Vol. 146, No. 1, 1987
July 15, 1987
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 7-12

THE EXPRESSION OF FUNCTIONAL ERYTHROPOIETIN RECEPTORS ON AN INTERLEUKIN-3 DEPENDENT CELL LINE

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Received May 13, 1987

Summary: We report the expression of the erythropoietin receptors on an interleukin-3 dependent cell line. By the transfer into medium supplemented with erythropoietin, DA-1 cells were converted to an erythropoietin dependent growth state. [^{125}I] erythropoietin was used to detect receptors specific for this hormone on the cell surface. Binding studies revealed that erythropoietin bound to 131 ± 23 receptors/cell with a Kd of 0.54 ± 0.2 nM. When the cells were incubated at 37°C, trichloroacetic acid soluble radioactivity appeared in the medium after [^{125}I] erythropoietin binding began to decrease, suggesting that the decline represents the degradation of cell associated [^{125}I] erythropietin- receptor complexes.

Recently, receptor studies of hematopoietic growth factors have been reported (1-4). The receptor for IL-2, originally considered to be present only on T cells, is now known to be expressed on non-T hematopoietic cells (5). Furthermore, it has been shown that the receptor for mouse B cell stimulating factor-1 exists on a variety of hematopoietic cells (6). These findings suggest the expression of the receptors for specific growth factors on a wide range of hematopoietic cells.

Abbreviations: IL-2; interleukin-2. IL-3; interleukin-3. Epo; erythropoietin HEPES; N-(2-Hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid. TCA; trichloroacetic acid. CM; conditioned medium. CFU-E; colony forming unit-erythroid.

0006-291X/87 $1.50

We have noted that, in the presence of Epo, an IL-3 dependent cell line, DA-1, retained its viability in liquid culture, and a small population proliferated in semisolid methylcellulose culture. DA-1 cells were then cultured continuously in medium supplemented with 1 unit/ml of Epo. The viable cells continued to grow and developed into an Epo dependent cell line that was designated DA-1(cl.14). In this paper, we demonstrated the expression of Epo receptor on DA-1(cl.14) cells.

Materials and Methods

Cell Culture: DA-1 and DA-1(cl.14) cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 100 units/ml of penicillin-G and 100 μg/ml of kanamycin (complete medium) supplemented with WEHI-3CM or Epo at 37°C with 5% Co_2.

Materials: Recombinant human Epo was kindly provided by Chugai Pharmaceutical Inc. (Tokyo, Japan). The purity of recombinant Epo was > 99.8%, and its biological activity was 200,000 units/mg protein as determined by polycythemic mouse method. [^{125}I] Epo was purchased from Amercham Japan Inc. (Tokyo, Japan). Specific radioactivity of labeled-Epo was 400-500 Ci/mmol. Effective radioactivity binding to cells was > 90% of the total activity.

Proliferative Response to Epo: Cells($2x10^5$) in a total volume of 1 ml of complete medium were cultured in 24-well plate for 7 days with 0.5-2.0 units/ml of Epo or 15% (v/v) of WEHI-3CM. Every day, the number of viable cells was counted by the trypan blue dye exclusion method. In the cell proliferation assay, $5x10^4$ cells were placed in 96-well microtiter plate and cultured in 200 μl of complete medium with various concentrations of Epo or WEHI-3CM for 48 hours. The cells were pulsed with [^{3}H] thymidine (2 Ci/mol, Amersham) for the last 6 hours followed by precipitation onto filters, and the radioactivity was counted with a liquid scintillation counter.

Radiolabeled Epo Binding to DA-1(cl.14) cells: Binding assay was performed as described previously (7) with modification. Serially diluted radiolabeled Epo in a total volume of 0.2 ml of RPMI 1640 medium containing 25 mM HEPES, pH 7.2., and 1 mg/ml of bovine serum albumin were incubated at 4, 25 and 37°C for the indicated periods. After incubation, cells were centrifuged. To the 40 ul of supernatant in Eppendorf tube, 10 μl of 50%TCA was added. After centrufuging the tubes, the radioactivity in supernatant was counted (TCA soluble radioactivity). Cell pellets were washed and resuspended in 150 μl of binding medium, and centrifuged through a 150 μl layer of 10% sucrose in phosphate buffered saline, pH 7.4. The tips of the tubes containing the cell pellet were cut off and the radioactivity was counted in a gamma counter. Nonspecific binding was determined by incubating cells with a 200-fold excess amount of unlabeled Epo. Specific binding was obtained by subtracting the nonspecific binding.

Results

As shown in Fig.1, DA-1(cl.14) cells proliferated in the presence of either Epo or WEHI-3CM. The doubling-time was 29 hours with Epo and 23 hours with WEHI-3CM. The parental DA-1 cells maintained in WEHI-3CM differed in

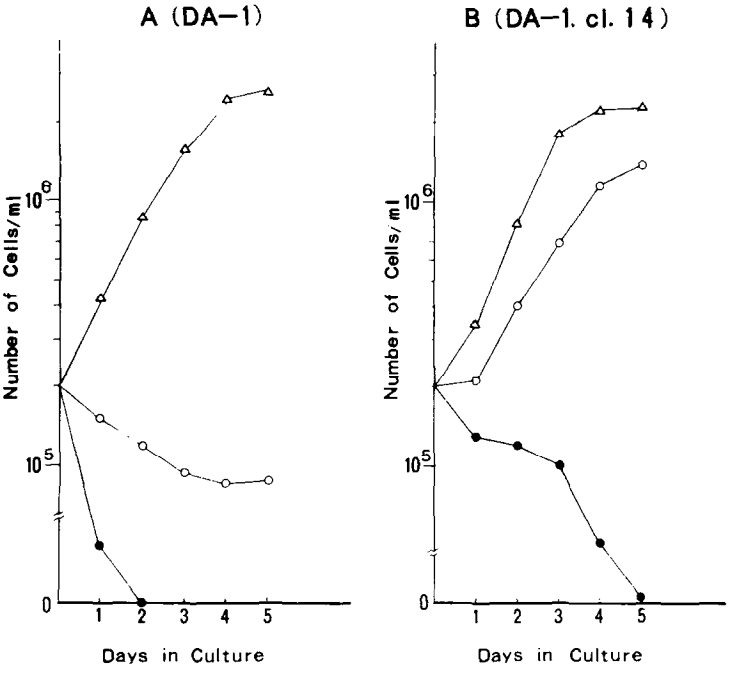

Figure 1. Growth response of IL-3-dependent cells to Epo. DA-1 and DA-1(c1,14) cells ($2x10^5$/ml) were cultured in complete medium alone (control ●-●), medium supplemented with 1 unit/ml of recombinant human Epo (○-○) or 15% (v/v) of WEHI-3 CM (△-△). Growth was determined by counting the number of viable cells after 1 to 5 days using the trypan blue dye exclusion method.

the way they responded to Epo. [^{3}H] thymidine incorporation was increased dose-dependently (data not shown).

[^{125}I] Epo exhibited specific binding to DA-1(cl.14) cells, and the binding saturable with increasing concentrations of radiolabeled Epo (Fig. 2). When the cells were incubated at 37°C, specific binding became maximum after about 30 min and then decreased, at which time TCA soluble radioactivity increased in the medium. This suggests the degradation of [^{125}I] Epo-receptor complexes. Therefore, the following binding assays were performed at 25°C. Fig. 3 illustrates typical equilibrium binding data for radiolabeled Epo, where Scatchard analysis (8) of the data indicates that there are single classes of binding sites for Epo. From the three binding experiments using three different radiolabeled preparations, the apparent Kd was 0.54 $\pm$ 0.2nM with 131 $\pm$ 23 specific binding sites/cell. As shown in Fig. 4, increases in the concentrations of unlabeled Epo resulted in the

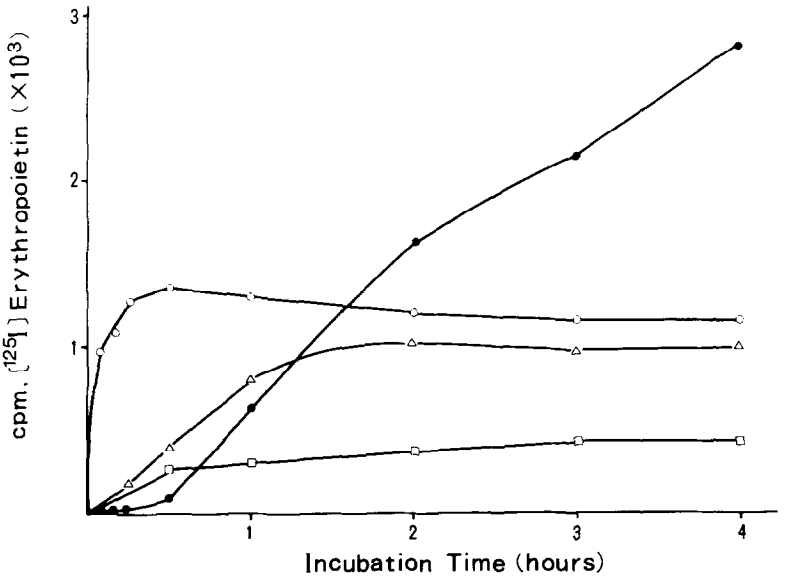

Figure 2. Time and temperature dependence of iodinated Epo binding to 1x10⁷ viable DA-1(cl.14) cells, and kinetics of iodinated Epo binding and degradation at 37°C. Specific binding at 37°C (○-○) and TCA soluble radioactivity (●-●). Cells were incubated with iodinated Epo (0.5nM). Nonspecific binding was measured in the presence of a 200-fold excess amount of unlabeled Epo. Specific binding at 25°C (△-△) and 4°C (□-□) are also shown. TCA radioactivity was determined by subtracting the TCA radioactivity in the supernatant obtained after incubation with unlabeled Epo.

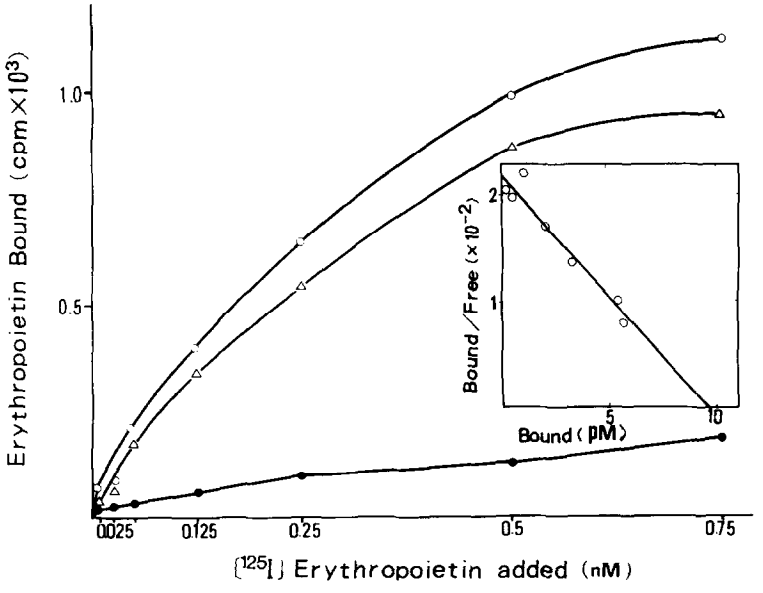

Figure 3. Equilibrium binding of [125I] Epo to DA-1(cl. 14) cells. Cells (1x10⁷) were incubated with various concentrations of [125I] Epo for 2 hours at 25°C. Total binding (○-○), nonspecific binding (●-●) and specific binding (△-△). Nonspecific binding was measured in the presence of a 200-fold molar excess of unlabeled Epo. The inset shows a Scatchard plot of the binding data; Y=0.022-0.0024X; r=0.98; Kd=0.42nM; 110 receptors/cell.

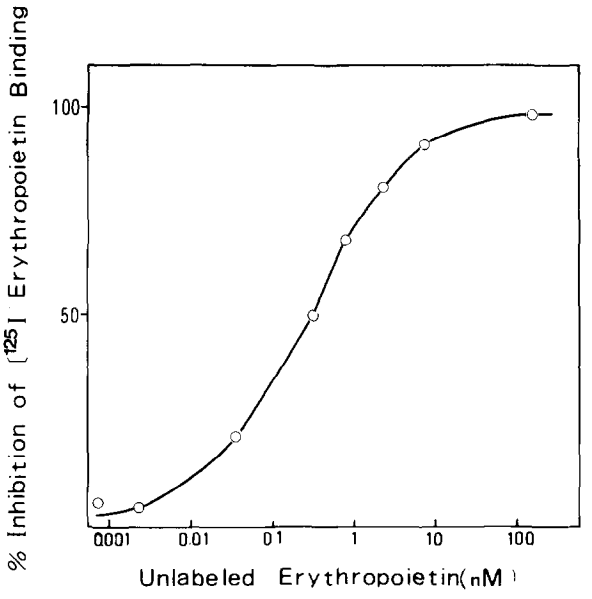

Figure 4. Inhibition of $[^{125}I]$ Epo binding to DA-1(cl.14) cells by unlabeled Epo. Cells ($1x10^7$) were incubated with iodinated Epo (0.5nM) and various concentrations of unlabeled Epo. Incubation was for 2 hours at 25°C and binding was assayed as described under "Materials and Methods".

inhibition of the binding of $[^{125}I]$ Epo to DA-1(cl.14) cells. In addition, the binding of $[^{125}I]$ Epo was not blocked by WEHI-3CM (data not shown).

Discussion

This study demonstrates the expression of Epo receptor on nonerythroid hematopoietic cells. The number of Epo receptor was 131 $\pm$ 23 /cell with a Kd of 0.54 $\pm$ 0.2nM. CFU-E enriched fetal mouse liver cells express two classes of receptors for Epo with different affinities, high and low.(9). Our data, however, suggest a single class of binding sites for Epo. This disparity between CFU-E and DA-1(cl.14) may be due to differences in the cell lineage.

When DA-1(cl.14) cells were incubated at 37°C, the total cell associated radioactivity decreased, and this was followed by an increase in TCA soluble radioactivity, suggesting that the decrease reflects degradation of the cell associated $[^{125}I]$ Epo-receptor complexes (10).

After DA-1 cells were transferred into medium supplemented with Epo, they developed into an Epo growth dependent cell line. IL-3 dependent cell lines

have been converted to an IL-2 dependent growth state by culture in medium supplemented with IL-2 (11). Our findings can be interpreted along two general lines. First, some IL-3 dependent cell lines may represent progenitors of cells destined to mature into erythroid lineage. Second, a number of progenitor cells in the bone marrow may constitutively express receptors for several growth factors. Their growth and/or defferentiation may be regulated by the availability of a specific lymphokine in the microenvironment (12,13). This interpretation is more likely because we know that some IL-3 dependent cell lines(eg.FDCP-2) respond to Epo. These findings suggest that a small number of receptors specific for different growth factor are expressed on cells of different lineages. In addition, DA-1(cl.14) cells are thought to be useful to investigate the mechanisms of Epo actions, including the structure of the receptor and the signal transport in the cytoplasm.

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