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Monoclonal Antibody CFC-6, which Binds to Helix II, Inhibits Erythropoietin-Induced Bioactivity

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Abstract : It was discovered that monoclonal anti-erythropoietin (EPO) antibody CFC-6 can neutralize EPO-induced cell activation. To know the binding site of CFC-6, recombinant human erythropoietin (rhEPO) was digested with Glu-C, followed by a separation using high performance liquid chromato graphy (HPLC). Each HPLC fraction was blotted on the nitrocellulose membrane and the membrane was treated with anti-EPO antibody CFC-6 and anti-mouse antibody which is modified with peroxidase. Only one spot showed the color and the fraction was sequenced by Edman degradation. The results suggest that CFC-6 recognizes amino acid sequence V63-W-Q-G-L-A-L-L-S-E72 which is a part of helix II of the EPO molecule. Binding of CFC-6 to EPO may inhibit EPO binding to its receptor, which implies that the antibody binding site and the receptor binding site are close or overlapping.
Key words : binding site, recombinant human erythropoietin (rhEPO), receptor.

Erythropoietin is a glycoprotein which has 165 amino acids and its molecular size is 30,400 dalton (Jacobs *et al.*, 1985, Lee *et al.*, 1996). The molecular structure is known as a four helix bundle structure (Boissel *et al.*, 1993). This hemopoietic regulating molecule was first discovered in 1906 by some animal experiments (Carnot and Deflandre, 1906). Among the members of the hemopoietic regulating factor family, uniquely, erythropoietin stimulates the proliferation and differentiation of the erythroids. Erythropoietin binds to its receptor which facilitates the receptor dimerization, and triggers cellular signaling (Watowich *et al.*, 1992). Treatment of erythropoietin to the anemia of chronic renal disease has been proven to be effective clinically (Winearls *et al.*, 1986). For the purification and assay of the EPO, monoclonal antibody is necessary.

A tertiary structure of EPO has been predicted using computer modeling and mutant study (Sytkowski and Donahue, 1987). The structure was proposed as a four-helix bundle model as shown in growth hormone (GH) and granulocyte colony stimulating factor, even though the structure was not determined with crystallography or NMR data. Therefore, it is not unusual to think that an EPO-erythropoietin receptor (EPOR) binding pattern might be similar to well-known growth hormone-growth hormone receptor (GH- GHR) binding (Ji *et al.*, 1995).

EPOR has a typical structure of cytokine receptors that are composed of a Cys-rich region and a W-S-X-W-S motif in the extracellular domain as well as of single transmembrane and cytoplasmic domains.

Homodimerization is one of the signal producing mechanisms of the cytokine receptor family (Ji *et al.*, 1995). X-ray crystallography study showed that a molecule of growth hormone is able to bind two molecules of growth hormone binding protein, which is the extracellular domain of the receptor (De Vos *et al.*, 1992). Point mutated EPO receptor exhibited constitutive activation of cell growth without EPO and that receptor was a homodimer which is covalently associated with a cysteine-cysteine disulfide bond (Watowich *et al.*, 1992). Identification of a functional domain in EPO was tried using site-specific anti-peptide antibodies (Sytkowski and Donahue, 1987, Fibi *et al.*, 1991) and deleted mutants of EPO (Boissel *et al.*, 1993). The results of these experiments proposed one receptor binding site which is located in amino acid 99-129. That site is between helices III and IV in the predicted tertiary structure model (Boissel *et al.*, 1993). Another receptor binding site has been unknown, so far.

In the present study, a trial was made to know a receptor binding site of EPO using anti-EPO monoclonal antibody.

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Materials and Methods

Materials

Endoproteinase Glu-C from *Staphylococcus aureus* V8 was obtained from Boehringer Mannheim Biochemicals (Mannheim, Germany). Iodoacetic acid was from Sigma Chemical Co. (St. Louis, USA) All other chemicals were of reagent grade.

Immunization

RhEPO, 20 μg, was injected into Balb/c mice by intraperitoneal route. Three more injections for additional immunization with incomplete Freund's adjuvant were performed every 4 weeks. Booster injections were followed during the last 4 days before fusion. Spleen was isolated from immunized Balb/c mice. Cells were fused with the myeloma cell line NS0 using polyethylene glycol (PEG) according to the standard methods (Galfre and Milstein, 1981). The final cell pellets were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) and HAT (0.1 mmol/ L-hypoxanthine, 0.004 mmol/ L-aminopterin, and 0.016 mmol/ L-thymidine) and splitted into 96-well culture plates. After 14 days, cell clones from positive wells were picked out, diluted and transferred to a new well. The supernatants were tested for antibody content as well as for anti EPO activity after two weeks. The final cloned cell line secreted IgG2a subtype antibody, CFC-6.

Production and purification of monoclonal antibodies

Cell lines producing anti EPO monoclonal antibodies were cultured in Balb/c mouse abdominal cavity. Antibodies from ascites fluids were purified by affinity chromatography using protein A sepharose (Park *et al.*, 1987).

Neutralization of EPO-induced cell proliferation

Bioassay was carried out by the method of Krystal (1983). Rat spleen cells were harvested after injection of phenylhydrazine. After 22-72 hours incubation with increasing amounts of recombinant EPO, cellular growth was determined by ^{3}H-thymidine uptake.

Diluted rhEPO (0.1 pmol/ ml final concentration) was preincubated with diluted CFC-6 samples for 1 h at 37°C and added to 3×10^{5} cells/ well in 96-well plates. After 24 h incubation at 37°C, 1 μCi of ^{3}H-thymidine was added to each well. Incorporated ^{3}H-thymidine was measured after 3 h.

Glu-C endopeptidase digestion

Enzymatic cleavage by Glu-C was performed after reductive alkylation of rhEPO with iodoacetic acid. Glu-C and substrate mixture was incubated in 25 mM ammonium carbonate buffer, pH 7.8.

The substrate concentration was 0.2 mg/ml, and the endoproteinase Glu-C concentration was 10 μg/ml. Digested sample, 10 μl, was diluted with Tris-HCl buffer to a final volume of 20 μl and loaded onto a Nucleosil C_8 column. HPLC was performed for 30 min with linear gradient 0~100% B. Solution A contains 0.1% TFA in water and solution B is composed of 0.1% TFA and 70% acetonitrile in water. The collected fractions were dot-blotted using anti EPO monoclonal antibody, CFC-6.

Dot blotting and peptide sequencing

Peptide fractions from Glu-C digestions and intact EPO (5 μg) were transferred to a 0.2 μm membrane by vacuum blotting (HYBRI.DOT, BRL). Non-specific binding sites were blocked by incubation in PBS/ 0.1% Tween (PBS-T). Blots were incubated with the anti-EPO antibody (15 μg) in 10 ml of PBS-T containing 5% skim milk for 2 h. After washing with PBS-T and incubation with a horseradish peroxidase-labelled anti-mouse Ig antibody for 1 h, the blots were washed with PBS and developed with the ECL system (Amer- sham).

The amino acid sequence of the #53 fraction was determined in a Beckman 890C spinning cup sequencer using a 0.1 M Quadrol program. The peptide fragments were analyzed by automated Edman degradation in an Applied Biosystems 470A gas-phase sequencer. The phenylthiohydantoin (PTH) derivatives were identified by reverse phase HPLC with a Hewlett-Packard Model 1084B chromatography equipped with an Altex Ultrasphere ODS column. The aqueous phase was 15 mM sodium phosphate, pH 5.5, and the PTH-amino acids were eluted with a gradient of methanol/acetonitrile (17:3 v/v).

Results and Discussion

Receptors containing a single transmembrane domain transfer the signal by ligand-dependent dimerization. It was proposed that a growth hormone has two unique binding sites to recognize two identical receptor molecules (De Vos *et al.*, 1992). One of the two binding sites binds to ligand first, which makes the second binding site open. Therefore, the second binding site is opened only when the first binding site is occupied by the ligand.

Many studies have shown that the EPO receptor may have the same type of ligand-receptor interaction mechanism. So far, one of the two putative receptor binding sites was proposed by anti-peptide antibodies. Antibodies against peptide 99-118 and 111-129 showed an inhibitory effect against EPO-derived bioactivity

up to 65 % (Sytkowski and Donahue, 1987). These regions represent the C-terminal half of helix III and a loop between helices III and IV. Trials were made to find another binding site, but failed. EPO mutants which were deleted in various regions including inner helix, inter helical loop, N-terminal, and C-terminal regions could not provide the clear answer, because of improper folding and inadequate secretion of mutants.

Meanwhile, amino acids 152 through 166 were reported as an important region for the biological function of EPO (Fibi *et al.*, 1991). Though a similar method using antipeptide antibodies was used, the results of this group did not show the importance of peptide 110 through 123. These controversial points should be discussed by further investigation.

Other mutant studies have revealed that Arg-150, Lys-152, Arg-103, Ser-104 and Arg-14 are involved in binding EPO-R (Mattews *et al.*, 1996). Another group found the important residues, which are Arg-14, Arg-103, Ser-104, Leu-108 and Lys-152 (Wen *et al.*, 1994).

One of the anti-EPO monoclonal antibodies, which we have prepared, showed the inhibition of EPO-induced thymidine uptake (Fig. 1). This EPO-neutralizing activity gave us a hint which explains the relationship between the binding site of monoclonal antibody and the binding site of EPO receptor. To know the binding site of monoclonal antibody (CFC-6), rhEPO was digested with Glu-C (Fig. 2), and all the fractions were dot-blotted using CFC-6. Only #53 in Glu-C digest showed positive color reaction (Fig. 3). This fraction was analyzed by Edman degradation. It has the sequence V63-W-Q-G-L-A-L-L-S-E72. CFC-6 binding peptide is located in the middle of the putative helix II of the EPO molecule. The helical wheel structure of this peptide (Fig. 3)

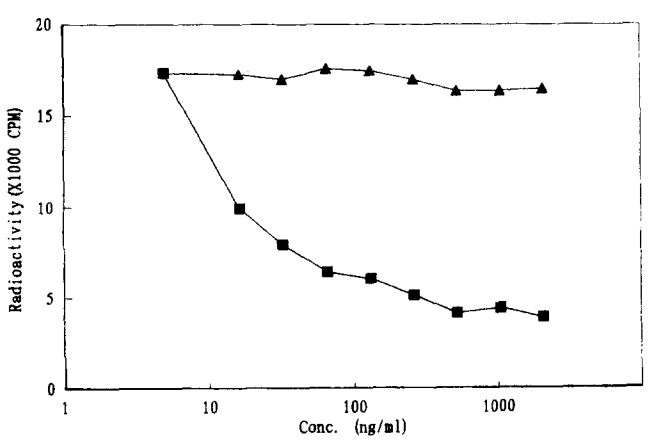

Fig. 1. Inhibition curve of the monoclonal antibody CFC-6 against EPO-derived thymidine uptake. CFC-6 monoclonal antibody (■—■) inhibits the ^{3}H-thymidine uptake of rat spleen cells. IC_{50} of CFC-6 is around 20 ng/ml. Purified mouse IgG was used as a control (▲—▲).

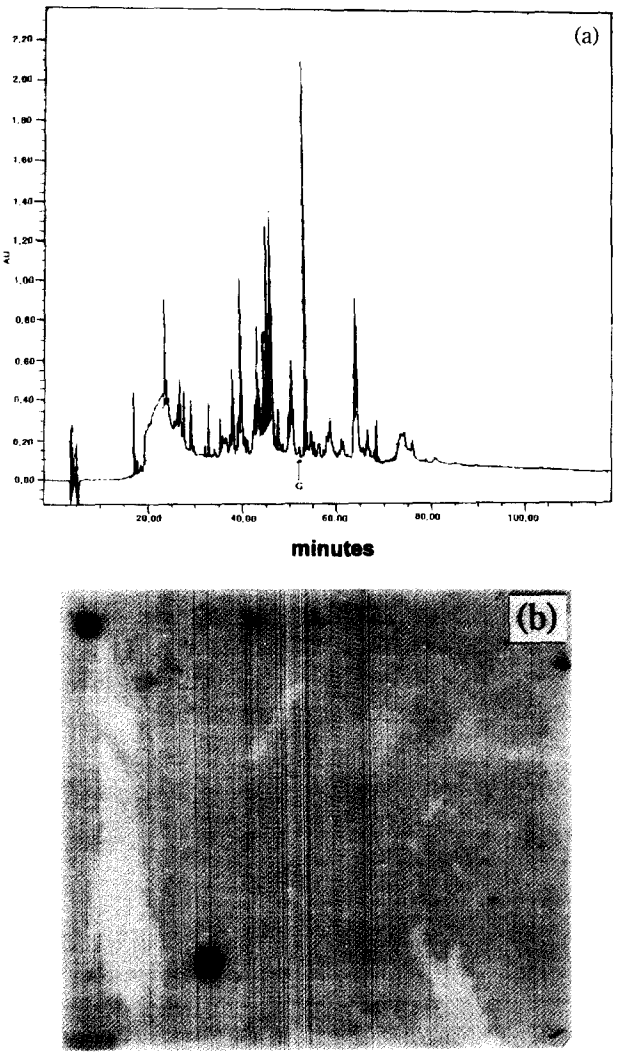

Fig. 2. Peptide map of EPO and dot blotting using CFC-6 monoclonal antibody. (a) EPO was treated with Glu-C and separated by C_8 column. Fractions were collected every 1 minute. (b) Each fraction was analyzed by dot blotting using CFC-6 monoclonal antibody as a primary antibody. Intact EPO (positive control) and fraction # 53 (denoted as G) showed the positive color reaction.

showed the cluster of hydrophilic amino acids (Q65 and E72). If we assume that hydrophilic residues are the outer region of the four helix bundle structure, this site may be an accessible region for antibody binding.

EPO-induced ^{3}H-thymidine uptake was inhibited depending on the concentration of CFC-6. The maximal inhibitory effect was 85% and IC_{50} of CFC-6 was around 20 ng/ml. Therefore, helix II may be a new receptor binding site of EPO. If this is the case, EPO has two receptor binding sites, as expected. The binding sites of EPO are a little different from GH receptor binding sites, and it is known that site 1 is composed of helix IV and a part of helix I, and site 2 is located in helix III and a part of helix I. Meanwhile, one of the re-

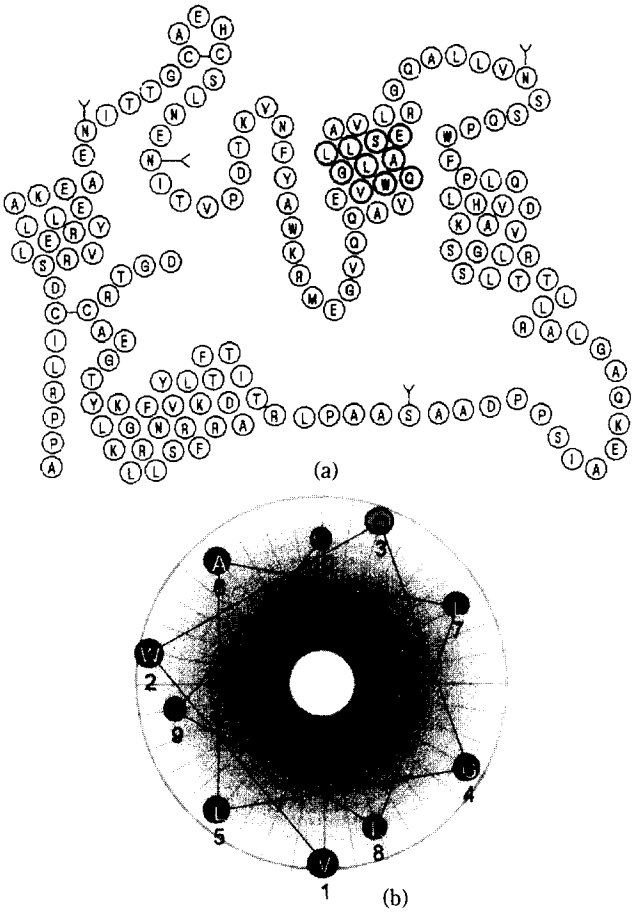

Fig. 3. CFC-6 binding region is located in the middle of the putative helix II of EPO (a). Its helical wheel structure showed the cluster of hydrophilic residues which may be a outer side of the four helix bundle structure (b). Helical wheel structure was predicted by DNA star program (DNA STAR, USA).

ceptor binding sites of EPO is composed of the C-terminal half of helix III and the central region of helix II , and another receptor binding site is located in helix I and IV. It seems that helix II and helix III are quite close in a space of three dimensions. It was reported that one of the monoclonal antibodies could recognize helix II and helix III simultaneously (Elliott *et al.*, 1996, Elliott *et al.*, 1997). Discovered 10mer peptide (63-72) may not be a putative receptor binding site. Binding of antibodies to helix II may sterically hinder the real receptor binding site. The other possibility is that the binding of antibody may induce conformational change of the EPO mole- cule as well as receptor binding sites, which also result in the inhibition of receptor binding. To study peptide-induced cell activation, we have chemically synthesized 10mer and 23mer peptides. Unfortunately, these peptides were extremely insoluble both in the aqueous solution or organic solvents. This result is well in accordance with other groups' results (Syt- kowski and Donahue, 1987). Even though the sequence of the peptides was same, we have observed the sol- ubility difference between enzyme-digested #53 peptide and chemically-synthesized 10mer peptide. The reason is unclear but the possible answer may be the presence of a secondary structure in the #53 peptide. During the preparation of this manuscript, similar results were reported (Elliott *et al.*, 1996, Elliott *et al.*, 1997). One of their neutralizing antibodies recognizes the conformational epitope containing amino acids 64 through 78 and 99 through 110.

A trial to find the peptide which acts as an agonist of EPO was made using the peptide phage library, and several potent peptides were discovered (Wrighton *et al.*, 1996, Livnah *et al.*, 1996). But the amino acid sequences of these peptides are not found in the primary sequence of EPO. It is still unknown whether two binding sites interact cooperatively or independently. In the case of GH, the initial binding site and second binding site interact allosterically and orderly. Another question is if homodimerization is absolutely necessary or if single occupation of the receptor could induce the signal, even in a tiny amount. Further studies to reveal the EPO-EPO receptor interaction have to be undertaken.

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