Identification and analysis of human erythropoietin receptors on a factor dependent cell line, TF-1

造血因子依存性に増殖する細胞株TF-1上のヒトエリスロポイエチン受容体の同定と解析

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ABSTRACT

We have recently established a novel cell line, TF-1, from bone marrow cells of a patient with erythroleukemia, that showed an absolute growth dependency on each of three hematopoietic growth factors: erythropoietin, granulocyte-macrophage colony-stimulating factor and interleukin 3. Erythropoietin stimulated the proliferation of TF-1 cells even at the physiological concentration (0.03 units/ml).

We performed binding experiments on TF-1 cells using radioiodinated erythropoietin. The binding of radioiodinated erythropoietin to TF-1 was specific, time- and temperature-dependent and saturable. Scatchard analysis of the saturation binding data suggested the existence of a single class of binding sites (Kd = 0.40 nM; number of binding sites = 1630/cell). TF-1 cells were usually maintained in RPMI 1640 containing 10 % fetal bovine serum and 5 ng/ml granulocyte-macrophage colony-stimulating factor. The Kd and the number of the erythropoietin receptors were not changed by incubating the cells with interleukin 3, although culturing the cells in the presence of erythropoietin resulted in down-modulation of erythropoietin receptors. The chemical cross-linking study demonstrated that two molecules with apparent molecular weights of 105 KD and 90 KD were the binding components of erythropoietin. Present data suggest that human erythropoietin receptors are very similar to the previously reported murine erythropoietin receptors.

INTRODUCTION

Erythropoietin (EPO) is a hematopoietic growth factor which induces proliferation and differentiation of erythroid cells[1,2]. Recently, cDNA for human EPO has been cloned and expressed in mammalian cells[3,4]. A large amount of recombinant human EPO (rHuEPO) has made it possible to investigate the receptors for EPO. Up to now the receptors for EPO have been analyzed mainly in murine systems such as in Friend murine erythroleukemic cells[5-11], mouse spleen cells[12] and fetal mouse liver cells[13, 14].

More recently, human EPO receptors have been studied using purified erythroid precursor cells (colony-forming unit-erythroid: CFU-E)[15], and an erythroleukemic cell line, K562[16]. The impurity of target cells and the existence of intrinsic EPO, however, have been the major problems for CFU-E system and the extremely low number (4-6 sites/cell) of EPO receptors expressed on K562 presents an obstacle for further investigation such as cross-linking studies.

We have recently established a novel cell line, designated as TF-1, from bone marrow cells of a patient with erythroleukemia that showed a definite growth dependency on EPO, granulocyte-macrophage colony-stimulating factor (GM-CSF)[17] and interleukin 3 (IL-3) (Kitamura et al., manuscript submitted). In the present paper, we outline an investigation into the biophysical and biological features of functional EPO receptors expressed on TF-1.

MATERIALS AND METHODS

Cells A factor-dependent human cell line, TF-1, (tri-factor-dependent), which was previously designated as MFD-1[17], was established from the

bone marrow cells of a patient with erythroleukemia. It was cultivated in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10 % fetal bovine serum (Hyclone, Logan, UT) and 5 ng/ml GM-CSF at 37°C in humid air containing 5% CO_2. The culture period was over 13 months at the time we submitted the paper.

The establishment and characterization of this cell line will be published elsewhere (Kitamura et al., manuscript submitted). Briefly, TF-1 is a unique cell line that shows a growth dependency on each of three hemopoietic growth factors; GM-CSF, EPO[17] and IL-3. Morphological features, cytochemical studies (PAS positive, peroxidase negative and esterase negative) and surface markers of the cells (My 7, My 9, My 10 and HLA-DR positive, glycophorin negative) suggested the very immature erythroid origin of TF-1. Moreover, clonal chromosonal abnormality (54X) and monotonous surface markers indicated the clonality of TF-1 cells.

Growth factors Purified rHuEPO (180,000 unit/A280) was kindly supplied by Kirin-Amgen Inc. (Thousand Oaks, CA)[4]. Purified recombinant human GM-CSF (4×10^7 U/mg) was wirmfuly furnished by Sumitomo Pharmaceutical Co. (Tokyo, Japan)[18]. Purified recombinant human IL-3 ($2\text{-}4 \times 10^7$ U/mg) was generous gift from Dr. Steven C. Clark[19].

Assay of cell proliferation The proliferation of cells was examined by two methods. First, colorimetric assay for cell growth was performed essentially as described by Mosmann et al.[20]. To summarize, after culturing the cells in a 96-flat bottom-well microtiterplate (Falcon) for 48 hr in the presence or absence of rHuEPO at various concentrations, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) was added (final concentration: 0.5 mg/ml), and the incubation was continued for the following 6 hr. The insoluble purple

reaction product, which was produced by MTT reduction, was then dissolved in isopropanol containing 0.04 N HCl, and the absorbance was measured with an Titertek Multiscan MC Autoreader (Flow Laboratories) using test and reference wavelengths of 570 and 630 nm respectively. MTT reduction is proportional to the number of viable cells[20].

Second, tritiated thymidine (^{3}H-TdR) uptake was also performed as a measure of the number of the cells in cell cycle. In brief terms, cells that had been deprived of growth factors for 4 hr were cultured in the presence or absence of rHuEPO, and the culture was continued for the following 24 hr in the presence of ^{3}H-TdR (0.1 μCi /10^{4} cells, 6.7 Ci/mmol; New England Nuclear). The culture was harvested with an automatic cell harvester and the amounts of radioactivity associated with cellular DNA were measured with a scintillation counter.

Radioiodination of EPO Purified rHuEPO was labeled with ^{125}I-sodium iodide (1.87 Ci/mmol, Amersham Japan Inc., Tokyo, Japan) using the chloramine-T method[21] with a minor modification as described[8,13]. Specific activities of iodinated rHuEPO (^{125}I-EPO) obtained through our procedure was 30-55 μCi/μg without loss of biological activity as described[8].

Binding method of ^{125}I-EPO Binding was performed as described previously[8,10,13,14]. In short, cells were incubated with ^{125}I-EPO in the presence or absence of a 200-fold excess of unlabeled EPO and were separated using n-butyl phthalate after the incubation time. The radioactivity of the cell pellets was counted by γ-ray counter (Clinigamma, L.K.B.-Wallac). Nonspecific binding was defined as bound ^{125}I-EPO that remained in the presence of the 200-fold excess of unlabeled EPO. Specific binding was determined by subtracting the nonspecific binding from the total binding. Nonspecific binding was

found to be less than 10 % of the total binding (data not shown).

Dissociation of bound ^{125}I-EPO In order to examine the reversibility of ^{125}I-EPO binding, TF-1 cells, which had been incubated with ^{125}I-EPO for 2 hr at 15°C were divided into two aliquots, with an excess amount of unlabeled EPO being added to one aliquot to a final concentration of 170 nM.

Modulation of EPO receptors by incubating with EPO and IL-3 TF-1 cells grown in the presence of 5 ng/ml GM-CSF were washed three times and cultured for various interval in the presence of 5 unit/ml (5 U/ml) EPO, 5 ng/ml GM-CSF or 5 ng/ml IL-3, or in the absence of growth factors. After this culturing period, acid-wash was performed to clear off the surface-bound EPO as described [8] with minor modifications. In short, the cells were suspended in 50 mM glycine buffer (pH 3.0) containing 150 mM NaCl, and immediately Hanks' balanced salt solution containing 20 mM HEPES, pH 7.4 (HBSS) was added to the cell suspension. The cells were washed by HBSS for additional two times and ^{125}I-binding experiments were performed on these acid-washed cells. To examine the efficiency of the acid-wash procedure, we conducted an experiment to wash the previously ^{125}I-EPO-labeled cells by this procedure and found that there was little ^{125}I-EPO associated with the cells after the acid-wash procedure.

Chemical cross-linking studies Cells (1×10^7) were incubated with ^{125}I-EPO (750 pM) with and without a 200-fold excess of unlabeled EPO at 15°C for 90 min. The cells were then washed three times with HBSS, and resuspended in 400 µl of HBSS. The cell suspension was kept on ice, and disuccinimidyl suberate (DSS; Pierce Chemical Co., Rockford, IL) was added to a final concentration of 200 µM. After 15 min of incubation on ice, the cross-linking reaction was quenched by the addition of 1 ml of

ice-cold 50 mM Tris HCl (pH 8.0) containing 150 mM NaCl and 2 mM EDTA. The cells were subsequently pelletted by centrifugation at 4,000 x g for 30 sec. The resulting cell pellet was solubilized in 0.5 % NP-40, 20 mM Tris HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride and 500 units/ml aprotinin. After 10-min incubation on ice, the soluble fraction was collected by centrifugation at 15,000 x g for 10 min. These samples were mixed with 1/5 volume of 5-fold-concentrated Laemmli's sample buffer[22] containing 100 mM dithiothreitol (DTT), boiled for 5 min and were analyzed by $NaDodSO_4$/PAGE[22] The dried gels were finally exposed to Kodak XAR-5 film with a Dupont Lightning Plus Screen for 7 days at -70° C.

RESULTS

Proliferation of TF-1 cells TF-1 cells proliferated in the presence of rHuEPO in a dose-dependent manner (Fig. 1), and deprivation of rHuEPO from culture media resulted in their rapid death within 2 to 3 days (data not shown). The colorimetric assay correlated well with ^{3}H-TdR uptake and half-maximum stimulation of the cell proliferation was obtained at about 0.3 U/ml (U/ml). Stimulation of the cell growth was observed in the presence of EPO at low concentrations (0.03 U/ml).

Binding of ^{125}I-EPO to TF-1 cells To clarify whether the biological effect of EPO was mediated through the specific receptor for EPO, we conducted binding experiments on TF-1 cells using ^{125}I-EPO. The specific binding paralleled to the number of cells (data not shown). The binding of ^{125}I-EPO to TF-1 required 60 min at 15°C to reach equilibrium (Fig. 2). A slow dissociation of bound ^{125}I-EPO was observed by the addition of an excess amount of unlabeled EPO to the cells that had been incubated with ^{125}I-EPO at 15°C for 2 hr (Fig 2).

As shown in Fig. 3, ^{125}I-EPO binding decreased when incubated with increased concentrations of unlabeled EPO. The other growth factors and lymphokines, including human GM-CSF, G-CSF, IL-1, IL-2, IL-3, transferrin and insulin, did not compete the ^{125}I-EPO binding to TF-1 at 15°C and at 37°C even when added to the binding mixture at the concentration of at least 50-fold molar excess (data not shown).

When TF-1 cells were incubated with ^{125}I-EPO at increasing concentrations, the specific binding was saturable (Fig. 4, inset). Scatchard analysis[23] of the saturation binding data exhibited a linear form, suggesting the existence of a single class of binding sites (Fig. 4). The value of the apparent dissociation constant (Kd) was 0.40 $\pm$ 0.08 nM, and the calculated number of binding sites was 1630 $\pm$ 90 per

cell from five separate experiments.

Down modulation of EPO receptors on TF-1 cells We next conducted several experiments to investigate the modulation of EPO receptors by human IL-3, GM-CSF and EPO. As shown in Table I, culturing cells in the presence of 5 U/ml EPO for 24 hours diminished the number of EPO receptors down to the level of two-thirds without modulating the Kd of the EPO receptors, compared with cells maintained in the presence of GM-CSF. The down-modulation of the EPO receptors on TF-1 cells started at least 30 minutes after the start of incubation with EPO, reached to a maximal level within 3-6 hours (50 % suppression) and reversed to a lower level after 24 hours (30 % suppression). Factor deprivation or culturing the cells in the presence of 5 ng/ml IL-3 for 24 hr, however, did not alter the binding capacity of the cells (Table I).

Cross-linking study of ^{125}I-EPO to TF-1 cells The cross-linking of ^{125}I-EPO to TF-1 revealed two major radioactive bands corresponding to a molecular mass of 140 KD and 125 KD under reduced conditions (Fig. 6, lane A). These bands were not found when binding was carried out in the presence of a 200-fold excess of unlabeled EPO as shown in lane B. Another radioactive band corresponding to a molecular mass of 60 KD was not competed by adding an excess of unlabeled EPO (Fig. 6), suggesting that this might be due to a nonspecific binding or aggregation of ^{125} I-EPO. Therefore, the molecular weights of EPO binding component were calculated to be about 105 KD and 90 KD. We performed the cross-linking studies threetimes with the same results except for the fuzzy band seen in lane B at approximately 75 Kd. This fuzzy band was not seen in the other two eperiments and was thought to be an artifact.

DISCUSSION

Human EPO is well known to cross-react with mouse EPO receptors. EPO receptors expressed on murine cells were analyzed using radiolabeled rHuEPO[5-14]. There have been no appropriate systems for analysis of human EPO receptors. EPO receptors expressed on K562 were in extremely low abundance and were not functional[16]. Purified CFU-E was considered to be a very good tool for investigating the physiological human EPO receptors [15], but it featured two problems; the impurity of target cells of EPO binding and the existence of EPO in the culture system. In the present study, we investigated the functional human EPO receptors expressed on a factor-dependent cell line, TF-1[17], that was cultivated in the presence of GM-CSF but not EPO using radioiodinated rHuEPO. Our investigation included Scatchard analysis of binding data, down-modulation of EPO receptors and chemical cross-linking studies.

Scatchard analysis of the binding data showed the existence of a single class of binding sites (Kd = 0.40 nM; number of binding sites = 1630/cell). This value of apparent Kd is almost identical to that of the MEL cell reported by us[14] and by Mayeux et al.[5], and that of murine spleen cells reported by Mufson and Gesner[12]. More recently, Fraser et al. demonstrated high-affinity EPO receptors were expressed on the human erythroleukemic cell line, K562[16]. Although the Kd value (0.27-0.29 nM) of EPO receptors expressed on K562 was similar to that of TF-1 reported here, the number of EPO receptors was extremely low (4-6/cell)[16]. This difference probably accounts for the distinctive effects of EPO on K562 and TF-1. Specifically, EPO stimulates short-term proliferation of TF-1 at a concentration as low as 0.03 U/ml, which is in the physiological concentration range[24,25]. In contrast, K562 cells do not respond to EPO even at a higher concentration, and proliferate independently.

Sawyer et al. have recently demonstrated the existence of two classes of EPO receptors on Friend virus-infected mouse spleen cells which could be induced to differentiate into mature erythroid cells. They reported that the Kd of EPO receptor having the higher affinity was 0.09 nM [7]. We also reported the existence of two classes of murine EPO receptors on fetal mouse liver cells[13] and another Friend murine erythroleukemic cell clone TSA8[10], both of which were induced to differentiate into more mature erythroid cells and the Kd of EPO receptors with the higher affinity is 0.25-0.45 nM. Although the implication of the existence of two classes of receptors with different affinities is far from understood at all, other molecules, associated with EPO receptors, might modulate the affinity of EPO receptors as was the case in the IL-2 receptor system[26]. Other research has surfaced indicating that some IL-3 dependent mouse cell lines responded to EPO and expressed EPO receptors[27,28]. It is interesting to note that TF-1 cells, which show growth-dependency on human IL-3 (Kitamura et al., manuscript submitted) as well as these IL-3 dependent mouse cell lines proliferate in the presence of EPO and possess only a single class of EPO receptors. These IL-3-dependent immature cells possibly have EPO receptors in premature forms. In our preliminary data, hemin or -aminolevlic acid induced hemoglobin synthesis of TF-1, although EPO could not induce it. We are now trying to investigate the modulation of EPO receptors in the differentiation course of TF-1.

Chemical cross-linking studies have indicated that murine EPO receptors appeared to consist of two species with estimated molecular masses of 110 and 95 KD[14]. In this article, we analyzed the human EPO receptors expressed on TF-1, and demonstrated that EPO bound to two kinds of molecules, which had molecular weights almost identical with

those of murine EPO receptors as previously reported[14]. These results suggest that human EPO receptors have a very similar structure with murine EPO receptors.

In some subclones of TF-1 cell line, the numbers of EPO receptors were about twice that compared with the parent TF-1 cells in spite of the invariable Kd and molecular weight of EPO receptors (data not shown). Such clones will be useful for further analysis or purification of human EPO receptors. Moreover, a confirmed strict growth-dependency of TF-1 cells on EPO would facilitate an investigation into the signal transduction of EPO receptors, and would be of interest to study the modulation of growth factor receptors as a model system for hematopoietic progenitor cells.

REFERENCES

1. Sherwood JB: The chemistry and physiology of erythropoietin. In: Harris RS(ed) Vitamins and hormones. Orland FL, Academic Press Inc, 41:161, 1984

2. Goldwasser E, Krantz SB, Wang FF: Erythropoietin and erythroid differentiation. Raven Press, New York:103, 1985

3. Jacobs K, Shoemaker C. Rudersdorf R. Neill SD, Kaufman RJ, Mufson A, Seehra J, Jones SS, Hewick R, Fritsch EF, Kawakita M, Shimizu T, Miyake T: Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature 313: 806, 1985

4. Lin F-K, Suggs S, Lin C-H, Browne JK, Smalling R, Egrie JC, Chen KK, Fox GM, Martin F, Stabinsky Z, Badrawi SM, Lai P-H, Goldwasser E: Cloning and expression of the human erythropoietin gene. Proc Natl Acad Sci USA 82:7580, 1985

5. Mayeux P, Billat C, Jacquot R: Murine erythroleukemia cells (Friend cells) possess high-affinity binding sites for erythropoietin in murine erythroid precursor cells. Blood 69:1485, 1987

6. Sawyer ST, Sanford B. Goldwasser E. Binding and receptor-mediated endocytosis of Erythropoietin in friend virus-infected erythroid cells. J Biol Chem 262:5554, 1987

7. Sawyer ST, Sanford B, Krantz SB, Luna J: Identification of the receptor for erythropoietin by cross-linking to Friend virus-infected erythroid cells. Proc Natl Acad Sci USA 84:3690, 1987

8. Fukamachi H, Tojo A, Saito T, Kitamura T, Nakata M, Urabe A, Takaku F: Internalization of radioiodinated erythropoietin and ligand-induced modulation of its receptor in murine erythroleukemia cells. Int J Cell cloning 5:209, 1987

9. Todokoro K, Kanazawa S, Amanuma H, Ikawa Y: Specific binding of

erythropoietin to its receptor on responsive mouse erythroleukemia cells. Proc Natl Acad Sci USA 84:4126, 1987

10.Kitamura T, Tojo A, Fukamachi H, Akahane K, Saito T, Urabe A, Takaku F: Characterization of the erythropoietin receptor on a Friend murine erythroleukemia cell clone, TSA8. Acta Haematologica Japonica, 51, 677-685, 1988

11.Krants SB, Goldwasser E: Specific binding of erythropoietin to spleen cells infected with the anemia strain of friend virus. Proc Natl Acad Sci USA 81:7574, 1984

12.Mufson RA, Gesner TG: Binding and internalization of recombinant human erythropoietin in murine erythroid precursor cells. Blood 69:1485, 1987

13.Fukamachi H, Saito T, Tojo A, Kitamura T, Urabe A, Takaku F: Binding of erythropoietin to CFU-E derived from fetal mouse liver cells. Exp Hematol 15:833, 1987

14.Tojo A, Fukamachi H, Kasuga M, Urabe A, Takaku F: Identification of erythropoietin receptors on fetal liver erythroid cells. Biochem Biophys Res Com 148:443, 1987

15.Sawada K, Krantz SB, Kans JS, Dessypris EN, Sawyer ST, Glick AD, Civin CI: Purification of human colony-forming units-erythroid and demonstration of specific binding of erythropoietin. J Clin Invest 80:357, 1987

16.Fraser JK, Lin F-K, Berridge MV: Expression and modulation of specific, high affinity binding sites for erythropoietin on the human erythroleukemic cell line K562. Blood 71:104, 1988

17.Kitamura T, Kuwaki T, Chiba s, Chao-Jung Tsao, Mitani K, Tojo A, Tange T, Urabe A, Takaku F: Demonstration of erythropoietin receptors on a novel multi-factor dependent human cell line, MFD-1. Blood Suppl

1:175a, 555, 1987

18.Metcalf D, Begley CG, Johnson GR, Nicola NA, Vadas MA, Lopez AF, Williamson DJ, Wong GG, Clark SC, Wang EA: Biologic properties in vitro of a recombinant human granulocyte-macrophage colony-stimulating factor. Blood 67:37, 1987

19.Yang Y-C, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Giannotti JS, Leary AC, Kriz R, Donahue RE, Wang GG, Clark SC: Human IL-3 (Multi-CSF) : Identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. Cell 47:3, 1986

20.Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55, 1983

21.Greenwood FC, Hunter WH, Glover JS: The preparation of ^{131}I-labeled human growth hormone of high-specific radioactivity. Biochem J 89:114, 1963

22.Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680, 1970

23.Scatchard, G: The attraction of proteins for small molecules and ions. Ann N.Y. Acad Sci 51:660, 1949.

24.Sherwood, J.B. and Goldwasser, E: A radioimmunoassay for erythropoietin. Blood 54:885, 1979

25.Urabe A, Saito T, Fukamachi H, Kubota M, Takaku F: Serum erythropoietin titers in the anemia of chronic renal failure and other hematological states. Int J Cell Cloning 5:202, 1987

26.Sharon M, Klausher RD, Cullen BR, Chizzonite R, Leonard WJ: Novel interleukin-2 receptor subunit detected by cross-linking under high-affinity conditions. Science 243:859, 1986

27.Sakaguchi M, Koshihara Y, Tsuda H, Fujimoto K, Shibuya K, Kawakita

M, Takatsuki K: The expression of functional erythropoietin receptors on and interleukin-3 dependent cell line. Biochem Biophys Res Commun 146:7, 1987

28.Tsao C-J, Tojo A, Fukamachi H, Kitamura T, Saito T, Urabe A, Takaku F: Expression of the functional erythropoietins on interleukin-3-dependent murine cell lines. J Immunol 140:89, 1988

Table I Modulation of EPO receptors on TF-1 cells

Factors[*1]		GM-CSF	IL-3	EPO	No factor
Kd[*2]	Exp.1 [*4]	0.32	0.35	0.35	0.31
	2	0.37	0.40	0.38	0.38
Number[*3]	Exp.1	1640	1580	1080	1600
	2	1820	1720	1120	1720

[*1]. Cells are usually cultured in the presence of 5 ng/ml GM-CSF. The Scathard analyses were performed after after cells were cultured in the presence of 5 ng/ml IL-3 or 5 unit/ml EPO, or in the absence of any factors.

[*2]. Dissociation constant of EPO receptors on TF-1 cells

[*3]. Number of EPO receptors on TF-1 cells

[*4]. Two independent experiments (Exp. 1 and 2) were performed

FIGURE LEGENDS:

Fig. 1 Growth dependency of TF-1 cells on rHuEPO. Cells ($1x10^4$) were cultured for two days in RPMI 1640 containing 20 % FCS with and without rHuEPO at various concentrations. Closed circles: the number of viable cells was examined by MTT reduction as described in the "Materials and Methods" section. Open circles: the number of the cells in cell cycle was examined by ^{3}H-TdR uptake. Briefly, cells that had been deprived of growth factors for 4 hr were cultured in the presence or absence of rHuEPO, and ^{3}H-TdR (0.1 μCi) was added in the following 24 hr. The cultured cells were then harvested, caught onto glass fiber filters and counted in a scintillation counter. The results shown are the mean ± SD of triplicates.

Fig. 2 Time course of changes in ^{125}I-EPO binding and dissociation. Cells ($2 x 10^5$) were incubated with ^{125}I-EPO (1.5 nM; $2.0 x 10^5$ cpm) at 15°C. Specific binding is defined as the difference between cpm bound in the absence of unlabeled EPO, and in the presence of a 200-fold excess of unlabeled EPO. The cells were divided into two aliquots after a 2 hr-incubation at 15 C; a 300-fold excess of unlabeled EPO was then added to one aliquot, with incubation being continued for the following four hours (broken line). The results shown are the mean ± SD of triplicates. The experiment was performed twice with similar results.

Fig. 3 Competition for ^{125}I-EPO binding by unlabeled EPO. Cells ($2 x 10^6$) were incubated with ^{125}I-EPO (1.5 nM; $2.0 x 10^5$ cpm) with and without various amounts of unlabeled EPO at 15°C for 90 min. The results shown are the mean ± SD of triplicates.

Fig. 4 Scatchard analysis of the saturation binding of ^{125}I-EPO to TF-1 cells. Various amounts of ^{125}I-EPO were incubated with TF-1 cells (2 x 10^5) at 15°C for 90 min with and without unlabeled EPO. Scatchard transformation of the binding data is shown. Each point is the mean of duplicate determinations. Inset: Saturation binding data of the same experiment. The experiment was repeated five times with similar results.

Fig. 5 Time course of down-modulation of EPO receptors expressed on TF-1 cells by EPO. ^{125}I-EPO binding to TF-1 cells was examined after culturing the cells in the presence of 5 U/ml EPO for the indicated periods. The results shown are the mean of duplicates. This figure is a representative of three separate experiments.

Fig. 6 Cross-linking of ^{125}I-EPO to TF-1 cells. Radioiodinated EPO (750 pM, 1.0 x 10^5 cpm) was cross-linked to TF-1 cells (2 x 10^6) with (lane B) and without (lane A) a 200-fold excess of unlabeled EPO by incubating with DSS (200 µM). Subsequent NaDodSO$_4$/PAGE was performed under reduced conditions.

Fig. 1

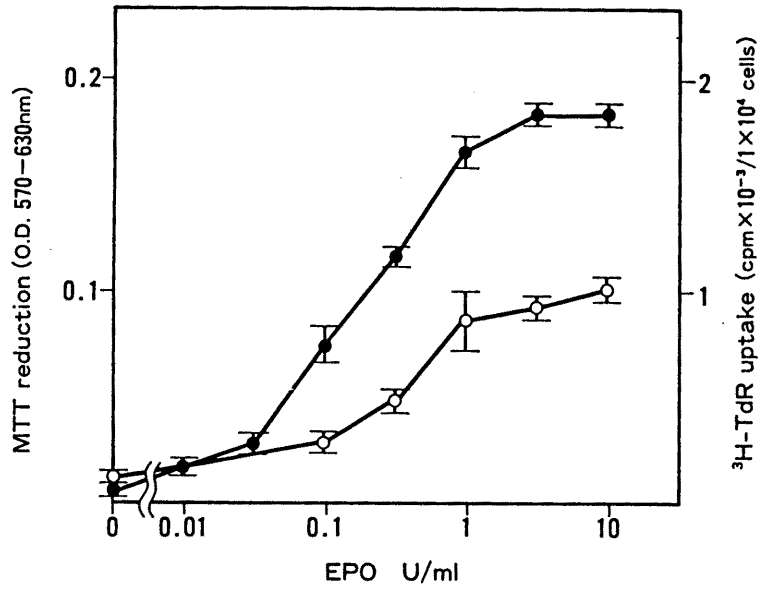

MTT reduction (O.D. 570−630nm)
0.2
0.1
2
1
^{3}H-TdR uptake (cpm×10^{-3}/1×10^{4} cells)
0
0.01
0.1
1
10
EPO U/ml

Fig. 2

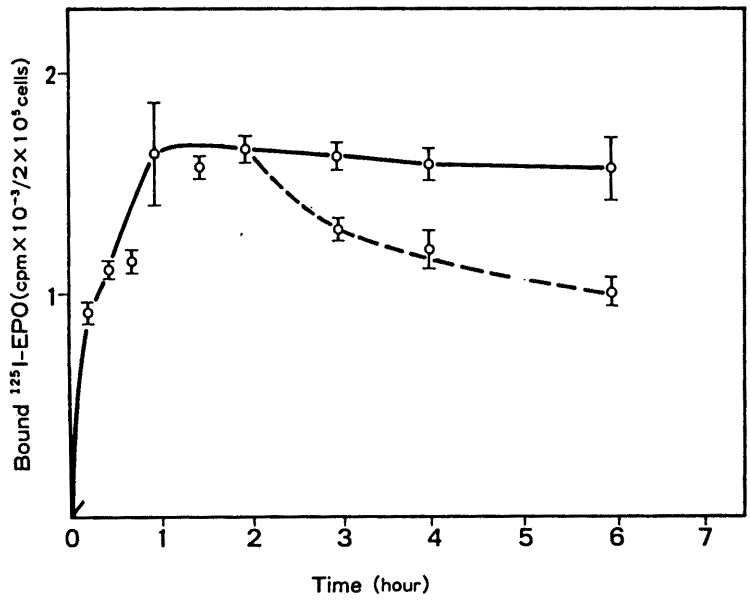

Bound ^{125}I-EPO(cpm×10^{-3}/2×10^{5}cells)
2
1
0
1
2
3
4
5
6
7
Time (hour)

Fig. 3

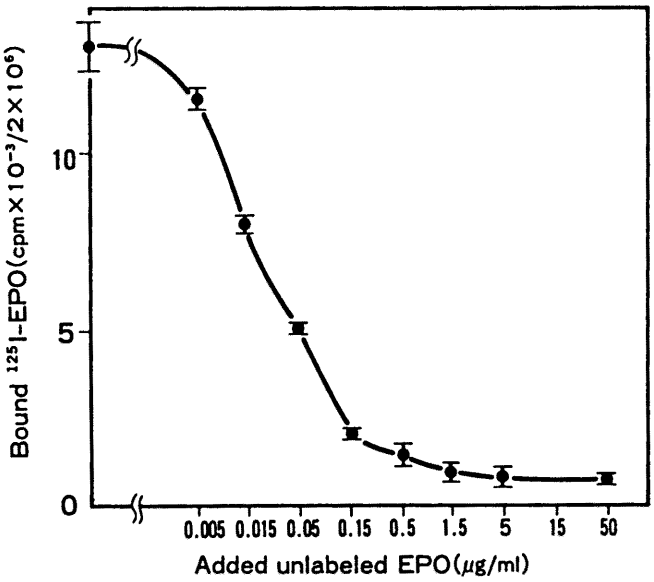

Bound ^{125}I-EPO(cpm×10^{-3}/2×10^6)
10
5
0
0.005 0.015 0.05 0.15 0.5 1.5 5 15 50
Added unlabeled EPO(μg/ml)

Fig. 4

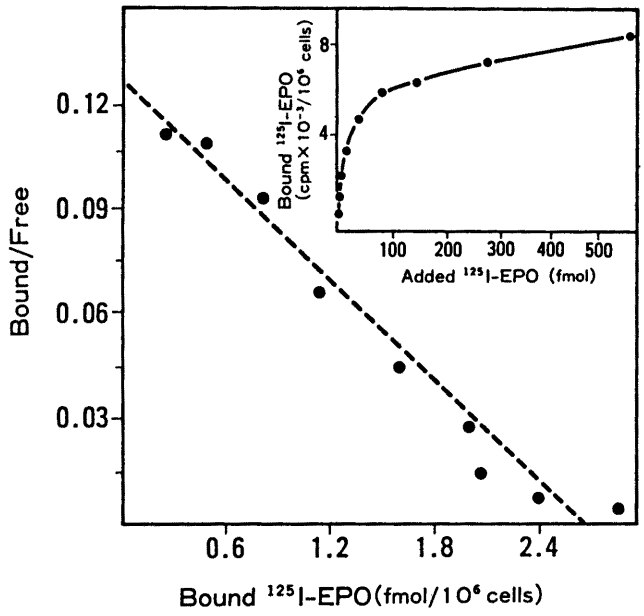

Bound/Free
0.12
0.09
0.06
0.03
0.6 1.2 1.8 2.4
Bound ^{125}I-EPO(fmol/10^6 cells)
Bound ^{125}I-EPO (cpm×10^{-3}/10^6 cells)
8
4
100 200 300 400 500
Added ^{125}I-EPO (fmol)

Fig. 5

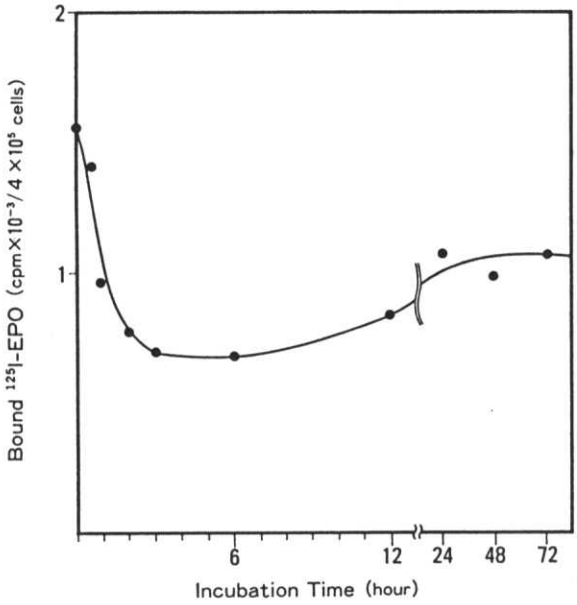
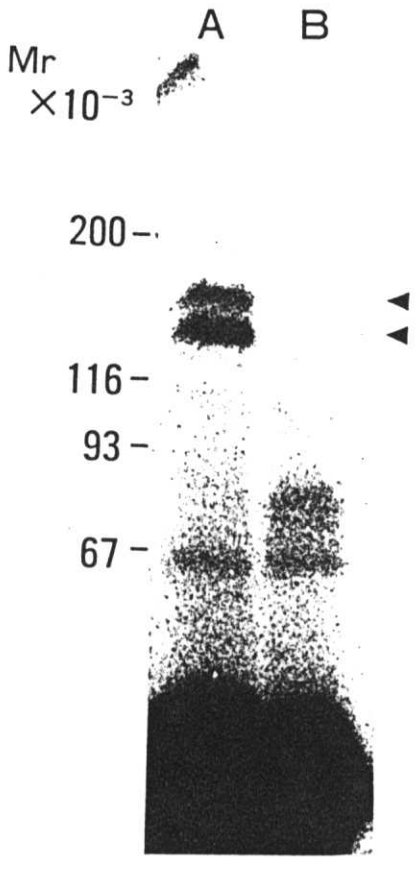

2
1
Bound ^{125}I-EPO (cpm×10^{-3}/4×10^5 cells)
6
12
24
48
72
Incubation Time (hour)

Fig. 6


A
B
Mr
×10^{-3}
200
116
93
67