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IDENTIFICATION OF ERYTHROPOIETIN RECEPTORS ON FETAL LIVER ERYTHROID CELLS

Arinobu Tojo, Hiromi Fukamachi, Masato Kasuga, Akio Urabe, and Fumimaro Takaku

The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Tokyo 113, Japan

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SUMMARY: Erythropoietin (EPO) has a central role in the growth and development of erythroid cells. Using a biologically active radioiodinated derivative, EPO receptors were identified on fetal mouse liver cells mostly consisting of erythroid cells. ^{125}I-EPO was cross-linked to two receptor forms with apparent molecular masses of 110 and 95 kilodaltons, respectively and both having similar affinity toward EPO.

Erythropoietin (EPO) is a hemopoietic growth factor which primarily controls the production of red blood cells *in vivo* (1). It appears certain to have hemopoietic specificity toward committed but undifferentiated erythroid progenitor cells *in vitro* (2). The interactions of EPO with these target cells induce a series of biochemical events and related morphological changes leading to terminal differentiation (3). By analogy to other polypeptide growth factor systems, its action is apparently mediated by specific receptors that bind it at the cell surface. Chang *et al.* were the first to provide evidence for EPO receptors on rat bone marrow cells (4). Subsequently, Krantz and Goldwasser directly demonstrated their presence on Friend virus-infected mouse spleen cells (5). However, in studying the nature of EPO receptors on normal progenitor cells, there is a major limitation such that EPO-sensitive cells represent only a minor percentage of the

ABBREVIATION: FMLC, fetal mouse liver cells; DSS, disuccinimidyl suberate; DSP, sulfosuccinimidyl propionate.

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total number of bone marrow cells. For example, using a fluorescent derivative of EPO, Weiss and Goldwasser found that not more than 1.5% of normal rat bone marrow cells could be labeled (6). An enriched population of normal EPO-sensitive cells was found to be present in fetal mouse liver cells (FMLC) on day 12 - 13 of gestation, consisting mainly of erythroid cells (7). We recently prepared the biologically active iodinated EPO derivative and characterized its receptors on FMLC (8). In the present report, an examination was made of the molecular characteristics of EPO receptors on FMLC by chemical cross-linking. The EPO receptors identified by this method appeared to consist of two species with estimated molecular masses of 110 and 95 kD, respectively.

MATERIALS AND METHODS

Mice. Pregnant ICR mice were sacrificed on day 12 or 13 of gestation and the fetal livers were removed. They were dispersed into a single cell suspension in α-medium (GIBCO, Gland Island, NY) by being passed through 26-gaguge needles repeatedly. The cells were washed twice with α-medium and once with α-medium containing 20 mM HEPES (pH 7.4), 0.1% bovine serum albumin and 100 μg/ml of bacitracin (binding buffer).

Growth Factors. Recombinant human EPO and granulocyte colony-stimulating factor (rhG-CSF) were gifts from Kirin-AMGen (Thousand Oaks, CA). Recombinant murine interleukin-3 (rmIL3) and granulocyte-macrophage colony-stimulating factor (rmGM-CSF) were generously provided by Dr. K. Arai, DNAX Research Institute (Palo Alto, CA).

Iodination of EPO. EPO was iodinated by a chloramine-T method as described elsewhere (9). The radioiodinated derivative had specific activity varying from 38 to 55 μCi/μg with full biological activity.

Binding Experiments. FMLC ($1-2 \times 10^7$) in binding buffer (0.5 ml) were placed in 1.5 ml Eppendorf tubes followed by the addition of ^{125}I-EPO (usually $15 - 20 \times 10^4$ cpm) with or without competitors. Incubation was performed either at 37°C for 45 min in a fully humidified incubator or at 15°C for 180 min in a cooling water bath. Following incubation the mixture was chilled on ice, sedimented and washed once with ice-cold phosphate-buffered saline (PBS) to obtain the cell pellet.

Chemical Cross-linking. The cell pellet was resuspended in 0.5 ml of cold PBS, and disuccinimidyl suberate (DSS; Pierce Chemical Co., Rockford, IL) freshly prepared in acetonitrile was added at 0.2 mM unless otherwise indicated. Another cross-linker, sulfosuccinimidyl propionate (DSP; Pierce Chemical Co.,) was also used at the same concentration. The cross-linking reaction was allowed to proceed on ice for 15 min and then quenched by adding 1 ml of cold Tris-buffered (25 mM, pH 7.4) saline (0.15 M) with 1 mM EDTA. The tubes were centrifuged at 4°C for 30 sec in a Beckman Microfuge B. The cell pellet was lyzed in 0.5% NP-40, 25 mM Tris (pH 7.4), 1 mM phenylmethylsulfonyl fluoride and 500 trypsin inhibitor units/ml of aprotinin (60 μl per 10^7 cells). After 30 min incubation on ice, the detergent-soluble fraction was collected by centrifugation at 15,000 x g for 10 min.

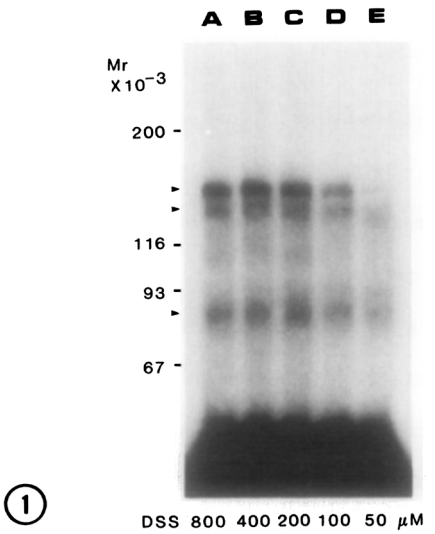
Electrophoresis and Autoradiography. Affinity-labeled samples were mixed with 1/5 volume of 5-fold concentrated Laemmli's sample buffer

(10), and boiled for 3 min in the presence of 50 mM dithiothreitol. Electrophoresis was performed according to Laemmli (10). Fixed, stained and dried gels were subjected to autoradiography for 1 to 3 weeks using Kodack X-Omat AR film with Dupont Lightning Plus screens.

RESULTS

When ^{125}I-EPO was cross-linked to FMLC with increasing concentrations of DSS, the three cross-linked bands appeared at relative molecular masses of 145 kD, 130 kD and 80-90 kD, respectively, under reducing conditions. These three bands increased in intensity with increasing amounts of DSS, reaching maximal intensity at 0.2 mM DSS. However, even at the highest amount of DSS (0.8 mM) most of the cell-bound radioactivity migrated as uncoupled ^{125}I-EPO (35 kD) (Fig. 1).

The ^{125}I-EPO affinity -labeling of the three molecular species was not affected by incubation of FMLC with excess amounts of colony-stimulating factors including rmIL3, rmGM-CSF and rhG-CSF (Fig. 2, lanes



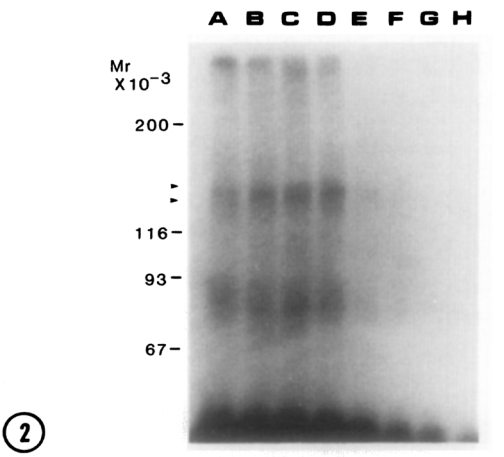


Figure 1. Effect of increasing concentrations of DSS on ^{125}I-EPO cross-linking to FMLC. ^{125}I-EPO was cross-linked to FMLC by incubation with indicated amounts of DSS as described in methods. Lanes A, 800; B, 400; C, 200; D, 100; E, 50 μM.

Figure 2. Molecular specificity of ^{125}I-EPO cross-linking to FMLC. FMLC were incubated at 37°C with ^{125}I-EPO without a competitor (A) or with rmIL3 (B), rmGM-CSF (C) or rhG-CSF at 1,000 u/ml. Competition was also brought about by increasing amounts of unlabeled EPO: Lanes E, 3; F, 10; G, 30; H, 150 nM. Subsequent analysis was conducted according to the described methods.

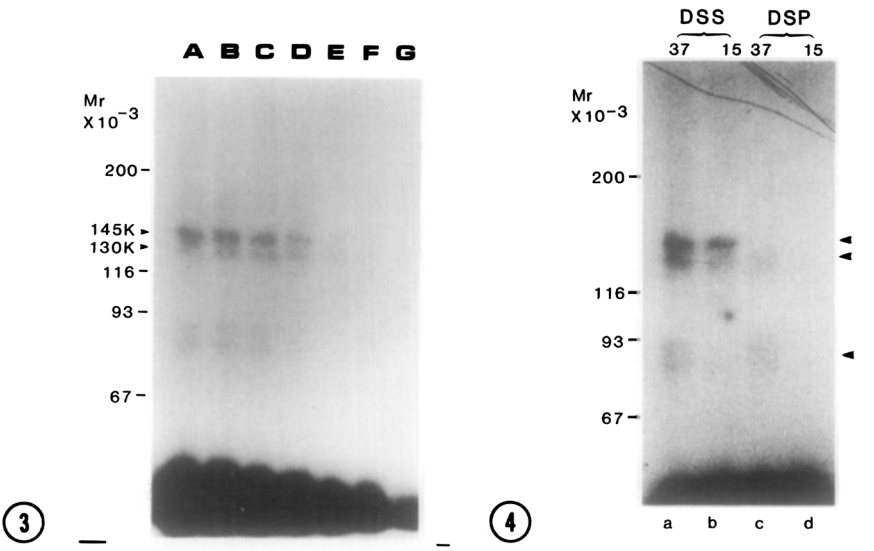


Figure 3. Dependence of cross-linked complex formation on concentration of ^{125}I-EPO. FMLC were incubated with increasing concentrations of ^{125}I-EPO at 15°C and then cross-linked as described in methods. Lanes A, 3200; B, 1600; C, 800; D, 400; E, 200; F, 100; G, 50 pM of ^{125}=EPO.

Figure 4. ^{125}I-EPO cross-linking to FMLC by different cross-linkers and under different binding conditions. Binding was performed at 37°C for 45 min (lanes a and c) or at 15°C for 180 min (lanes b and d). Cross-linking was performed with DSS (lanes a and b) or DSP (lanes c and d) as described in methods.

b-d). In contrast, when increasing concentrations of unlabeled EPO were added during incubation with ^{125}I-EPO, the radioative bands corresponding to both the three receptor species and uncoupled ^{125}I-EPO decreased in intensity in a similar dose-dependent manner.

Labeling of the receptor complexes appeared to attain saturation by incubation of FMLC with increasing amounts of ^{125}I-EPO at 15°C (Fig. 3). In this study, the various receptor forms yielded apparently identical affinity toward EPO; a half maximal effect was noted at approximately 400 pM ^{125}I-EPO (Fig. 3 lane D).

Next, FMLC were incubated with ^{125}I-EPO at different temperatures, and then cross-linked with different types of chemical cross-linkers. Under reducing conditions, the DSS-mediated cross-linking of ^{125}I-EPO to its receptor generated the 145 kD and 130 kD bands regardless of the incubation temperatures (Fig. 4 lanes a and b). This is essentially

consistent with the results shown in Fig. 3. DSP-mediated ^{125}I-EPO-receptor comlexes could be cleaved under reducing condition at the disulfide bridges inherent in the cross-linker. Thus, a trace (lane c) or no (lane d) radioactivity remained associated with the receptor molecules.

DISCUSSION

This report describes the molecular features of EPO receptors on fetal liver erythroid cells. As shown for other peptide hormone-receptor systems, the divalent agents DSS and DSP cause cross-linking of bound ^{125}I-EPO to unique cellular components. Following typical binding experiments, the labeling of EPO receptors produced two major radiolabeled bands (145 kD and 130 kD) possibly corresponding to ^{125}I-EPO-receptor complexes. A class of 80 - 90 kD component was also detected in the cross-linking studies. However, this form is likely to represent proteolytic products of 145 and 130 kD receptor forms either internalized on ligand binding or degraded during incubation, since labeling of this form is highly variable from one experiment to another, and incubation at 37°C which allows internalization of ^{125}I-EPO (9) increased the frequency of appearance of this species. The 145 and 130 kD receptor forms appear to have similar affinity for EPO in the picomolar range at which EPO is biologically active. Scatchard analysis of the binding data with FMLC revealed two classes of binding sites, one with high affinity (0.4 nM) and the other with low affinity (3 nM)(8). However, the structural basis of EPO receptors which can distinguish between high- and low-affinity binding sites remains unknown in the present study.

In regard to the relationship between the 145 kD and 130 kD species, there are several possibilities. First, the two species may be different subunits of a single receptor complex. Recently a series of reports have shown IL2 receptor complexes to be formed from two distinct

molecules which bind independently IL2 (11-13). Second, the 145 kD species may be degraded into the 130 kD species by specific and limited proteolysis . For example, the insulin receptor (14), and epidermal growth factor receptor (15) have been reported to undergo such a process. Third, a single receptor protein may undergo unique posttranslational modification. Finally, some covalent attachment to receptor neighboring proteins may occur. We cannot entirely exclude any of these possibilities in the present experiments and further study including peptide mapping analysis should be conducted for confirmation.

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