Activation of the Erythropoietin (EPO) Receptor by Bivalent Anti-EPO Receptor Antibodies

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Oligomerization of cytokine receptors including the erythropoietin (EPO) receptor has been advanced as a model for activation. If homodimerization of the EPO receptor activates it, then bivalent antibodies raised to the extracellular domain of the EPO receptor should also homodimerize and activate. Mouse monoclonal antibodies (IgG) raised to the soluble, extracellular domain of the human EPO receptor (EPOR) were found that would stimulate thymidine uptake of an human EPO-dependent cell line, UT-7/EPO. Dose response curves showed bell shapes where activity was low at low and high concentrations. Monovalent (Fab) fragments bound to the receptor but did not stimulate thymidine uptake, which indicates that two antibody binding sites are required for activation. The anti-EPOR antibodies stimulated the formation of burst forming unit erythroid colonies from human CD34+ cells purified from peripheral blood. This indicates that homodimerization of the EPO receptor by anti-EPOR antibodies is sufficient for both proliferation and differentiation of erythroid progenitor cells and that the constraints on dimerization necessary for activation are rather loose.

Erythropoietin (EPO)† is a glycoprotein hormone that is the primary regulator of erythropoiesis. It stimulates erythroid progenitors to proliferate and differentiate via binding to and activation of an EPO receptor expressed on the surface of cells. The murine and human EPO and EPO receptor genes have been cloned (1–6). The human EPO gene encodes a 508-amino acid protein that includes a 25-amino acid signal peptide, a 225-amino acid extracellular domain, a 22-amino acid transmembrane domain, and a 236-amino acid cytoplasmic domain. The EPO receptor is a member of a family of cytokine receptors that includes receptors for prolactin, growth hormone, interleukins 2–7, granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor, leukemia inhibitory factor, thrombopoietin ligand, and ciliary neurotrophic factor (7–11). This family is characterized by regions of similarity in their extracellular and intracellular domains. The family is also characterized by a lack of an identifiable protein tyrosine kinase domain in their intracellular region. Activation of the receptors by ligand binding induces a cascade of signaling events including phosphorylation of the EPO receptor (12), activation of the JAK-STAT pathway (13, 14), activation of PI3 kinase (15, 16), and activation of the RAS-MAPK pathway (17). Down modulation of the signal transduction pathway is also effected by binding of the protein phosphatase SH-PTP-1 to the C-terminal region (18).

Activation of many different receptors is thought to occur by oligomerization of the receptors (for reviews see Refs. 19 and 20). One of the most studied systems is growth hormone receptor where complexes have been shown to consist of two receptors bound to one ligand (21, 22). In this case the ligand is thought to act as a cross-linker bringing the receptors into close proximity whereupon they bind and interact with signaling molecules. EPO exists as a monomer in solution but is thought to contain two receptor binding sites. Thus it also may act as a cross-linker bringing two receptor subunits together. However, reports of putative EPOR subunits cloud the issue. Support for EPOR activation by dimerization comes from several sources. Oligomerization of the EPO receptor has been reported, although this can occur in the absence of EPO (23). In addition a murine EPO receptor with an Arg129 to Cys mutation has been reported to be constitutively active via cross-linking through the cysteines (24, 25). A retrovirus expressing this constitutively active form of the EPO receptor causes polycythemia in mice and infected cells can differentiate in the presence of a burst promoting activity and serum in the absence of EPO (26), which suggests that dimerization may support both proliferation and differentiation of erythroid precursor cells.

In order to extend the studies on the mechanism of EPOR activation, we decided to test for a non-EPO reagent that could cross-link and thus perhaps activate EPO receptors in a controlled manner. One method that may homodimerize receptors is by interaction of two receptors with a monoclonal antibody raised to the extracellular domain of the EPO receptor. This is possible because an antibody (e.g. IgG) contains two antigen binding sites. However, there have been numerous reports of anti-EPO receptor antibodies, but none were reported to activate. We have screened monoclonal antibodies raised to the extracellular domain of the EPO receptor for their ability to activate the EPO receptor. Four antibodies out of 96 that recognized EPO receptor in ELISA were identified that had this property. One of these was found to support formation of BFUe colonies from erythroid precursors. This suggests that a subset of anti-EPOR antibodies can homodimerize the EPO receptor. This homodimerization is sufficient for activation resulting in signals that support both proliferation and erythroid differentiation.

EXPERIMENTAL PROCEDURES

Immunization and Hybridoma Preparation—Balb/c mice (Charles Rivers Laboratories, Wilmington, MA) were immunized with subcutaneously injected soluble EPO receptor. Soluble EPO receptor was purified from medium conditioned by CHO cells expressing a cDNA encoding the EPO receptor signal peptide and the first 225 amino acids of the human EPO receptor (Met1-Pro249 as shown in 2) by affinity and
conventional chromatography (V. Parker, Ken Aoki and Tom Strickland (Amersham Corp.). After the final lyophilization, the spleenocytes were fused to Sp2/0-Ag14 mouse myeloma cells (American Type Culture Collection, Rockville, MD accession no. CRL 1581) using the polyethylene glycol procedure as described (27, 28). Tissue culture supernatants of hybridomas selected on HAT medium were tested by ELISA for specific antibody reactivity to rHuEPOs. Ninety six hybridomas which were positive in ELISA (96) were subjected to further screening and the antibody titers measured by the various antibodies was then determined colorometrically using ABTS Peroxidase single component substrate (Kirkegaard and Perry Laboratories, Inc.). Hybridomas were selected at passage one if optical density scored greater than 5-fold above background.

**ELISA Assays—** Purified, soluble EPO coated microtiter plates blocked with 5% BSA were used. Samples diluted in PBS/1% BSA were added to each well, incubated at room temperature then rinsed with wash solution (Kirkegaard and Perry Laboratories, Inc.). Wells were then treated with 50 mM heavy- and light- chain specific horseradish peroxidase conjugated secondary antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN). The immunoreactivity measured by the various antibodies was then determined colorometrically using TBS-Tween (20 mM Tris, pH 7.5 0.5M NaCl, 0.09% Tween 20). HRP-labeled goat anti-mouse IgG in PBS-Tween was incubated in each well for 45 min. at RT. Membranes were washed as before and the amount of antibody bound was determined using an ECL kit (Amersham Corp.). Each well was scored - to + + + based on intensity of dots.

**BIAcore Analysis of Anti-EPO Antibodies—** Real-time biospecific interaction analysis (BIA, Pharmacia Biosensor AB, Uppsala, Sweden) is based on surface plasmon resonance (SPR) (29) and was used to screen the ELISA positive supernatants. Soluble rHuEPO was covalently coupled to the sensor chip CM5 via primary amines. Each analysis cycle included an injection of 20 µl of hybridoma supernatant, followed by an injection of 100 µl of 35 µCi/ml [3H]thymidine (1 mCi/ml, 20 Ci/mmol) in a 96-well tissue culture plate. The SPR response is measured in Resonance Units (RU). Results of screening 96 wells which were positive in ELISA are shown in Table I. In these experiments, background binding is about 20 RU.

**FACscan Analysis—** The initial antibody screen used CHO cells transfomed with human EPO receptor sequences (Met-Tyr-Tyr competent (2)). CHO cells scraped from tissue culture dishes were washed and resuspended as single cells in a 96-well round bottom plate. Cells were suspended in either a negative control media or in one of the anti-EPO hybridomas supernatants at 4 °C for 1 h. After the incubation, cells were washed with PBS/BSA and then resuspended in a solution of fluorescein isothiocyanate labeled goat anti-mouse monoclonal antibody (Southern Biotech, Birmingham, AL). The cells were incubated again at 4 °C for 1 h, washed, and analyzed on a Becton Dickenson FACscan (San Jose, CA), and the data were analyzed using Cellquest software. To test purified antibody preparations, UT-7 cells (30) were used. Cells were suspended in either PBS/BSA (control) or rHuEPO at a concentration of 500 µg/ml (EPO block) at 4 °C for 45 min. Cells were washed twice with PBS/BSA and incubated a final time at 4 °C with phycoerythrin-labeled goat anti-mouse monoclonal antibody (Southern Biotech, Birmingham, AL). After a wash and resuspension the cells were analyzed by FACscan as described above.

**Thymidine Uptake Proliferation Assays—** Thymidine uptake (UT-7/EPO cells (31)) were grown in medium (1 × Iscove’s modified Dulbecco’s medium with 1-glutamine/25 mM HEPES buffer and 3024 mg/liter sodium bicarbonate/10% (v/v) fetal bovine serum/1% (v/v) l-glutamine/penicillin-streptomycin solution (Irvine Scientific)/1 unit/ml rHuEPO) to approximately 3 × 10⁶ cells/ml. Cells were plated in triplicate to 100 µl in assay medium (1 × RPMI medium 1640 without l-glutamine (Life Technologies, Inc.), sodium pyruvate, human transferrin, lipids, human insulin, bovine serum albumin, and 100 ng/ml stem cell factor. The medium contained no serum, hydrocortisone, proteins, or growth factors other than those listed. Duplicate samples contained CD34⁺ cells (10,000 cells/ml), stem cell factor (100 µg/ml), and a combination of sample and medium totaling 1 ml. The 35 × 10⁶-mm tissue culture plates were incubated at 37 °C with 10% CO₂ in a humidified tissue culture incubator. Erythroid colonies (orange to red in color) were scored after 21 days.

The positive signals came from mAbs 58, 71, 73, 74, and 87. The positive signals were confirmed by FACscan analysis. Positive signals were defined as those that resulted in a mean cell fluorescence greater than controls labeled only with the secondary antibody. The positive signals from mAbs 58, 71, 73, 74, and 75 (Table I) were confirmed using FACscan results. The positive signals from mAbs 58, 71, 73, 74, and 75 (Table I) were confirmed using FACscan results.
Characterization of anti-EPO receptor antibodies

Tissue culture medium conditioned by hybridomas secreting the indicated antibodies were tested with the assays indicated. Supernatants containing all the antibodies shown gave a positive signal in ELISA assays. ++, +++, and + indicate positive responses with +++, indicating those having the greatest effect. -- indicates a response less than or equal to the response of control medium. NT indicates samples that were not tested. ? indicates a sample that could not be assigned a response.

| Antibody | BIAcorea | FACS mean fluorescenceb | Immunodot blotc | Inhibition of EPO activityd | Stimulation of UT-7-EPO cells2 |
|----------|---------|----------------------|----------------|--------------------------|-----------------------------|
| 1        | 98      | --                   | ++             | --                       | --                          |
| 6        | 9       | --                   | --             | --                       | --                          |
| 16       | 15      | --                   | ++             | --                       | --                          |
| 23       | 4       | --                   | ++             | --                       | --                          |
| 30       | 270     | --                   | --             | --                       | --                          |
| 31       | 16      | --                   | --             | --                       | --                          |
| 37       | 16      | --                   | --             | --                       | --                          |
| 39       | 574     | --                   | ++             | --                       | --                          |
| 50       | 345     | --                   | ++             | --                       | --                          |
| 58       | 15      | 14.99                | --             | +                       | ?                           |
| 62       | 814     | --                   | --             | --                       | --                          |
| 65       | 5       | --                   | +++            | --                       | ?                           |
| 67       | 1000    | --                   | --             | --                       | --                          |
| 69       | 877     | --                   | --             | --                       | --                          |
| 70       | 789     | --                   | ++             | --                       | ?                           |
| 71       | 1584    | 23.55                | ++             | +                       | ++                          |
| 72       | 1190    | --                   | --             | --                       | --                          |
| 73       | 354     | 13.71                | --             | +                        | --                          |
| 74       | 408     | 18.53                | --             | --                       | --                          |
| 77       | 434     | --                   | ++             | --                       | --                          |
| 83       | 1025    | --                   | --             | ++                       | --                          |
| 87       | 4       | 12.81                | --             | --                       | --                          |
| 88       | 4       | --                   | ++             | --                       | --                          |
| 89       | 1       | --                   | ++             | --                       | --                          |
| 90       | 9       | --                   | --             | --                       | --                          |

* Binding to EPO receptor is measured by BIAcore analysis. Values are reported in response units using a BIAcore chip with attached soluble EPO receptor.

** FACS analysis of supernatants. EPO receptor transfected CHO cells were immunostained as described in the legend to Fig. 1. Using conditioned medium containing the antibody of interest, then labeled with fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody.

*** Immunodot blots using denatured EPOR as antigen. Wells were scored ++ to -- based on intensity of dots for individual hybridoma supernatants.

**** Inhibition of [3H]thymidine uptake by UT-7-EPO cells. 50 milliunits of EPO and varying amounts of antibody were incubated with cells. After an overnight incubation, cells were pulse labeled with [3H]thyridine, and the amount of radioactivity taken up was determined. A positive response was defined as one that progressively inhibited thymidine uptake with increasing amounts of antibody.

***** Stimulation of [3H]thymidine uptake by UT-7-EPO cells. Varying amounts of antibody were incubated with cells. After an overnight incubation, cells were pulse labeled with [3H]thymidine, and the amount of radioactivity taken up was determined. A positive response was defined as one that had a progressive increase in thymidine uptake with increasing amounts of antibody.

Antibodies bound at or close to the EPO binding site. Positive FACscan signals were also observed for OCIM1 cells (34) and 32D cells (35, 36) that had been transformed with a human EPO receptor. The untransformed 32D cells gave no signal. This indicates that the binding detected in FACscan is specific for cells expressing an EPO receptor.

To establish further that the antibodies bound at the EPO binding site, cold displacement assays were done. OCIM1 cells were incubated with 125I-EPO and varying amounts of cold rHuEPO or purified anti-EPO antibodies. As shown in Fig. 1, unlabeled rHuEPO displaced 125I-EPO from cells as expected. mAbs 71, 58, and 74 also displaced rHuEPO, albeit not as effectively as rHuEPO. mAb 73 had a modest effect, if any, and a control antibody had no effect. This suggests that the antibodies 58, 71, and 74 competed with rHuEPO for binding to the EPO receptor. Thus, these antibodies do in fact specifically recognize surface expressed EPO, and they bind in a way that interferes with rHuEPO binding.

mAbs Raised to EPO Can Stimulate Thymidine Uptake in EPO-dependent Cell Lines—Homodimerization of the EPO receptor is thought to be mechanism for its activation. We reasoned that because IgG antibodies have two identical binding sites, some antibodies may be able to dimerize two EPO receptor monomers and thus activate them. We therefore tested the hybridoma supernatants containing anti-EPO mAbs to see if any would stimulate thymidine uptake in the EPO-dependent cell line UT-7/EPO. Two antibodies were identified from this initial screen that stimulated thymidine uptake, mAbs 71 and 73 (Table 1). Several others gave ambiguous or weak responses. mAb 71 recognized denatured rHuEPO and mAb 73 did not. Both mAb 71 and mAb 73 recognized surface receptor as measured by FACscan.

The assay was also performed in the presence of rHuEPO to identify neutralizing antibodies. Several inhibited rHuEPO activity including mAbs 58 and 71. This result was confirmed for mAb 71 by testing a purified preparation for neutralizing activity (see Fig. 5). Other antibodies inhibited weakly included Abs 65, 88, and 89.

To test the antibodies further, we performed dose response experiments with purified antibodies. The four best FACscan positive antibodies including mAb 71 and mAb 73 were used in this study. As shown in Fig. 2 all four antibodies could stimulate thymidine uptake in UT-7/EPO cells. Several different negative control antibodies were also tested, and no positive response was observed (data not shown). The two strongest activators, mAbs 58 and 71 showed bell-shaped dose response curves with maximum stimulation at approximately 1 µg/ml. Stimulation by antibodies required higher concentrations than was required for rHuEPO stimulation. The EC50 for rHuEPO, mAb 71, mAb 58, mAb 74, and mAb 73 were approximately 5 pm, 50 pm, 250 pm, and >500 nm, respectively. mAb 58

**FIG. 1.** Cold displacement of rHuEPO from OCIM1 cells. OCIM1 cells and 125I-EPO were incubated for 3 h with the indicated amounts of rHuEPO ( ), mAb 71 ( ), mAb 73 ( ), mAb 74 ( ) and mAb 58 ( ). and a control antibody, anti-HER2-501 ( ). Cells were rinsed free of unbound reagents, and the cell bound radioactivity was determined.
differed from rHuEPO and mAb 71 in that it was unable to stimulate to the same extent. The maximum amount of thymidine incorporation was half that of mAb 71.

To establish that stimulation of thymidine uptake by mAb 71 was due to activation of the EPO receptor, we performed an experiment with soluble human EPO receptor to see if it would inhibit activation of UT-7/EPO cells. Soluble EPO would be expected to bind EPO or mAb 71, thus preventing them from binding EPOR on the surface of the cells. When this experiment was done, the soluble EPO inhibited activation of UT-7/EPO cells by rHuEPO in a dose-dependent manner. In a similar manner, activation of UT-7/EPO cells by mAb 71 was also inhibited (data not shown). These results suggest that stimulation of thymidine uptake in UT-7/EPO by mAb 71 was due to activation of human EPOR and was not due to other nonspecific effects.

Monovalent Fabs Do Not Activate the EPO Receptor—The bell-shaped activation curves for mAbs 71 and 58 are consistent with inhibition at high doses because the excess antibody to EPOR would not allow dimerization. That is, when antibody is in excess, EPOR-mAb complexes would be present primarily in 1:1 ratios instead of 2:1. This result suggests that activation is due to dimerization of receptors. If this is the case, then monovalent Fabs prepared from an activating antibody would be unable to stimulate thymidine uptake in UT-7/EPO cells because they could not form 2:1 EPOR-mAb complexes. Monovalent Fabs were prepared from mAbs 71 and 73.

Fig. 3 shows that neither Fab 71 nor Fab 73 would stimulate thymidine uptake in UT-7/EPO cells. Both of the Fabs can still inhibit binding of rHuEPO to surface expressed EPO receptor as shown in Fig. 4. mAb 71 and Fab 71 also act as an EPO antagonist at high concentrations as shown in Fig. 5. This indicates that the Fabs retain the ability to bind to EPO but lack the ability to activate EPOR. This suggests further that two binding sites on anti-EPOR antibodies are required for EPOR activation.

Anti-EPOR mAb 71 Supports Growth and Differentiation of Erythroid Precursors—EPO has several different activities including stimulation of proliferation, stimulation of differentiation, and inhibition of apoptosis. If dimerization of the EPO receptor is a necessary and sufficient event for these activities, then an activating antibody should support the formation of erythroid colonies from hematopoietic precursors because all three activities must be present for this to occur. To test this possibility, BFUe assays were performed using purified CD34+ cells from human blood. The medium contained stem cell fac-
tor, insulin, and BSA but had no serum or other proteins. rHuEPO under these conditions supported the growth and differentiation of BFUe from these cells. In a similar manner mAb 71 also supported growth of these cells (Fig. 6). Control samples lacking rHuEPO or mAb 71 as well as a sample containing a similar dose of a control monoclonal antibody did not result in any erythroid colonies. We found that stem cell factor in combination with either rHuEPO or mAb 71 was required for erythroid colony formation. No erythroid colonies were observed with stem cell factor alone. This result suggests that the cytokine requirements for BFUe growth and differentiation are similar for rHuEPO and mAb 71. The colonies grown in the presence of mAb 71 were fewer in number and were smaller in size than rHuEPO grown BFUe. However, they had an orange to red color consistent with the presence of hemoglobin. Fig. 7 shows representative colonies from this experiment. mAb 71 showed a bell-shaped dose response curve in this assay similar to that seen with mAb 71 in the thymidine uptake assay (Figs. 2 and 3). The amount of mAb 71 required for maximal stimulation of colony formation and stimulation of thymidine uptake in UT-7/EPO cells was also the same (1–5 μg/ml). However, the dose range that would stimulate colony formation was narrower. These results suggest that dimerization of the receptor by an anti-EPOR antibody is sufficient to send both proliferative and differentiation signals.

**DISCUSSION**

We report here the identification of monoclonal antibodies raised to the extracellular domain of the human EPO receptor. A subset of these, about 4%, stimulated thymidine uptake in EPO factor-dependent cell lines. The stimulation could be blocked by the addition of soluble EPO receptor to the assay mix. This indicates that the activation requires binding to surface expressed EPOR.

Homodimerization of EPOR has been hypothesized as the mechanism of its activation. Our studies support this proposal. Only bivalent anti-EPO receptor antibodies could activate cells. Monovalent antibodies (Fab) could bind EPOR but could not activate. In addition, the dose response curves showed reduced activation at both low and high doses. This is most easily explained by reduced dimerization at high antibody concentrations. At high antibody concentration, complexes would consist primarily of 1 antibody bound to each receptor. This complex would be inactive. At lower concentrations, complexes would exist in one antibody per two receptors resulting in activation.
stimulate formation of hemoglobinized BFUe colonies from purified CD34+ cells. These results were seen in serum free medium where the only proteins present were insulin, stem cell factor, and BSA. This suggests that other added extraneous proteins in the medium are not responsible for nor contribute to the activity. Thus mAb 71 can stimulate differentiation as well as proliferation. Cells in the process of differentiation from BFUe to CFUe to erythrocytes are reported to undergo apoptosis in the absence of EPO (38–40). The fact that viable cells were seen in BFUe colonies stimulated by mAb 71 suggests that the antibodies also blocked apoptosis. Thus homodimerization of the EPO receptor with antibodies appears to induce all the necessary signal transduction pathways for growth and differentiation to erythroid cells.

This ability of an antibody to support growth of BFUe cells is intriguing in light of reports of additional subunits associated with EPOR. Hydrodynamic studies suggest that EPOR is multicomponent (42). One possible component is a non-EPOR 100-kDa protein that can be cross-linked to EPO (43–45). There is also a report suggesting that an additional factor present in some cell lines can increase the affinity of the EPO receptor (46). If these factors are important for signaling, they must be present in complexes with the EPOR when activated by mAb 71. The fact that mAb 71 can support the differentiation of BFUe colonies also suggests that these factors need not contact EPO or anti-EPOR mAbs to be activated because mAb 71 was raised to purified extracellular domain and is thus unlikely to have the ability to bind to the other accessory EPOR proteins.

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