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DANUTA MAGIELSKA-ŻERO, AMALIA GUZDEK, JOANNA BERETA, ANNA KURDOWSKA, KRYSTYNA CIĘSZKA and ALEKSANDER KOJ

EFFECTS OF DEXAMETHASONE AND INSULIN ON THE ACUTE PHASE RESPONSE OF MORRIS HEPATOMA CELLS AND OF RAT HEPATOCYTES IN CULTURE *

Department of Animal Biochemistry, The Jan Zurzycki Institute of Molecular Biology, Jagiellonian University, Al. Mickiewicza 3; 31-120 Kraków, Poland

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Dexamethasone and insulin stimulate production of several plasma proteins in primary cultures of adult rat hepatocytes but inhibit their production in primary cultures of Morris hepatoma cell line 7777W. The acute phase response elicited in cultured cells by crude cytokines from activated rat peritoneal macrophages is considerably higher in hepatocytes in the presence of hormones, and especially of dexamethasone. In hepatoma cells the hormones enhance the cytokine-induced formation of fibrinogen and cysteine proteinase inhibitor but are without significant effect on suppression of albumin and α-fetoprotein synthesis by macrophage supernatants.

Numerous plasma proteins are synthesized and secreted to the medium by cultured hepatocytes [1, 2] or hepatoma cells [1, 3, 4, 5]. The pattern of produced proteins depends not only on cell genotype and differentiation stage but is profoundly influenced by the composition of the culture medium, including hormones [1, 2, 6 - 14].

The actue phase response is a highly complex early reaction to a variety of injuries resulting from bacterial or parasitic infection, mechanical or thermal trauma, malignant proliferation or ischaemic necrosis. Only recently it has been possible to identify the hormone-like cytokines responsible for the changes which occur in the liver cells.

Inflammatory cytokines, such as interleukin 1, tumor necrosis factor

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or hepatocyte stimulating factor, produced by activated monocyte-macrophages or some other cells [15], inhibit synthesis of albumin but increase synthesis of α_1-acid glycoprotein, fibrinogen and other "positive" acute phase proteins [1, 2, 4, 16 - 19]. In rat hepatocytes the acute phase response critically depends on the presence of corticosteroids, while the requirement for this hormone is less stringent in the case of human mouse liver cells [1]. Here we compare the effects of insulin[1] and dexamethasone on production of seven plasma proteins by primary cultures of adult rat hepatocytes and rat hepatoma cells (line Morris 7777W) in the absence and presence of crude cytokines from rat peritoneal macrophages.

MATERIALS AND METHODS

Crude cytokines from thioglycollate-elicited rat peritoneal macrophages were obtained as described earlier [18].

Hepatocytes were isolated as described by Koj *et al.* [18] from livers of adult male Wistar rats weighing approximately 200 g and were cultured (1.2×10^6 cells/well) in 35 mm flat-bottom cluster plates (Nunclon, Kamstrup, Denmark) pretreated with collagen (Flow Lab., McLean, U.S.A.) [2, 18]. Williams E medium (Gibco, Paisley, Scotland) containing 5% foetal calf serum, heparin (5 units/ml, Sigma, St. Louis, MO, U.S.A.) and antibiotics (penicillin 300 units/ml, streptomycin 10 μg/ml and neomycin 20 μg/ml) was used for hepatocyte culture.

Morris 7777W hepatoma cells isolated from Buffalo rats as described by Magielska-Żero *et al.* [5] were cultured (2.5×10^6 cells/well) in Eagle's minimal essential medium supplemented with 5% foetal calf serum, heparin (2.5 units/ml) and the antibiotics listed above.

After initial preincubation at 37°C under 5% CO_2 (2 h for hepatocytes and 16 h for hepatoma cells) the medium with unattached cells was replaced by 1 ml of fresh medium containing either no hormones or 1 μM dexamethasone (Sigma), 1 μM insulin (Polfa, Tarchomin, Poland) or an equimolar mixture of the two hormones. In some experiments lower concentrations of hormones were used as indicated. Immediately after addition of the medium the cells were supplemented with 0.25 ml of either 0.1% bovine serum albumin in phosphate-buffered saline (BSA/PBS, control culture) or with serial dilutions of macrophage-derived cytokines (stimulated culture) [18]. The cells were cultured usually for 2 days with daily replacement of the medium and BSA/PBS or cytokine solution. The collected media

[1] Abbreviations used: DEX, dexamethasone; INS, insulin; ALB, albumin; AFP, α-fetoprotein; FBG, fibrinogen; CPI, cysteine proteinase inhibitor; AGP, α_1-acid glycoprotein; API, α_1-proteinase inhibitor; A2M, α_2-macroglobulin; BSA/PBS, 0.1% bovine serum albumin in phosphate-buffered saline; AP-protein, acute phase protein.

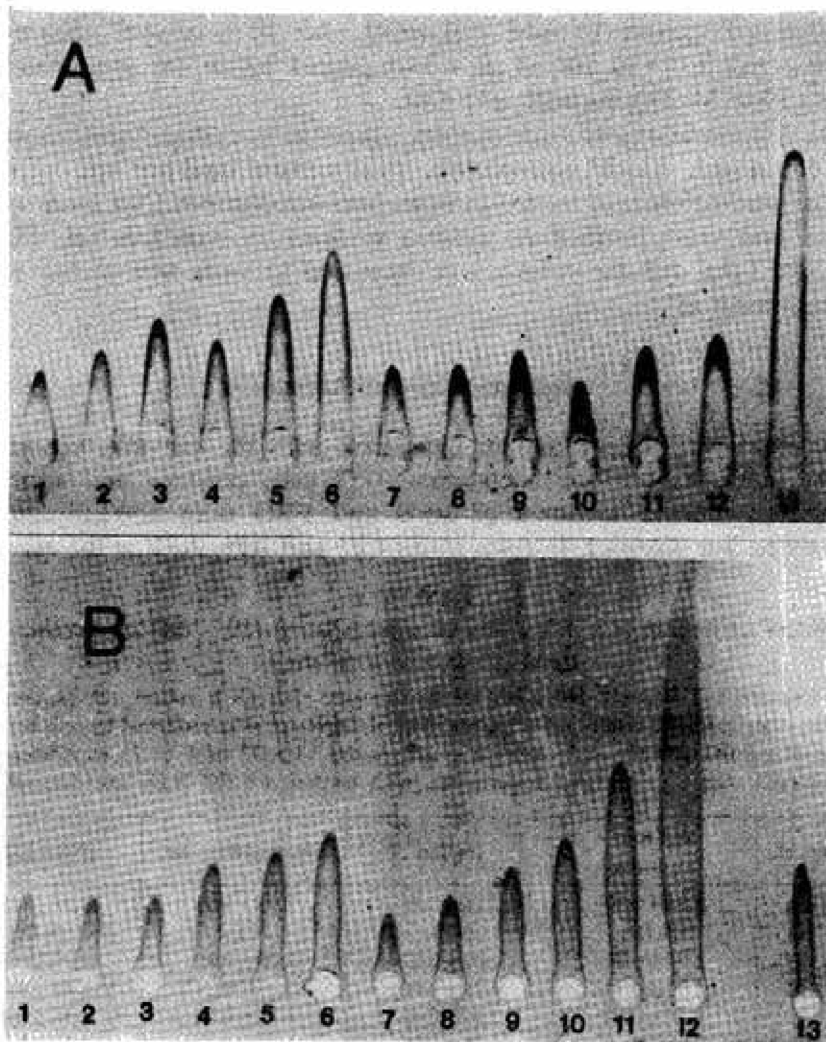

Plate 1. The effect of hormones on secretion of albumin by Morris hepatoma 7777W cells (A) and rat hepatocytes (B) after 2 days in culture

A. 1% agarose contained 100 μl monospecific antibodies against rat albumin. Wells were loaded with 10 μl of 20-fold concentrated culture media: 3, 6, 9, 12 — from control cultures; 1 - 3, media without hormones; 4 - 6, with 1 μM dexamethasone; 7 - 9, with 1 μM insulin; 10 - 12, media with both hormones. Wells: 1, 4, 7, 10, media from cells cultured in the presence of 200 μl, and wells 2, 5, 8, 11, in the presence of 40 μl of crude cytokines from rat macrophages. Well 13, protein standard (1.5 μg of rat albumin).
B. 1% agarose contained 90 μl monospecific antibodies against rat albumin. Wells were loaded with 6 μl of 12.5-fold concentrated culture media: 3, 6, 9, 12 — from control cultures; 1 - 3, media without hormones; 4 - 6, with 1 μM insulin; 7 - 9, with 1 μM dexamethasone; 10 - 12, media with both hormones. Wells: 1, 4, 7, 10, media from cells cultured in the presence of 200 μl, and wells 2, 5, 8, 11, in the presence of 20 μl of crude cytokines from rat macrophages. Well 13, protein standard (0.26 μg of rat albumin).

were dialysed against 15 mM NH_4HCO_3 for 24 h at 4°C, freeze-dried and dissolved in 50 to 100 μl of 4-fold diluted buffer for immunoelectrophoresis (0.08 M Tris/barbital, pH 8.8).

The concentrations of α-fetoprotein, albumin, α_1-cysteine proteinase inhibitor, fibrinogen, α_1-acid glycoprotein, α_1-proteinase inhibitor and α_2-macroglobulin were determined by rocket immunoelectrophoresis with monospecific antisera and using purified rat plasma proteins as standards (cf. Plate 1, and [20]). Total cellular protein was measured in each well at the end of the experiment [5].

RESULTS AND DISCUSSION

As shown in Table 1 and in agreement with the previous reports [5], primary cultures of Morris hepatoma 7777 cells produce and secrete to the medium smaller amounts of ALB and FBG than normal adult rat hepatocytes, but comparable amounts of CPI and API. AFP is the exception

Table 1

The effects of insulin (10^{-6} M) *and dexamethasone* (10^{-6} M) *on production of some acute phase proteins.*

Morris hepatoma 7777 cells and adult rat hepatocytes after 48 h culture (cf. Materials and Methods) were used. The results are expressed in μg of a given protein produced by cells equivalent to 1 mg of cellular protein, and reported as the means (±S.D.) of 4 to 15 experiments with hepatoma cells (a), or 3 to 6 experiments with hepatocytes (b). N.D., not detectable.

Proteincell		Without hormones	Insulin	Dexamethasone	INS+DEX
AFP	(a)	2.74±0.30	2.16±0.56	1.96±0.40	1.96±0.52
	(b)	N.D.	N.D.	N.D.	N.D.
ALB	(a)	1.56±0.26	1.02±0.22	2.08±0.80	1.36±0.36
	(b)	3.08±0.78	4.29±0.90	3.98±0.24	8.43±2.80
CPI	(a)	2.58±0.60	1.50±0.44	1.76±0.62	1.58±0.44
	(b)	0.88±0.19	1.38±0.10	1.57±0.48	1.53±0.52
FBG	(a)	1.06±0.14	0.30±0.06	0.61±0.16	0.41±0.20
	(b)	3.74±1.58	5.05±1.10	5.65±2.10	4.48±1.26
API	(a)	1.67±0.44	1.06±0.26	1.72±0.34	1.37±0.46
	(b)	1.43±0.04	1.54±0.36	1.86±0.23	3.66±1.22
AGP	(a)	N.D.	N.D.	N.D.	N.D.
	(b)	0.93±0.40	1.48±0.28	2.83±0.93	3.13±1.59
A2M	(a)	N.D.	N.D.	N.D.	N.D.
	(b)	N.D.	N.D.	3.89±0.96	3.77±0.62

being not detectable in hepatocytes while constituting the primary secretory protein product of hepatoma cells. On the other hand, Morris hepatoma 7777W cells do not produce measurable amounts of AGP and A2M, even in the presence of corticosteroids, which are known to be essential for full expression of these two proteins [1, 9, 21].

In rat hepatocytes insulin and dexamethasone increased synthesis of all tested plasma proteins in all experiments carried out so far. In the case of albumin synthesis the effect of the two hormones was additive (Table 1; and [20]). In contrast, production of the majority of secretory proteins by Morris hepatoma cells was suppressed by insulin and dexamethasone, fibrinogen being the most affected and API the least responsive. The reduced output of plasma proteins by hepatoma cells cultured with hormones cannot be explained by indirect effects, such as changes in cell proliferation during culture, since all the results have been recalculated per 1 mg of cellular protein. In addition, it was shown that insulin or dexamethasone do not affect [^{3}H]thymidine incorporation into Morris hepatoma cells cultured for 3 days (not shown).

The suppression of AFP production by dexamethasone was reported earlier in Morris hepatoma lines [8, 22] and by insulin in a Reuber hepatoma line [12]. However, the response to hormones varies depending on cell line: in McA-RH8994 rat hepatoma cells DEX was found to stimulate AFP production at the stage of gene transcription [22]. An opposite response of certain proteins to dexamethasone was observed by Ivarie & O'Farrell [6] in HTC and FAZA rat hepatoma cells.

The data shown in Table 1 suggest that fibrinogen synthesis in Morris hepatoma cells is suppressed by insulin or dexamethasone alone as well as by a mixture of the two hormones. This effect of insulin has not been reported earlier for any hepatoma line.

Immediately after plating Morris hepatoma cells were refractory to the added hormones, probably as a result of alteration of the cell membrane receptors. Moreover, in response to insulin and dexamethasone, synthesis of all tested proteins progressively decreased during the second and third days of culture (Fig. 1). This is clearly different from the situation observed with normal adult rat hepatocytes [2].

Although some effects of hormones were noticed at 10^{-9} or 10^{-8} M the most efficient were concentrations in the range of 10^{-7} or 10^{-6} M (Fig. 1). Similar dose response curves were obtained for ALB and A2M (not shown). Thus, sensitivity of Morris hepatoma 7777W cells to insulin and dexamethasone is similar to that reported for adult rat hepatocytes [2, 11, 13, 14]. Evaluation of results is somewhat complicated by the fact that lack of these hormones in primary hepatocyte culture leads often to progressive deterioration of cell morphology, and the cells begin to detach from the substratum even in the presence of serum ([16] and A. Guzdek, unpublished). Replenishment of the medium with insulin and dexamethasone restores the capacity of rat hepatocytes to produce higher amounts of the proteins tested [2]. Mode of action of DEX and INS may be different as suggested by the elegant studies of Nawa *et al.* [13] who demonstrated that dexamethasone increases the relative rate of transcription of the ALB

gene in cultured rat hepatocytes while insulin at 0.1 μM concentration acts at a post-transcriptional step.

The results shown in Table 2 indicate that addition of supernatants from stimulated rat macrophages to hepatocyte culture in the presence of dexamethasone and insulin elicited a tyical acute phase response as described elsewhere [2, 18]. Lack of hormones, and especially of DEX, reduced this

Table 2

Influence of crude cytokines from rat macrophage supernatant on the synthesis of some proteins

Morris hepatoma cells (a) and rat hepatocytes (b) were cultured for 2 days in the presence or absence of hormones. The results of control cultures without cytokines were assumed as 100% (means of 4 to 14 estimations in hepatoma cells and 3 to 8 estimations in hepatocytes). For other details see Table 1

Proteincell		Without hormones	Insulin	Dexamethasone	INS+DEX
AFP	(a)	53	81	69	77
	(b)	N.D.	N.D.	N.D.	N.D.
ALB	(a)	67	79	48	68
	(b)	83	84	66	43
CPI	(a)	107	150	214	218
	(b)	113	134	144	152
FBG	(a)	97	174	305	304
	(b)	110	105	156	211
API	(a)	79	96	93	103
	(b)	93	123	109	101
AGP	(a)	N.D.	N.D.	N.D.	N.D.
	(b)	116	117	139	122
A2M	(a)	N.D.	N.D.	N.D.	N.D.
	(b)	N.D.	N.D.	165	191

reaction both with a negative AP-protein (ALB) and with positive AP-proteins such as FBG, CPI and AGP. It is worth underlining here that A2M was not detectable in the absence of DEX, in agreement with the reports of several authors [1, 2, 4]. On the other hand, the highest relative inhibition of AFP synthesis in Morris hepatoma cells occurred in the absence of DEX and INS (Table 2). However, the two hormones, and especially dexamethasone, enhanced induced synthesis of CPI and FBG. Thus, in respect to the two latter proteins hepatoma cells respond to hepatocyte stimulating factors similarly as adult hepatocytes.

The reason for requirement of DEX for inducible synthesis of several acute phase proteins in rat hepatocytes or hepatoma is still not fully understood [4, 10, 23] although recent studies of Klein *et al.* [24] with activation of AGP gene, and Nawa *et al.* [13] with ALB gene suggest the existence

in the cell of a specific regulatory protein distinct from glucocorticoid receptor.

Macrophage cytokines depressed ALB synthesis in hepatoma cells independently of the presence of hormones in the medium (Table 2). This observation is in discrepancy with our results reported earlier [5] but in the present experiments a new clone of Morris hepatoma 7777 cells was used. These cells produce in control culture five times more albumin than does the former clone. Thus, not only expression of the protein gene but also its regulation appear to be unstable in transformed hepatocytes.

The described effects of macrophage cytokines and hormones on synthesis

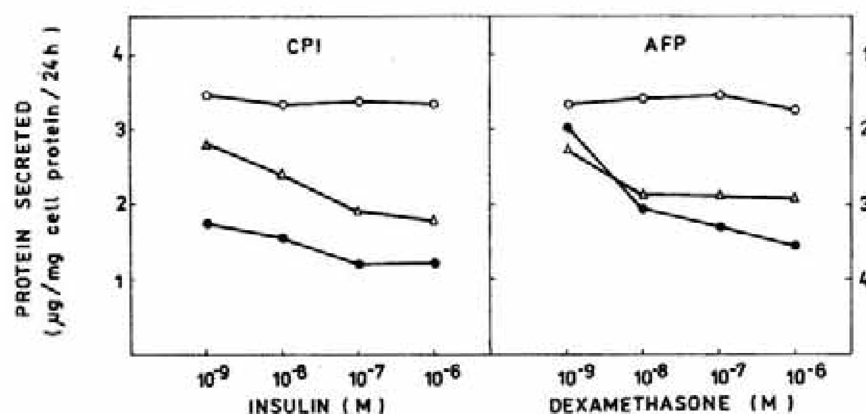

Fig. 1. Synthesis of cysteine proteinase inhibitor (CPI) and α-fetoprotein (AFP) in Morris hepatoma. The cells cultured for 24 h (○), 48 h (△) and 72 h (●). The results derive from a single experiment

of plasma proteins by Morris hepatoma cells in comparison with adult rat hepatocytes indicate that the basic mechanisms controlling the acute phase reaction are similar in normal and neoplastic liver cells. Elucidation of the observed differences in response of these two types of cells to dexamethasone, insulin and inflammatory cytokines will require quantitative determinations of transcription rates of appropriate genes and analysis of their structure and expression [23, 24].

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