twice in Ham's F-12 medium containing 10% fetal calf serum (binding medium). Five $\times 10^6$ cells in 100 μl of binding medium containing ^{125}I-Epo at various concentrations was incubated with or without 0.2 μM (40-fold or more excess) unlabeled Epo at 10°C for 4 hr. In the binding kinetics experiments, cells (5 ×

Abbreviations: Epo, erythropoietin; ^{125}I-Epo, ^{125}I-labeled Epo; FVA, anemia-inducing Friend virus complex; IL-3, interleukin 3; CFU-E, colony-forming erythroid precursor cells.

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10^6 in 100 μl of binding medium) were incubated with 120 pM ^{125}I-Epo at either 10°C or 37°C. Following incubation, aliquots of cells were filtered through a glass filter (Whatman GF/A). The filters were washed with 12 ml of phosphate-buffered saline, dried, and the radioactivities retained on the filters were determined. Results were corrected for nonspecific binding of ^{125}I-Epo obtained in the presence of excess unlabeled Epo and represent specific binding of ^{125}I-Epo.

Affinity Labeling of ^{125}I-Epo to Its Receptor. Cells were incubated with 0.75 nM ^{125}I-Epo with or without 75 nM unlabeled Epo for 30 min at 37°C, and crosslinking of bound ^{125}I-Epo to its receptor was done essentially as described by Pilch and Czech (10). After being washed twice with ice-cold phosphate-buffered saline, the cell surface proteins were crosslinked with 0.3 mM disuccinimidyl suberate (Nakarai) for 60 min at 4°C. Cells were washed with 50 mM Tris·HCl, pH 6.8, and the cell surface proteins were extracted in 50 mM Tris·HCl, pH 6.8, containing 1.5% Triton X-100. ^{125}I-Epo crosslinked complexes were analyzed by $NaDodSO_4$/PAGE using 10% acrylamide separating gels with or without 5% 2-mercaptoethanol, according to the method of Laemmli (11). Gels were dried and subjected to autoradiography on Kodak XAR-5 film at −70°C in the presence of an intensifying screen.

RESULTS

Isolation of Epo-Responsive Cell Line. Mouse erythroleukemia cells induced by FVA were maintained in suspension, and clonal cell lines were isolated after limiting dilution. The potential of the clonal cell lines to differentiate in the presence of Epo was examined by incubating cells with recombinant Epo (0.5 unit/ml) in suspension cultures for 4 days and monitoring the percentage of hemoglobin-positive cells. Following several recloning procedures, a cell line, SKT6, that showed the highest response to Epo (over 70%) was established and maintained in suspension over 1.5 years. It was necessary to use the fetal calf serum containing negligible Epo activity to keep the SKT6 cells highly responsive to Epo in the long-time culture.

Colony-Forming Assay for SKT6 Cells. When SKT6 cells were cultured in methylcellulose in the presence or absence of 2 units of Epo per ml for 3–4 days, they formed erythroid colonies of 8–32 cells, similar to those derived from relatively mature erythroid progenitor cells (CFU-E) from normal bone marrow (Fig. 1). The plating efficiency was about 40%. The cells within colonies were relatively large and homogeneous in size. Colonies formed in the presence of Epo were morphologically indistinguishable from those formed without Epo. Only in the presence of Epo, however, were the hemoglobin-positive colonies formed.

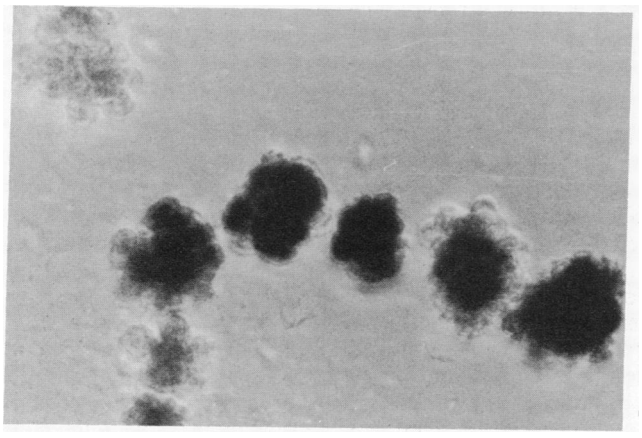

FIG. 1. CFU-E-like colonies from Epo-responsive SKT6 cells. SKT6 cells were plated in methylcellulose in the presence of Epo (2 units/ml) as described. Colonies with hemoglobin were stained by benzidine (0.2%) after 4-day culture. Several hemoglobin-positive and one hemoglobin-negative colonies are seen.

Fig. 2 shows the kinetics of hemoglobinized colony formation with and without Epo. The number of colonies formed was about the same whether or not Epo was present. The rate of colony formation was slow compared with normal CFU-E, which forms hemoglobin-positive colonies 2 days after plating. A high percentage (≈75%) of the colonies formed were hemoglobin-positive in the presence of 2 units of Epo per ml after 3–4 days, whereas only 1–4% of the colonies formed were hemoglobin-positive in the absence of Epo. A small number of partly stained colonies consisting of hemoglobin-positive and -negative cell clusters were also detected.

Supplement of recombinant mouse IL-3 (30 units/ml) and/or human holotransferrin (10 ng/ml) in methylcellulose culture did not affect the percentage of hemoglobin-positive colonies, the kinetics of colony formation, or the morphology of the colonies, whether or not Epo was present. Some decline of the percentage of hemoglobin-positive colonies was observed 5 days after plating as shown in Fig. 2, but it was suppressed by the addition of transferrin. No burst-forming erythroid progenitor (BFU-E)-like colonies were observed even after 2-week culture with recombinant IL-3 and Epo.

Effect of Epo Concentration on Formation of Hemoglobin-Positive Colonies. The SKT6 cells were cultured with various Epo concentrations for 4 days in methylcellulose, and the percentage of hemoglobinized colonies was determined. As shown in Fig. 3, over 60% of the colonies formed were hemoglobin-positive at 0.1 unit of Epo per ml, which indicates that the newly established cell line SKT6 is highly responsive to Epo. Beyond 0.1 unit of Epo per ml, the percentage of hemoglobin-positive colonies did not significantly increase. The maximum level was achieved at 2.0 units/ml and remained stable in the range examined (up to 4.0 units/ml). In this range about 75% of the colonies formed were hemoglobin-positive.

Time Course of Binding for ^{125}I-Epo. Radioiodination of Epo by chloramine-T (12) or Bolton–Hunter reagent (13)

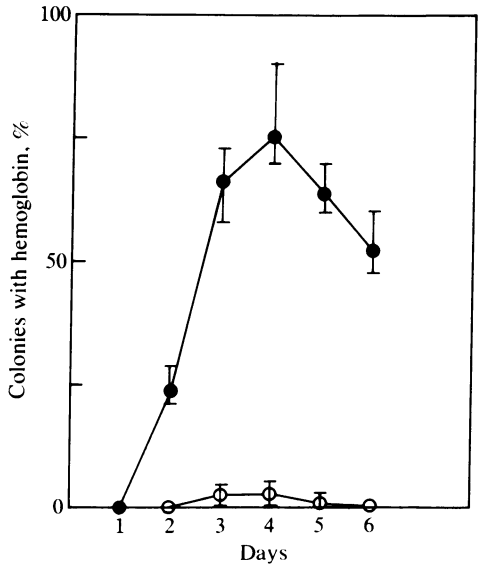

FIG. 2. Kinetics of hemoglobin-positive colony formation in the presence or absence of Epo. SKT6 cells were plated in methylcellulose in the presence (●) or absence (○) of 2 units of Epo per ml. The percentages of colonies with hemoglobin were determined by benzidine staining at the indicated time after plating. Figures are averages of the data from five independent experiments.

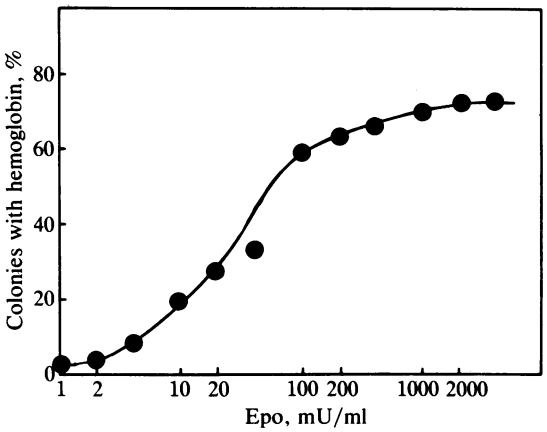

FIG. 3. Effect of Epo concentration on the formation of hemoglobin-positive colonies. Various concentrations of Epo were cultured with SKT6 cells in methylcellulose for 4 days, and the hemoglobin-positive colonies were stained by benzidine. Each point is the mean of duplicates. mU, milliunits.

caused significant losses of its biological activity. The two-phase labeling method with chloramine-T (14) and the lactoperoxidase/glucose oxidase method (15) both resulted in low incorporation of ^{125}I into Epo. However, Epo could be iodinated to high specific radioactivity with no detectable loss of differentiation-inducing activity for SKT6 cells when the IODO-GEN method was used.

Specific binding of ^{125}I-Epo to SKT6 cells showed different kinetics at different temperatures (Fig. 4). The binding was rapid at 37°C and appeared to be maximal by 15 min. However, this maximal level slightly declined after 3 hr. This observation could be explained by the receptor internalization and degradation. At 10°C the rate of binding was significantly slower and plateaued at 4 hr. The maximal binding was nearly that observed at 37°C. In all subsequent experiments binding was measured at 10°C and at 4 hr to ensure the equilibrium binding but minimize the possibility of receptor turnover.

Binding of ^{125}I-Epo to SKT6 and Other Cells. Fig. 5*A* shows the specific binding of ^{125}I-Epo to SKT6 cells as a function of ^{125}I-Epo concentration. Values shown are averages of data from five independent experiments. Specific binding increased with increasing concentrations of ^{125}I-Epo and was largely inhibited by the presence of excess unlabeled Epo. The specific binding of ^{125}I-Epo saturated at higher concentrations of the ligand. Scatchard analysis of the data showed that SKT6 cells had a single class of high-affinity receptor with an apparent K_d of 0.15 nM (Fig. 5*B*). The estimated number of receptors was ≈470 per cell.

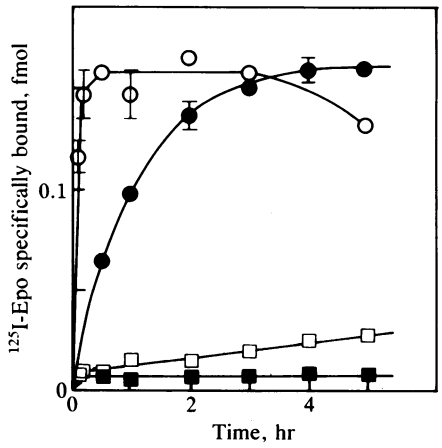

FIG. 4. Kinetics of specific binding of ^{125}I-Epo to SKT6 cells. Five × 10^6 cells were incubated with ^{125}I-Epo (120 pM) in 100 μl of binding medium at 37°C (○) or 10°C (●). Results are the average of duplicate determinations. Specific binding is reported in fmol per 10^6 cells. Nonspecific binding of ^{125}I-Epo at 37°C (□) or 10°C (■) was determined in the presence of 0.2 μM of unlabeled Epo.

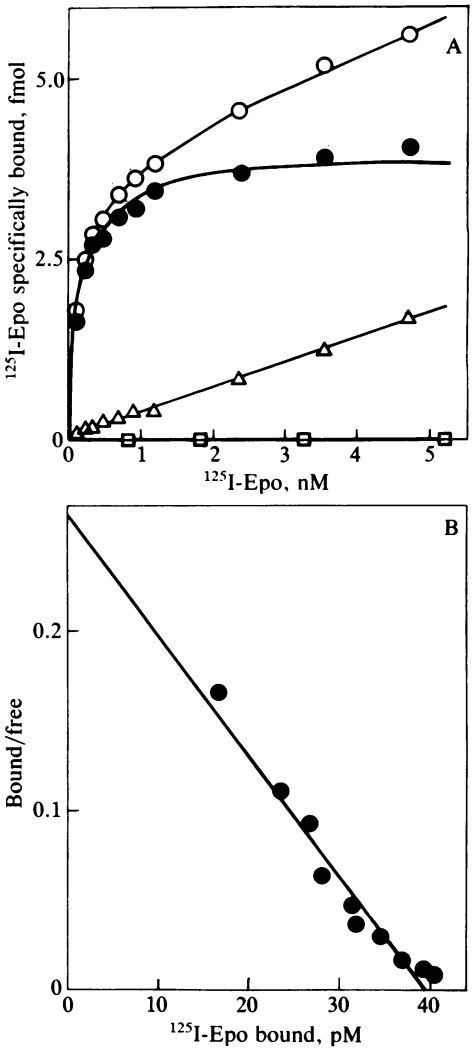

FIG. 5. Binding of ^{125}I-Epo to SKT6 cells. Cells (5 × 10^6 in 100 μl) were incubated at 10°C for 4 hr. Specific binding was determined by subtracting the binding in the presence of 0.2 μM (40-fold or more excess) unlabeled Epo. (*A*) Binding curves of SKT6 cells. Total binding (○); nonspecific binding (△); specific binding (●). Binding to WEHI-3 cells (□) is also shown. (*B*) Scatchard plot of the same data. The average of data from five independent experiments is shown.

No specific binding of ^{125}I-Epo was observed for any of the following cell lines; human erythroleukemia HEL and K-562 cells, mouse myelomonocytic leukemia WEHI-3 cells, human promyelocytic leukemia HL-60 cells, mouse IL-3 dependent P cell-like IC-2 cells (16) and mouse reticulum sarcoma FVTCT cells (17). Even when the concentration of ^{125}I-Epo was increased to 5 nM, no specific binding could be detected on these cells.

Binding of ^{125}I-Epo to three Epo-unresponsive erythroleukemia cell lines derived from cells of mouse spleen infected with polycythemia-inducing Friend virus was also examined. Interestingly enough, ^{125}I-Epo bound specifically to T3-Cl-2-0 (8), K-1 (8), and GM86 (clone 745) (18) cells. The binding to these cells was saturable, and the affinity and the receptor number were similar to those of SKT6 cells (data not shown).

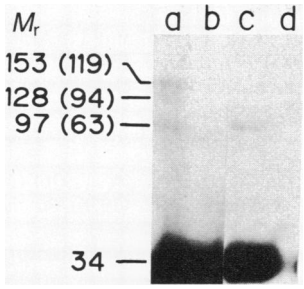

FIG. 6. Affinity labeling of ^{125}I-Epo to SKT6 cells and T3-Cl-2-0 cells. Cells were allowed to bind ^{125}I-Epo and then treated with disuccinimidyl suberate. Cell lysates prepared by Triton X-100 were fractionated by NaDodSO$_4$/PAGE. An autoradiograph in the absence of 2-mercaptoethanol is shown. Lanes a and b, SKT6 cells; lanes c and d, T3-Cl-2-0 cells. Lanes b and d, crosslinking carried out in the presence of excess unlabeled Epo. $M_r \times 10^{-3}$ values for the receptors are indicated in parentheses.

This indicates that mouse erythroleukemia cells induced by either anemia- or polycythemia-inducing Friend virus possess Epo binding sites on their membranes, whether or not they are responsive to Epo.

Affinity Labeling of ^{125}I-Epo to Its Receptor on SKT6 Cells. When SKT6 cells were chemically crosslinked with ^{125}I-Epo under binding conditions, the Epo receptor was specifically labeled and could be identified by NaDodSO$_4$/PAGE and autoradiography. This resulted in the labeling of three crosslinked species that migrated as polypeptides of M_r ≈153,000, ≈128,000, and ≈97,000 (Fig. 6, lane a); 2-mercaptoethanol did not affect the pattern. All three bands were absent if excess unlabeled Epo was included during the crosslinking reaction (lane b). Assuming that these bands represented receptor crosslinked to one ^{125}I-Epo molecule, apparent M_r values of the receptors were ≈119,000; ≈94,000; and ≈63,000. The 34,000 material represents noncrosslinked ^{125}I-Epo.

Similar results were also obtained when another crosslinking reagent, ethylene glycol bis(succinimidyl succinate), was substituted for disuccinimidyl suberate. No crosslinked product, however, was detected when dimethyl suberimidate was used.

The possibility that smaller M_r species (128,000 and/or 97,000 bands) were proteolytic cleavage products of larger M_r species (153,000 and/or 128,000 bands) was addressed by including a mixture of protease inhibitors in the extraction buffer. All species were found in similar amounts (data not shown).

Affinity Labeling of ^{125}I-Epo to Epo-Unresponsive Cells. The crosslinked complex identified from Epo-unresponsive T3-Cl-2-0 cells was seen to migrate as a single iodinated band with M_r 97,000 (Fig. 6, lane c), the same as the smallest band from SKT6 cells, under both reducing and nonreducing conditions. This band was not present when cells were incubated with an excess of unlabeled Epo (lane d). The same results were obtained from the other two Epo-unresponsive Friend erythroleukemia cell lines K-1 and GM86, but no crosslinked product was observed from the other human and mouse tumor cell lines described above. The calculated M_r of the receptor on these Epo-unresponsive cells was 63,000.

DISCUSSION

Study of the mechanism of receptor-mediated erythroid differentiation has been hampered by three major factors: (*i*) difficulty in obtaining a large quantity of purified Epo; (*ii*) difficulty in preparing the biologically active ^{125}I-Epo; and (*iii*) lack of a sufficient number of homogeneous Epo-responsive cells. Through recombinant DNA technology a sufficient quantity of pure human Epo can be produced. ^{125}I-Epo that has full biological activity as well as high specific radioactivity was prepared by the Iodo-Gen method as described here. The first two problems were thus resolved.

The third problem was overcome by establishing a stable permanent cell line that is highly responsive to Epo. Cells responsive to Epo have been identified in the bone marrow, fetal liver, and mouse spleen infected with FVA (19–21). It was, however, difficult to obtain a sufficient number of pure target cells from these heterogeneous cell populations. Isolation of Epo-responsive Friend erythroleukemia cell line (TSA8) has been reported (22). The response of TSA8 cells to Epo, however, was much lower than that of SKT6 cells; only 45% of the colonies were hemoglobin-positive in the presence of Epo. TSA8 cells also had a high background, i.e., 20% of the colonies were positive without Epo. Moreover, according to the latest report (23), Epo response of TSA8 cells was much lower than the percentage stated; only 13–18% were positive with Epo. TSA8 cells may still be useful for studying the mechanism of erythroid differentiation induced by chemicals, but not differentiation induced by Epo. The SKT6 cells described here, on the other hand, have been maintained over 1.5 years and still exhibited a differentiation-inducible character—namely, high response to Epo (75%) and low background (1–4%) (Fig. 2). Consequently, this cell line proved to be suitable for studying Epo action at subcellular and molecular levels.

As shown in Fig. 3, even a low concentration of Epo (0.1 unit/ml—i.e., 42 pM) was enough to induce differentiation in most of the SKT6 cells. According to the binding curve (Fig. 5*A*), about 69 Epo molecules bind to its membrane receptor per cell at 42 pM Epo. In other words, biological response can occur on SKT6 cells at a lower receptor occupancy (14% of total receptors). Under normal physiological conditions Epo exists in the circulation at a concentration of 0.015–0.030 unit/ml of serum, or about 9 pM (24). This indicates that only 15 Epo molecules bind to the receptor per cell (i.e., only 3% receptor occupancy). These observations suggest that the signal generated by Epo binding to its specific receptor must be very potent.

The binding of [^{3}H]Epo to mouse spleen cells infected with FVA (FVA cells) has been reported (21). In contrast to the report, the binding of ^{125}I-Epo to SKT6 cells was of high affinity (K_d = 0.15 nM) compared with the binding of [^{3}H]Epo to FVA cells (K_d = 5.2 nM), whereas the number of binding sites per cell was about the same. In addition, the binding of [^{3}H]Epo to FVA cells was slow, requiring 2 hr to reach a stable maximum at 37°C. These discrepancies may be due to the difference between the established Epo-responsive cell line (SKT6 cells) and the heterogeneous primary cells (FVA cells).

Membrane components that specifically bind with ^{125}I-Epo were identified by affinity labeling; three receptor species were found from SKT6 cells, and one was found from unresponsive erythroleukemia cells. Considering the existence of three receptor species in SKT6 cells, we advance three possible interpretations of these data. (*i*) Three different Epo receptors may exist in SKT6 cells, but this is not likely because Scatchard analysis showed the receptors to have only a single class of binding sites. (*ii*) The smaller M_r bands may have been proteolytic cleavage products of larger M_r bands. This, however, is also unlikely because a mixture of protease inhibitors had no effect on the appearance of any of the species. (*iii*) Some proportion of surface receptors may closely associate with other membrane components. For instance, the 153,000 band may represent the 128,000 receptor complex (94,000 receptor + 34,000 Epo) coupled to a third protein of 25,000. Alternatively, an Epo binding subunit of 63,000, which was found in all Friend virus-induced erythroleukemia cells examined here, might specifically associate

with two other effector subunit molecules of 25,000 and 31,000, and the binding subunit itself may be inactive as a receptor unless it associates with the other subunits. Such a complex subunit structure of Epo receptor molecules might bring about the complex crosslinking pattern. Further study is required to clearly define the fine structure of Epo receptor on SKT6 cells and Epo-unresponsive cells. This may clarify the detailed molecular mechanisms of Epo-induced erythroid differentiation and of erythroleukemia transformation.

Friend erythroleukemia cells have been used to study the mechanism of cell differentiation because they differentiate into more mature erythroid cells by various chemical inducers (25). However, this model system obviously does not represent the receptor-mediated erythroid differentiation occurring *in vivo*. Drastic changes of protooncogene (c-*myb*, c-*myc* and c-*fos*) expression upon Epo induction were observed on SKT6 cells (K.T., unpublished data). Those changes appeared, however, to be entirely different from those observed on Epo-unresponsive erythroleukemia cell differentiation induced by chemicals (26, 27). Studies of the molecular mechanism of erythroid differentiation should be based on receptor-mediated pathway rather than chemically induced bypass. Further investigation on Epo-induced cell response of SKT6 cells may reveal the detailed mechanisms of receptor-mediated signal transduction at the molecular level.

We thank Dr. F. Takaku for most helpful discussions and Drs. T. Kaneko and K. Arai for generously supplying recombinant hormones.

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