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Induction of the Receptor for Erythropoietin in Murine Erythroleukemia Cells after Dimethyl Sulfoxide Treatment[1]

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ABSTRACT

Biologically active ^{125}I-labeled human recombinant erythropoietin (EPO) was used to demonstrate specific receptors for this erythroid-specific hemopoietic growth factor on the cell surface of murine erythroleukemia cell clone B8. The binding of radioiodinated EPO to these cells was time and temperature dependent, specific, saturable, and reversible. During erythroid differentiation by dimethyl sulfoxide, B8 cells displayed a rapid and marked increase in the amount of specific ^{125}I-EPO binding before the appearance of hemoglobin-containing cells. Scatchard analysis of the saturation binding data revealed that B8 cells had a single class and low number (350 to 650) of EPO receptors per cell with an apparent K_d of 1.2 to 1.4 nM. In addition, the number of EPO receptors on B8 cells was increased twice by induction with DMSO for 1 day, but the binding affinity of EPO toward its receptors did not change significantly. Affinity cross-linking experiments with disuccinimidyl suberate demonstrated two radiolabeled components with apparent molecular weights of 145,000 and 130,000 under both reducing and nonreducing conditions. Labeling of the two components was inhibited by incubation of cells with unlabeled EPO. These results suggest that some murine erythroleukemia cells potentially express EPO receptors as a differentiation marker of erythroid lineage, which contain two polypeptides with molecular weights of 109,000 and 94,000.

INTRODUCTION

EPO,[3] a well-known polypeptide growth factor, is required for the proliferation and differentiation of late erythroid progenitor cells *in vivo* and *in vitro*. As with peptide hormones in general, these effects appear to be mediated through the interaction of the ligand with specific receptors on the cell surface of target cells. Recently, human urinary EPO was purified to homogeneity (1), and furthermore human EPO complementary DNA has been molecularly cloned and expressed in mammalian cells (2, 3). Thus, availability of large quantities of purified EPO has made it possible to investigate the mechanism of action of EPO. Binding studies with tritiated EPO by Krantz and Goldwasser (4) demonstrated the presence of receptors for EPO on Friend virus-infected murine spleen cells. Recently, Sawyer *et al.* (5) have prepared radioiodinated human recombinant EPO with full biological activity using an IODO-GEN method and characterized the EPO receptors on the same population of cells as Krantz and Goldwasser used. We have also prepared biologically active radioiodinated EPO using a chloramine-T method and reported its binding to fetal mouse liver cells (6, 7) and EPO-responsive MEL cells (7).

MEL cells are virally transformed erythroid progenitor cells thought to be arrested at or before the proerythroblastic stage of erythroid differentiation. Treatment of MEL cells with DMSO or other chemical inducing agents results in a series of morphological and biochemical changes that can mimic some aspects of normal red cell maturation (8), which is regulated by EPO in a physiological state.

In the present study, we have demonstrated specific binding of ^{125}I-EPO to a MEL cell clone B8 (9) and characterized some properties of the binding sites and specifically labeled polypeptides that exhibit properties expected for the EPO receptor on B8 cells, using the divalent cross-linking reagent DSS. Under these conditions we report that ^{125}I-EPO seems to bind solely to M_r 109,000 and M_r 94,000 proteins.

MATERIALS AND METHODS

Chemicals and Reagents. Human recombinant EPO used in this study was produced in Chinese hamster ovary cells as described (3) and generously provided by Kirin-AMGen, Inc. (Thousand Oaks, CA). This preparation has been purified to homogeneity with a specific activity of 180,000 units/A_{280} of EPO determined by radioimmunoassay (10). Reagents used specifically for this study were purchased from the following sources. Phenylmethylsulfonyl fluoride, aprotinin, and DMSO were from Sigma Chemical Co. (St. Louis, MO). DSS was purchased from Pierce Chemical Co. (Rockford, IL). Reagents for SDS-PAGE were obtained from Bio-Rad Laboratories (Richmond, CA).

Radioiodination of EPO. EPO was labeled with sodium [^{125}I]iodide (Amersham International, Amersham, United Kingdom) using the chloramine-T procedure described by Greenwood *et al.* (11) with a minor modification (7). Briefly, EPO (5 μg), 0.5 mCi of Na^{125}I, and chloramine-T (3.75 μg/ml) in 30 μl of 0.3 M sodium phosphate (pH 7.4) were placed in a 1.5-ml polypropylene tube. The iodination reaction was continued for 10 min at 22°C and stopped by addition of 5 μl of sodium metabisulfite (60 μg/ml). Labeled EPO was separated from free ^{125}I by column chromatography using a Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). The fraction containing EPO was pooled and stored at 4°C. This procedure produced labeled EPO of specific activity varying 38 to 55 μCi/μg. When tested in an erythroid colony formation assay using mouse bone marrow cells, labeled EPO preparations were found to retain nearly all of their biological activity (7).

Culture and Differentiation Induction of MEL Cells. A MEL cell clone B8 transformed by infection of Friend spleen focus-forming virus (9) was maintained in RPMI 1640 medium containing 10% fetal calf serum (Gibco, Grand Island, NY), penicillin (100 units/ml), and streptomycin (100 μg/ml). For induction of erythroid differentiation, logarithmically growing B8 cells were suspended at 5×10^5/ml in 75-cm^2 culture flasks (Iwaki Glass, Tokyo, Japan). DMSO was added at 1% (v/v) at Day 0 to the cell suspension. A portion of the suspension was discarded at Day 2, and fresh medium containing DMSO was added to maintain a constant cell density of 1×10^6/ml. On Days 0 to 5, the cells were harvested for ^{125}I-EPO binding, and a small part of the cell suspension was prepared for cytochemical analysis. The degree of differentiation was measured by scoring the ratio of benzidine-staining positive cells. One hundred cells were counted for three separate preparations.

Assay for Binding of ^{125}I-EPO to B8 Cells. Just before use, B8 cells were sedimented, washed twice, and resuspended in HBSS containing 0.1% bovine serum albumin and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4 (binding buffer). Cells (5×10^6) were incubated with ^{125}I-EPO (800 pM; 10 to 12×10^4 cpm) in 100 μl of

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[3] The abbreviations used are: EPO, erythropoietin; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; MEL, murine erythroleukemia; CSF, colony-stimulating factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HBSS, Hanks' balanced salt solution; CFU-E, colony-forming unit-erythroid.

binding buffer. Incubation was carried out in Falcon No. 2063 tubes. At the end of incubation, duplicate 40-μl aliquots of the incubation mixture (containing 2×10^6 cells) were then transferred onto a cushion of phthalate oil in 500-μl polyethylene centrifuge tubes. The cells were sedimented by centrifugation for 30 s in Beckman Microfuge B. The supernatant was then aspirated, and the tubes were cut off just above the cell pellet and assayed for cell-associated radioactivity in an Aloka Autogamma ARC-251 instrument. Nonspecific binding measured in the presence of 150 nM unlabeled EPO was subtracted from total binding unless otherwise indicated. All binding determinations were performed in duplicate or quadruplicate, and experiments were repeated at least twice. For statistical analysis of the data when comparing results with or without DMSO treatment, the Student *t* test was used.

Dissociation Experiments. B8 cells (5×10^6) in 200 μl of binding buffer were incubated with 800 pM ^{125}I-EPO in 1.5-ml Eppendorf tubes at 15°C for 180 min. At the end of this incubation, the cells were pelleted and washed once with ice-cold binding buffer, and cell-bound ^{125}I-EPO was allowed to dissociate by incubation in 1.0 ml of binding buffer containing 50 nM unlabeled EPO at 15°C or at 37°C. After dissociation, the cells were sedimented and washed once. The radioactivity that remained bound was determined as described above.

Affinity Labeling Protocol. Cells (1 to 3×10^7) were incubated with ^{125}I-EPO (800 pM) in 500 μl of binding buffer, in both the presence and absence of unlabeled EPO for 180 min at 15°C. After washing twice with ice-cold HBSS, the cells were resuspended in the same volume of buffer without bovine serum albumin. To cross-link the bound ^{125}I-EPO, DSS freshly prepared in DMSO was added to a final concentration of 0.2 mM and incubated at 15°C for 15 min (12). The reaction was quenched with three volumes of 10 mM Tris buffer, pH 7.4, containing 1 mM EDTA and 150 mM NaCl. After 5 min, the cells were pelleted and solubilized in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.4, containing 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1000 trypsin inhibitor units per ml of aprotinin. After discarding insoluble materials, trichloroacetic acid was added to a final concentration of 5%. The trichloroacetic acid pellets were washed in ethanol:ether (3:1, v/v), air-dried, and dissolved in the sample buffer of Laemmli (13) in either the presence or absence of 50 mM dithiothreitol. Samples were boiled for 3 min in the sample buffer and subjected to SDS-PAGE, using 8% polyacrylamide gels in a discontinuous buffer system (13). After electrophoresis, the gels were fixed, stained with Coomassie blue, dried, and autoradiographed with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) at −70°C using intensifying screens. Apparent molecular weights were calculated by using standard proteins.

RESULTS

Time-Course and Reversibility of ^{125}I-EPO Binding. In a ligand binding assay, specific binding of ^{125}I-EPO to B8 cells was demonstrated. Time-courses of specific ^{125}I-EPO binding are shown in Fig. 1. At 37°C, binding was rapid and appeared to be maximal by 40 min. However, this level of binding declined by 60 min, and steady-state binding was not shown at this temperature. At 15°C, the rate of binding was decreased, but steady-state binding was reached at 3 h. Accordingly, further binding study was performed at 15°C for 3 h of incubation. Binding at 2°C was much less than at 15°C even after 21 h of incubation.

Dissociation of cell-bound ^{125}I-EPO was rather slow when preincubated cells were washed free of unbound radioligand and further incubated at 15°C (Table 1). On the other hand, dissociation of prebound ^{125}I-EPO appeared progressive in cells incubated at 37°C, whereas prolonged incubation at 37°C produced a significant level of degraded ^{125}I-EPO (data not shown).

Differentiation-related Changes in ^{125}I-EPO Binding to B8 Cells. Fig. 2 illustrates changes in ^{125}I-EPO specific binding and the ratio of benzidine-staining positive cells during erythroid differentiation induced by DMSO. Untreated cells showed

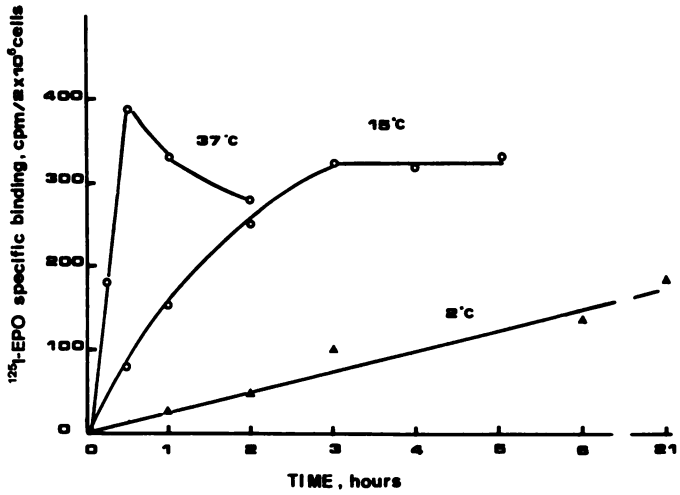

Fig. 1. Time and temperature dependence of ^{125}I-EPO specific binding to B8 cells. Uninduced cells (5×10^6) were incubated with ^{125}I-EPO (800 pmol) in the presence or absence of 150 nM unlabeled EPO. Specific binding per 2×10^6 cells was determined as described in "Materials and Methods." *Points*, mean of duplicate determinations.

Table 1 *Dissociation of ^{125}I-EPO prebound to B8 cells*

B8 cells (5×10^6) were incubated with ^{125}I-EPO (800 pmol) at 15°C for 3 h. Cells were then washed, and cell-bound ^{125}I-EPO was allowed to dissociate by incubation in binding buffer containing excess unlabeled EPO under the indicated conditions. The radioactivity that remained bound was determined.

Dissociation conditions		Cell-associated radioactivity (% of prebound cpm)
Temperature (°C)	Time (min)	
15	30	86.9 ± 1.4[a]
15	120	72.3 ± 2.7
15	240	64.3 ± 0.4
37	30	68.3 ± 1.0

[a] Mean ± SD of triplicate determinations.

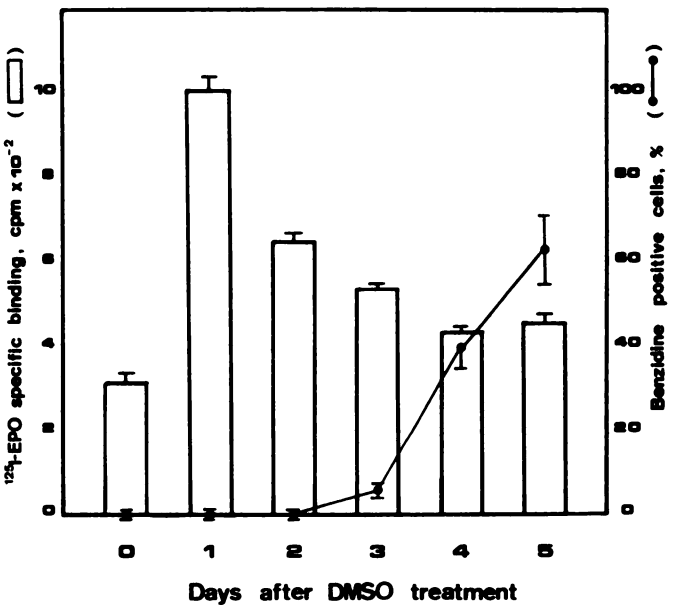

Fig. 2. Changes in specific ^{125}I-EPO binding to B8 cells and the ratio of benzidine-positive cells during erythroid differentiation. B8 cells were treated with 1% (v/v) DMSO and harvested after the indicated incubation periods. The cells were incubated with ^{125}I-EPO in the presence or absence of 150 nM unlabeled EPO. Specific binding per 2×10^6 cells was determined. *Columns*, mean of quadruplicate determinations; *bars*, SD. A portion of the cells was directly stained with benzidine dye, and the ratio of positive cells was scored as the percentage of the total (100 cells examined). *Columns*, average of triplicate preparations (●); *bars*, SD.

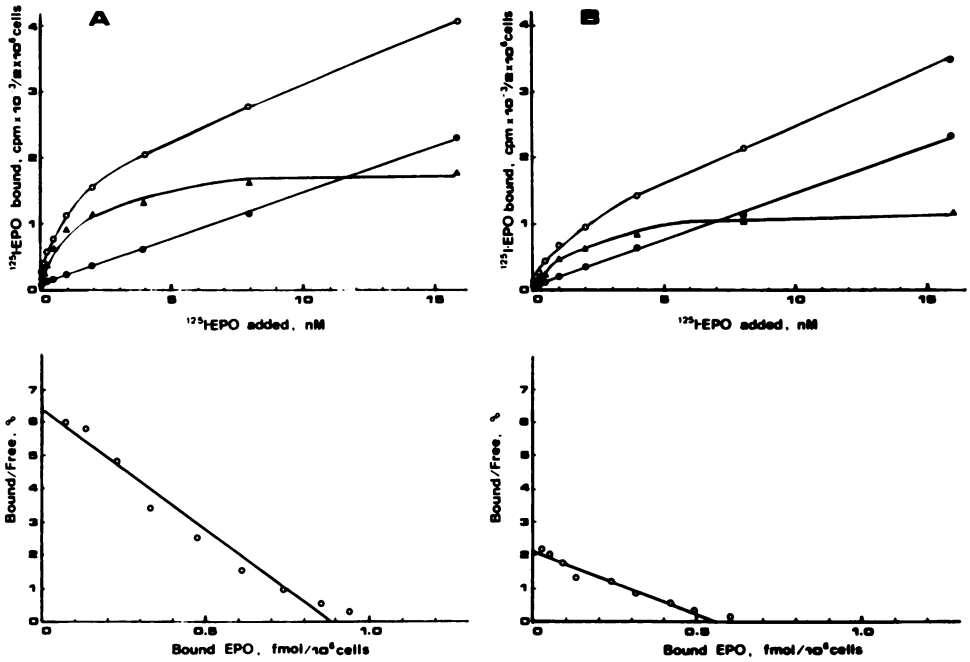

Fig. 3. Saturation analysis of ^{125}I-EPO binding to induced or uninduced B8 cells (*top*) and Scatchard analysis of the binding data (*bottom*). B8 cells were cultured in the presence (*A*) or absence (*B*) of 1% (v/v) DMSO for 24 h. In binding assays, the cells (5×10^6) were incubated with increasing concentrations of ^{125}I-EPO, with or without 150 nmol of unlabeled EPO at 15°C for 3 h. Specific binding per 2×10^6 cells (▲) was plotted by subtracting nonspecific binding (○) from total binding (●). *Points*, mean of duplicate determinations.

a modest amount of specific binding. A marked increase of ^{125}I-EPO specific binding was observed in cells at Day 1 after treatment with DMSO, and thereafter specific binding gradually decreased up to Day 5. In contrast, differentiated erythroid cells producing hemoglobin appeared only after Day 3 and progressively increased in ratio. In further experiments, B8 cells 1 day after DMSO treatment were used unless otherwise indicated. Association and dissociation kinetics values of ^{125}I-EPO binding to DMSO-induced B8 cells were similar to the data shown in Fig. 2 and Table 1 (data not shown).

Saturation Analysis of ^{125}I-EPO Binding to B8 Cells. To clarify the observation obtained above, we tried saturation analysis of ^{125}I-EPO binding to both DMSO-induced and uninduced B8 cells. Fig. 3, *top*, shows typical saturation binding data for ^{125}I-EPO at 15°C. When increasing amounts of ^{125}I-EPO were incubated with DMSO-treated and untreated cells, specific binding increased to an apparent saturation. Scatchard analysis of the binding data for both B8 cell populations yielded straight lines, indicating a single class of binding sites for EPO (Fig. 3, *bottom*). Repeated experiments with at least three different batches of ^{125}I-EPO gave similar results to those described here. The apparent dissociation constants and the receptor number deduced from the five separate experiments were listed in Table 2. The result suggested that the apparent increase in specific ^{125}I-EPO binding by DMSO induction was caused for a major part by a change in the binding sites in B8 cells. On the other hand, the binding affinity of EPO towards its receptor did not change significantly by DMSO induction.

Table 2 *Dissociation constants and receptor numbers of EPO binding to DMSO-induced and uninduced B8 cells*

These parameters were determined from Scatchard plots of five separate saturation binding assays.

	No. of receptors/cell[a]	Dissociation constants (nM)[b]
DMSO (−)	329 ± 72[c]	1.45 ± 0.28
DMSO (+)	626 ± 93[d]	1.21 ± 0.64

[a] Determined from the intercept on the abscissa of the Scatchard plot.
[b] Apparent dissociation constant determined from the slope of the Scatchard plot.
[c] Mean ± SD.
[d] $P < 0.01$ *versus* DMSO (−).

Affinity Labeling of the EPO Receptor in B8 Cells. Next, we attempted to affinity label the receptor for EPO using a chemical cross-linking reagent. To clarify the protein(s) to which EPO binds on the cell surface of B8 cells, 800 pmol of ^{125}I-EPO were incubated with the DMSO-treated cells with or without excessive unlabeled EPO, washed, and covalently coupled with DSS. The autoradiogram of these preparations analyzed by SDS-PAGE under reducing conditions revealed three ^{125}I-labeled bands with molecular weights of approximately 145,000, 130,000, and 36,000 in the absence of unlabeled EPO (Fig. 4, *Lane a*). The radioactivity in the M_r 36,000 band is probably ^{125}I-EPO that was not covalently cross-linked to its receptor, since it comigrates with the original ^{125}I-EPO (data not shown). A M_r 130,000 band was preferentially labeled in all experiments performed on B8 cells. ^{125}I-EPO affinity labeling of DMSO-induced B8 cells was competed by unlabeled EPO in a dose-dependent fashion (Fig. 4, *Lanes b* and *c*). There appears no difference between the displacement patterns for these two components, suggesting their similar affinity for ^{125}I-EPO. Under nonreducing conditions (Fig. 4, *lanes d* and *e*) these two species also migrated with the apparent same molecular mass.

DISCUSSION

In this paper, we have demonstrated and characterized the cell surface receptor for EPO on a MEL cell clone B8. Furthermore the putative receptor molecule has been identified with ^{125}I-EPO and the chemical cross-linking reagent. The radiolabeled EPO derivative used here was confirmed to be biologically active on mouse bone marrow cells (7). ^{125}I-EPO in this study shows relatively low or moderate specific activity (0.8 to 1.0 mol of iodine per molecule). In our preliminary experiments, ^{125}I-EPO with high specific activity (>100 μCi/μg) caused a considerable increase in nonspecific binding. In these cases, the presence of high-molecular-weight aggregates was demonstrated by gel filtration chromatography (data not shown).

Among the growth factors tested including a family of CSFs, only EPO could compete for the binding of ^{125}I-EPO to B8

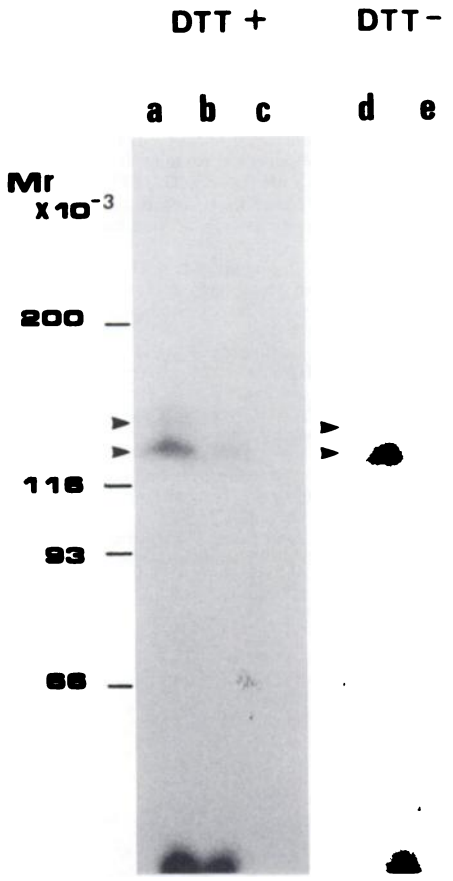

Fig. 4. ^{125}I-affinity labeling of EPO receptors in B8 cells. B8 cells (2×10^7) with DMSO treatment were incubated with ^{125}I-EPO in the presence of the indicated concentrations of unlabeled EPO. After cross-linking with DSS, the samples were analyzed by SDS-PAGE under reducing (*Lanes a* to *c*) and nonreducing (*Lanes d* and *e*) conditions, followed by autoradiography. Unlabeled EPO concentrations are as follows: *Lanes a* and *d*, 0; *Lane b*, 4 nM; *Lanes c* and *e*, 150 nM. *DTT*, dithiothreitol.

cells as well as to fetal mouse erythroid cells (Ref. 6; Footnote 4). Walker *et al.* (14) have recently proposed that, in mouse bone marrow cells, the interaction of each of four CSFs with its specific receptor might be down-modulated by other species of CSFs in a hierarchical manner. We would suppose that the binding of EPO to normal erythroid progenitor cells may undergo similar regulation by multipotent CSFs such as interleukin 3, since *in vitro* erythroid colony formation was reported to be modulated by addition of interleukin 3 (15). To elucidate this interesting issue, further studies are needed on a population of physiological erythroid progenitor cells such as fetal mouse liver cells.

Nicola and Metcalf (16) previously showed that ^{125}I-granulocyte-CSF bound to responsive but not unresponsive leukemic cell lines. In the present study, specific ^{125}I-EPO binding was exhibited in unresponsive MEL cells. B8 cells did not respond to EPO under the various conditions tested. Although we cannot entirely exclude the possibility that EPO receptors on B8 cells function under appropriate conditions, they may be interpreted as properties associated with a specific stage of erythroid differentiation. Of particular interest in this case is that the number of EPO receptors, but not the binding affinity of EPO toward its receptor, was increased in B8 cells 1 day after DMSO treatment. In combination with our recent studies with fractionated fetal mouse liver cells showing that the highest amount of ^{125}I-EPO binding was observed in a population enriched in CFU-E (6), it is likely that 1 day-induced B8 cells may have some properties specific for the CFU-E stage. Mayeux *et al.* (18) reported that another clone of MEL cells decreased in the number of EPO binding sites by differentiation induction for 4 days. However, they did not report EPO binding in the early phase of differentiation.

The finding of a single class and extremely low number of EPO receptors is similar to those previously shown on EPO-unresponsive MEL cells (5, 17). Our data of the dissociation constant (K_d 1.21 to 1.45 nM) are also comparable to the values of Mayeux *et al.* reported (0.5 nM) (17) and that Sawyer *et al.* reported (1.3 nM) (5). Recently, it has been revealed that EPO-responsive erythroid cells display two classes of binding sites for EPO, one with high affinity and the other with low affinity (5–7). Taking it into account, the EPO receptors we observed would correspond to the low affinity binding sites. This finding supports the assumption that only the high-affinity receptors appear to be responsible for the growth signal delivered upon binding of the growth factor. To the contrary, Todokoro *et al.* (18) have found a single class of EPO receptors with a K_d of 0.15 nM on both responsive and unresponsive MEL cells. At present there seems to be no reasonable explanation for this discrepancy with the exception that the cell populations used were different from each other. More extensive data about EPO binding should be accumulated to address this issue.

The affinity-labeled M_r 145,000 and 130,000 species have the properties characteristic of the EPO receptor in binding studies on B8 cells. (*a*) The ^{125}I-EPO associated with the M_r 145,000 and 130,000 components is clearly visualized in DMSO-induced cells but not in uninduced cells (data not shown). (*b*) The affinity labeling of these two bands is displaced by unlabeled EPO in a dose-dependent manner. Assuming that only one EPO molecule (M_r 36,000) is covalently coupled to the receptor, the receptor protein would have a molecular weight of 109,000 and 94,000, respectively. Our results also suggest that these receptor molecules do not consist of subunits linked together by disulfide bridges, as evidenced by similar electrophoretic mobility between reducing and nonreducing conditions. Sawyer *et al.* (19), have recently cross-linked ^{125}I-EPO to the two M_r 100,000 and 85,000 components in membrane preparations from EPO-sensitive erythroid cells. This result appears compatible with our data in spite of a little difference in molecular mass. Todokoro *et al.* (18) have detected three M_r 63,000, 94,000, and 119,000 receptor species on EPO-responsive MEL cells and only the M_r 63,000 species on EPO-unresponsive cells. However, it is likely that this smallest molecule is a proteolytic fragment of larger receptor species, since they incubated the intact cells with ^{125}I-EPO at 37°C before cross-linking, at which temperature the degradation products of EPO receptors are easier to develop than at the 15°C in our study. The structural basis of the EPO receptor which distinguishes the difference between functional and nonfunctional binding sites still remains to be resolved. Nevertheless, the findings reported herein supported the existence of the EPO receptors in a MEL cell clone B8 and raise some suggestions in regard to the differentiation stage of these cells.

[4] Unpublished data.

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