Transcriptional regulation of transferrin and albumin genes by retinoic acid in human hepatoma cell line Hep3B

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Transferrin and albumin, which are both secreted from the human hepatoma cell line Hep3B, were regulated transcriptionally by retinoic acid (RA) in a dose-dependent manner. The cell growth rate was little affected under the same conditions. The treatment of Hep3B cells with RA (10 μ M for 48 h) resulted in an 8-fold increase in transferrin protein synthesis, a 10-fold increase in the steady-state transferrin mRNA level, and a 5-fold increase in its transcriptional rate. The same treatment led to 4-fold decrease in albumin synthesis, as well as a 7-fold decline in the steady-state albumin mRNA level and a 4-fold decrease in the transcriptional rate. Cycloheximide and actinomycin D blocked the action of RA, suggesting that RA may regulate transferrin and albumin gene expression indirectly in human liver cells.

INTRODUCTION

Retinoic acid (RA) exhibits a variety of potent effects on cell growth and differentiation, such as the suppression of carcinogenesis *in vivo* [1,2], and the regulation of pattern formation in developing and regenerating limbs [3–5]. Specific cellular genes induced by RA have been identified and cloned from differentiating HL-60 promyelocytic leukaemia cells [6], cultured human epidermal keratinocytes [7], tracheal epithelial cells [8], mouse teratocarcinoma cells [9–13], and human embryonal carcinoma cells [14].

The molecular mechanisms of RA action on cell growth and differentiation have not yet been elucidated. However, four different RA receptors (RARs) have been cloned and shown to be members of the steroid and thyroid hormone nuclear receptor superfamily [15–19]. This provides a clue as to the molecular mechanism of RA action on gene expression. The liver has been shown as a major storage organ of retinoids, and it also expresses all four types of RAR [15–20]. However, how RA affects gene expression in the liver cell has not yet been studied. On the other hand, one of the RAR genes has been shown to be the integration site of hepatitis B virus in one human hepatoma tissue [21]. Aberrant transcripts of RAR (2.5 kb mRNA) were found in six out of seven hepatoma cell lines, while none were found in normal adult and fetal livers [22]. This suggests some correlation between RAR expression and the process of hepatocellular carcinogenesis.

In this study we have used cultured human hepatoma cell line Hep3B as a model to investigate the effects of RA on gene expression in the human liver cell. This cell line maintains most of the features that are found in matured human hepatocytes, including morphology and plasma protein secretion [23,24]. Our studies revealed that RA only slightly affected the growth rate of Hep3B cells, but stimulated the expression of the transferrin gene, while simultaneously repressing the expression of the albumin gene.

EXPERIMENTAL

Reagents

RA, actinomycin D and cycloheximide were purchased from

Sigma. The rabbit polyclonal antibodies against human albumin and transferrin were obtained from Bio-Yeda Ltd (Rehovot, Israel).

Cell culture

The human hepatoma cell line Hep3B was maintained in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories), supplemented with 5% fetal calf serum and antibiotics in a humidified atmosphere of 5% CO₂ at 37 °C. The medium was changed every 2 days.

Radioimmunoprecipitation and gel electrophoresis

The Hep3B cells were treated with different concentrations of freshly prepared RA (10⁻⁵–10⁻⁹ M) in DMEM containing 5% fetal calf serum for 30 h, then the medium was discarded and fresh methionine-free DMEM was added along with 50 μ Ci of [³⁵S]methionine/ml, and incubated for a further 18 h without RA. After labelling, the culture medium was collected. Radioactivity of the labelled secreted proteins was determined by trichloroacetic acid precipitation and liquid scintillation counting. Proteins were separated by SDS/12% PAGE. Radioimmunoprecipitation of transferrin and albumin were performed as follows. The labelled secreted proteins were immunoprecipitated with specific rabbit anti-(human plasma protein) antibodies. The immunoprecipitated proteins were adsorbed on to Protein A-Sepharose and washed with TNE buffer (Tris/HCl 50 mM, NaCl 500 mM, EDTA 1 mM, pH 7.5). The final pellet was resuspended in SDS sample buffer and heated at 100 °C for 3 min [25]. It was then analysed by SDS/12% PAGE and autoradiographs were quantified with a laser densitometer (Bio-med Instruments Inc.; model SL-2D/1D u.v.).

RNA isolation and Northern blot analysis

Total cellular RNA was isolated by lysing cells with guanidinium isothiocyanate. The RNA was pelleted through a CsCl cushion and precipitated with ethanol. The total RNA was electrophoresed on a 1.0% denaturing formaldehyde/agarose gel, and then transferred on to a nitrocellulose paper. The bound RNA on nitrocellulose paper was treated as follows for hybridization with the probe at 42 °C. The hybridization buffer was 50% (v/v)

Abbreviations used: RA, retinoic acid; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; RAR, retinoic acid receptor.

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formamide, $5\times$ Denhardt's solution, $5\times$ SSPE ($1\times$ SSPE = 0.15 M-NaCl, 10 mM- NaH_2PO_4 , 1 mM-EDTA, pH 7.4), 10% dextran sulphate, 0.1% SDS and 200 μg of denatured salmon sperm DNA/ml. The probes were specific albumin and transferrin cDNA inserts [26,27], labelled with dCTP by nick translation. After hybridization, the filters were washed in $0.1\times$ SSC/0.1% SDS at 55°C , and then autoradiographed at -70°C . The autoradiographs were quantified by a laser densitometer.

Nuclei isolation and run-on assays

Nuclei were isolated from the Hep3B cells according to the method described by Brown *et al.* [28]. Cells were homogenized in a Dounce homogenizer with buffer A [10 mM-Tris/HCl (pH 8.0), 5 mM-dithiothreitol (DTT), 0.3 M-sucrose and 0.1% Triton X-100]. Nuclei pellets were obtained by centrifugation at 160000 g at 1°C for 90 min. The pellets were then washed with buffer C (containing 50 mM-Tris/HCl, pH 8.0, 0.1 mM-EDTA, 5 mM- MgCl_2 and 0.5 mM-DTT) and then resuspended in the storage buffer (40% glycerol, 50 mM-Tris/HCl, pH 8.0, 5 mM- MgCl_2 and 0.1 mM-EDTA), and stored at -70°C before use. Transcription assays were performed as previously described by Matrisian *et al.* [29]. Nuclei (5×10^7) were incubated in the reaction buffer [containing 50 mM-Tris/HCl, pH 8.0, 5 mM- MgCl_2 , 100 mM-KCl, 3 mM- MnCl_2 , 0.1 mM-EDTA, 1 mM-DTT, 15% glycerol, 40 units of placental ribonuclease inhibitor, 1 mM each of ATP, GTP, CTP, and 200 μCi of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ (3000 Ci/mmol)] for 45 min at 26°C . The labelled RNA products were purified using DNAase, proteinase K and ethanol/ammonium acetate precipitation. The labelled RNA species were then hybridized for 3 days on to a nitrocellulose filter immobilized with various DNAs, including pBR322, actin, albumin, transferrin, RAR α , and RAR β cDNA fragments. After 3 days, filters were washed, air-dried and autoradiographed at -70°C .

RESULTS

Effect of RA on Hep3B cell growth

Hep3B cells were incubated in the presence of RA ($10\text{ }\mu\text{M}$) for up to 5 days, with the medium being changed daily. As seen in Fig. 1, the growth rate of Hep3B cells was only slightly inhibited (about 30% decrease) by the addition of RA. The treated Hep3B

Fig. 1. Effect of RA on the growth of Hep3B cells

Cells were plated on to a 6-well plate at a density of 2.5×10^5 cells/well in DMEM containing 5% fetal calf serum (FCS). After 24 h the medium was changed to DMEM with 5% FCS alone (●) or with 5% FCS plus RA ($10\text{ }\mu\text{M}$) (○). The medium was changed and cell numbers were counted daily using a haemocytometer. Each point represents the average of three experiments, with s.d. $< 10\%$. The experiment presented here was performed three times with similar results.

Fig. 2. Effect of RA on protein synthesis in Hep3B cells

(a) Effect on protein secretion. Cells were plated and treated with medium containing RA, as described in the legend of Fig. 1. RA concentrations used were: lane 1, 0; 2, 10^{-5} M; 3, 10^{-6} M; 4, 10^{-7} M; 5, 10^{-8} M; 6, 10^{-9} M. After 30 h, the medium was changed and cells were cultured in the fresh medium containing $[\text{S}^{35}]\text{methionine}$, without fetal calf serum, for another 18 h. The culture medium was collected, and the radioactivity was counted after trichloroacetic acid precipitation by liquid scintillation counting. Labelled protein (10^5 c.p.m.) was loaded and analysed by SDS/12%-PAGE and autoradiography, as described in the Experimental section. (b) Radio-immunoprecipitation of secreted proteins. Cells were treated without (lanes 1-3) or with RA ($10\text{ }\mu\text{M}$) (lanes 4-6) and labelled with $[\text{S}^{35}]\text{methionine}$, as described in (a). The same amount of $[\text{S}^{35}]\text{methionine}$ -labelled secreted protein (10^5 c.p.m.) was then immunoprecipitated using normal rabbit serum (lanes 1 and 4), or rabbit specific anti-(human serum albumin) (lanes 3 and 6) or anti-(human transferrin) (lanes 2 and 5) antibodies, as described in the Experimental section. The immunocomplex was absorbed using Protein A-Sepharose, and then washed with TNE buffer and analysed by SDS/PAGE and autoradiography.

cells showed no morphological changes in comparison with the control cells.

Effect of RA on plasma protein secretions in Hep3B cells

Hep3B cells were treated with RA ($10\text{ }\mu\text{M}$) for 30 h in DMEM with 5% fetal calf serum, and then labelled with $[\text{S}^{35}]\text{methionine}$ for another 18 h in serum-free DMEM. ^{35}S -labelled proteins present in the culture medium were subsequently analysed by SDS/PAGE followed by autoradiography (Fig. 2a). It appeared that RA treatment did not change the total amount of protein synthesis in the cells, but stimulated the production of a 78 kDa protein, while simultaneously repressing a 67 kDa protein. The 78 kDa and 67 kDa proteins were identified by using specific rabbit anti-(human transferrin) and -(human albumin) anti-

Fig. 3. Dose- and time-dependent effects of RA on transferrin and albumin transcription in Hep3B cells

(a) Cells were plated in DMEM with 5% fetal calf serum overnight. RA was then added in to the medium at the indicated concentrations (10^{-9} – 10^{-5} M), and the cells were continuously cultured for 48 h. Total cellular RNA was extracted from both untreated and RA-treated cells, separated on a 1.0% formaldehyde/agarose gel, blotted on to nitrocellulose filters and hybridized to transferrin- or albumin-specific cDNA probes as described in the Experimental section. The filters were then subsequently hybridized with an 18 S rRNA probe to ensure that equal amounts of RNA were present in the different lanes. (b) An identical experiment was performed as described above, except that the RNA was extracted at different time intervals after a single dose of RA ($10 \mu\text{M}$) was added, and analysed by Northern blot hybridization.

bodies. As seen in Fig. 2(b), the 78 kDa protein [which showed a 7.8 ± 1.6 fold (mean $\pm$ S.D., $n = 3$) increase on treatment with $10 \mu\text{M}$ -RA], and the 67 kDa protein (which showed a 4.3 ± 1.1 fold decrease on RA treatment) were identified as human transferrin and albumin respectively.

Effects of RA on transferrin and albumin mRNA levels

To investigate the mechanism of regulation of transferrin and albumin synthesis by RA, the level of transferrin and albumin mRNA in RA-treated Hep3B cells was examined. Northern blot analysis detected the mRNAs of transferrin and albumin as 2.1 kb and 2.3 kb mRNA species respectively. The sizes of these two RNAs are consistent with the reported sizes of transferrin and albumin mRNAs found in the human liver [30,31] (Fig. 3). It was also found that RA treatment increased transferrin mRNA in a dose-dependent manner (Fig. 3a), with a 10.3 ± 2.5 -fold increase occurring at 48 h after RA ($10 \mu\text{M}$) treatment. A 7.4 ± 2.0 -fold suppression of the steady-state albumin mRNA level by $10 \mu\text{M}$ -RA was also obtained (Fig. 3a). The induction of trans-

Fig. 4. Nuclear run-on analysis of transferrin and albumin gene transcription rates in Hep3B cells after RA treatment

Hep3B cells were cultured and treated (lane 2) or not (lane 1) with RA ($10 \mu\text{M}$) for 48 h as described. The nuclei were then isolated from control and RA-treated cells, and transcription was performed in the presence of [α - ^{32}P]UTP as described in the Experimental section. The labelled RNAs were hybridized on to nitrocellulose filters containing transferrin, albumin, actin (positive control), pBR322 (negative control), RARα and RARβ cDNA fragments, as described in the Experimental section.

Fig. 5. Effect of protein and RNA synthesis inhibitors on the regulation by RA of transferrin and albumin mRNA levels

Hep3B cells were cultured in DMEM with 5% fetal calf serum for 48 h, and preincubated with (lanes 2 and 4) or without (lanes 1 and 3) $25 \mu\text{g}$ of cycloheximide/ml (a) or $5 \mu\text{g}$ of actinomycin D/ml (b), for 30 min. RA ($10 \mu\text{M}$) (lanes 3 and 4) or a control solvent (lanes 1 and 2) was then added to the cells and incubation was continued for another 24 h. After RA treatment the RNA was extracted and analysed using Northern blot analysis, as described in the legend to Fig. 3.

ferrin mRNA was detected after 8 h of RA treatment, whereas the suppression of albumin mRNA was detected after 24 h of RA treatment (Fig. 3b).

Quantitative assay of transferrin and albumin mRNA transcription rates

Since changes in the mRNA level may be controlled at either the transcriptional or the post-transcriptional level, the rate of transcription of both the transferrin and albumin genes was examined by a nuclear run-on assay. Nuclei from the control and RA ($10\ \mu\text{M}$, for 48 h)-treated cells were isolated, and their transcription reaction was allowed to continue in the presence of [α - ^{32}P]UTP. The labelled RNA was hybridized on to nitrocellulose filters, which contained different DNA fragments, including pBR322, actin, transferrin, albumin, RAR α , and RAR β cDNA inserts. The results showed that RA could indeed up- and down-regulate the transcription rates of the transferrin and albumin genes respectively (Fig. 4). When the hybridization signals were quantified by densitometry, this revealed that RA caused a 4.9 ± 1.2 -fold increase in the transcription rate of the transferrin gene and a 4.1 ± 0.8 -fold decrease in albumin gene transcription, compared with the transcription rate of the actin gene. In addition, the transcription rate of the RAR α gene was stimulated whereas that of the RAR β gene was suppressed by RA. Therefore the changes in the transferrin and albumin mRNA levels were largely due to regulation by RA of the transcription rate.

Effect of protein and RNA synthesis inhibitors

To determine whether the regulation of transferrin and albumin mRNA by RA is dependent upon *de novo* protein synthesis, Hep3B cells were preincubated for 30 min with $25\ \mu\text{g}$ of cycloheximide/ml (which inhibited the protein synthesis up to 95%; results not shown) prior to RA addition. The effects of RA on transferrin mRNA and albumin mRNA were almost completely abolished on cycloheximide treatment (Fig. 5a). The effect of the RNA synthesis inhibitor actinomycin D on the regulation of transferrin and albumin mRNA by RA was also tested. The actinomycin D treatment completely blocked the RA-induced increase in transferrin mRNA and decrease in albumin mRNA (Fig. 5b).

DISCUSSION

Our results showed that RA can activate transferrin protein and mRNA synthesis, while repressing albumin protein and mRNA synthesis, in Hep3B cells in a time- and dose-dependent manner. Nuclear run-on assays demonstrated that the transferrin gene was transcriptionally up-regulated, while the albumin gene was transcriptionally down-regulated, by RA treatment. The fact that actinomycin D completely abolished the effect of RA on both the albumin and the transferrin genes further supports the view that the regulatory effect of RA is mainly at the transcriptional level. The RA regulation of the transcription rate, however, did not completely account for the change in the steady-state level of mRNA for either transferrin or albumin. This discrepancy may reflect inherent differences in the nature of the two assays. It may also be partly due to the post-transcriptional alterations which occurred in the albumin and transferrin mRNAs after synthesis.

The increase in transferrin mRNA was first observed at a RA concentration of $1\ \text{nM}$. This increase in transferrin mRNA steadily rose with increasing concentrations of RA (up to $10\ \mu\text{M}$). This correlates with the results of a dose-response study of the effect of RA on rat growth hormone [24], in which the production of growth hormone was stimulated by RA at concentrations less than $1\ \mu\text{M}$. In contrast, the decrease in albumin mRNA was observed at various RA concentrations.

Recent reports suggest that RA acts by first binding to a nuclear RAR [16], which then associates with hormone-

responsive elements in the target gene, resulting in the activation or inhibition of transcription [32]. Four types of RAR with different affinities for RA ($K_d\ 10^{-10}$ – $10^{-5}\ \text{M}$) have been identified in mice and humans [15,16,18–20]. Since different RA concentrations were required for regulating transferrin and albumin gene expression, this suggests that RA may act through different RA receptor isoforms to regulate the expression of these two genes. Since the mechanism of RA action is similar to that of steroid hormones [15,16], it is possible that RA could regulate the promoter activity of both albumin and transferrin genes through direct interaction of the RAR with its response element in both genes. However, the relatively slow action of RA on transferrin and albumin gene expression (8–24 h) favours an alternative hypothesis, in which the transcriptional regulation of the transferrin and albumin gene expression may be a secondary response to RA in Hep3B cells. This hypothesis is further supported by the observation that the effects of RA on both transferrin and albumin gene expression were completely abolished by a protein synthesis inhibitor. Similar observations were seen in some retinoid-responsive genes of F9 cell culture, such as laminin B1 or collagen IV genes [33,34]. These genes were induced after a relatively long time (18–24 h) after exposure to retinoids, were also sensitive to cycloheximide, and might be secondary to the induction of the primary response genes, such as ERA-1 [35]. Another example can be seen in the expression of the tissue plasminogen activator gene in F9 teratocarcinoma cells [36], in which the induction of the transcription factor SP1 was a prerequisite for the induction of plasminogen activator gene expression by RA.

Both albumin and transferrin gene expression has been shown to be regulated by various agents, such as cell density [37], dexamethasone [37], human growth hormone [38] and temperature [39]. Oestrogen can also regulate the expression of these two genes in a similar fashion in transgenic mice [40]. Several laboratories have shown that the RAR can activate gene transcription though both the palindromic thyroid hormone [20,31] and the vitamin D response element [18]. However, no such sequence similarity was found in the transferrin and albumin promoters [41,42].

The physiological significance of our finding is not clear at the present time. However, our observations demonstrate for the first time that RA may exert an important regulatory effect on albumin and transferrin gene expression in the human liver. Whether the simultaneous regulation of albumin and transferrin gene expression was mediated through some common or distinct RA-regulated transcriptional factors requires further investigation.

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