Volume 298, number 2,3, 169–172 FEBS 10734 February 1992

Developmental changes in erythropoietin receptor expression of fetal mouse liver

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Received 15 November 1991; revised version received 20 December 1991

Erythropoietin (EPO) stimulates proliferation and differentiation of late erythroid precursor cells (CFU-E) and thereby determines the rate of erythropoiesis. Liver is the major erythropoietic site in a fetus. We dealt with developmental changes in CFU-E and EPO receptor (EPO-R) of fetal mouse liver. The affinity of the EPO-R to EPO was unchanged during fetal development. The popupation size of CFU-E, the number of EPO-R per liver cell, and EPO-R mRNA decreased as gestation proceeded, in a pattern indicating that the expression of EPO-R on erythroid precursor cells in fetal mouse liver is governed mostly by the process of mRNA production.

Erythropoietin receptor; Fetal liver; Erythropoietin receptor mRNA; Developmental change

1. INTRODUCTION

During the development of fetal mice with their gestation period of 21 days, erythropoiesis is first detectable at day 7 or 8 in the yolk sac [1]. The erythropoietic site shifts from the yolk sac to the fetal liver, which becomes the main erythropoietic organ during gestation; erythropoiesis in the liver starts on day 10.5 [2]. Spleen and bone marrow are responsible for murine erythropoiesis after birth but erythropoiesis in these organs is not detected until day 15 of gestation [3]. Erythropoietin (EPO) is a glycoprotein that is the major physiological regulator of erythropoiesis; EPO supports survival of the late erythroid precursor cells and enhances their proliferation and differentiation through binding to its specific receptor [4]. The CFU-E, late erythroid precursor cells, are the main targets of EPO [4].

Investigation of the binding characteristics of EPO receptor (EPO-R) on the erythroid cells of hamster yolk sac has indicated that the number of EPO-R per cell and the affinity of EPO-R to EPO change during hamster ontogeny [5]. Interestingly, the binding affinity decreased as gestation proceeds; the affinity on day 13 is one-thirtieth that on day 8 [5]. The binding properties of EPO-R and the products of cross-linking between EPO and EPO-R have been studied in fetal mouse liver cells at a certain stage of gestation [6–8] but the developmental changes in the properties of EPO-R have not been reported. Here we report on the binding characteristics of EPO-R, the expression of EPO-R mRNA, and the number of CFU-E in fetal liver cells during mouse ontogeny.

Abbreviations: EPO, erythropoietin; CFU-E, colony-forming unit-erythroids; EPO-R, EPO receptor; rHuEPO, recombinant human EPO; PBS, phosphate-buffered saline, pH 7.4.

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2. MATERIALS AND METHODS

2.1. *rHuEPO and cells from mice*

Recombinant human EPO (rHuEPO) was produced in baby hamster kidney cells [9] and isolated [10] as described previously. Radioiodination of rHuEPO (about 30 μCi/μg EPO) was done as reported elsewhere [6]. Mice (ICR, unless otherwise indicated) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). For preparation of reticulocytes, male mice (8 weeks old) received 5 intraperitoneal injections of 50 mg/kg phenylhydrazine solution (5 mg/ml in PBS) on 5 consecutive days. On day 2 after the last injection, blood was withdrawn and the cell preparation enriched reticulocytes (>90%) was used to isolate RNA. For preparation of spleen enriched EPO-responsive cells [11], mice were made anemic by injections of phenylhydrazine as described elsewhere [12]. For the same purpose, C3H mice received injections of transplantable erythroblastic cells [13] as reported previously [14]. Spleens from these mice were used to isolate RNA.

2.2. *Cell culture*

Cells were cultured in a humid 5% CO_2 atmosphere at 37°C. All media for cell culture contained 100 IJ/ml penicillin and 100 μg/ml streptomycin. Friend erythroleukemia cells, TSA8 [15], and mouse myelomonocytic leukemia cells, M1 [16], were maintained in IMDM (Gibco-BRL Life Technologies Inc., Tokyo) supplemented with 10% fetal calf serum (Wittaker Bioproducts Inc., MD) and in RPMI 1640 (Gibco-BRL Life Technologies Inc., Tokyo) supplemented with 10% horse serum (Wittaker Bioproducts Inc., MD), respectively.

2.3. *Binding of EPO with fetal mouse liver cells*

Binding of [^{125}I]rHuEPO with fetal mouse liver cells was done as described previously [6]. A single-cell suspension (4×10^6 cells) of livers obtained from fetuses at different stages of gestation was incubated

with [^{125}I]rHuEPO for 3 h at 15°C in the presence or absence of a 200-fold excess of unlabeled rHuEPO. Binding was assayed at 10 concentrations of [^{125}I]rHuEPO, ranging from 0.02 to 4.8 nM. Binding mixtures contained 0.1% NaN_3, an inhibitor of the receptor-mediated internalization of the ligand. Specific binding was calculated by subtraction of the nonspecific binding (binding in the presence of unlabeled EPO) from total binding (binding in the absence of unlabeled EPO). Data on specific binding were analyzed by the method of Scatchard [17] to estimate the number of binding sites and the dissociation constants of ligand–receptor complexes. One Scatchard plot was made with liver cells of fetuses from one pregnant mouse (~10 fetuses/mouse). Three plots of each gestation time were made to estimate the binding parameters.

2.4. *RNA preparation and blot analyses*

Total RNA was prepared by the guanidinium thiocyanate-CsCl method [18]. Northern blot analysis was done by the method of Thomas [19]. Details of analyses by Northern blotting and slot blotting were as described in a previous paper [20]. The probe for blot analyses of EPO-R mRNA was prepared by isolation of a 1.7 kb *Kpn*I fragment from a plasmid (the kind gift of Dr. D'Andrea) that contained cDNA of mouse EPO-R [21], and the probe was labeled with ^{32}P by multipriming of the fragment.

2.5. *Assay of CFU-E*

The CFU-E in the fetal livers were assayed by the method of Iscove et al. [22]. The cells (2×10^4 to 2×10^5 cells) from fetal livers at different stages of gestation were cultured in methylcellulose for 2 days in the presence of 2 U/ml rHuEPO. Erythroid colonies that were stained with benzidine and that consisted of 8 or more cells were counted. Triplicate methylcellulose cultures of fetal liver cells from two pregnant mice at each gestation time were done.

3. RESULTS AND DISCUSSION

Scatchard analyses of EPO binding to EPO-R on a variety of erythroid precursor cells including cell lines have shown that certain cells express single-affinity receptors but other cells express high- (K_d = ~100 pM) and low- (K_d = ~500 pM) affinity receptors (reviewed in [23]) and the mechanism of the presence of binding sites with different affinities is not known. Here we assayed binding to fetal mouse liver cells in a wide range of EPO concentrations (0.02–4.8 nM) so that both low- and high-affinity receptors could be detected. Fig. 1 shows a representative Scatchard plot. There was a deviation from linearity at high concentrations of the ligand, suggesting the presence of low-affinity sites. The deviation, however, was not always reproducible and it was difficult to estimate binding parameters of low-affinity sites. The occupancy of high-affinity sites by EPO induces the growth and differentiation of CFU-E [4], so we deal here only with high-affinity binding sites. The affinity of fetal mouse liver cells to EPO was unchanged in various stages of gestation, but the number of binding

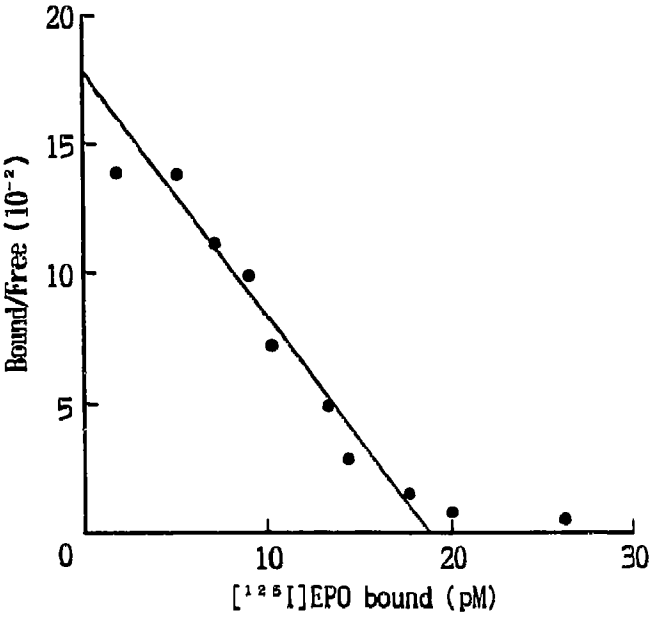

Fig. 1. Scatchard plot of EPO binding to liver cells of a fetal mouse on day 13 of gestation.

Table I

Percentage of CFU-E in mouse fetal liver and binding parameters of EPO to mouse fetal liver cells

Day of gestation	K_d (pM)	Receptors/liver cell	CFU-E (% in liver cells)
12	106 ± 8	376 ± 14	–
13	111 ± 9	419 ± 15	5.86 ± 0.20
14	114 ± 9	335 ± 14	–
15	137 ± 11	241 ± 12	–
16	94 ± 6	126 ± 8	1.01 ± 0.16
19	100 ± 9	40 ± 5	0.32 ± 0.06

– = not determined. Values are means ± SD.

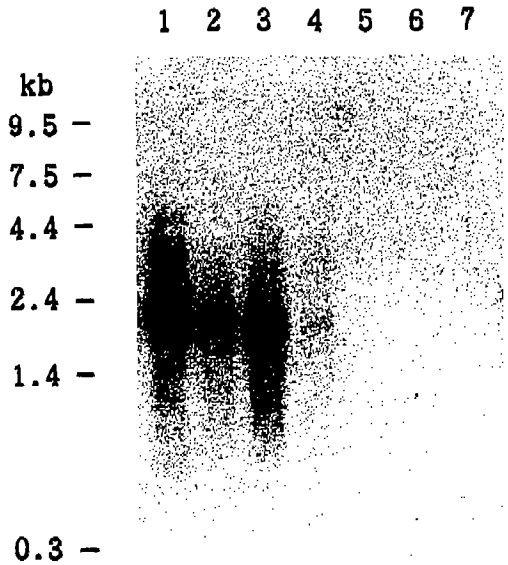

Fig. 2. Northern blot analysis of EPO-R mRNA. Poly(A)-selected RNA (10 μg) was used. (Lane 1) Fetal mouse liver on day 13 of gestation; (lane 2) TSA8, mouse erythroleukemic cell line; (lane 3) spleen of an anemic adult mouse; (lane 4) ELM, transplantable mouse erythroleukemic cell line; (lane 5) adult mouse liver; (lane 6) M1, mouse myelomonocytic cell line; (lane 7) mouse reticulocytes.

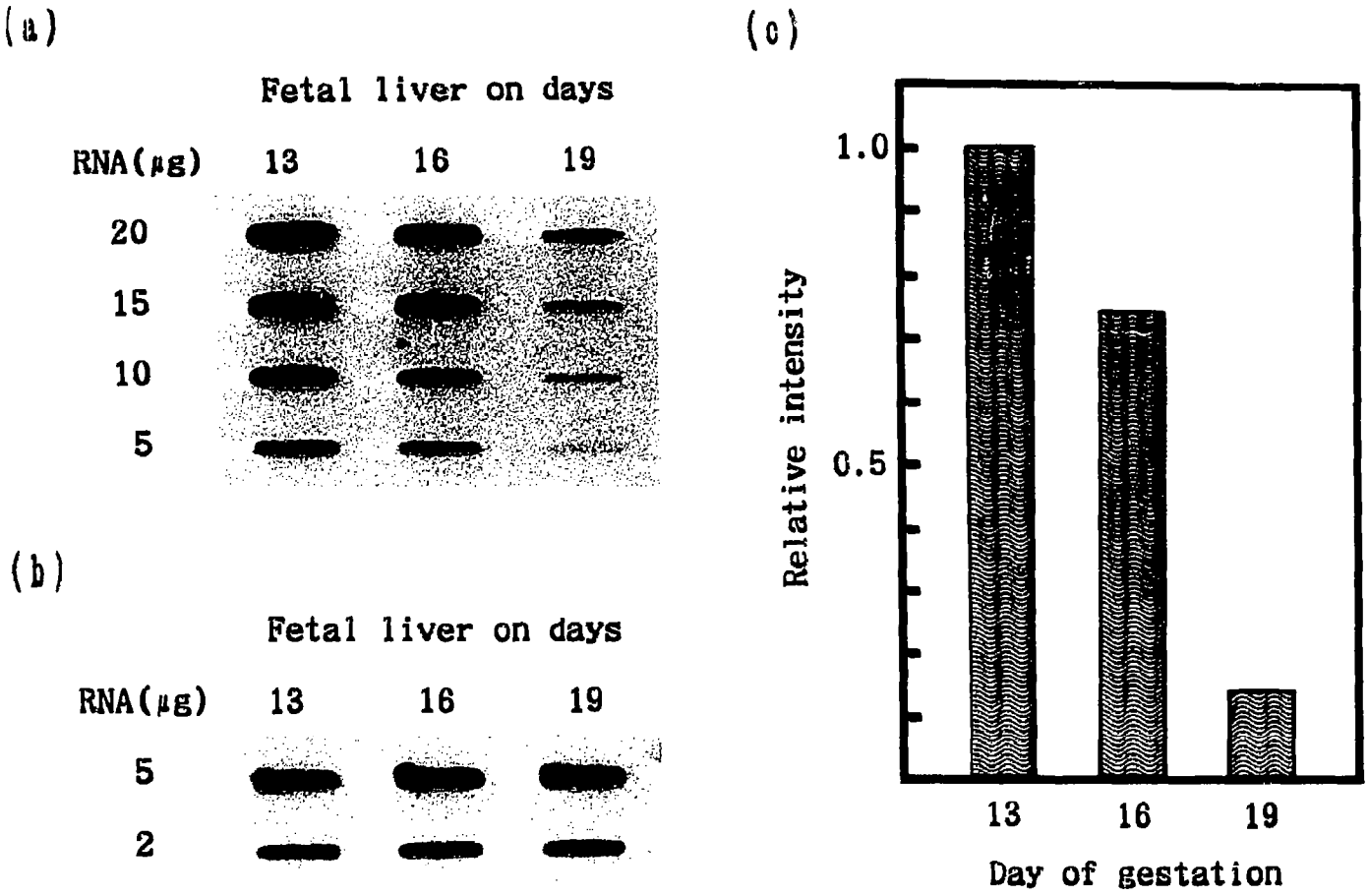

Fig. 3. Slot-blot analysis of EPO-R mRNA of fetal mouse liver. (a) Hybridization with a ^{32}P-labeled probe for EPO-R mRNA. (b) Hybridization with a ^{32}P-labeled probe for actin mRNA. (c) Intensity of the bands. Intensity was measured with a densitometer with 10 μg of RNA and the value for a fetus on day 13 was defined as 1.0.

sites per liver cell between days 13 and 19 of gestation decreased as the gestation advanced (Table 1).

CFU-E in fetal mouse liver were assayed by their ability to form erythroid colonies in vitro in the presence of EPO. The calculated population size of CFU-E at days 13, 16, and 19 of gestation is shown in Table I; the proportion of CFU-E declined from 5.86% on day 13 of gestation to 0.32% on day 19. This decline was roughly proportional to that of the number of EPO binding sites per liver cell. CFU-E are erythroid precursor cells that express EPO-R at the highest number [18], and therefore the developmental change in the number of EPO-R per liver cell could arise from the change in the percentage of CFU-E.

To identify a major step to control expression of EPO-R on the cell surface, we studied EPO-R mRNA in fetal livers of mice at different stages of gestation. EPO-R mRNA with a size of 2.1 kb has been identified in some erythroleukemic cell lines and in spleen cells of anemic adult mice [21,24] but EPO-R mRNA of fetal liver cells has not been analyzed. First, we detected EPO-R mRNA of fetal mouse liver cells by Northern blotting and compared it with mRNAs in adult mouse cells and cell lines (Fig. 2). A single band at the position of 2.1 kb was found with poly(A)-selected RNA from fetal mouse liver cells on day 13 of gestation (lane 1 in Fig. 2). The size was consistent with that found with TSA8 (a mouse erythroleukemic cell line) (lane 2), and spleen cells of an adult mouse made anemic by phenylhydrazine treatment (lane 3), and ELM (a transplantable mouse erythroleukemic cell line) (lane 4). These cells have been shown to express EPO-R on their cell surfaces by assays of EPO binding [6–8]. EPO-R mRNA was undetectable in adult mouse liver cells, M1 (a mouse myelomonocytic cell line), and mouse reticulocytes (lanes 5–7); these cells did not express EPO-R on their cell surfaces [6]. Second, to examine the developmental changes in the amount of EPO-R mRNA, slot-blot analysis was done with total RNA prepared from fetal mouse livers on days 13, 16, and 19 of gestation (Fig. 3a). The intensity of bands on each day of gestation, measured with a densitometer, was proportional to the amount of RNA used (data not shown). The amount of EPO-R mRNA decreased as the gesta-

tion advanced (Fig. 3a), but that of actin mRNA remained constant (Fig. 3b). Densitometric measurements of the intensity of the bands clearly show the gestation age-dependent decrease in EPO-R mRNA (Fig. 3c). Thus, the number of EPO-R per fetal liver cell could be directly related to the amount of EPO-R mRNA. These results indicate that the binding affinity of EPO-R on erythroid precursor cells did not change during the development of the mouse fetus and that the significant contribution of translational and/or post-translational regulation to EPO-R expression on the cell surface is unlikely.

Acknowledgements: We thank Yoko Tanaka for help in the preparation of the manuscript. This study was supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

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