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Characterization of erythropoietin receptor on erythropoietin-unresponsive mouse erythroleukemia cells

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A membrane receptor for erythropoietin was identified in various erythropoietin-unresponsive mouse erythroleukemia cells. Scatchard analyses of the binding of human ^{125}I-labeled erythropoietin to T3C1-2-0, K-1, GM86 and 707 cells showed the presence of a single class of binding sites with apparent K_d values of 0.27–0.78 nM, which are slightly higher than those of erythropoietin-responsive cells. The number of binding sites varied from 110 to 930 per cell. Crosslinking of ^{125}I-erythropoietin to its binding sites with disuccinimidyl suberate revealed the existence of a single binding protein with molecular mass of 63 kDa. No binding sites with higher molecular mass, as observed in erythropoietin-responsive cells, were detected, nor was any specific binding observed to the non-erythroid hematopoietic cell or to the human erythroleukemia cells examined.

Introduction

Erythropoietin (EPO) is a glycoprotein factor that acts specifically on relatively mature erythroid progenitor cells and induces their differentiation into mature erythrocytes [1]. EPO has been purified to homogeneity from the urine of patients with aplastic anemia [2]. Clones of complementary and genomic DNA encoding human EPO, and of mouse genomic EPO DNA were isolated, and their molecular structures were determined [3–6]. Very little, however, is known about the molecular mechanism of the erythroid cell differentiation induced by this hormone.

We and others have characterized the membrane receptor for EPO on EPO-responsive cells, i.e., on mouse erythroleukemia SKT6 cells [7] and on mouse spleen cells infected with anemia-inducing Friend virus complex [8]. The results suggested the existence of a complex subunit structure of EPO-receptor molecules. EPO-unresponsive mouse erythroleukemia cells, on the other hand, have single binding sites for EPO [7]. Here, we describe the results of detailed analyses of the EPO-receptor molecules in various EPO-unresponsive Friend erythroleukemia cells, and discuss the relationship between EPO-receptor activity and its subunit structure.

Materials and Methods

Binding of ^{125}I-EPO to cells

Purified recombinant human EPO (over 70000 units/mg, M_r 34000) was kindly supplied by Drs. T. Kaneko and J. Koumegawa (Kirin Brewery),

Abbreviations: EPO, erythropoietin; HEL, human erythroleukemia.

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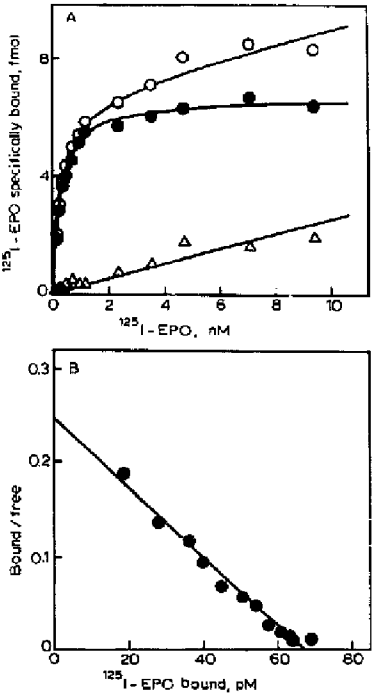

Fig. 1. Binding of ^{125}I-EPO to K-1 cells. Cells ($5 \cdot 10^6$ cells/100 µl) were incubated at 10°C for 4 h. Specific binding was determined by subtracting the binding in the presence of 0.2 µM unlabeled EPO from observed total binding. (A) Binding curves: total binding (○); nonspecific binding (△); specific binding (●). (B) Scatchard plot of the specific binding data. Each points represent an average of four determinations.

and was radiolabeled using IODO-GEN (Pierce) and carrier-free Na^{125}I (Amersham) [9]. ^{125}I-EPO was purified using Sephadex G-25 equilibrated with phosphate-buffered saline containing 0.02% Tween-20. The specific radioactivity of ^{125}I-EPO was $9.5 \cdot 10^5$ Bq/µg. ^{125}I-EPO retained full biological activity when assayed by measuring the hemoglobinized colony formation of SKT6 cells [7]. EPO-unresponsive mouse erythroleukemia cell lines K-1 [10], T3C1-2-0 (GM979) [10], GM86 (745) [11] and 707 [11] were maintained in Ham's F-12 medium containing 10% fetal calf serum. Equilibrium binding of ^{125}I-EPO to cells was measured as described [7]. Briefly, cells ($5 \cdot 10^6$) were incubated with ^{125}I-EPO in 100 µl of Ham's F-12 medium containing 10% fetal calf serum with or without 0.2 µM (40-fold or more molar excess) of unlabeled EPO at 10°C for 4 h. Aliquots of cells were filtered through Whatman GF/A glass filters, and the filters were washed with phosphate-buffered saline. The radioactivity remaining on the filters was counted.

Crosslinking of ^{125}I-EPO to the receptor

Affinity crosslinking of ^{125}I-EPO to membrane receptor was done essentially as described by Pilch and Czech [12]. Cells were incubated with 0.75 nM ^{125}I-EPO with or without 75 nM unlabeled EPO for 30 min at 37°C, washed with phosphate-buffered saline twice at 4°C, and then affinity crosslinked with 0.3 mM disuccinimidyl suberate for 90 min at 4°C. After washing with 50 mM Tris-HCl (pH 6.8), cells were solubilized in 50 mM Tris-HCl (pH 6.8) containing 1.5% Triton X-100 in the presence of 10 µM pepstatin, 10 µM leupeptin, 10 µM antipain and 2 mM phenylmethylsulfonyl fluoride. ^{125}I-EPO-crosslinked complexes were analyzed by 10% SDS-polyacrylamide gel electrophoresis and subsequent autoradiography.

Results

Specific binding of ^{125}I-EPO to EPO-unresponsive cells

^{125}I-EPO bound specifically to various EPO-unresponsive mouse erythroleukemia cell lines, K-1, T3C1-2-0 (GM979), GM86 (745) and 707. The binding at 10°C increased during approximately the first 4 h of incubation and remained stable thereafter. The plateau reflected an equilibrium state of reversible binding, since the addition of unlabeled EPO led to a large decrease in the specifically bound radioactivity (data not shown). Fig. 1 illustrates typical equilibrium binding data for ^{125}I-EPO (only the data for K-1 cells are shown here). The binding was strongly inhibited by a large molar excess of unlabeled EPO (Fig. 1A). A Scatchard plot of the data (Fig. 1B) gave a single line, indicating the existence of a single class of affinity for binding sites. The apparent dissociation constants (K_d) and numbers

TABLE II

BINDING PARAMETERS OF ^{125}I-EPO TO CULTURED CELL LINES

Equilibrium binding curves were determined on the indicated cell lines. Scatchard analyses were then performed as shown in Fig. 1. Data represent mean ± S.E. for four determinations.

Cells	Characteristics	K_d (nM)	Number of binding sites per cell
Mouse			
K-1	erythroleukemia	0.28 ± 0.03	930 ± 140
T3C1-2-0	erythroleukemia	0.62 ± 0.07	690 ± 160
GM86	erythroleukemia	0.27 ± 0.07	110 ± 20
707	erythroleukemia	0.78 ± 0.08	120 ± 30
WEHI-3	myelomonocyte	–	none
IC-2	IL-3-dependent P cells	–	none
FVTCT	reticulum cell sarcoma	–	none
14-1	NIH/3T3 cells expressing Friend spleen focus-forming virus	–	none
Human			
K562	erythroleukemia	–	none
HEL	erythroleukemia	–	none
HL60	myeloid	–	none

of binding sites per cell were obtained from four independent experiments, and the results are summarized in Table I. The cell lines examined here had approx. 110–930 binding sites per cell (Table I), and dissociation constant (K_d) values were in the range of 0.27–0.78 nM.

The ^{125}I-EPO did not bind to the other mouse and human hematopoietic cell lines examined: IC2 (mouse IL-3-dependent P cells) [13], WEHI-3 (mouse myelomonocytes) [14], K562 and HEL (human erythroleukemia cells) [15,16], and HL60 (human myeloid cells) [17]. In order to exclude the possibility that Friend virus gene products are directly involved in the binding of ^{125}I-EPO, we examined the binding of ^{125}I-EPO to FVTCT cells (cells of a mouse reticulum sarcoma induced by Friend virus) [18] and 14-1 cells (mouse NIH/3T3 fibroblast cell transfected with polycythemia-inducing Friend spleen focus-forming virus). No binding to these cells was observed (Table I).

Affinity crosslinking

^{125}I-EPO was crosslinked to intact K-1, T3C1-2-0, GM86 and 707 cells with disuccinimidyl suberate. The complex thus formed migrated on SDS-polyacrylamide gel as a single distinctive band with a molecular mass of 97 kDa under both reducing (Fig. 2, lanes a, d, e, g) and nonreducing (Fig. 2, lanes h, i) conditions. As for the results under nonreducing conditions, only those for K-1 and T3C1-2-0 cells are shown. All cell lines gave the same pattern. It should be noted that the intensities of the bands in autoradiographs reflect the number of binding sites per cell. The complex

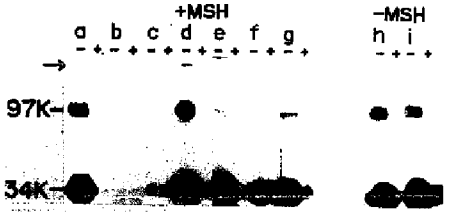

Fig. 2. Affinity crosslinking of EPO receptors in various cell lines. Cells were allowed to bind ^{125}I-EPO and then crosslinked with disuccinimidyl suberate. Cell lysates were applied to 10% SDS-PAGE, and the autoradiographs are shown. + and − indicate the presence and absence of unlabeled EPO during the binding reaction. Arrow indicates the position of minor band of 250 kDa. Lanes a–g; 2-mercaptoethanol (MSH) was included in the loading buffer. Lanes h and i, in the absence of a reducing reagent. Lanes a and h, T3C1-2-0 cells; lanes b, IC2 cells; lane c, 14-1 cells; lanes d and i, K-1 cells; lane e, 707 cells; lane f, FVTCT cells; lane g, GM86 cells.

was not detected when excess unlabeled EPO was included during the reaction. Assuming that this band represented a receptor crosslinked to one ^{125}I-EPO molecule, the apparent molecular mass of the receptor was 63 kDa. The band with a molecular mass of 34 kDa represents non-crosslinked ^{125}I-EPO. Another minor band with a high molecular mass (approx. 250 kDa) was observed on gels both in the presence and absence of reducing reagent (Fig. 2). This band, however, was not detected in EPO-responsive SKT6 cells [7]. ^{125}I-EPO did not form a covalent complex when incubated with the other cell lines mentioned above (Fig. 2, lanes b, c, f).

Discussion

The present study clearly indicated the presence of EPO-binding sites on various EPO-unresponsive mouse erythroleukemia cells. The dissociation constant (K_d) values of ^{125}I-EPO binding to EPO-unresponsive cells ranged from 0.27 to 0.78 nM, which is slightly higher than that of EPO-responsive SKT6 cells (0.15 nM) [7]. Recently, Sawyer et al. [19] reported that spleen cells infected with anemia-inducing Friend virus had high- and low-affinity receptors for EPO, but EPO-unresponsive mouse erythroleukemia cells (clone 745) had only the low-affinity binding site. We did not detect the two classes of EPO receptor in EPO-responsive SKT6 cells, but the K_d value of SKT6 cells (0.15 nM) corresponds to the K_d values of their higher-affinity sites of EPO-responsive spleen cells (0.07–0.18 nM) [7,19]. Sawyer et al. stated that the K_d values of the lower-affinity binding sites ranged from 0.55 to 1.33 nM, which is similar to the values of EPO-unresponsive cells examined here (0.27–0.78 nM). They also reported that 745 cells had 760 binding sites per cell with a K_d of 1.3 nM. In contrast to this, our results here showed that GM86 cells (originally a cell line identical to clone 745) had 110 binding sites per cell with a K_d of 0.27 nM, which is close to the K_d of their higher-affinity site. These discrepancies may be due to the differences in experimental conditions (e.g., incubation temperature and time, and EPO concentrations) and cells used (heterogeneous primary spleen cell suspension vs. established cell lines). Furthermore, sialidase-treated EPO binds to target cells with higher affinity than does untreated EPO [20], indicating that loss of terminal sialic acid of EPO increases the binding affinity to the receptor. These data do not allow us to make a conclusion as to the existence of high and low affinity in the EPO receptor.

According to Fraser et al. [21] human erythroleukemic K562 cells expressed 4–6 EPO-binding sites per cell. Detection of such a small number of binding sites in the presence of high background requires great care. Here, we concluded that there existed no specific binding of ^{125}I-EPO to either K562 or HEL cells. These HEL cells do not have membrane receptors for EPO, and therefore may be EPO-unresponsive. On the contrary, all mouse erythroleukemia cells examined here are totally EPO-unresponsive while they do have EPO-binding sites.

Crosslinking studies demonstrated that all EPO-unresponsive erythroleukemia cells examined here had the same single EPO-binding site with a molecular mass of 63 kDa, but no other species with higher molecular mass as was observed in EPO-responsive cells. Sawyer et al. described that the EPO receptor on EPO-unresponsive 745 cells was identical to that on EPO-responsive cells, although no data were shown [8]. Sasaki et al. [22], however, detected different crosslinked products in the same 745 cells, i.e., the molecular mass of EPO-binding sites was similar to that which we have reported here. The following facts strongly indicate that 63 kDa species are the specific EPO-binding sites. Firstly, the distinctive crosslinked materials were detected in all mouse erythroleukemia cells only in the absence of unlabeled EPO (Fig. 2). Secondly, the intensity of the crosslinked bands correlated well with the number of EPO-binding sites per cell (Fig. 2 and Table I). Thirdly, a mixture of proteinase inhibitors was included during sample preparations, as described in Materials and Methods, and thus the 63 kDa species are not the degradation products of high molecular mass receptors. Finally, no crosslinked band was detected in cells which have no EPO-binding sites (Fig. 2, lanes b, c and f), and therefore it is obvious that the 63 kDa species is not serum albumin or any other protein present in the binding buffer.

The biologically active form of the EPO recep-

tor seems to be composed of EPO-binding subunits and effector subunits specifically associated with the EPO-binding subunit. Signal transduction on EPO-receptor-mediated erythroid cell differentiation may be performed with this type of sophisticated EPO-receptor complex. On the contrary, EPO-binding sites on EPO-unresponsive erythroleukemia cells exist in a completely different form, and thus they may be functionally inactive. It was recently reported that EPO stimulates adenylate cyclase activity of the membranes of erythroblasts [23]. We found that protein kinase-C activity is not affected by the addition of EPO to EPO-responsive SKT6 cells (Todokoro, K. and Sugimoto, Y., unpublished data).

Further analyses are awaited to define the biochemical and physiological functions of the EPO-receptor complex. Molecular cloning of the gene coding for the EPO receptor may be done by using these mouse erythroleukemia cells and will reveal the fine structure and the functions of this hormone receptor.

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