Effect of Tunicamycin Treatment on Ligand Binding to the Erythropoietin Receptor: Conversion From Two Classes of Binding Sites to a Single Class

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Scatchard analyses of erythropoietin (EPO) binding to its receptor (EPO-R) have shown that some erythroid cells display a biphasic nature of the ligand-saturation curve, indicating the presence of two classes of binding sites with different affinities. The biochemical basis accounting for this observation is unknown. We found that the culture of a single phase with high-affinity sites. Mouse EPO-R is glycosylated at one asparagine residue in the extracellular region. The mutant EPO-R, in which asparagine residue responsible for N-glycosylation was replaced with glutamine residue, was expressed on BHK cells. Unexpectedly, mutant EPO-R was similar in ligand binding to wild-type EPO-R. BHK cells that expressed mutant EPO-R showed biphasic Scatchard plots that were converted to single-phase plots with only high-affinity sites by tunicamycin treatment. These results indicate that the N-linked sugar of EPO-R is not involved in the manifestation of two classes of binding sites, and that there is a yet unidentified glycoprotein crucial for the ligand-saturation characteristics of EPO-R.

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(5'-GGACCTCCAGTACGGTCTCAGAGC-3'), and mutation primer R (5'-AGGTATGCTGAAAACTGTCACCGGAGGC-3') were synthesized by a DNA synthesizer ABI 380 A (Applied Biosystems, Los Angeles, CA). The first PCR was performed between primers N and R and between primers F and C using pXM190 as a template. Each PCR product was precipitated with 0.6 vol of 2.5 mol/L NaCl containing 20% polyethylene glycol (PEG) and the precipitated products were then mixed. The mixture was used as a template for the second PCR between primers N and C. Each of the total 30 cycles of PCR consisted of incubation for 1 minute at 95°C for denaturation, 2 minutes at 55°C for annealing, and 3 minutes at 72°C for elongation. The PCR product of 429-bp length was digested with HindIII and PstI. The HindIII site is included in primer N. The digest was ligated with the fragment of pUC18 digested with HindIII and PstI. Mutations were confirmed with the dideoxy sequencing method. The HindIII site was blunt-ended by a Klenow fragment and ligated with a KpnI linker.

The KpnI ligated with the fragment of pUC18 digested with HindIII and PstI. The transfectants expressing the wild-type or the N-glycosylation-defective mutant EPO-R were established.

Protein synthesis and N-glycosylation. BHK cells (3 x 10^5 cells/well) were precultured in 6-well tissue culture plates (3 mL culture medium/well) for 24 hours. For the assay of protein synthesis, L-[35S]Met was added at 30 μCi/mL to the culture medium and the culture was continued for 2.5 hours with or without either 500 μmol/L cycloheximide or 1.3 μg/mL tunicamycin. For the assay of glycoprotein synthesis, D-2-[3H]Man was added at 30 μCi/mL to the culture medium and the culture was continued for 2.5 hours with or without 1.3 μg/mL tunicamycin. The cells were washed three times with PBS and lysed with 1.3 mL of 250 mmol/L Tris-HCl, pH 7.4, containing 1% Triton X-100, 25 mmol/L NaCl, and 250 mmol/L MgCl2. The incorporation of radioactivity into 5% trichloroacetic acid-insoluble materials was measured by the filter paper method using 50 μL lysate per well.

Anti-N-terminal mouse EPO-R antiserum. A peptide (AP-SPSLDPKFESKAC) was synthesized. Amino terminal 15 amino acids of this peptide correspond to those of mouse EPO-R. The rabbit anti-N-terminal mouse EPO-R antiserum was prepared as previously described, using this peptide conjugated to KLH as an antigen. The N-terminal peptide-directed antibodies in the serum were purified with CH Sepharose 4B gel on which the antigen peptide cross-linked to ovalbumin was fixed. The serum was gently mixed with the gel at 4°C overnight. The gel was washed thoroughly with PBS and the bound antibodies were eluted from the gel with 250 mmol/L acetic acid, pH 2.5, containing 500 mmol/L NaCl. The eluted fraction was neutralized with 1 mol/L Tris-HCl, pH 8.5. The antibodies were concentrated by ultrafiltration, replacing the buffer with PBS.

Preparation of rHuEPO-fixed gel. For preparation of the EPO-fixed gel, 150 mg rHuEPO in 100 mmol/L NaHCO3, was gently mixed with 9 mL CH Sepharose 4B gel at 20°C for 1 hour and at 4°C overnight. The gel was pelleted by centrifugation and was suspended in 100 mmol/L Tris-HCl, pH 8.0, to block the remaining sites on the gel. The gel was washed with an acidic solution (50 mmol/L acetic acid containing 500 mmol/L NaCl) and then with the basic solution (50 mmol/L Tris containing 500 mmol/L NaCl) four times each. The EPO-fixed gel was kept in PBS containing 0.1% NaN3 at 4°C before use.

Western blotting of EPO-R expressed by BHK cells. Wild-type EPO-R and N-glycosylation-defective mutant EPO-R, solubilized from BHK cells, were concentrated by using the EPO-fixed gel and then identified with Western blotting technique. Approximately 10^6 cells were lysed by incubation at 4°C for 1 hour in 2 mL buffer A (PBS containing 0.5% [wt/vol] CHAPS, 10 μmol/L APMSF, 10 μmol/L leupeptin, and 1 mmol/L EGTA). The lysate was centrifuged at 12,000g for 30 minutes at 4°C. The supernatant was mixed with 15 μL rHuEPO-fixed gel (15 mg rHuEPO/mL gel) overnight at 4°C. The gel was pelleted by centrifugation and washed three times with 500 μL of buffer A. Proteins bound to the EPO-fixed gel were solubilized in a 50 μL sodium dodecyl sulfate (SDS)-buffer consisting of 60 mmol/L Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1.4 mol/L 2-mercaptoethanol, and 0.001% bromophenol blue. Solubilized proteins were separated by electrophoresis with SDS-5% polyacrylamide gel. Western blotting was performed according to the method of Burnette, with some modification. Brieﬂy, the polyacrylamide gel was immersed in 50 mL of a transfer buffer consisting of 48 mmol/L Tris, 39 mmol/L glycine, 1.3 mmol/L SDS, and 20% methanol for 15 minutes, with two changes of buffer. The proteins in the gel were transferred to a 0.45-μm nitrocellulose ﬁlter at 1.2 mA/cm2 for 40 minutes. The nitrocellulose ﬁlter was immersed in 15 mL of block

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was washed three times with buffer B and was immersed in a 15 mL

...detection system.

...antibodies purified from the rabbit antiserum). After incubation for 1 hour at room temperature, the filter was washed three times with buffer B containing peroxidase-fixed goat antirabbit IgG (1 μg/mL) for 1 hour at room temperature and washed five times with buffer B. Antigens (EPO-R) were visualized by using the ECL Western blotting detection system.

RESULTS

Effect of tunicamycin treatment on binding of 125I-rHuEPO on human erythroleukemia RM10 cells. Figure 1 shows Scatchard plots of the ligand-saturation curves of human erythroleukemia RM10 cells. The plot was clearly biphasic (Fig 1A). There were 14 binding sites/cell of high-affinity sites (kd = 12 pmol/L) and 63 sites/cell of low-affinity sites (kd = 614 pmol/L), which were consistent with previous results. The Scatchard plot of the cells that had been cultured for 12 hours in the presence of tunicamycin gave a straight line (Fig 1B). The drug-treated cells had 59 binding sites/cell with a kd of 142 pmol/L, indicating that the low-affinity sites disappeared upon tunicamycin treatment of the cells. Two other experiments showed similar Scatchard plots. Averaged kinetic parameters of three experiments for the drug-untreated cells were 15 ± 4 sites/cell of high-affinity sites (kd = 14 ± 6 pmol/L) and 72 ± 9 sites/cell of low-affinity sites (kd = 594 ± 22 pmol/L), and those for the drug-treated cells were 51 ± 8 sites/cell with a kd of 112 ± 23 pmol/L. Mouse EPO-R is N-glycosylated at position 51 in the extracellular region. The nucleotide sequence predicts that human EPO-R also has one N-glycosylation site at the same position. One possibility to account for the tunicamycin-induced conversion of the ligand-saturation curve of human erythroleukemia cells is that the EPO-R with no N-linked sugar has a higher affinity to EPO than N-glycosylated EPO-R and the cells before the drug treatment express both N-glycosylated and unglycosylated EPO-R molecules, showing the biphasic nature of Scatchard plot.

Binding of 125I-rHuEpo to BHK cells. To test the above possibility, a plasmid was constructed for expression of the murine mutant EPO-R in which Asn at position 51 responsible for N-glycosylation was replaced with Gln. Stable transformants of BHK cells that expressed the wild-type EPO-R or the N-glycosylation-defective mutant EPO-R were established. Three clones of each EPO-R type were arbitrarily chosen and the binding of EPO was examined. The parental BHK cells that were mock-transfected or transfected with the plasmid lacking EPO-R cDNA showed no specific binding of 125I-rHuEPO. Scatchard plots of BHK cells (W1, W2, and W3) expressing the wild-type EPO-R were all biphasic, similar to the plots of COS cells. It is noted that a biphasic nature of Scatchard plots was also found for all BHK cells (M1, M2, and M3) with the mutant EPO-R. These results and calculated parameters are summarized in Table 1. Dissociation constants for high- and low-affinity sites of the mutant EPO-R were similar to those of the wild-type.

We examined whether the wild-type EPO-R was indeed glycosylated. Three BHK clones were used for experiments: W1 that expressed the wild-type EPO-R, W1 that were cul-
Fig 2. Western blotting of wild-type EPO-R and the N-glycosylation-defective mutant EPO-R expressed by BHK cells. EPO-R solubilized from BHK cells was concentrated with an EPO-fixed gel (see Materials and Methods). The preparation of parental BHK cells as a control was also obtained by identical procedures. The samples eluted from the EPO-fixed gel were subjected to SDS-polyacrylamide gel electrophoresis and the separated proteins were transferred to nitrocellulose filter. The EPO-R protein was detected using rabbit antibodies against the N-terminal peptide of murine EPO-R (see Materials and Methods). Lanes 1 and 5, parental BHK cells with no EPO-R; lanes 2 and 6, W1 cells with the wild-type EPO-R; lanes 3 and 7, W1 cells cultured for 12 hours in the presence of 1.3 μg/L tunicamycin; lanes 4 and 8, M1 cells with the mutant EPO-R lacking the N-glycosylation site; lane 9, mouse erythroleukemia TSA8 cells. Lanes 1 through 4 and 9, 1 minute of exposure; lanes 5 through 8, 5 minutes of exposure of lanes 1 through 4.

The EPO-R on these cells was solubilized and the solubilized EPO-R was concentrated by a column containing the immobilized EPO. The concentrated EPO-R was detected with Western blotting using the antibodies against the N-terminal region of murine EPO-R (Fig 2). No bands were detected for the parental BHK cells (lane 1). The wild-type W1 gave two bands of 68 and 66 Kd (lane 2), with the intensity of the 68-Kd protein band being much stronger than that of the 66-Kd band. The only 66-Kd band was found when the wild-type W1 cells were cultured for 12 hours in the presence of tunicamycin (lane 3). These results indicate that the 68-Kd band is the N-glycosylated form of EPO-R and the 66-Kd band is the unglycosylated form. A very faint band with 66 Kd was found for the N-glycosylation-defective mutant M1 cells (lane 4), which became clearly visible upon longer exposure (lane 8). Nonspecific bands of 55 and 41 Kd also appeared upon longer exposure (lanes 5 and 8). Although the N-glycosylation-defective mutant EPO-R showed a faint band even upon longer exposure (lane 8), the cell surface expression of EPO-R was confirmed by the Scatchard analysis of these transfected cells (Table 1). Mouse erythroleukemia TSA8 cells used as a control yielded the 68-Kd band (lane 9). We concluded from these results that wild-type EPO-R expressed by BHK cells is mostly N-glycosylated, but N-glycosylation has no effect on ligand-saturation curves.

Thus, the tunicamycin-induced conversion of Scatchard plot on RM10 cells may not be attributable to the disappearance of the glycosylated form of EPO-R. There may be an additional glycoprotein that affects the affinity of EPO-R to the ligand. If this is the case, the tunicamycin treatment of BHK cells expressing EPO-R would change the biphasic nature of the ligand-saturation curves.

Turnover of EPO-R on BHK cells. To find the appropriate culture time of BHK cells in the presence of tunicamycin, and M1 that expressed the N-glycosylation-defective mutant EPO-R. The EPO-R on these cells was solubilized and the solubilized EPO-R was concentrated by a column containing the immobilized EPO. The concentrated EPO-R was detected with Western blotting using the antibodies against the N-terminal region of murine EPO-R (Fig 2). No bands were detected for the parental BHK cells (lane 1). The wild-type W1 gave two bands of 68 and 66 Kd (lane 2), with the intensity of the 68-Kd protein band being much stronger than that of the 66-Kd band. The only 66-Kd band was found when the wild-type W1 cells were cultured for 12 hours in the presence of tunicamycin (lane 3). These results indicate that the 68-Kd band is the N-glycosylated form of EPO-R and the 66-Kd band is the unglycosylated form. A very faint band with 66 Kd was found for the N-glycosylation-defective mutant M1 cells (lane 4), which became clearly visible upon longer exposure (lane 8). Nonspecific bands of 55 and 41 Kd also appeared upon longer exposure (lanes 5 and 8). Although the N-glycosylation-defective mutant EPO-R showed a faint band even upon longer exposure (lane 8), the cell surface expression of EPO-R was confirmed by the Scatchard analysis of these transfected cells (Table 1). Mouse erythroleukemia TSA8 cells used as a control yielded the 68-Kd band (lane 9). We concluded from these results that wild-type EPO-R expressed by BHK cells is mostly N-glycosylated, but N-glycosylation has no effect on ligand-saturation curves.

Fig 3. Turnover of EPO-R expressed on BHK cells. W1 cells expressing wild-type EPO-R were cultured in the presence of 500 μmol/L cycloheximide. At the indicated times, the specific binding was assayed with 1 nmol/L (●) and 200 pmol/L (○) 125I-rHuEPO. Each point is the mean ± SD of triplicate assays.
When the specific binding was tested at 200 pmol/L, there was an increase of the binding at 1 hour of the culture. This increase was reproducible, but the reason behind it is unknown. After 2 hours of the culture, the binding decreased in a time course similar to that seen at 1 nmol/L; at 3.5 hours of the culture, about one-half of the binding disappeared. At 12 hours of the culture, no significant binding was observed when the binding was tested at either 1 nmol/L or 200 pmol/L (not shown). With BHK cells (M1) expressing mutant EPO-R we have not traced a culture time-dependent change in the specific binding, but we have confirmed that the specific binding disappeared when M1 cells were cultured for 12 hours in the presence of cycloheximide. Thus, all of the EPO-R molecules on the plasma membrane are renewed within 12 hours of the culture. Most (>95%) of the cells cultured for 12 hours in the presence of 500 μmol/L cycloheximide were viable, judging from staining with trypan blue. The decline in the EPO binding upon culture of the cells with the drug was not due to cell death.

Binding of 125I-rHuEPO to BHK cells treated with tunicamycin. The BHK cells that expressed wild-type EPO-R (W1) or mutant EPO-R (M1) were cultured in the presence or absence of tunicamycin and the ligand-saturation curves were drawn. The Scatchard plots are shown in Fig 4. The plots of W1 and M1 cells cultured for 16 hours in the absence of tunicamycin were biphasic (Fig 4A and D) and the calculated kinetic constants (kd values and binding sites) were consistent with those shown in Table 1. When W1 cells were cultured for 8 and 16 hours in the presence of tunicamycin, the ligand-saturation curves gave linear Scatchard plots, leaving only high-affinity sites (Fig 4B and C). Such conversion of the ligand-saturation curve occurred in M1 cells cultured for 8 hours in the presence of the drug (Fig 4E). Total binding sites decreased in both W1 and M1 cells upon culture in the presence of the drug. Tunicamycin not only inhibits N-glycosylation but also protein synthesis; under the conditions used here, the drug inhibited N-glycosylation and protein synthesis by 80% and 50%, respectively. Inhibition of both pathways may be responsible for the decrease in EPO binding sites of W1 cells expressing wild-type EPO-R, because the retardation of N-glycosylation impairs posttranslational processes of many glycoproteins, including their translocation to plasma membrane. The decrease in EPO binding sites of M1 cells expressing mutant EPO-R would be due to the inhibition of protein synthesis. The cells cultured for 12 hours in the presence of tunicamycin were morphologically similar to the drug-untreated cells and were viable.

DISCUSSION

In some erythroid cell lines, including primary erythroid precursor cells, EPO interacts with EPO-R with biphasic equilibrium binding kinetics, reflecting two classes of binding sites with high and low affinity. The biphasic Scatchard plot of a human erythroleukemia cell line, RM1, was converted to a single phase with only high-affinity sites by cultivating with tunicamycin. Similar results were obtained with BHK cells that expressed EPO-R cloned from a murine erythroid cell line. One putative N-glycosylation site exists in the extracellular region of both murine and human EPO-Rs and N-glycosylation has been shown in murine EPO-R. Modulation of the EPO binding by tunicamycin has been reported in murine erythroleukemia cell lines. These cells showed a straight line of Scatchard plot. The affinity increased five-fold in Friend cells and 1.7-fold in Rauscher cells upon culture of the cells with the drug. From these data, we thought that N-glycosylation defect of EPO-R might govern the ligand-saturation kinetics. However, this possibility was excluded by the result that the N-glycosylation-defective mutant EPO-R was similar to the wild-type receptor in their ligand-binding kinetics before and after drug treatment. These results indicate the presence of an accessory protein that affects the ligand-binding kinetics of EPO-R. The biphasic Scatchard plot of COS cells expressing EPO-R cloned from a murine erythroleukemia cell line that showed a straight line of Scatchard plot could be explained by the presence of such a protein.

There are two ways for such a protein to function as an affinity converter of EPO-R, the converter could be either positive or negative. First, cloned EPO-R has low affinity and its interaction with the putative converter protein forms high-affinity sites; glycosylation of the converter protein affects affinity of EPO-R to EPO. Second, EPO-R has high affinity and the converter protein exerts an inhibitory effect on the interaction of EPO-R with the ligand. This inhibitory protein may be rapidly metabolized and the culture of cells with tunicamycin results in the disappearance of the putative protein, leaving only high-affinity sites. The disappearance of the inhibitory protein could be derived from inhibition of N-glycosylation or that of protein synthesis or both. The soluble EPO-R lacking transmembrane and cytoplasmic regions binds to EPO with very low affinity (kd = ~17 nmol/L)7; also, rat pheochromocytoma cells (PC12 cells) express EPO-R with similar low affinity, which immunologically cross-reacts with the erythroid EPO-R.48 Taking these data together, we propose the hypothesis that EPO-R can be present in three forms with different affinities to EPO: low affinity (kd = ~20 nmol/L), middle affinity (kd = ~50 nmol/L), and high affinity (kd = ~50 pmol/L). The cloned EPO-R alone may express low-affinity sites. The interaction with a putative converter glycoprotein results in an expression of the binding sites with middle or high affinity. Interaction of EPO-R with the unglycosylated form of a converter protein constructs high-affinity sites, whereas the glycosylated form yields middle-affinity sites. The cells manifesting Scatchard plots with biphasic nature may express both unglycosylated and glycosylated forms. Cells with high-affinity sites of EPO-R have not been found. To show the presence of such a protein, we are currently testing the effect of the membrane fractions on the EPO binding to the soluble EPO-R and also to the EPO-R of PC12 cells.

EPO-R is a member of the cytokine receptor super family. Oligomerization of the receptors in this family appears to play a key role in constructing high-affinity sites and also providing the first step in the signal transduction pathway. The receptors for interleukin-2, interferon-γ, and granulocyte-macrophage colony-stimulating factor consist of heterosubunits, whereas granulocyte colony-stimulating factor, prolactin receptor, and growth hormone receptor
form homodimers. The formation of hetero-oligomeric or homodimeric structure has been proposed for EPO-R, but the relationship between these structures and ligand-saturation characteristics remains to be investigated.

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REFERENCES


Fig 4. Scatchard plots of wild-type EPO-R and the N-glycosylation–defective mutant EPO-R on BHK cells cultured with or without tunicamycin. In (A), (B), and (C), W1 cells with the wild-type EPO-R were used. In (D) and (E), M1 cells with the mutant EPO-R lacking the N-glycosylation site were used. (A) and (D) were cultured for 16 hours in the absence of tunicamycin; (B), (C), and (E) were cultured with 1.3 µg/L tunicamycin for 8, 16, and 8 hours, respectively. Cell numbers used for one assay were 6.5 X 10⁶ in (A), 5.4 X 10⁶ in (B), 8.4 X 10⁶ in (C), and 1.4 X 10⁶ in (D) and (E). In (A), (C), and (D), each point is the mean of duplicate assays. In (B) and (E), each point is the mean ± SD of triplicate assays.
N-LINKED SUGAR OF ERYTHROPOIETIN RECEPTOR


Effect of tunicamycin treatment on ligand binding to the erythropoietin receptor: conversion from two classes of binding sites to a single class

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