Anti-Erythropoietin Receptor (EPO-R) Monoclonal Antibodies Inhibit Erythropoietin Binding and Neutralize Bioactivity

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We have generated six high affinity monoclonal antibodies (MoAbs) to the human erythropoietin receptor (hEPO-R) polypeptide. All six MoAbs bind to the extracytoplasmic domain of the hEPO-R, and all immunoprecipitate 35S-labeled hEPO-R from metabolically labeled Ba/F3-hEPO-R cells. Four of the MoAbs neutralize the EPO-dependent growth of Ba/F3-hEPO-R cells, whereas two MoAbs are non-neutralizing. None of the MoAbs inhibit the EPO-dependent growth of Ba/F3-hEPO-R cells, whereas two MoAbs are non-neutralizing. Of the four neutralizing MoAbs, the one-half maximal inhibition occurs at MoAb concentrations ranging from 1 nmol/L to 50 nmol/L. These MoAbs also compete with radiolabeled EPO for hEPO-R binding. The two non-neutralizing MoAbs fail to inhibit EPO-dependent growth or compete with EPO-binding, even at antibody concentrations as high as 500 nmol/L. The four neutralizing MoAbs, designated group I, compete with each other for an epitope of the hEPO-R polypeptide required for EPO-binding. The two non-neutralizing MoAbs recognize discrete epitopes, and are designated group II and group III MoAbs. In conclusion, this is the first description of MoAbs specific for the hEPO-R. The MoAbs, which recognize three discrete epitopes, may be useful in characterizing the spectrum of cells that display the hEPO-R and in further defining the role of EPO in hematopoiesis.

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ERYTHROPOIETIN (EPO) is the primary regulator of mammalian erythropoiesis. EPO mediates its activity by binding to a specific receptor (EPO-R) expressed on the surface of immature erythroblasts. Although murine1,2 and human3,4 EPO-R cDNAs have been isolated, there remain several unresolved questions regarding the quaternary structure of the EPO-R complex.

The EPO-R exists in both high- and low-affinity states on the surface of FVA erythroblasts5,6 (erythroblasts infected with the anemia strain of the Friend Spleen Focus-Forming Virus) and transfected COS cells.7 A structural explanation for these two affinity states has not been established. Furthermore, when radiolabeled EPO is crosslinked to the EPO-R, two major cross-linked complexes of approximately 140 Kd and 100 Kd are observed.8 No correlation exists between the presence of the two crosslinked complexes and the two receptor affinities. For instance, murine erythroleukemia (MEL) cells demonstrate only the lower affinity receptor for an epitope of the hEPO-R polypeptide required for EPO-binding. The two non-neutralizing MoAbs recognize discrete epitopes of hEPO-R, which do not contribute to the EPO binding site.

MATERIALS AND METHODS

Growth factors. Highly purified recombinant human EPO was stored at −80°C (1 mg/mL). EPO was radiolabeled by the iodine monochloride technique to a specific activity of 1,000 cpm/fmol as previously described.9 Radiolabeled EPO retained full biological activity. WEHI-conditioned media was used as a source of murine interleukin-3 (IL-3).10

Generation of MoAbs. Monoclonal anti–hEPO-R–antibody-producing hybridoma cell lines were generated from two separate fusions using either mouse or hamster splenocytes. Balb/c mice were immunized with the purified secreted human EPO-R (shEPO-R) polypeptide.5 This secreted hEPO-R was generated by insertion of a termination codon at the beginning of the transmembrane domain of the hEPO-R cDNA; Chinese hamsters (CytoGen Research and Development, West Roxbury, MA) were immunized with Chinese hamster ovary (CHO) cells expressing the cell surface human EPO-R. Animals were boosted with immunogen on the 4 days before fusion, splenocytes were harvested, and hybridization with p36x6Ag8.653 myeloma cells was performed as previously described.9 Hybridoma supernatants were collected and assayed for the presence of antihuman EPO-R antibodies using standard enzyme-linked immunosorbent assay (ELISA) as the initial assay and either radioimmune precipitation or bioassay using FDCP-e cell proliferation70 as the secondary screening procedure. Twelve monoclonal anti–hEPO-R-antibodies were derived, from which a panel of 6 MoAbs were selected for further study.

Purification of MoAbs. Cell culture or ascites fluid generated from each hybridoma cell line (approximately 100 mL) was clarified by centrifugation at 1,500g × 10 minutes and filtered through a 0.45-mm filter. Ig-containing fractions were prepared on Protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described.9 The specificity of the anti–EPO-R MoAbs was

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Ten fore injection over the surface (hatched box). (B) Ba/F3 cells ex-
lized on the sensor surface using a carboxylated dextran matrix.
pressed the human EPO-R polypeptide were metabolically labeled
with 35S-methionine and cysteine for 2 hours. labeled proteins

RU value is the average of six independent binding assays. As con-

Specifically, for these studies purified secreted EPO-R (2.8 mg/
mL in PBS) (approximately 100 nmol/L) was immobilized to a

carboxylated dextran matrix. Unreacted groups were blocked
by the injection of 30 µL of ethanolamine, pH 8.5, followed by 15 µL
of 30 mmol/L NaOH to remove noncovalently bound peptide.
MoAb (100 nmol/L) was allowed to interact with the sensor sur-
face. Runs were performed at 25°C at a flow rate of 3 µL/min. The
surface was regenerated by injecting a 50-nmol/L HCL solution
over 2 minutes (6 µL injection).

Cell culture. Ba/F3 cells, a murine IL-3-dependent line, were
grown as previously described.1 Ba/F3 cells were electroporated
with either PXM-mEPO-R1 or PXM-hEPO-R2 as previously de-
scribed.7

Immuno~Precipitation of radio~labeled EPO-R. Immuno~
precipitation of radiolabeled EPO-R was performed as previously
described1 with a few modifications. Ba/F3 hEPO-R cells were meta-
bolically labeled with 35S-methionine and 35S-cysteine (35S-Express;
New England Nuclear, Boston, MA). Cell lysates were prepared,
and labeled proteins from an equal volume of cell lysate were im-
muno~precipitated with 10 µg of the indicated MoAb. Proteins were
eluted in gel sample buffer and run on 20% polyacrylamide/sodium
dodecyl sulfate gels in the presence of 10 mmol/L dithiothreitol.

EPO or IL-3-dependent growth assay. Ba/F3-hEPO-R or Ba/
F3-mEPO-R were grown in EPO media as previously described.1
To assay factor-stimulated growth, cells were washed twice in Plain
media (RPMI + 10% fetal calf serum with no supplemental growth
factor) and resuspended in Plain media. Cells (5 x 10^6 cells/100 µL)
were added to multiple wells of a 96-well plate. Supplemental EPO,
IL-3, or MoAb was added as indicated and growth was measured
after 48 hours by the dimethylthiazol diphenyl-tetrazolium bro-
mide (MTT) reduction assay.7

Radiolabeled EPO-binding assay. Ba/F3-hEPO-R cells (1 x
10^6) were incubated with a single, saturating concentration of radio-
labeled human EPO (200 pmol/L) in 200 µL of Plain media for 10
hours at 4°C. An excess of unlabeled EPO (10 nmol/L) and/or an
anti-EPO-R MoAb (100 nmol/L) was added throughout the incu-
bation. Each MoAb was run in triplicate.

Flow cytometric analysis. Ba/F3-hEPO-R cells were incubated
with the indicated anti-hEPO-R MoAbs (5 nmol/L) and were
stained with fluorescein isothiocyanate (FITC)-conjugated anti-
mouse secondary antibody. Cells were analyzed by FACScan (Becton
Dickinson, San Jose, CA).

BIACORE epitope exclusion assay. The secreted hEPO-R was im-
mobilized to the sensor surface as described above.11 Fourteen mi-
croliters of the first MoAb (100 nmol/L in PBS) was injected at a

confirmed by standard ELISA and/or radioimmunosorhent assay
(RIA) procedure. The six MoAbs used in this study were numbered:

mh2er 16.5.1 (MoAb no. 1), mh2er 13.2.2 (MoAb no. 2), mh2er
11.6.1 (MoAb no. 3), hhler 36.4.1 (MoAb no. 4), mh2er 7.9.2
(MoAb no. 5), and mh2er 19.2 (MoAb no. 6). MoAbs no. 1, 2, 5,
and 6 were all of subclass IgG1. MoAb no. 3 was IgA. MoAb no. 4
was hamster in origin (see above). All MoAbs were stored as 1
mg/mL stocks in phosphate-buffered saline (PBS). As controls, a
neutralizing anti-EPO MoAb (5.5.11)11 and a neutralizing anti-mur-
line IL-3 MoAb (Genzyme Corp, Boston, MA) were used.

Assay of anti-EPO-R MoAb binding. Standard conditions for
assaying MoAb binding with the BIAcore apparatus (Pharmacia)
were used.11 The Biacore system allows a measure of antibody-anti-
gen interactions in real time. The system uses surface plasmo
resonance to detect changes in optical properties at the surface of a
thin gold film. Initially, an antigen (EPO-R) is covalently cou-

Fig 1. Anti-EPO-R MoAbs bind specifically to the human EPO-R
polypeptide. (A) The secreted hEPO-R polypeptide was immobi-
lized on the sensor surface using a carboxylated dextran matrix.
Ten µL of the indicated MoAb solution (100 nmol/L) was injected at
a 3 µL/min flow rate. The surface was regenerated by injecting 5 µL
of 50 nmol/L HCl. The antibody binding is shown in RU units. Each
RU value is the average of six independent binding assays. As con-

The lower arrow identifies the EPO-R polypeptide. The upper arrow
identifies a proteolytic fragment of the EPO-R that was present in
some immunoprecipitations and was confirmed by Western blot
analysis using an antiserum against the amino terminal of the EPO-
R polypeptide (not shown).
flow rate of 7 μL/min. After binding, 14 μL of the second MoAb (10 nmol/L in PBS) was injected at the same flow rate. RU values were recorded for all 36 pairs of the six MoAbs tested.

RESULTS

Specificity of anti-EPO-R MoAbs. Initially, the specificity of the MoAbs against hEPO-R was assayed (Fig 1). For these experiments the secreted hEPO-R polypeptide (amino acids 1-223) was covalently coupled to a solid matrix, and binding of the anti–EPO-R MoAbs was tested directly using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden). This system has previously been used to assay association rates of MoAb/antigen interactions. Binding of MoAb to immobilized EPO-R peptide is measured in RU, which is proportional to the refractive index increment and therefore to the mass of protein bound to the surface. Figure 1A shows that all six MoAbs specifically bind to secreted hEPO-R (RU values ranging from 1540 to 2121), whereas a mock MoAb shows no specific binding. When the MoAbs were prebound in solution with an excess of secreted hEPO-R, binding to the solid matrix was inhibited (Fig 1A, hatched boxes).

The specificity of the MoAbs was further tested by immunoprecipitation of the full-length 35S-labeled human EPO-R, solubilized from metabolically labeled Ba/F3-hEPO-R cells (Fig 1B). The MoAbs immunoprecipitated a single band of 66 Kd (Fig 1B, upper arrow), consistent with the size of the human EPO-R described previously. MoAbs no. 1 and 5 demonstrate the most efficient immunoprecipitation of human EPO-R; MoAbs no. 2, 3, 4, and 6 only weakly immunoprecipitate the full-length polypeptide. Also, all six MoAbs recognize the full-length hEPO-R polypeptide by Western blot analysis (data not shown).

Neutralization of EPO-dependent growth by the anti-EPO-R MoAbs. To assay the bioactivity of the anti–EPO-R MoAbs, we generated EPO-dependent Ba/F3 subclones (Fig 2). Ba/F3 cells, which are murine IL-3 dependent, were stably transfected with either the human EPO-R or the murine EPO-R cDNAs and individual subclones were isolated by limiting dilution. Ba/F3-hEPO-R and Ba/F3-mEPO-R cells had similar EPO-dependent growth (one-half maximal growth at 50 mU/mL, or 5 pmol/L EPO). These cell lines also had similar IL-3-dependent growth (one-half maximal growth in 5% WEHI-conditioned media) (data not shown). We tested the ability of the anti–EPO-R MoAbs, at a single concentration of MoAb (100 nmol/L), to inhibit growth of Ba/F3-hEPO-R cells, growing in EPO or IL-3 (Fig 2B). MoAbs no. 1, 2, 3, and 6 all inhibited EPO-dependent growth, whereas MoAbs no. 4 and 5 only slightly inhibited EPO-dependent growth. None of the anti-EPO-R MoAbs inhibited IL-3-dependent growth, confirming that the MoAbs specifically bind to the human EPO-R but not to
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The MoAbs therefore specifically interact with the human and not the murine EPO-R polypeptide, even though these proteins have 82% amino acid identity.2,3

Binding of MoAbs to the cell surface EPO-R. To further assess the direct interaction of the anti-EPO-R MoAbs with the cell surface hEPO-R, we used a binding competition assay (Fig 3). Ba/F3–hEPO-R cells were incubated with a single concentration of 125I-EPO (200 pmol/L) either in the presence or absence of excess cold EPO, and with the various MoAbs. A subpopulation of MoAbs (MoAbs no. 1, 2, 3, and 6) inhibited 125I-EPO binding, whereas MoAbs no. 4 and 5 did not. The inhibition of EPO binding by the MoAbs therefore correlates well with their ability to neutralize the bioactivity of EPO (Fig 2B).

Because MoAb no. 5 failed to compete with radiolabeled EPO binding, we next tested its ability to bind directly to the cell surface hEPO-R using FACS analysis (Fig 4). For this analysis we compared MoAb no. 1 (neutralizing) and MoAb no. 5 (non-neutralizing) because these MoAbs are both murine and both of IgG1 subclass. MoAbs no. 1 and 5 had comparable binding to the Ba/F3–hEPO-R cells. Consistent with the data in Fig 3, EPO competed with MoAb no. 1 for EPO-R binding (Fig 4, A v B) but not with MoAb no. 5 (Fig 4, C v D), suggesting that these MoAbs recognize different epitopes of hEPO-R.

By FACS analysis (data not shown), all six anti-EPO-R MoAbs bind to the cell surface human EPO-R polypeptide but none bind to the murine EPO-R polypeptide. This result further confirms the specificity of the MoAbs for the human EPO-R and accounts for their failure to neutralize EPO-dependent growth of Ba/F3–mEPO-R cells (Fig 2C). The MoAbs therefore specifically interact with the human and not the murine EPO-R polypeptide, even though these proteins have 82% amino acid identity.2,3

**Fig 3.** Inhibition of EPO binding to hEPO-R by MoAbs. Ba/F3–hEPO-R cells (1 x 10^6) were incubated with a saturating concentration of 125I-EPO (200 pmol/L) in the presence (hatched box) or absence of cold EPO (10 nmol/L) (closed box). After an 8-hour incubation at 4°C, cells were washed and specific binding versus non-specific binding was assayed as described in Materials and Methods. Where indicated, Ba/F3–hEPO-R cells were preincubated with anti-EPO-R MoAb (100 nmol/L) for 18 hours at 4°C before the EPO binding assay.

**Fig 4.** Competition between EPO and anti-EPO-R MoAbs for cell surface hEPO-R binding. Ba/F3 hEPO-R cells were incubated with MoAb no. 1 (A and B) or MoAb no. 5 (C and D) (5 nmol/L) and stained with FITC-conjugated anti-mouse IgG antibody. Fluorescence was analyzed by FACS as described in Materials and Methods. The shaded area shows the staining of FITC-conjugated antimouse IgG alone and the unshaded area shows the staining of anti-EPO-R MoAbs and FITC-conjugated antimouse IgG. For the bottom two panels (B and D), staining was performed in the presence of excess EPO.
Fig 5. Dose-dependent inhibition of EPO-induced growth of Ba/F3-hEPO-R cells. Ba/F3-hEPO-R cells or Ba/F3-mEPO-R cells were incubated in EPO (10 pmol/L) or murine IL-3 (5% WEHI-conditioned media) in the presence of various concentrations of the anti–EPO-R MoAb as indicated. After 2 days an MTT reduction assay was performed. Results are expressed as the mean of triplicate values. Symbols represent Ba/F3-hEPO-R in EPO (closed triangles), Ba/F3-hEPO-R in WEHI (closed circles), Ba/F3-mEPO-R in EPO (open triangles), and Ba/F3-mEPO-R in WEHI (open circles). MoAbs examined were MoAb no. 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), and 6 (F).
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The first MoAb (100 nmol/L) was prebound to the sensor surface of the BIAcore apparatus as described in Materials and Methods. The first MoAb (100 nmol/L) was prebound to the sensor surface (MoAbs 1-6, columns). The surface was washed, and the second MoAb (10 nmol/L) was injected over the surface (MoAbs 1-6, rows). Binding of the second MoAb is shown as RU values. The shaded values demonstrate that each MoAb effectively competes with itself for surface binding (RU values > 265). The circled values are elevated RU values (> 900) that indicate that the second MoAb binds to a different epitope than the first MoAb.

The anti-EPO-R MoAbs should be useful in studying the EPO-binding pocket of the native EPO-R complex. The EPO-R is a member of the cytokine receptor superfamily. Although its crystal structure is not known, it is probably similar to the crystal structure of the growth hormone receptor, another member of this superfamily. The growth hormone receptor: (1) contains two distinct extracytoplasmic domains, both of which contribute residues to the growth hormone binding site; and (2) undergoes homodimerization after monomeric ligand binding. By analogy, we would predict that our group I antibodies recognize an epitope formed by two domains of the EPO-R polypeptide extracytoplasmic region.
ing by steric inhibition or by inducing a conformational change in the EPO-R that alters epitope recognition.

The cloned hEPO-R polypeptide may be only one component of the native EPO-R complex. Because group I MoAbs block EPO-binding, we can conclude that the hEPO-R polypeptide itself binds directly to EPO. Consistent with this observation, Harris et al.20 have recently demonstrated that hEPO-R fusion proteins bind directly to EPO. Also, a polyclonal antiserum raised against the extracytoplasmic domain of the human EPO-R polypeptide blocks EPO binding.20 We cannot rule out the existence of other EPO-R subunits that contribute to the EPO-binding pocket.

These MoAbs may be useful for future studies. First, they will be useful in signal transduction studies. Like EPO, they may induce EPO-R–associated tyrosine kinase activity,21 EPO-R phosphorylation,22 and EPO-R homodimerization.6 Secondly, they may be useful in analyzing and isolating early erythroblasts in bone marrow samples, as well as in distinguishing erythroleukemia (AML, M6 morphology), which comprises approximately 1% to 3% of human acute myeloblastic leukemia. Finally, because the MoAbs specifically recognize the hEPO-R and not the mEPO-R, chimeric receptors containing regions of the human and murine polypeptides should help delineate the specific epitopes recognized.

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