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Erythropoietin receptors induced by dimethyl sulfoxide exhibit positive cooperativity associated with an amplified biologic response

(erythrocytes/signal transduction/erythropoiesis/differentiation/Rauscher murine erythroleukemia cells)

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ABSTRACT **Erythropoietin triggers the differentiation of erythrocyte progenitors by binding to receptors on their plasma membrane. We report here that pretreatment of erythropoietin-responsive murine erythroleukemia cells with chemical inducers resulted in a striking increase in erythropoietin-specific hemoglobinization. This amplification of the erythropoietin biologic response was accompanied by the induction of a new population of high-density receptors (≈20,000 per cell) exhibiting marked positive cooperativity. Erythropoietin binding to new receptors displayed a convex upward Scatchard plot and a Hill coefficient (nH) of 6.75. Measurement of erythropoietin receptor mRNA demonstrated an initial decrease in receptor transcript followed by an approximately 2- to 3-fold increase after 24–48 hr. This increase in receptor message does not appear to account for the magnitude of the receptor up-regulation by dimethyl sulfoxide. We propose that this positive cooperativity reflects the interaction (clustering) of receptors, presumably through the formation of homooligomers or heterooligomers, and that this receptor interaction may amplify the erythropoietin signal transduction pathway.**

The mechanism by which erythropoietin–receptor binding is transduced to the nucleus of erythroid cells is under intense study. The elucidation of the biochemical properties of the receptor and its associated proteins in the plasma membrane is crucial to this endeavor.

Studies of erythropoietin binding to receptors on the plasma membranes of erythroid cells have yielded a diversity of results. Both normal and transformed cells have been examined, and the number of erythropoietin receptors detected per cell ranges from 4 (1) to ≈3000 (2). Moreover, analysis of the thermodynamics of binding has, in many cases, revealed the presence of two classes of receptors based upon their relative binding affinities toward erythropoietin (3–5). In contrast, some studies have revealed only one affinity class of receptors on specified erythroid cell types (6).

The description of an erythropoietin receptor cDNA cloned from Friend murine erythroleukemia cells has served to increase the controversy (7). Although thermodynamic measurements of erythropoietin binding to these receptors on the original Friend cells revealed a single affinity class, transfection of the cDNA clone into receptor-deficient COS cells resulted in the appearance of two affinity classes. Additionally, crosslinking studies using ^{125}I-labeled erythropoietin (^{125}I-erythropoietin) demonstrated two ^{125}I-erythropoietin–receptor complexes with different molecular weights. It has been proposed that such differences in the size of erythropoietin–receptor complexes detected by crosslinking may be due to posttranslational processing of a single receptor protein (8). In contrast, the existence of a receptor heterodimer consisting of two different proteins has also been suggested (9).

We have shown recently that pretreatment of erythropoietin-sensitive Rauscher murine erythroleukemia cells with the chemical inducer dimethyl sulfoxide (DMSO priming) results in a large amplification of the erythropoietin biologic response characterized by a severalfold increase in the number of cells responding, an increase in the rate of response, and a markedly left-shifted dose–response curve (10). Preliminary experiments demonstrated that DMSO priming also caused a marked increase in erythropoietin receptor density.

We have now compared the effects of DMSO priming on the erythropoietin response with those of two other chemical inducers, hexamethylene bisacetamide (HMBA), and sodium butyrate, and have characterized the erythropoietin–receptor interaction in detail. The results give new insight into possible mechanisms of erythropoietin receptor functioning.

MATERIALS AND METHODS

Cells. Rauscher murine erythroleukemia cells (11) were the generous gift of N. J. DeBoth (Erasmus University, Rotterdam). Clone R28 was derived by limiting dilution from its parent line (10). Clone PAN-4 was derived by sequential "panning" (12) on Petri dishes coated with streptavidin and biotinylated erythropoietin as follows. Petri dishes (Falcon 1024) were coated with 0.01 μg of streptavidin per ml (10 ml) in coating buffer (1.6 g of Na_2CO_3 and 2.9 g of $NaHCO_3$ per liter) for 16 hr at 23°C. The dishes were washed four times with sterile Dulbecco's phosphate-buffered saline (PBS). Biotinylated, biologically active recombinant human erythropoietin (rhEpo) (10 μg) in 10 ml of PBS/1% bovine serum albumin was added for 2 hr at 23°C. The solution was removed, and the dishes were washed five times with culture medium containing 1% fetal bovine serum (FBS). Ten milliliters of 1×10^6 cells per ml in Dulbecco's modified Eagle medium (DMEM)/1% FBS were added to the dish and were incubated for 1 hr at 4°C. Nonadherent cells were removed by

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Abbreviations: DMSO, dimethyl sulfoxide; HMBA, hexamethylene bisacetamide; rhEpo, recombinant human erythropoietin; FBS, fetal bovine serum.

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gentle swirling and aspiration. Adherent cells were removed with a plastic cell scraper, washed twice, and recultured at 37°C for 48 hr. They were then repanned and recultured for a total of four cycles. The resulting cells were designated PAN-4. Friend murine erythroleukemia cells (clone 745) (13) were the generous gift of Blanche Alter (Mt. Sinai School of Medicine). Throughout these experiments, R28 and PAN-4 appeared identical in all respects. Terminal differentiation was measured by detection of hemoglobin before or after treatment with erythropoietin and/or by staining with benzidine (14). DMSO, HMBA, and sodium butyrate were purchased from Sigma.

Erythropoietin. Highly purified rhEpo (15) was the generous gift of Elanex Pharmaceuticals (specific activity = 200,000 international units/mg). It was >99% pure by SDS/polyacrylamide gel electrophoresis (16). Erythropoietin was biotinylated with NHS-LC-biotin [sulfosuccinimidyl 6-(biotinamido) hexanoate; Pierce]. After quenching the reaction with 0.1 M glycine and dialysis to remove excess biotin reagent, the specific biological activity of the material was 80% that of the control, nonbiotinylated hormone (17). Absorption of the biotinylated rhEpo with immobilized streptavidin followed by bioassay revealed that >75% of the biological activity was absorbed out by streptavidin, thus demonstrating that biotinylated rhEpo was biologically active. Additional studies demonstrated that reaction of biotinylated rhEpo with excess streptavidin in solution yielded ≈50% of the specific biological activity of native erythropoietin, thus confirming that the rhEpo–biotin–streptavidin construct retained its capacity to recognize the erythropoietin receptor and could serve as an affinant in the panning procedure. rhEpo was labeled with ^{125}I using Iodo-Gen (Pierce) as described (4). The radiolabeled hormone exhibited full biological activity and contained 0.2–0.4 mol of ^{125}I per mol of rhEpo.

Receptor Binding. Cells were collected by centrifugation, washed twice with PBS, resuspended in DMEM/10% FBS/0.2% sodium azide (binding buffer), and incubated at 0°C for 30 min to inhibit energy-dependent receptor-mediated endocytosis. Cells (5×10^6) were added to glass test tubes, incubated for 5 min at 37°C, and then incubated with specified concentrations of ^{125}I-erythropoietin in the absence or presence of a 100-fold excess of unlabeled erythropoietin (total volume = 200 μl). All determinations were done on triplicate samples. After 30 min of incubation, which had been determined in preliminary experiments to achieve equilibrium, each cell suspension was transferred onto a 200-μl cushion of FBS in a polypropylene centrifuge tube. The cells were sedimented by centrifugation at 9000 rpm, 3 min (Beckman Microfuge 12). The tube contents were frozen and the tips were cut off for measurement of bound radioactivity by γ scintillation spectrometry. Specific binding at a given erythropoietin concentration was defined as the difference in bound radioactivity between the mean of samples incubated in the absence or presence of a 100-fold excess of unlabeled erythropoietin.

Total Cytoplasmic RNA Extraction and Northern Blot Analysis. Cytoplasmic RNA was prepared using guanidinium isothiocyanate (18). Forty micrograms of total RNA was subjected to electrophoresis in 1.2% agarose containing 5.5% formaldehyde and transferred to GeneScreen (Du Pont). The filters were hybridized sequentially with a ^{32}P-labeled synthetic oligomer complementary to nucleotides 256–305 of the Friend murine erythroleukemia cell erythropoietin receptor cDNA (7) and with a ^{32}P-labeled plasmid containing a cDNA of glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene (19). The synthetic oligomer was end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (20). The radiolabeled plasmid probe was generated by nick-translation (21). Relative molecular mass of the mRNA that hybridized to each of the probes agreed with previously published values.

RESULTS AND DISCUSSION

The biologic response of Rauscher cells to erythropoietin is markedly enhanced by pretreatment (addition, then removal) with DMSO (10). We have now obtained similar results with two other chemical inducers, HMBA and sodium butyrate. Cells were pretreated for 24 hr with 1% DMSO, 2 mM HMBA, or 1 mM sodium butyrate, washed, and then grown in the absence or presence of 10 international units of rhEpo per ml for 2 days. The percentage of erythropoietin-specific Hb^+ cells was determined (Table 1). Whereas cells without pretreatment were only 10% Hb^+, pretreatment resulted in large amplification of the response to 60%, 40%, and 50% Hb^+ cells, respectively, for the three agents.

A more detailed study of the effects of one of these agents, DMSO, has shown that this amplification of the erythropoietin response is accompanied by an increase in the rate of response—that is, pretreatment moves the maximum erythropoietin-induced hemoglobinization from 4 days of erythropoietin treatment to only 2 days. In addition, the erythropoietin sensitivity of the cells is increased by 10- to 20-fold (left-shifted dose–response curve) (10). These changes are accompanied by an increase in the erythropoietin receptor density of 5.6- to 6.6-fold (Table 1). In contrast to the results seen with Rauscher cells, identical experiments performed on Friend erythroleukemia cells (13), which do not hemoglobinize in response to erythropoietin, showed no effect of chemical inducers on the erythropoietin response (Table 1). A small effect on receptor density was observed, but it did not approach the magnitude seen with Rauscher cells. These observations prompted further investigation of the erythropoietin receptor in DMSO-primed cells.

The binding of ^{125}I-erythropoietin to receptors on nonpretreated Rauscher cells was concentration dependent and saturable (Fig. 1). A Scatchard analysis (22) revealed two slopes, consistent with two receptor populations (Fig. 2). Calculations from this analysis revealed that PAN-4 cells have ≈1000 higher-affinity receptors (K_d = 0.8 nM) and ≈2000 lower-affinity receptors (K_d = 8.1 nM) per cell. The results for R28 cells are strikingly similar (10). This density of receptors and the observation of two affinity populations are similar to those reported on other erythroleukemia cells (2)

Table 1. Effect of pretreatment with chemical inducers on erythropoietin-induced differentiation and receptor number of erythroleukemia cells

Cells	Pretreatment*	Epo-specific Hb^+ cells,† %	Epo receptors, no. per cell
Rauscher	None	10 ± 2	3,000 ± 100
	DMSO	60 ± 10	20,000 ± 400
	HMBA	40 ± 10	19,000 ± 350
	Butyrate	50 ± 10	17,000 ± 700
Friend	None	0	210 ± 20
	DMSO	0	400 ± 50
	HMBA	0	350 ± 30
	Butyrate	0	380 ± 20

Epo, erythropoietin. Values are means ± SD from triplicate determinations.

*After pretreatment for 24 hr with 1% DMSO, 2 mM HMBA, or 1 mM sodium butyrate, cells were washed and incubated in the absence or presence of 10 international units of rhEpo per ml for 48 hr.

†Hb^+ cells were detected by staining with benzidine. Epo-specific Hb^+ values were determined by subtracting the % Hb^+ cells detected in replicate cultures pretreated with chemical inducers but incubated in the absence of Epo from that obtained in cultures incubated in the presence of Epo.

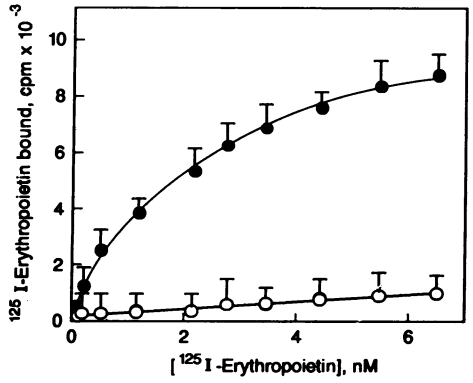

FIG. 1. ^{125}I-erythropoietin binding to receptors on PAN-4 cells. ●, Specific binding; ○, nonspecific binding. Each point is the mean of triplicate determinations ± SD. Binding was carried out as described in the text.

and contrast with 350 receptors per cell found on the parent, uncloned Rauscher line (not shown).

Erythropoietin receptors of Rauscher cells were increased dramatically by DMSO priming (Fig. 3, Table 1). At lower ^{125}I-erythropoietin concentrations the binding to DMSO-primed cells was identical to that of unprimed cells. However, binding at higher ^{125}I-erythropoietin concentrations revealed the presence of an apparently new population of high-density receptors on DMSO-primed cells. This unexpected observation was made consistently on R28 and PAN-4 cells in several experiments using cells of widely different passage number.

We analyzed the binding curve of DMSO-primed PAN-4 cells by the method of Scatchard (Fig. 4). As shown in Fig. 4 *Inset*, the Scatchard analysis of the portion of the binding curve involving lower concentrations of ^{125}I-erythropoietin (corresponding to the left side of Fig. 3) shows a single slope consistent with 1000 receptors per cell with a K_d = 1.0 nM, virtually identical to that of the higher-affinity receptor population of non-DMSO-primed cells (K_d = 0.8 nM) (Fig. 2). In marked contrast, the Scatchard analysis of the DMSO-induced, high-density receptor population (Fig. 4) reveals a pronounced convex upward geometry. This finding is considered to be diagnostic of positive cooperativity—that is, an increasing affinity of the receptor for erythropoietin with increasing site occupancy (24). A Hill plot of these data (Fig. 5, line A) exhibits a coefficient (nH) of 6.75, indicative of marked positive cooperativity among these DMSO-induced erythropoietin receptors. In contrast, the lower density non-cooperative receptor population found on DMSO-primed cells exhibits a Hill coefficient of nH = 0.84 (Fig. 5, line B) comparable to that found for the receptors on non-DMSO-primed cells (nH = 0.82, not shown). Because of this ther-

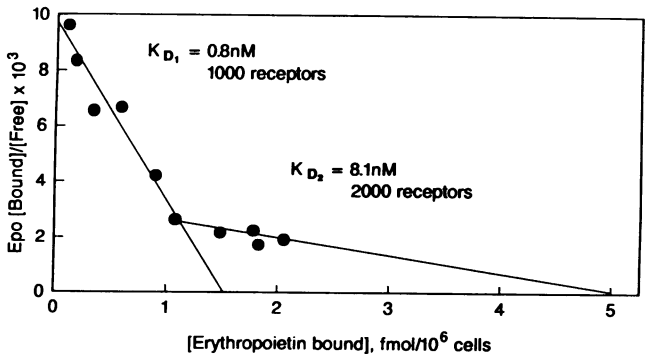

FIG. 2. Scatchard analysis of erythropoietin receptors on PAN-4 cells. Data are from Fig. 1.

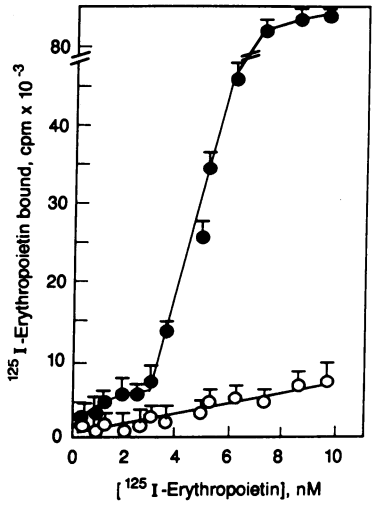

FIG. 3. Erythropoietin receptors on PAN-4 cells are increased dramatically by DMSO priming. PAN-4 cells were incubated in the presence of 1% DMSO for 24 hr prior to the binding study. ●, Specific binding; ○, nonspecific binding. Values are means of triplicates ± SD.

modynamic characteristic, an equilibrium dissociation constant for the DMSO-induced, high-density receptors could not be calculated from these data. However, the binding curve revealed saturation to be approached at ≈20,000 receptors per cell, a 7-fold increase over nonprimed cells. The association of the appearance of this new high-density receptor class with an amplified erythropoietin biologic response (Table 1) strongly suggests that the high-density cooperative receptor class is biologically functional. Although investigators have observed some effect of DMSO treatment on the erythropoietin receptor density of other erythroleukemia cells (2, 23), the magnitude of the density increase seen here, the appearance of marked positive cooperativity, and the amplification of erythropoietin's biological effect are unique.

Positive cooperativity is a common property of steroid receptors (25). These receptor proteins apparently can accommodate more than one ligand, thereby resulting in an allosteric effect of one ligand binding on another. In contrast, examples of positive cooperativity among receptors of polypeptide hormone ligands are rare. The insulin receptor can exhibit modest positive or negative cooperativity under specified conditions of binding (26). Recently, McLane and

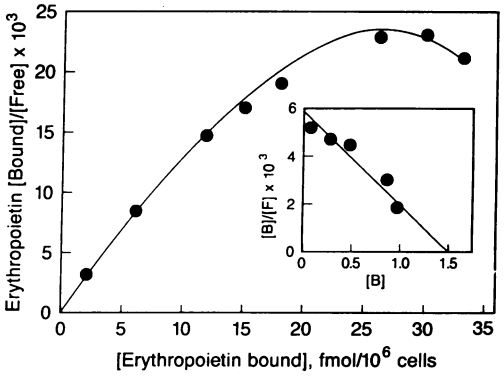

FIG. 4. Scatchard analysis of erythropoietin receptors on DMSO-primed PAN-4 cells. Note the convex upward geometry diagnostic of positive cooperativity (23) in the Scatchard analysis of DMSO-induced, high-density receptors. (*Inset*) Analysis of binding at low ^{125}I-erythropoietin concentrations. Note the single negative slope consistent with 1000 receptors per cell, K_d = 1.0 nM. [B]/[F], [bound]/[free].

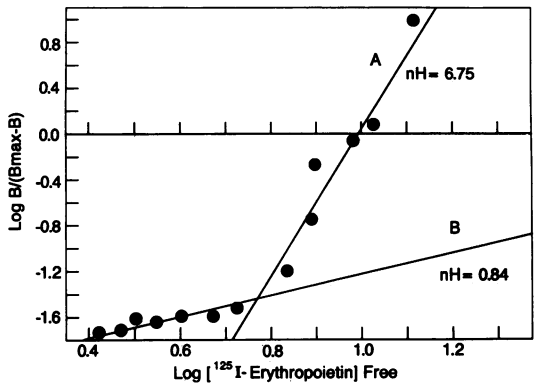

FIG. 5. Hill analysis of erythropoietin receptors on DMSO-primed PAN-4 cells. Line A: DMSO-induced high-density receptors; Hill coefficient (nH = 6.75) indicative of strong positive cooperativity. Line B: analysis of noninduced receptors; Hill coefficient (nH = 0.84) indicates noncooperativity.

Pawelek (27) demonstrated that S91 mouse melanoma cells expressing receptors for β-melanocyte-stimulating hormone exhibit a pronounced increase in receptor density and positive cooperativity during the G_2 phase of the cell cycle. Interestingly, this was associated with an increased response of these cells to the hormone, analogous to the data on the erythropoietin receptor reported here. Anagnostou *et al.* (28) have demonstrated functional erythropoietin receptors on endothelial cells. These receptors, which appear to exhibit positive cooperativity spontaneously, are of a very different size than the erythropoietin receptor found on erythroid cells. The relationship of these endothelial cell erythropoietin receptors to those found on erythroid cells remains to be elucidated.

Our measurement of the degree of positive cooperativity using the Hill analysis and the resultant calculation of the Hill coefficient (nH) reveals a very high value (6.75) for the DMSO-induced erythropoietin receptors. This Hill coefficient is substantially higher than others reported for receptor systems but is comparable to that of nH = 5.07 reported for methyltrienolone binding to canine prostatic cells (25) and nH = 4.5 found for binding of the endogenous activator protein to gastric H^+,K^+-ATPase (29).

We addressed one possible mechanism for the DMSO up-regulation of the erythropoietin receptor by measuring erythropoietin receptor mRNA (Fig. 6). DMSO priming resulted in an initial decrease in receptor message followed by an increase of approximately 2- to 3-fold after 24–48 hr. No change was seen in glyceraldehyde-3-phosphate dehydrogenase, a control, housekeeping gene. This observed increase in receptor message does not appear to account for the magnitude of the receptor up-regulation.

In addition to a DMSO-induced increase in receptor transcript levels, whether due to an effect on gene transcription or on mRNA processing, other possibilities exist that might

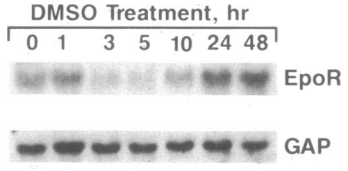

FIG. 6. Effect of DMSO on erythropoietin receptor mRNA in PAN-4 cells: Northern blot analysis (EpoR) probed with 50-mer synthetic oligonucleotide probe homologous to Friend cell receptor cDNA. GAP, glyceraldehyde-3-phosphate dehydrogenase. Note decrease in receptor mRNA after 3 hr of DMSO treatment followed by increase in 24–48 hr. Receptor transcript = 2 kilobases, consistent with results published for Friend cells (7).

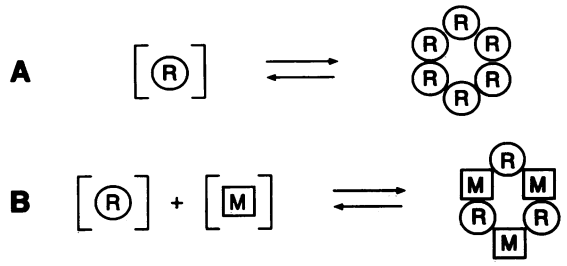

FIG. 7. Possible mechanisms of receptor–receptor interaction (cooperativity) through formation of oligomers (clusters). R, receptor binding unit; M, hypothetical accessory molecule. Although the stoichiometries depicted are arbitrary, the magnitude of the Hill coefficient (nH = 6.75) implies several subunits.

explain the increase in receptor binding unit density. Post-translational processing of receptor protein leading to stabilization within the cell and/or an increased efficiency of transport to the plasma membrane surface may play a role. Additionally, DMSO may exert a direct effect on the plasma membrane itself, thereby "unmasking" otherwise "buried" receptor binding units. The recent description of protamine uncovering otherwise hidden epidermal growth factor receptors on 3T3 and A431 cells and isolated plasma membranes from these cells would lend credence to such a mechanism (30).

Recently, Lammers *et al.* (31) demonstrated transphosphorylation between chimeric receptors for insulin and epidermal growth factor, indicating the physical association of receptor molecules and a functional consequence thereof. Importantly, these investigators emphasized the possible role of such intermolecular interactions in amplifying signal transduction events. Since the erythropoietin receptor lacks a kinase domain, we speculate that the receptor interaction or "clustering" (32, 33) reflected by the positive cooperativity may occur through oligomer formation involving receptor protein and one or more accessory proteins (Fig. 7). Presumably, the accessory protein would exhibit a functional domain capable of initiating the cytosolic signal. Our demonstration that erythropoietin activates the protooncogene c-*myc* (34) through a protein kinase C-dependent pathway (35) and that phosphorylation/dephosphorylation events are triggered by the erythropoietin–receptor interaction (36, 37) indicates that kinase activation follows rapidly after receptor activation. Further analyses of these alternatives and the possibility that natural modulators of erythropoiesis may induce a similar erythropoietin–receptor interaction during normal erythrocyte differentiation will help identify the initial events in erythropoietin's signal pathway and the macromolecules participating in it.

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