EFFECT OF GLUCOCORTICOIDS ON FETOPROTEIN PRODUCTION BY AN ESTABLISHED CELL LINE FROM MORRIS HEPATOMA 8994

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SUMMARY: Glucocorticoids at concentrations equal to or higher than 10^{-7}M lead to an increase of alpha-fetoprotein production by an established cell line from Morris hepatoma 8994. These cells also secreted $alpha_M$-fetoprotein into the culture medium but only after addition of at least $4x10^{-7}$M hydrocortisone or $5x10^{-8}$M dexamethasone. The effects on both fetoproteins were observed in spite of a decrease of cell multiplication and an increase of cell detachment.

Several tissue culture systems have been established synthesizing stable amounts of alpha-fetoprotein in vitro. For rat alpha-fetoprotein (AFP), these are fetal hepatocytes (1) or cells from tumors, for example Yoshida sarcoma (2) or hepatoma AH66 (3) and AH70B(4). We recently isolated and cultured a cell line from Morris hepatoma 8994, known to give rise to the highest serum AFP concentration in vivo among the 39 transplantable hepatomas tested (5). The properties and characteristics of the cell line will be published elsewhere (J.E. Becker *et al*, in preparation). In the present paper we report that in spite of a marked alteration in the course of cell growth, the glucocorticoids exert a positive control on the *in vitro* production by the 8994 cells of two serum fetoproteins described by Stanislawski-Birencwajg (6).

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MATERIALS AND METHODS

The cell line is cultured in plastic tissue culture flasks (Corning 25 cm^2) at 37°, without agitation, in 4ml medium, in an atmosphere of 5% CO_2 and 95% air. The medium consists of Swim's S-77 medium (7) with 4 mM glutamine and supplemented with 5% fetal calf serum and 20% horse serum (Gibco, Grand Island, NY).

Sterile solutions of dexamethasone ("Decadron Phosphate" Merck Sharpe and Dohme, West Point, Pa) and hydrocortisone ("Solucortef", Upjohn Company, Kalamazoo, Mi) were made in S-77 medium and added during the exponential phase of the growth curve. All the experiments with hormones were done under conditions in which there was a single change of medium at the zero-time and a total time course of 48 hours.

The immunological reagents were prepared in Dr. Jose Uriel's laboratory as described (6), with the modifications mentioned in (8). The alpha-fetoprotein concentrations in the culture media were estimated by electro-immunodiffusion (9), using a monospecific rabbit antiserum and a standard of alpha-fetoprotein whose concentration was determined with the pure preparation isolated by Aussel et al (10). The presence of $alpha_M$-fetoprotein (for terminology, see ref. 11) was demonstrated by a sensitized double immunodiffusion technique (12). No attempt was made to quantify the amount of this fetoprotein because of the lack of a standard.

The cell number was counted with a hemocytometer. The floating cells were collected by centrifugation of the medium and the attached cells harvested after trypsinization.

RESULTS AND DISCUSSION

Figure 1 shows the AFP accumulation in the culture medium during 48 hours of the exponential phase of the cell growth. If the medium is not changed during this period, AFP accumulates more slowly after the first day. With a change every 24 hours, the rate of AFP accumulation remains very rapid, perhaps higher on the second day than the first.

Figure 2 shows the AFP accumulation with various doses of glucocorticoids added at zero time. Figures 2A and 2B correspond to the same experiment, Fig. 2C to another. In the presence of doses higher than $1x10^{-7}M$ for hydrocortisone or $5x10^{-8}M$ for dexamethasone, the concentration of AFP secreted into the medium rises above the control. Nevertheless, in our cell line growth is inhibited by the glucocorticoids (Table 1). The detachment of some cells can partially but not completely

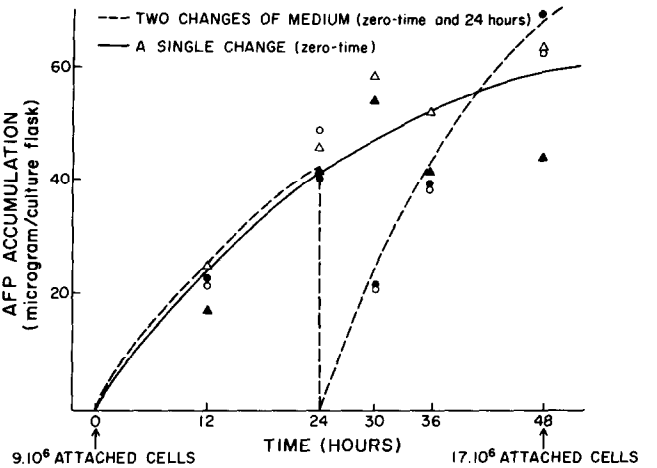

FIGURE 1. ALPHA-FETOPROTEIN ACCUMULATION IN 8994 CELL LINE CULTURE MEDIUM DURING 48 HOURS. The medium was changed either at zero-time (triangle) or at zero-time and at 24 hours (circle). Each flask contained 4 ml of medium. Samples of media of two cultures were taken at every time point for each series.

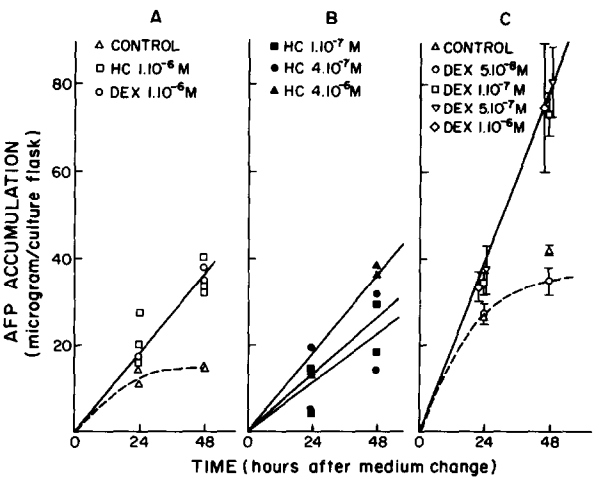

FIGURE 2. ALPHA-FETOPROTEIN ACCUMULATION IN 8994 CELL LINE CULTURE MEDIUM AFTER ADDITION OF GLUCOCORTICOIDS. The glucocorticoids at different concentrations were added with the single change of medium at zero-time. Each flask contained 4 ml of medium. Each point is the mean of two or three samples (if three, the S.E.M. is shown).

account for this. An inhibition at the level of DNA synthesis might be involved as in the case of hepatoma 5123 *in vitro* (13).

The response of the 8994 cells to various doses of hormones is expressed in micrograms of AFP accumulated in 48 hours per 10^6 cells

TABLE I

EFFECT OF GLUCOCORTICOIDS ON CELL GROWTH OF 8994 CELL LINE

		CELLS ($\times 10^6$) ATTACHED	FLOATING	TOTAL	PERCENT DETACHED CELLS	CELL MULTIPLICATION FACTOR
CONTROL	Zero time	1.24		1.24		(1.00)
	48 hours	3.22	0.03	3.26	0.9	2.63
HC(M)	1×10^{-7}	2.92	0.04	2.96	1.3	2.39
	4×10^{-7}	1.91	0.285	2.195	13.0	1.77
	1×10^{-6}	1.15±0.03	0.87±0.10	2.02±0.11	43.1	1.63
	4×10^{-6}	0.595	0.845	1.44	58.7	1.16
DEX(M)	1×10^{-6}	0.55	1.055	1.605	65.7	1.29
CONTROL	Zero time	2.39±0.27		2.39±0.27		(1.00)
	48 hours	7.51±0.20	0.20±0.01	7.71±0.21	2.6	3.23
DEX(M)	5×10^{-8}	7.26±1.03	0.72±0.50	7.98±1.08	9.0	3.34
	1×10^{-7}	5.90±0.15	0.72±0.12	6.62±0.26	10.9	2.77
	5×10^{-7}	4.16±0.41	1.06±0.21	5.22±0.24	20.3	2.18
	1×10^{-6}	4.63±0.48	1.12±0.22	5.75±0.26	19.5	2.41

The medium was changed and glucocorticoids (hydrocortisone = HC or dexamethasone = DEX) were added at the zero-time for a period of 48 hours. Each number of cells is the mean for 2 or 3 culture flasks with S.E.M. calculated where possible. The percentage of detached cells is the number of floating cells divided by the total number of cells and multiplied by 100. The cell multiplication factor is obtained by dividing the total number of cells harvested at 48 hours by the number of attached cells present at zero-time.

(total number: attached plus floating) (Fig. 3). In the controls, the AFP production is 5.2 ± 0.3 µg/48 h/10^6 cells. Expression of the data on a per 10^6 cells basis is for comparison only and is not intended to indicate homogeneity of the cell population with regard to AFP production. One AH66 clonal cell line produced 2.4 to 4.0 µg AFP/72h/10^6 cells (14), while another (3) produced as much as 28 µg/24h/10^6 cells.

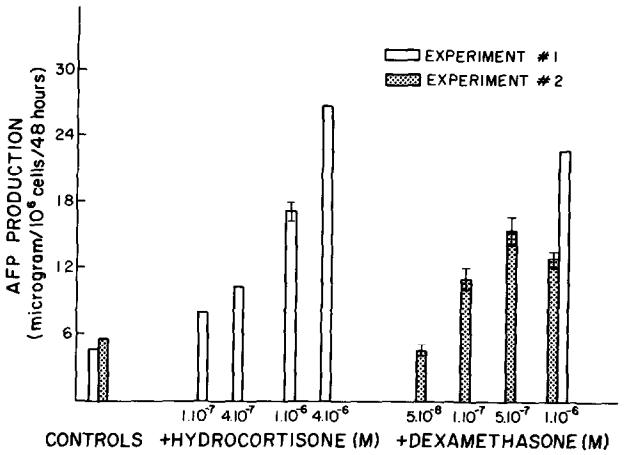

FIGURE 3. ALPHA-FETOPROTEIN PRODUCTION BY 8994 CELL LINE IN RESPONSE TO INCREASING DOSES OF GLUCOCORTICOIDS. For protocol, see legend of FIGURE 2.

A certain variation is also found among the three AFP producing cell strains derived from Yoshida sarcoma: between 0.3 and 1.2 µg AFP/24h/10^6 cells (2).

Nevertheless, the basal AFP production by 8994 cells is doubled with $4x10^{-7}$M hydrocortisone or $1x10^{-7}$M dexamethasone and tripled with $1x10^{-6}$M hydrocortisone or $5x10^{-7}$M dexamethasone. Dexamethasone was reported to have no effect on AFP production by AH66 cells (14). In vivo, Belanger et al (15) observed that prednisolone increased the AFP serum concentrations in hepatoma-bearing rats while glucocorticoids accelerated the physiological decrease of AFP in the serum of young rats.

Another response of the cells is the appearance of $alpha_M$-fetoprotein in the culture medium (Table 2). This fetoprotein was never detected in the control medium, but it appears in 48 hours or less after addition of glucocorticoids. Doses of hydrocortisone lower than $4x10^{-7}$M did not effect this phenomenon.

As far as we know this is the first time that the requirement of

TABLE II

FREQUENCY OF ALPHA$_M$-FETOPROTEIN PRODUCTION IN THE CULTURE MEDIUM OF 8994 CELL LINE AFTER ADDITION OF GLUCOCORTICOIDS

		CULTURES SHOWING PRESENCE OF ALPHA$_M$FETOPROTEIN	
	DOSE (M)	At 24 hours	At 48 hours
HC	4×10^{-6}	2/2	2/2
	1×10^{-6}	1/3	3/3
	4×10^{-7}	0/2	2/2
	1×10^{-7}	0/2	0/2
DEX	1×10^{-6}	2/2	5/5
	5×10^{-7}	ND	3/3
	1×10^{-7}	ND	3/3
	5×10^{-8}	ND	3/3
CONTROL		0/2	0/5

The frequency is expressed as the number of cultures with detectable alpha$_M$-fetoprotein in the medium compared with the total number of cultures tested. The detection test was done on samples taken at 24 or 48 hours -(ND = not done). See legend of Table I for experimental protocol and abbreviations.

glucocorticoids for alpha$_M$-fetoprotein has been demonstrated *in vitro*. This finding is consistent with the *in vivo* observation of the absence of this protein in the serum of adrenalectomized rats and its presence in such rats if treated with corticosteroids (16).

If a cancer cell produces a fetoprotein, its synthesis could take place either continually or during resting periods (17) or during the mitotic cycle. In the latter case this synthesis could be restricted to a part of every cycle. With respect to AFP, fetal hepatocytes in culture may synthesize or at least release it during G_2 (1) whereas in Yoshida sarcoma cells there are indications of its synthesis from the late G_1 to

the late S phase (18). Thus, at any given time in the non-synchronous culture of the 8994 cell line, there may be only a fraction of the population synthesizing AFP. Whether this fraction is selected by glucocorticoids via a specific growth inhibition of the rest of the population, or whether the hormones act by increasing the amount of fetoprotein synthesized per cell or by recruiting cells for this synthesis remains unsolved. But our data taken with the report of a suppression of DNA synthesis in hepatoma cells exposed to glucocorticoid hormone in vitro (13) and with the in vivo observation of a block in the hepatocyte proliferative cycle in G_1 by corticosterone after partial hepatectomy (19), suggest that the production of two fetoproteins is not obligatorily linked with cell proliferation. According to theories considering oncogeny as a "retrodifferentiation" (20) or a "blocked ontogeny" (21), the transitory stage of differentiation associated with AFP synthesis can be reached by either process. Hepatomas synthesizing AFP (5) can be considered "locked in" to this stage while others that do not (5) are "locked in" before or subsequent to this stage. In contrast, normal liver appears to proceed rapidly beyond this point in the course of its differentiation (8,22,23). Attempts to verify the "locked in" concept imply characterization of such stages by additional transitory markers.

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