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Role of Retinoic Acid in Maturation of Fetal Liver Cells *in vitro*

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Regulation of the biosynthesis of α-fetoprotein and albumin was studied in a temperature-sensitive fetal rat hepatocyte line (RLA209-15) which exhibits a differentiated phenotype when grown at 40°C. Retinoic acid inhibited α-fetoprotein production but increased albumin production. This retinoid also changed the proportion of three forms of α-fetoprotein; the biosynthesis of the 73,000- and 69,000-dalton variants, which are indistinguishable from authentic rat α-fetoprotein, was inhibited and an additional 65,000-dalton variant was induced. It has previously been shown that α-fetoprotein production decreases during maturation whereas albumin production increases. Our data suggest that retinoic acid induces maturation of fetal liver cells *in vitro*. Further, the 65,000-dalton α-fetoprotein variant may be characteristic of liver maturation.

The alterations in the expression of α-fetoprotein (AFP) and albumin during development are an example of eukaryotic gene regulation (1, 2). AFP and albumin are two major plasma proteins synthesized by mammalian liver (3, 4). AFP is the major serum protein in the developing fetus; its plasma concentration decreases dramatically following birth (1). Serum albumin concentrations increase throughout fetal development and reach high levels in adult life (2, 4). The expression of AFP serves as a marker for differentiated fetal hepatocytes, whereas the expression of albumin serves as a marker for differentiated fetal and adult hepatocytes.

Study of the control mechanisms of gene expression during development requires fetal cells that both express the differentiated fetal functions and are able to develop further. Rat fetal liver RLA209-15 cells that retain differentiated hepatic functions have been established by transforming normal

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liver cells with a temperature-sensitive A (tsA) mutant of simian virus 40 (SV40) that is ts in the gene required for the maintenance of transformation (5). RLA209-15 cells exhibit a normal, differentiated phenotype at the nonpermissive temperature (40°C) and synthesize high levels of AFP and albumin that are characteristic of fetal hepatocytes. The AFP variants synthesized by RLA209-15 cells at 40°C are two polypeptides of 73,000 and 69,000 daltons that co-migrate with authentic rat AFP (6). The RLA209-15 fetal liver cell line provides a suitable in vitro model system to study the molecular basis of maturation.

Retinoids (vitamin A) elicit many biological responses from cells both in vivo and in vitro. They have been shown to inhibit cell transformation initiated by a variety of agents (7, 8, 9). In addition, retinoids induce differentiation of embryonal carcinoma (10, 11), melanoma (12), and choriocarcinoma (13) cells in vitro. In the present work, we found that retinoic acid may be one of the substances used to regulate maturation in vivo.

MATERIALS AND METHODS

Cells and culture conditions

The SV40 tsA mutant-transformed rat fetal liver cell line, RLA209-15 (5) was grown in α-modified minimal essential medium supplemented with streptomycin, penicillin, and 4% fetal bovine serum (αMEM-4). Cultures were initially grown at 33°C and were shifted to 40°C after 3 to 4 days growth at 33°C (day 0). Stock solution of all-trans retinoic acid (Eastman-Kodak, Co., Rochester, NY) and retinyl palmitate were prepared at 100 mM in dimethyl sulfoxide and were added at the time of the temperature shift (day 0). Stock solution of retinyl phosphate (100 mM) was in 300 mM ammonium acetate containing 4% bovine serum albumin. Cells were counted with a Celloscope 112TH (Particle Data, Inc., Elmhurst, IL).

Radioimmunoassays

AFP and albumin were determined in culture media by double-antibody radioimmunoassays as previously described (5). The sensitivities of the assays were 0.2 ng for AFP and 0.5 ng for albumin. Complete medium not exposed to cells contains no detectable AFP or albumin. The statistical methods of Rodbard (14) were used for quality control and dose interpolation.

Biosynthesis of AFP

Cultures were labeled for 3 h by incubation in methionine-free medium to which L-[^{35}S]methionine at 100 μCi/ml (1370 Ci/mmol, Amersham Corp., Arlington Heights, IL) had been added (6, 15). Cell lysates and medium samples prepared as previously described (15) were stored at -70°C until analyzed.

AFP-specific polypeptides in the cell lysates and medium samples were isolated by immunoprecipitation with rabbit antiserum against rat AFP as described previously (15). The immunoprecipitates were washed as described by Roberts and Roberts (16), solubilized in sodium dodecyl sulfate (SDS) sample buffer, heated for 5 min at 95°C, and used for electrophoresis in a 10% polyacrylamide slab gel containing SDS (17). Radioactivity was visualized by fluorography (18). Apparent molecular weights were determined using the following [^{14}C] methionine-labeled protein standards obtained from Amersham: myosin (200,000 daltons), phosphorylase B (92,500 daltons), bovine serum albumin (69,000 daltons), ovalbumin (46,000 daltons), and carbonic anhydrase (30,000 daltons).

RESULTS AND DISCUSSION

It has been demonstrated that the ts rat fetal liver cell line, RLA209-15, exhibits a normal, differentiated phenotype characterized by increased levels of both AFP and albumin when grown at the nonpermissive temperature (40°C) (5). In the current experiments, AFP production was inhibited by retinoic acid in RLA209-15 cells grown at 40°C (Fig. 1). This inhibition was slightly greater with 10 μM retinoic acid than with 1 μM. Albumin production, however, was increased in these cells by retinoic acid (Fig. 1). Significantly more albumin was produced at 10 μM retinoic acid than at 1 μM. The observed effects were not artifacts of cell growth in retinoic acid; the growth rates and saturation densities for RLA209-15 cells grown in control

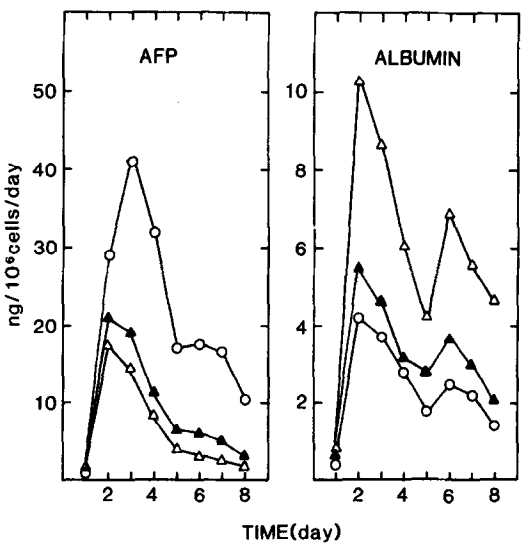

Figure 1. Effects of retinoic acid on AFP and albumin production by RLA209-15 cells. Cultures were initially plated at 33°C and were shifted to 40°C after 4-day growth at 33°C. Retinoic acid was added at the time of temperature shift (day 0). The corresponding medium was changed and collected every day. AFP and albumin in the culture media were determined by radioimmunoassays. O, control; Δ, 1 μM retinoic acid; Δ, 10 μM retinoic acid.

medium were the same as the cells grown in 1 or 10 μM retinoic acid. Cells grown in medium containing retinoic acid underwent no apparent morphological alteration under the light microscope.

We have previously demonstrated (6) that RLA209-15 cells grown at 40°C synthesize high levels of two extracellular AFP variants of 73,000 and 69,000 daltons, which co-migrate with the two fully processed AFPs from rat amniotic fluid. The two intracellular AFP-specific polypeptides at 40°C are the 70,000- (major) and 59,000-dalton (minor) polypeptides. The 70,000-dalton polypeptide is the precursor of the 73,000- and 69,000-dalton extracellular AFPs. The extracellular effect of retinoic acid was to increase the biosynthesis of an anti-AFP-precipitable polypeptide of 65,000 daltons, but inhibit the biosynthesis of anti-AFP-precipitable polypeptides of 73,000 and 69,000 daltons (Fig. 2). The principal intracellular retinoic acid effect was to decrease the biosynthesis of the anti-AFP-precipitable polypeptide of 70,000 daltons (Fig. 2). In agreement with the radioimmunoassay results, these effects were dependent on concentration. Retinoic acid had greater effects on AFP biosynthesis at 10 μM than at 1 μM (Fig. 2). The retinoic acid effects were reversible. Removal of retinoic acid increased the biosynthesis of the 73,000- and 69,000-dalton polypeptides but decreased the biosynthesis of the 65,000-dalton polypeptide (data not shown).

We have also tested other retinoids for their effects on AFP biosynthesis at 40°C (Fig. 3). Retinyl palmitate, a naturally occurring long-chain fatty acid ester of retinol (19), did not affect AFP biosynthesis. This result is consistent with those of Strickland and Mahadavi (10), who found that retinyl palmitate cannot substitute for retinoic acid in the induction of differentiation of teratocarcinoma stem cells in vitro. In contrast, retinyl phosphate, the phosphorylated form of retinol that acts as a carrier of specific mannosyl residues (20, 21), had the same effects as retinoic acid.

AFP often exhibits molecular heterogeneity due to structural variation in the carbohydrate chains (22, 23). We have previously demonstrated that the nonglycosylated form of the 73,000- and 69,000-dalton AFP variants is a

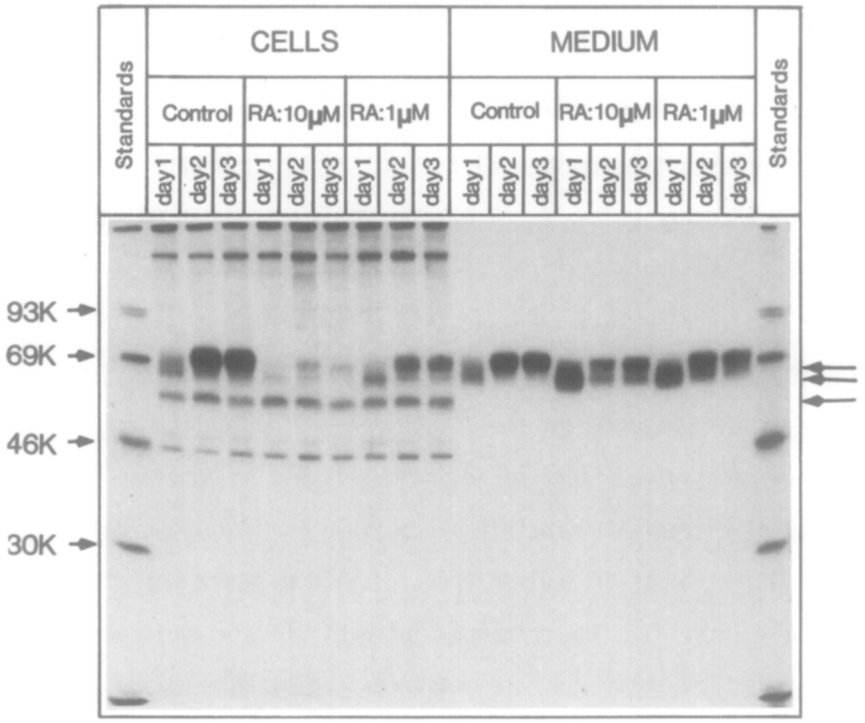

Figure 2. Biosynthesis of AFP in RLA209-15 cells in the presence or absence of retinoic acid. Cultures were initially plated at 33°C and were shifted to 40°C after 4-day growth at 33°C. Retinoic acid (RA) was added at the time of temperature shift (day 0) and the corresponding medium was changed every day. Each day, cells to be collected were labeled with L-[^{35}S] methionine for 3 h. Cell lysates and medium samples were immunoprecipitated with rabbit anti-AFP serum. Anti-AFP-precipitable polypeptides were analyzed by SDS-gel electrophoresis and fluorography. Arrows indicate AFP-specific polypeptides. Polypeptides from 4 x 10^6 cells were applied to each gel.

polypeptide of 66,000 daltons and the nonglycosylated form of the 65,000-dalton variant is a polypeptide of 48,000 daltons (6). In the present experiments, we examined the effects of retinoic acid in the presence of tunicamycin, an inhibitor of protein glycosylation (24). As shown previously, the major nonglycosylated form of AFP in control cultures grown at 40°C in tunicamycin was a polypeptide of 66,000 daltons (Fig. 4). In the presence of tunicamycin, retinoic acid inhibited the biosynthesis of the 66,000-dalton AFP-specific polypeptide but induced the biosynthesis of the 48,000-dalton polypeptide. Therefore, retinoic acid modified the biosynthesis of the polypeptide chain of AFP.

Our results suggest that retinoic acid induced maturation of these fetal liver cells in culture. AFP and albumin are both markers for differentiated

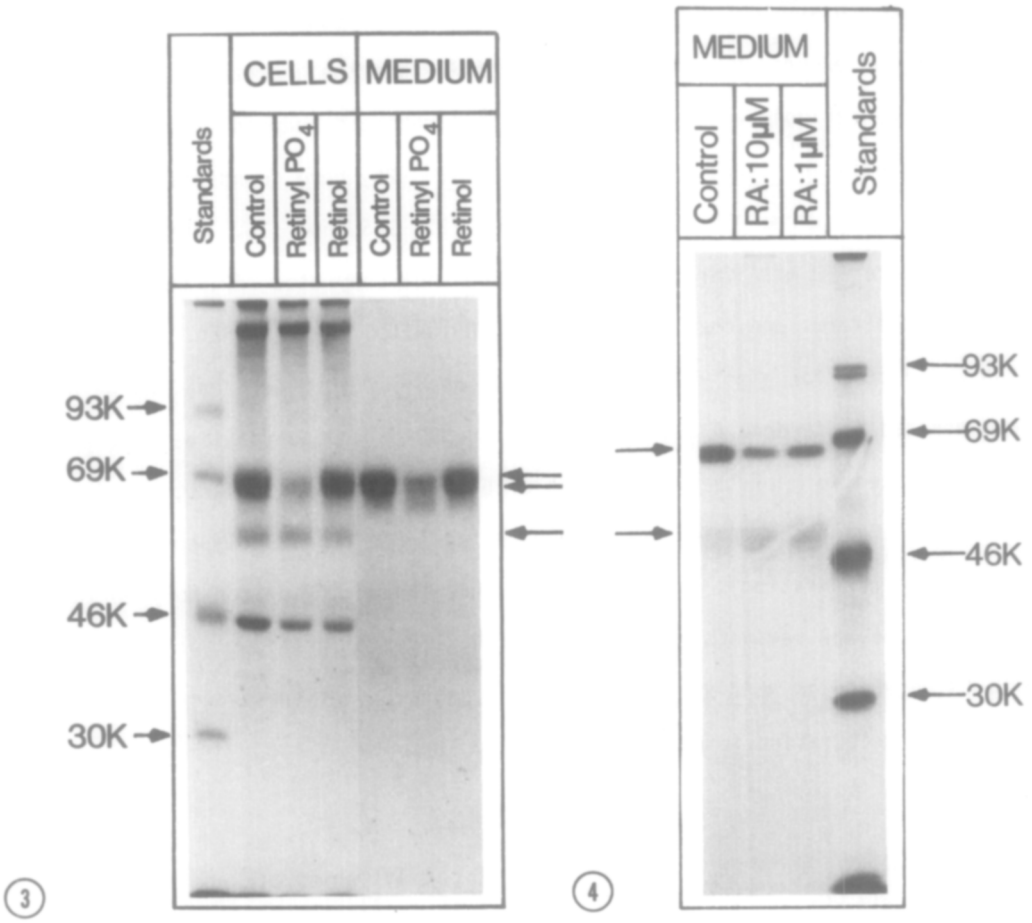

Figure 3. Effects of retinyl phosphate and retinyl palmitate on AFP biosynthesis in RLA209-15 cells. Cultures were initially plated at 33°C and were shifted to 40°C after 4-day growth at 33°C. The effectors (each at 1 μM) were added at the time of temperature shift (day 0) and the corresponding medium was changed every day. After an additional 3-day incubation at 40°C, cells were labeled with L-[^{35}S] methionine for 3 h. Cell lysates and medium samples were immunoprecipitated with rabbit anti-AFP serum. Anti-AFP-precipitable polypeptides were analyzed by SDS-gel electrophoresis and fluorography. Arrows indicate AFP-specific polypeptides. Polypeptides from 4 x 10^6 cells were applied to each gel.

Figure 4. Effects of retinoic acid on AFP biosynthesis in the presence of tunicamycin. Cultures were initially plated at 33°C and were shifted to 40°C after 4-day growth at 33°C. Retinoic acid (RA) was added at the time of temperature shift (day 0). After an additional 3-day incubation at 40°C, cells were labeled with L-[^{35}S] methionine for 3 h in the presence of tunicamycin (1 μg/ml). Before labeling, cultures were preincubated with tunicamycin for 4 h. Medium samples were immunoprecipitated with rabbit anti-AFP serum. Anti-AFP-precipitable polypeptides were analyzed by SDS-gel electrophoresis and fluorography. Arrows indicate AFP-specific polypeptides. Polypeptides from 4 x 10^6 cells were applied to each gel.

fetal hepatocytes and consequently, the synthesis of both proteins is inhibited following transformation and is increased when the fetal cells are induced to differentiate (5). However, a selective decrease in AFP mRNA

occurs at birth whereas albumin mRNA increases continuously until adult life (25). In our experiments the inhibition of AFP and the increase of albumin synthesis by retinoic acid suggests that this retinoid induced maturation of RLA209-15 cells in vitro. Recently, Gal et al. (26) have found that in adult liver there are three AFP mRNA molecules of 20S, 14S, and 11S. The 20S mRNA is the mature AFP mRNA for the 66,000-dalton unglycosylated AFP, and its glycosylated forms are the 73,000- and 69,000-dalton AFP variants. The 14S mRNA would code for a polypeptide of approximately 48,000 daltons. As we have demonstrated (reference 6 and Fig. 4) the 48,000-dalton polypeptide is the unglycosylated form of the AFP variant of 65,000 daltons. In fact, a 14S RNA was found to be the mRNA for the 65,000-dalton AFP-specific polypeptide (Chou and Ito, in preparation). It is possible that the 65,000-dalton polypeptide is one of the AFP variants synthesized during liver maturation. The retinoid effects observed in RLA209-15 cells are a consequence of the induction of fetal liver maturation in culture.

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