Inhibition of Synthesis of α-Fetoprotein by Glucocorticoids in Cultured Hepatoma Cells

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ABSTRACT α-Fetoprotein (AFP) synthesis was studied in the presence and absence of glucocorticoids in rat hepatoma Mc-A-RH-7777 cells. Radioimmunoassay of media from cell cultures grown in the presence of glucocorticoid (dexamethasone or cortisol) showed a reduction in AFP, an increase in albumin, and no significant change in transferrin accumulation, as compared to controls. Labeling experiments with L-[^{35}S]methionine indicated that in both cells and media of dexamethasone-treated cultures there was a 50–80% reduction in polypeptide precipitated by anti-AFP serum, as compared with controls; no change was seen in polypeptide precipitated by anti-transferrin serum. Pulse and pulse-chase experiments demonstrated that dexamethasone inhibited the synthesis of AFP but not its secretion. The half-time for secretion of AFP in the presence and absence of dexamethasone was 43 min.

α-Fetoprotein (AFP) is a major plasma glycoprotein synthesized by mammalian embryonic liver and yolk sac (1, 8–10, 18). The concentrations of AFP are higher in fetal or neonatal sera and in amniotic fluid but fall as the liver develops and matures. Nonpregnant adult mammals have extremely low circulating AFP levels (12, 16). Elevated serum concentrations of AFP have been associated with developmental, regenerative, and carcinogenic processes (1, 19). As an oncodevelopmental protein, AFP can serve as a marker for the detection of tumors, prenatal neural tube defects, or other fetal abnormalities (1, 4, 19, 21).

During pregnancy, the levels of glucocorticoids increase with gestation and increase rapidly during spontaneous labor (7, 14). Glucocorticoids induce the development and maturation of enzyme systems, promote differentiation, and play a role in the initiation of labor at term (13). Administration of glucocorticoids to newborn mice has been shown to cause an early fall in serum AFP levels (6). The inverse relationship between levels of glucocorticoids and AFP and the role of glucocorticoids in hormone-mediated differentiation made these hormones good candidates for direct regulators of AFP synthesis. The effects of glucocorticoids on AFP synthesis in rat hepatoma cells may serve as a model of gene regulation during development and neoplasia.

MATERIALS AND METHODS

Cells and Culture Conditions

Mc-A-RH-7777 rat hepatoma cells (2) were obtained from Dr. V. R. Potter and were grown in α-modified minimal essential medium supplemented with 4% fetal bovine serum (αMEM-4). Cells were maintained at 37°C in 95% air–5% CO_2. We used in this study hepatoma cells in logarithmic phase of growth. Glucocorticoids were added 2 d after subculturing (day 0) and the medium was changed every day.

Radioimmunoassays

AFP, albumin, and transferrin were determined in culture media by double-antibody radioimmunoassays as previously described (5). Purified preparations of rat AFP, rat albumin, and rat transferrin were used as standards and also radioiodinated for use as tracers in the respective assays. Rat AFP was kindly provided by Dr. J. F. Chiu and antisera against AFP were raised (in rabbits) to this highly purified rat AFP. Rat albumin, rat transferrin, rabbit antiserum against rat albumin, and rabbit antiserum against rat transferrin were obtained from N. L. Cappel Laboratories, Inc. (Cochran, PA). Sheep antiserum against rabbit γ-globulin was used as the precipitating antibody. The sensitivities of the assays were 0.2–10 ng for AFP, 0.5–25 ng for albumin, and 0.1–5 ng for transferrin. Less than 0.01% cross-reactivity exists between AFP, albumin, and transferrin in the respective radioimmunoassays. Complete medium not exposed to cells had no detectable AFP, albumin, or transferrin.

Labeling and Extraction of Cells

Cells were grown in 25-cm^2 flasks in αMEM-4. Before labeling, the cultures were rinsed with 5 ml of αMEM-4 lacking methionine and incubated with 5 ml of this medium for 1 h. Then they were incubated for various times with L-[^{35}S]methionine at 100 μCi/ml (1,370 Ci/mmol, Amersham Corp., Arlington Heights, IL) in methionine-free αMEM-4. Labeling was terminated by the addition of L-methionine (0.1 ml, 5 mg/ml) in αMEM-4. For pulse-chase experiments, the cells were washed with αMEM-4 containing L-methionine and incubated for various times in αMEM-4. After incubation, the medium was removed, and the cells were washed with phosphate-buffered saline (PBS) and lysed by the addition of 1 ml of PBS containing 1% Triton X-100, 0.5% sodium

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deoxycholate, 0.1% SDS, and 100 μg of trypsin inhibitor. The lysates were sonicated for 2 min at maximal power with a magnetostrictive oscillator (model DF-101; 250 W, 10-KC; Raytheon Co., Manchester, NH), then treated with 5 μl of 3% protamine sulfate. After 10 min on ice, the precipitates were removed by centrifugation for 1 h at 18,000 rpm in a Sorvall rotor RC2B (DuPont Instruments–Sorvall Biomedical Div., DuPont Co., Newtown, CT). Samples of medium was brought to 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, by the addition of concentrated solutions. Cell lysates and medium samples were stored at −70°C until analysis.

Immunoprecipitation and PAGE

AFP-specific and transferrin-specific polypeptides in the cell lysates and medium samples were detected by the direct immunoprecipitation assay with rabbit antiserum against rat AFP or rat transferrin. Samples of cell lysates and media were cleared of nonspecific radioactive components by precipitation with human serum and rabbit anti-human serum. The resulting precipitated proteins were removed by centrifugation. Then 5 μg of rat AFP or of rat transferrin were added to each sample as carrier. Excess antibody to rat AFP or rat transferrin was added and the mixtures were incubated for 30 min at room temperature and overnight at 4°C. Precipitation was ~90% complete for both proteins. The immunoprecipitates were sedimented in a Beckman microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) for 20 min and washed as described by Roberts and Roberts (15). The washed pellets were solubilized in SDS sample buffer. The amount of radioactivity in each of the immunoprecipitates was determined by counting portions of the solubilized samples in a liquid scintillation counter. Other portions of the immunoprecipitates were heated for 2 min at 100°C and used for electrophoresis in a 10% polyacrylamide slab gel containing SDS (11). Radioactivity was visualized by fluorography by the method of Bonner and Laskey (3). Apparent molecular weights were determined using the following [^{14}C]methionine-labeled protein standards obtained from Amersham: myosin (200,000), phosphorylase B (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300).

RESULTS

Effects of Glucocorticoids on the Production of AFP, Albumin, and Transferrin

Both cortisol and dexamethasone inhibited the accumulation of AFP in the culture media from rat hepatoma Mc-A-RH-7777 cells grown in either serum-containing or serum-free medium; the results with cells grown in serum-containing medium were shown in Fig. 1. The peak level of AFP in control as well as in cortisol- and dexamethasone-treated cultures was reached in 3 d. The production of AFP was coupled to cellular proliferation because these hepatoma cells were growing logarithmically from day 1 to day 3 (data not shown). When cell proliferation subsided after day 3, AFP production declined. This was expected, because it has been demonstrated that AFP production in primary fetal hepatocytes was proportional to the number of replicating cells (20). Both glucocorticoids increased the level of albumin (Fig. 1). Dexamethasone had a longer-lasting effect on albumin levels: after the peak, albumin levels dropped precipitously in the cortisol-treated cultures but not in the dexamethasone-treated cultures. Neither glucocorticoid affected the levels of transferrin significantly (Fig. 1). Although we used cortisol and dexamethasone at 10^{-5} M and 10^{-6} M, respectively, in this study, the two glucocorticoids, at 10^{-6} M and 10^{-7} M respectively, were almost as effective. Furthermore, progesterone did not block the ability of dexamethasone to inhibit AFP synthesis (data not shown).

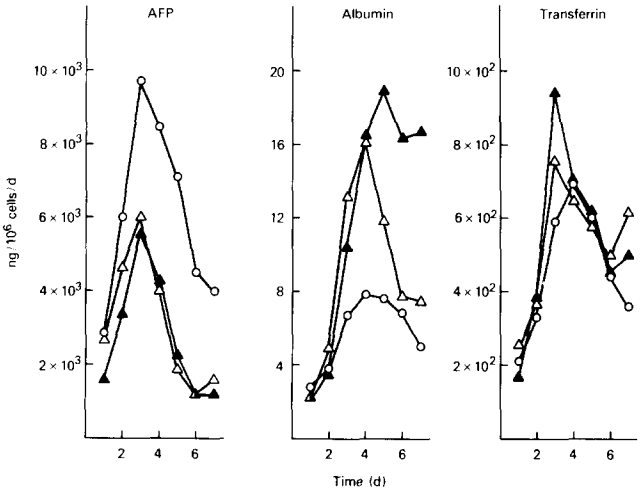

FIGURE 1 Effects of glucocorticoids on the production of AFP, albumin, and transferrin by hepatoma cells. Cells in 25-cm^2 flasks were grown in the absence and presence of either cortisol (10^{-5} M) or dexamethasone (10^{-6} M). Medium was changed and collected every 24 h. At the indicated times, media were assayed separately for AFP, albumin, and transferrin. (○) Control. (△) Cortisol. (▲) Dexamethasone.

Effects of Glucocorticoids on the Incorporation of L-[^{35}S]Methionine into AFP

A labeling experiment was performed to determine whether the observed inhibition of AFP accumulation in culture media by glucocorticoids resulted from inhibiting AFP biosynthesis. Each day, cells grown in the absence and presence of dexamethasone or cortisol were labeled for 30 min and 180 min with L-[^{35}S]methionine, and then cell lysates and medium samples were examined for anti-AFP-precipitable polypeptides. The 30-min labeling time was chosen because pulsing

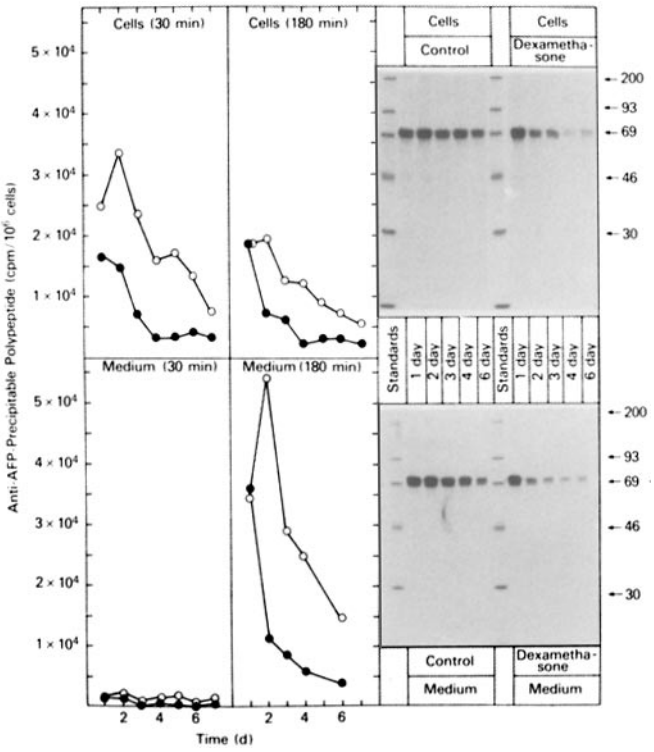

FIGURE 2 Effects of dexamethasone on the incorporation of L-[^{35}S]methionine into anti-AFP-precipitable polypeptides. Cells were grown in the absence and presence of 10^{-6} M dexamethasone, and medium was changed every day. Each day, cells were labeled with L-[^{35}S]methionine for 30 min and 180 min. Cell lysates and medium samples were immunoprecipitated with rabbit anti-AFP serum. Left, anti-AFP-precipitable radioactivity. (○) Control. (●) Dexamethasone. Right, autoradiograph of SDS gel electrophoresis of anti-AFP-precipitable polypeptides.

hepatoma cells with L-[^{35}S]methionine for 30 min measured mainly intracellular AFP biosynthesis (Fig. 2). Secretion of AFP occurred only after a lag of 30 min. Pulse labeling of these cells with L-[^{35}S]methionine for 180 min, however, measured both intracellular synthesis and extracellular accumulation of AFP (Fig. 2). Parallel experiments were carried out with cortisol and dexamethasone; the results with cortisol (data not shown) were similar to those with dexamethasone.

Pulse labeling of hepatoma cells with L-[^{35}S]methionine in the presence of dexamethasone showed inhibition of intracellular AFP synthesis after 30-min and 180-min labeling (Fig. 2). There was no appreciable secretion of anti-AFP-precipitable polypeptides into medium in either control or dexamethasone-treated cultures after 30-min labeling (Fig. 2). However, after labeling for 180 min, significant amounts of radioactive anti-AFP-precipitable polypeptides had accumulated in the medium; dexamethasone inhibited also the extracellular accumulation of newly synthesized AFP. Gel electrophoresis showed inhibition in all anti-AFP-precipitable bands. Dexamethasone did not affect the processing of AFP in hepatoma cells. The molecular weights of the fully processed AFP chains in medium of both control and dexamethasone-treated cultures were 69,000 and 73,000 daltons (Fig. 2).

The glucocorticoid effect was specific for AFP. The incorporation of L-[^{35}S]methionine into total trichloroacetic acid-precipitable radioactivity was not affected by dexamethasone or cortisol (data not shown). Furthermore, the synthesis and accumulation of L-[^{35}S]methionine-labeled anti-transferrin-precipitable polypeptides were not altered by dexamethasone (data not shown).

Kinetics of AFP Biosynthesis in the Absence and Presence of Glucocorticoid

Pulse and pulse-chase experiments were conducted to ascertain whether dexamethasone inhibited AFP synthesis or only its secretion. Dexamethasone decreased the rate of intracellular AFP biosynthesis and the rate of AFP accumulation in medium (Fig. 3). Dexamethasone did not affect AFP secretin by hepatoma cells. The kinetics of disappearance of intracellular anti-AFP-precipitable polypeptides and the kinetics of their appearance in medium were similar in dexamethasone-treated cultures and control cultures (Fig. 4). The half-time of secretion of AFP in cultures grown in the presence or absence of dexamethasone was 43 min (Fig. 5).

DISCUSSION

Glucocorticoids are seen to inhibit AFP biosynthesis in the rat hepatoma Mc-A-RH-7777 cells in vitro. Radioimmunoassays of media of cultures grown in the presence and absence of glucocorticoids indicate that the effect of glucocorticoids is a specific inhibition, as no such effect is seen in the levels of transferrin, and albumin levels are stimulated. This inverse relationship between albumin synthesis and AFP synthesis has

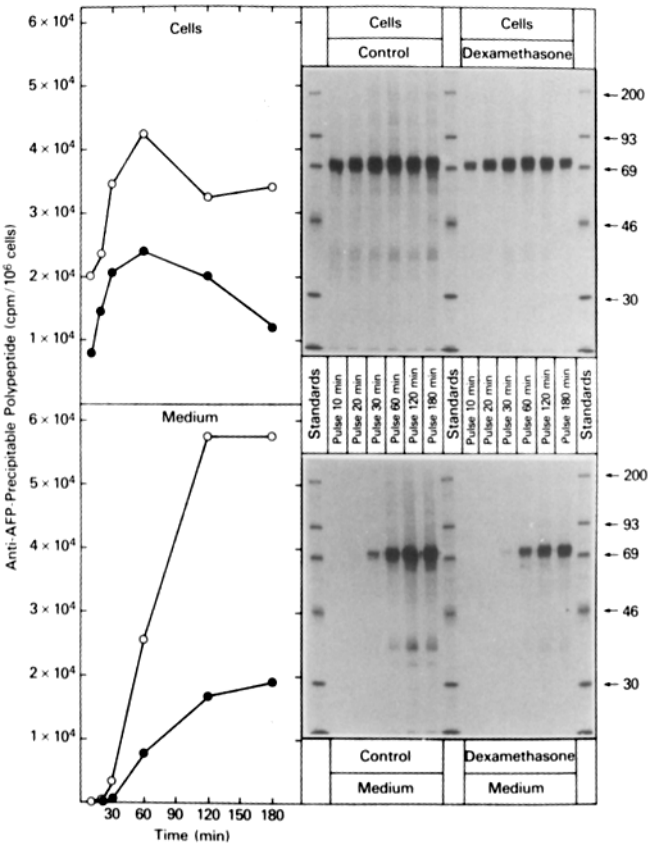

FIGURE 3 Effects of dexamethasone on pulse labeling of AFP. Cells were labeled with L-[^{35}S]methionine for various periods in the absence and presence of 10^{-6} M dexamethasone. Cell lysates and medium samples were immunoprecipitated with rabbit anti-AFP serum. Left, anti-AFP precipitable radioactivity. (○) Control; (●) Dexamethasone. Right, autoradiograph of SDS gel electrophoresis of anti-AFP-precipitable polypeptides.

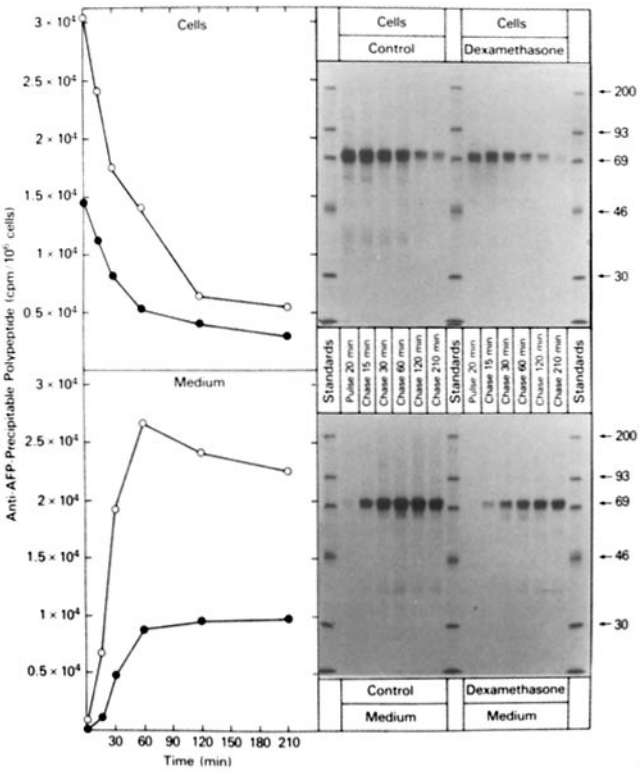

FIGURE 4 Pulse-chase labeling of AFP in the absence and presence of dexamethasone. Cells were labeled for 20 min with L-[^{35}S]methionine in the absence and presence of 10^{-6} M dexamethasone, then chased for 15, 30, 60, 120, and 210 min in fresh medium in the absence and presence of dexamethasone. Cell lysates and medium samples were immunoprecipitated with rabbit anti-AFP serum. Left, anti-AFP-precipitable radioactivity. (○), Control. (●) Dexamethasone. Right, autoradiograph of SDS gel electrophoresis of anti-AFP-precipitable polypeptides.

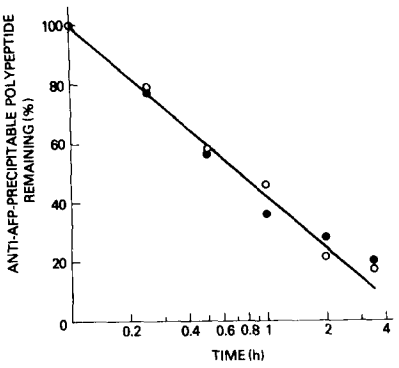

FIGURE 5 Disappearance of anti-AFP-precipitable radioactivity from cells in the absence and presence of dexamethasone. The experimental conditions were the same as in Fig. 4. (○) Control; (●) Dexamethasone.

been documented in vitro and in vivo as concomitant with maturation and cellular differentiation (1, 17).

Labeling experiments demonstrate the inhibitory effect of glucocorticoids on AFP levels on both intracellular biosynthesis and extracellular accumulation. The inhibitory effect of glucocorticoids is seen in all anti-AFP-precipitable bands. Pulse-chase experiments demonstrate that glucocorticoid does not affect the kinetics of AFP secretion. The half-time of secretion of AFP in both control and dexamethasone-treated cultures was 43 min.

The inhibition of AFP accumulation in media in response to glucocorticoids in this cell line has been reported previously (2). We have shown this effect to be initiated at the level of synthesis of AFP. Inhibition of AFP synthesis by glucocorticoids has been demonstrated in vivo in newborn mice and has been shown to take place at the transcriptional level (6). Our data support the view that the disappearance of mRNA coding for AFP seen in vivo is due to the direct regulatory effects of the glucocorticoid on the hepatocytes and is not a secondary result of the systemic effects of the hormone.

The effect of glucocorticoids on AFP in this system may serve as a model for inhibitory regulation. Further investigation of this system may elucidate the processes involved in hormone effects on maturation and cellular differentiation in both normal and neoplastic development.

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