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Retinoic acid up-regulates erythropoietin production in hepatoma cells and in vitamin A-depleted rats

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Abstract

Retinoic acid (RA) stimulated the production of erythropoietin (Epo) in a human hepatoma cell line, HepG2 cells. The stimulation was due to the accumulation of Epo mRNA. The Epo production in HepG2 cells was also dependent on O_2 tension for cell culture but the enhancement of Epo production by RA was independent of O_2 tension, indicating that RA exerts its effect through a pathway different from O_2. Oral administration of RA to the vitamin A-depleted rats elevated the concentration of Epo in serum. These results suggest that RA up-regulates EPO production in vivo as well as in vitro.

Key words: Erythropoietin; Retinoic acid; Oxygen tension

1. Introduction

Epo is a major physiological regulator of erythropoiesis [1,2]. In the fetus, the primary site of Epo production is the liver [3] and is replaced by the kidney in adults [4]. Hypoxia induces Epo production by increase of Epo mRNA [5–10]. The *cis*-acting sequences of the Epo gene responsible for hypoxic induction were identified in a promoter region and in a 3′-flanking region [11–17].

RA, a derivative of vitamin A, exerts a wide variety of effects on vertebrate development, cellular differentiation and homeostasis. Most effects of RA are caused by the transcriptional control of the target genes, which is mediated by two classes of nuclear receptors, retinoic acid receptors (RAR-α, RAR-β and RAR-γ) and retinoid X receptors (RXR-α, RXR-β and RXR-γ) [18]. RARs and RXRs are members of the steroid/thyroid receptor superfamily, and function as ligand-dependent transcription factors by binding with the specific response elements of the target genes. Homo- and heterodimers of RARs and RXRs recognize direct repeats of the core motif (AGGTCA or its closely related motifs) with one, two or five nucleotide space (DR-1, DR-2 and DR-5, respectively) [19–23].

Although the sequence homologous to DR-2 is present in the 3′-enhancer region of Epo gene and the nuclear extract of Epo-producing cells contains a protein(s) bound to the DR-2 sequence [11,12], there is no reports showing that RA regulates Epo production. In this paper, we have examined the effect of RA on Epo production using human hepatoma cells (HepG2) cultured in the retinoid-depleted FCS and vitamin A-depleted rats.

2. Materials and methods

2.1. Cell culture and others

HepG2 cells were cultured at 37°C in the minimum essential medium supplemented with 10% FCS (M. A. Bioproducts, Walkersville, MD) at 21% O_2/5% CO_2/balance N_2 gas in a controlled atmosphere chamber (Napco Scientific Co., Tualatin, OR). Retinoid-depleted FCS was prepared by treatment with charcoal [24]. To deplete intracellular retinoids, after the cells were cultured in the retinoid-depleted FCS for three days, the cells were replated at 2×10^4 cells/cm^2 and cultured for another two days. Experiments were started by the cell culture at 21% O_2 at 1% O_2 in the fresh RA-depleted FCS added at indicated concentrations of RA. All-*trans* RA, retinol, retinal, and α- or β-carotene were dissolved in ethanol and diluted with the medium (< 0.1% final ethanol contents). To measure the cellular protein, the cells were washed with PBS, incubated in 1 ml of 0.1 M HCl for 1 min and then lysed with 1 ml of 1 M NaOH. Protein in the lysates was determined with commercial kits (Protein Assay; Bio-Rad Laboratories, Richmond, CA).

2.2. De novo synthesis of protein and RNA

The cells precultured to deplete RA in 6-well dishes were incubated at 21% O_2 or 1% O_2 in the RA-depleted FCS containing RA at 0, 1, and 100 nM. After incubation for 4 h, 50 μCi of [^{35}S]methionine/[^{35}S]cysteine mixture or [^{3}H]uridine was added and the cells were cultured for 20 h. Incorporation of the radioactive precursors into the trichloroacetic acid-soluble fraction was determined. The background was assessed by concurrent addition of inhibitors (10 μg/ml of cycloheximide for protein synthesis or 5 μg/ml of actinomycin D for RNA synthesis).

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Abbreviations: Epo, erythropoietin; RA, retinoic acid; FCS, fetal calf serum; PBS, 10 mM phosphate-buffered saline, pH 7.2; EIA, enzyme-linked immunoassay; MoAb, monoclonal antibody; RT-PCR, reverse transcription-polymerase chain reaction.

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2.3. Sandwich-type enzyme immunoassay of Epo

We have described a sandwich-type EIA for Epo measurement using two MoAb (R2 and R6) that bind Epo at different epitopes [25,26]. To measure small amounts of Epo, this EIA was further improved. To the 96 well-microtiter plates was added 100 μl of the R6 solution (10 mg/ml in 50 mM sodium carbonate buffer, pH 9.6). After a 2-h incubation, the unfixed MoAb was washed with washing buffer (PBS containing 0.05% Tween 20 and 0.02% NaN_3). To each well was added 100 μl of the standard recombinant human Epo [27] or sample containing 0.05% Tween 20 and 1 mM EDTA and the plates were incubated for 2 h. After washing, 100 μl of alkaline phosphatase-linked R2 (0.5 μg/ml in washing buffer) was added and incubated for 2 h to form ternary complexes (fixed R6·Epo·R2 linked with alkaline phosphatase). After washing, the activity of phosphatase trapped in the microtiter wells was measured colorimetrically using coupled enzymes as follows. In this method, NADP is dephosphorylated by alkaline phosphatase and the resulting NAD is amplified by coupling enzymes, alcohol dehydrogenase and diaphorase. To the washed well was added 100 μl of 0.1 M diethanolamine buffer, pH 9.5, containing 0.4 M NADP, 2 mM $MgCl_2$, and 0.02% NaN_3. After incubation for 20 min was added 200 μl of the enzyme solution containing 0.2 mg/ml alcohol dehydrogenase, 1.6 unit/ml diaphorase, 56 mg/ml ethanol and 350 mg/ml INT violet. The reactions for amplification were stopped with 50 μl of 0.4 M HCl and the produced formazan was determined by measuring the absorbency at 492 nm with an EIA reader. This improved assay measures Epo as low as 1 pg/ml.

2.4. Competitive RT-PCR for Epo transcripts

To estimate Epo mRNA, the competitive RT-PCR was used [28]. Total RNA was prepared by the acid guanidinium isothiocyanate/phenol/chloroform method [29]. Five micrograms of the denatured RNA was reverse-transcribed using the Epo-antisense primer (Epo-B; 5′-AGATGTCATTGCTGGCACTGGAGTGTCCAT-3′) (see Fig. 3A) [30]. The transcribed Epo cDNA was co-amplified by PCR in the presence of various amounts of genomic Epo DNA as a competitor. The primers used for amplification were Epo-A, 5′-GTCGGGCAG-CAGGCCGTAGAAGTCTGGCAG-3′ and Epo-B (see Fig. 3A). Each of 60 cycles of PCR consisted of incubation for 1 min at 94°C for denaturation, for 2 min at 62°C for annealing, and for 3 min at 72°C for elongation. The amplified DNA was fractionated with electrophoresis, stained with ethidium bromide, and analyzed densitometrically.

3. Results and discussion

3.1. Effect of RA on Epo production in HepG2 cells

The presence of retinoids in the intracellular pool and in the serum used for cell culture makes it difficult to evaluate effect of the exogenous RA on cultured cells. We stripped retinoids from FCS with charcoal, and precultured the cells in the medium containing retinoid-depleted FCS to remove intracellular retinoids before the start of the experiment. Fig. 1 shows the Epo production in HepG2 cells cultured for 48 h in the untreated or retinoid-depleted FCS at 21% O_2. The cells cultured in the untreated FCS produced 78 ± 6 ng/g protein (13.3 ± 2.2 pg/ml in the medium) and the addition of RA at 10 nM slightly stimulated Epo production (1.3-fold stimulation). When the cells were cultured in the retinoid-depleted FCS, the production was low but there was a distinct stimulation (3-fold enhancement) of Epo production by RA. The Epo production in the retinoid-depleted FCS plus RA was comparable to that in the untreated FCS plus RA. There was no significant difference in the total de novo protein synthesis with or without RA (data not shown). Therefore, the stimulation of

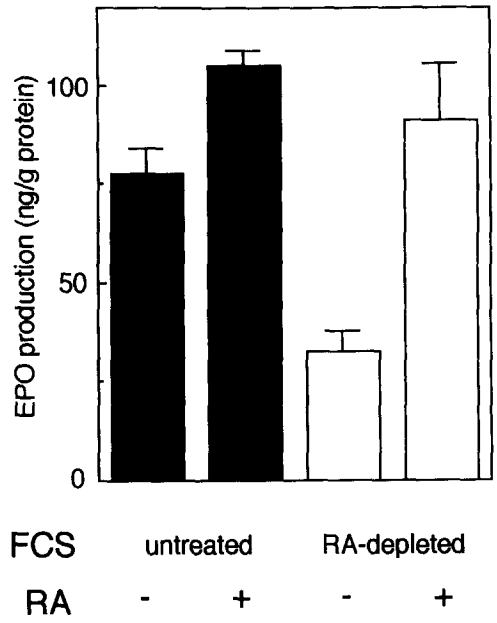

Fig. 1. RA stimulates Epo production in HepG2 cells. HepG2 cells were cultured in the untreated FCS (shaded columns) or RA-depleted FCS (white columns) (see section 2 of preparation of RA-depleted cells) at 21% O_2. Then the cells were cultured in 6-well dishes with or without 10 nM RA for 48 h. Epo in the culture media and cellular protein were determined. Each value is the mean ± S.D. in triplicate cultures.

Epo production by RA is not due to the activation of total protein synthesis in the cells.

Hypoxia is a major inducer of Epo production and the 3′ enhancer region required for the hypoxic induction contains a putative RA-responsive element [11]. To test whether the signal pathway for the stimulation of Epo production by RA interacts with that for the induction by hypoxia, we compared the effect of RA on Epo production in HepG2 cells at different O_2 tensions (21% O_2 and 1% O_2). RA stimulated the Epo production under 1% O_2 as well as 21% O_2 (Fig. 2) and the extents of induction at different RA concentrations were similar under both O_2 tensions (Fig. 2, inset). It thus appears that RA stimulates Epo production through the pathway independent of the hypoxic induction.

The stimulation of Epo production was detectable at 0.1 nM RA ($P < 0.01$) and the maximum enhancement was 3-fold (Fig. 2). The concentration (ED_{50}) of RA required for half the maximal effect was 0.7 nM (Fig. 2, inset). Retinol and retinal also stimulated Epo production but they were less potent than RA; ED_{50} values for retinol and retinal were 17.8 and 6.3 nM, respectively. The effect of retinol and retinal probably results from conversion of these retinoids to RA. There was no stimulation of Epo production by α- or β-carotene.

3.2. Effect of RA on Epo mRNA contents in HepG2 cells

Most effects of RA are due to transcriptional control of the target genes [18]. We examined the effect of RA

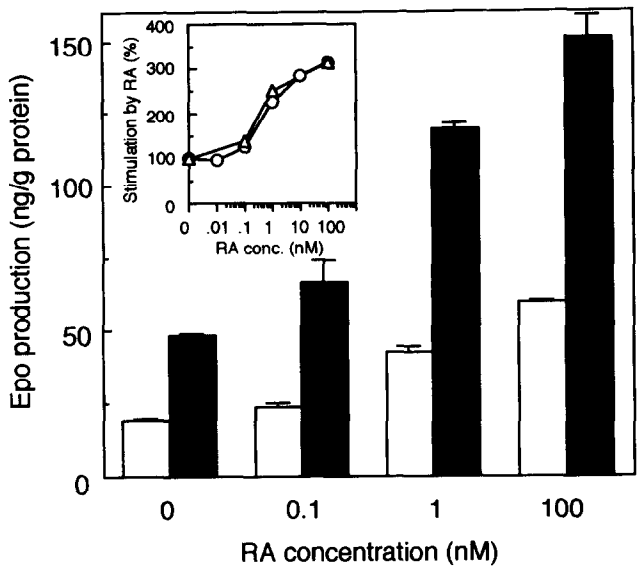

Fig. 2. Enhancement of Epo production by RA in HepG2 cells cultured under different O_2 tensions. After preculture of HepG2 cells to deplete RA as described in section 2, the cells were cultured in 6-well dishes in the RA-depleted FCS added at the indicated concentrations of RA at 21% O_2 (white bars) or 1% O_2 (hatched bars) for 24 h. Epo in the culture media and cellular protein were determined. Each value is the mean ± S.D. in triplicate cultures. In the inset, the amount of Epo produced in the cells cultured without RA under each O_2 tension is defined as 100% and each enhancement of Epo production by RA is represented in percent (○, 21% O_2; △, 1% O_2).

on the Epo mRNA contents by the competitive RT-PCR (Fig. 3A). Epo mRNA in the total RNA preparation was reverse-transcribed to Epo cDNA. Epo cDNA was amplified by PCR in the presence of various amounts of a competitor DNA, the genomic DNA fragment of Epo. The amplification yields the DNA fragments with 485 bp and 619 bp from Epo cDNA and Epo genomic DNA, respectively. The relative intensity of these two amplified products depends on the concentration ratios of Epo cDNA and Epo genomic DNA in the reaction mixtures for amplification, which allows the estimation of Epo cDNA. When total RNA was prepared from the cells cultured in the presence of 100 nM RA at 1% O_2, the specific band for Epo cDNA (485 bp) was detected and the content of Epo cDNA in the reaction mixture was estimated to be 5.0 ± 0.2 pg (n = 3; Fig. 3B). When the total RNA was prepared from the cells cultured without RA, the Epo cDNA content was almost the lower limit of detection (1 pg) (data not shown) . These results indicate that RA stimulates Epo production through accumulation of Epo mRNA. There was no significant difference in the total RNA synthesis in the cells cultured with or without RA (data not shown), indicating that RA accumulated Epo mRNA in a specific manner.

The DR-2 sequence (a RA-responsive element) is present in the 3′ flanking region of Epo gene [11–13,16,17]. Epo-producing cells express a protein(s) that binds to this sequence [11,12]. It is thus likely that the accumulation of Epo mRNA by RA is caused by the transcriptional activation of Epo gene due to the interaction of RA receptors to the DR-2 sequence, although the possibility that RA accumulates Epo mRNA through elongation of its lifetime has not been excluded. However, Blanchard et al. [11] have shown that the transient expression of the reporter gene linked to the 3′ enhancer region containing the DR-2 sequence does not respond to RA in Hep3B cells. The inconsistency with our results needs to be investigated further. It is noted that during preparation of this paper, Fandrey et al. [32] have shown thyroid hormone-induced stimulation of Epo production in HepG2 cells and isolated perfused rat kidneys.

3.3. *Effect of RA on serum Epo concentrations in vitamin A-depleted rats*

To know the in vivo effect of RA on Epo production, vitamin A-depleted rats were used, because the retinoid

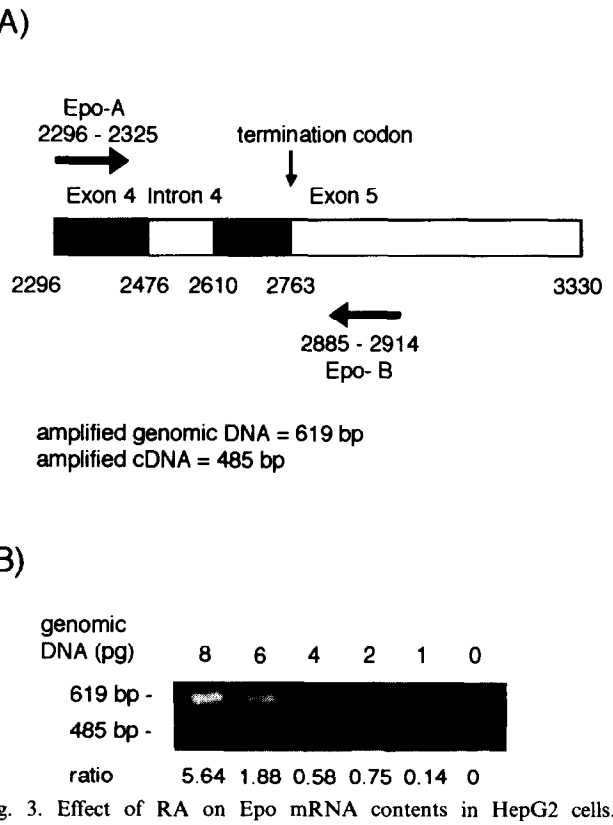

Fig. 3. Effect of RA on Epo mRNA contents in HepG2 cells. (A) Schematic drawing for a competitive RT-PCR. A horizontal column represents a part of the human Epo genomic DNA. Horizontal arrows show sense (Epo-A) and antisense (Epo-B) primers. Representing nucleotide numbers are based on the reference 30. Epo genomic DNA and Epo cDNA were amplified to yield 619 bp and 485 bp fragments, respectively. (B) After preculture of the cells to deplete RA as described in section 2, the cells were cultured in 100-mm dishes in RA-depleted FCS containing 100 nM RA for 24 h at 1% O_2. The indicated amount of Epo genomic DNA was added in each PCR reaction as a competitor. Ratios of the two bands (619 bp of Epo genomic DNA/485 bp of Epo cDNA) are presented in each lane. Each photograph shows representative data in triplicate cultures.

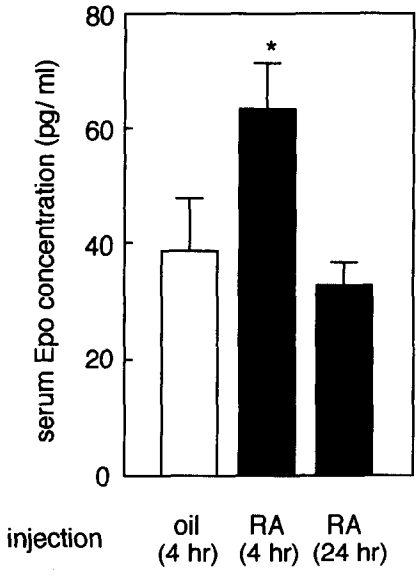

Fig. 4. RA elevates the serum Epo concentration in vitamin-A depleted rats. Vitamin A-depleted rats were prepared [31]. Male weaning rats of the Wistar strain (3-week-old) were depleted of endogenous retinoids by feeding them on a vitamin A-deficient diet, in which the other nutrients were sufficient to support normal growth based on the AIN-76 diet. After 30 days of depletion, these vitamin A-depleted rats were given intragastrically 100 μg RA in 0.2 ml soybean oil (dark-shaded columns) or 0.2 ml soybean oil alone (a white column), and were sacrificed after 4 h or 24 h. Epo in the sera was assayed. Four rats were used for the 24 h experiment and six rats were used for the 4 h experiments. Each value represents the mean ± S.D. The asterisk represents significant difference from the vitamin A-depleted rats given soybean oil alone ($P < 0.01$).

pool in the normal rats interferes with the examination of the effect of RA. After the breeding of rats with a vitamin A-deficient diet, RA was given intragastrically. The Epo concentration in rat serum increased ($P < 0.01$) 4 h after RA administration, and returned to the original level within 24 h when exogenous RA was almost completely catabolized (Fig. 4). These findings suggest that RA stimulates Epo production in Epo-producing cells in vivo. The RA-induced increase in serum Epo, however, was too small and our attempt to find whether RA elevated Epo mRNA in kidney or liver was unsuccessful. We could identify Epo mRNA in these tissues but the RA-induced changes in its level were within experimental error. Although hypoxia is a signal that can stimulate Epo production most intensively, RA may play an important role in the maintenance of an Epo level appropriate to the day-to-day production of red blood cells under normal, steady-state conditions.

Epo has been thought to exclusively act on erythroid cells in vivo but there is growing evidence that neurons respond to Epo [33,34]. A novel site for Epo production in brain has been found [35,36]; astrocytes produce Epo [36]. It is of interest whether RA stimulates brain Epo production, which is in progress using cloned astrocytes.

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