Vol. 99, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1981 Pages 196-204

Adrenal growth factors in the rat mammotropic pituitary tumor (MtT-F_4)

Anne-Marie Cathiard and José M. Saez

INSERM U. 162, Hôpital Debrousse, 69322 Lyon Cedex 1, France.

Received December 20, 1980

SUMMARY

Rat mammotropic pituitary tumor (MtT-F_4) crude extracts, prepared either in phosphate buffer or in preheated acetic acid 1 M, stimulated DNA synthesis of both mouse adrenal cell line (Y-1) and primary bovine adrenocortical cell cultures. Chromatography of both extracts on a carboxymethyl-Sephadex C-50 column gave one fraction with growth-promoting activity on bovine adrenal cells. The mitogenic activity of this fraction could not be related to the known hormones secreted by the tumor (prolactin, growth hormone or ACTH). A second mitogenic fraction was eluted from the column only when the extract was prepared in phosphate buffer. This fraction had some of the properties of fibroblast growth factor (FGF). These results suggest that the pituitary rat tumor MtT-F_4 secretes adrenal growth factors (other than ACTH) which could be responsible for the adrenal hyperplasia observed in rats bearing the tumor.

ACTH has paradoxical effects on adrenal cell growth, depending on the experimental conditions. In vivo high concentrations of ACTH are associated with increased DNA synthesis (1, 2) and DNA content (3), while in vitro ACTH inhibits DNA synthesis and replication of adrenal cells but increases RNA and protein synthesis (4-9). To explain this discrepancy, two hypotheses have been postulated. The first suggests that ACTH or some of its peptides could have mitogenic effects on adrenal cells, but that these effects are abolished by ACTH-stimulated accumulation of cAMP (2) and consequent inhibition of DNA synthesis in vitro. This hypothesis has been recently confirmed (9), but cannot completely explain the discrepancy between the in vivo and in vitro results. The second hypothesis postulates that in vivo ACTH may increase the effective delivery of growth factors to adrenocortical cells. However, such factors have still not been isolated (7, 10). A third possibility is that factors controlling adrenal growth are secreted simultaneously but independently of ACTH by the pituitary. One good model to investigate this hypothesis is the MtT-F_4 rat pituitary tumor, described by Furth (11). This tumor secretes prolactin, growth hormone and ACTH (12, 13) and the adrenal weight of rats bearing the tumor is greatly increased (14-16). Therefore we decided to investigate whether the extracts of these tumors contained some factors, other than

0006-291X/81/050196-09$01.00/0

ACTH, able to stimulate adrenal cell replication in vitro. In this paper we report the partial purification of two adrenal mitogenic factors from MtT-F_4 tumor.

MATERIALS AND METHODS

Rats bearing MtT-F_4 tumors were obtained from Dr. C. Sonnenschein, Tufts University, Boston, Massachusetts and have been propagated in this laboratory for three years by the intramuscular injection of tumor minces in the leg area of intact rats of Fisher 344 strain. Three to four weeks after transplantation the animals were sacrified by decapitation and tumors were removed, immediately homogenized in distilled water (Polytron setting 8, 10 sec.), immersed in liquid nitrogen and then lyophilized. Two methods were used for purification of the dry powder. The first was similar to that described by Gospodarowicz for purification of FGF (17) except that the $(NH_4)_2SO_4$ precipitation was omitted. The crude extract prepared by this method will be referred as pH 4.5 extract. In the second method the dry powder was dissolved in 1 M acetic acid preheated at 80°C, stirred for 5 min and then homogenized at 4°C (Polytron setting 8, 10 sec), filtered through gauze and centrifuged for 20 min. The supernatant will be referred as acetic acid extract. Each extract was dialysed against 4 liters of distilled water three times, using Spectrapor dialysis tubings (M.W. cut off 1000 to 2000) and then lyophilised. The dry extracts were dissolved in 10 mM sodium phosphate buffer pH 6 and purified on a carboxymethyl Sephadex-C50 column (2.7 x 30 cm) which had been equilibrated with 10 mM sodium phosphate buffer pH 6. The column was eluted with a stepwise increasing salt concentration : 0, 0.2 and 1 M NaCl in the same buffer.

The mitogenic activity of tumor extracts was tested by their ability to stimulate DNA synthesis of either mouse adrenal cell line Y-1 (18) or primary cultures of bovine adrenal cells in the second passage. The former were cultured as described (9) in Ham's F-10 medium complemented with 10 % heat-inactivated horse serum 2.5 %, heat-inactivated fetal calf serum (FCS), penicilline (50 U/ml) and streptomycine (25 µg/ml) at 37° in an atmosphere of 95 % air/5 % CO_2. Bovine adrenal cells were isolated as described by Gospodarowicz (19). The dispersed cells were washed three times with Ham's F-10 medium containing 5 % fetal calf serum. Cells were cultured in Ham's F-10 medium containing 10 % horse serum, 2.5 fetal calf serum, penicilline (50 U/ml) and streptomycine (25 µg/ml) at 37°C in a humidified atmosphere of 5 % CO_2. Both cells were subcultured in microtiter wells (diameter 0.6 cm) and the growth arrested by serum deprivation for 72 h (see legend Table 1). The medium was removed and replaced by the same fresh medium containing 0.005 % bovine serum albumin with or without the fraction indicated in Tables. [^{3}H] thymidine (30 Ci/mmol ; 0.2 µCi/well) was added 6 h later and the incubation continued for 18 h. The medium was then removed, and the cells washed with phosphate buffer saline and incubated for 5 min in phosphate buffer saline containing 0.2 % trypsine 1 mM EDTA. The cells were harvested using a Ilacon sheet processor (Ilacon Ltd, England) and filtered under vacuum through a glass fiber paper (Whatman GF/C). The filter was washed five times with phosphate buffer and ten times with ethanol and assayed for radioactivity in 5 ml scintillator 299 TM Packard.

The steroids in the medium were extracted by dichloromethane and measured by radioimmunoassay using an anti-cortisol antibody furnished by NEN. cAMP was extracted from the culture medium by ethanol and evaporated. The extract was acetylated (20) and the cAMP content measured in triplicate by radioimmunoassay (21). Prolactin and growth hormone were measured by the kit kindly provided by the rat Pituitary Hormone Distribution Program, NIAMD. Standards used were NIAMD rat prolactin RP1 and rat growth hormone RP1. ACTH was measured by the kit furnished by CEA, Saclay, France. $ACTH_{1-24}$ was a gift from Dr. W. Rittel and FGF was obtained from Collaborative Research Inc. Mass. USA.

TABLE 1

Effects of crude extracts from MtT-F_4 rat pituitary tumors upon DNA synthesis of Y-1 and bovine adrenal cells

		[^{3}H]thymidine uptake into DNA (% of control)	
		Y-1 cells	Bovine adrenal cells
Control		100 ± 15	100 ± 32
10 % fetal calf serum		1100 ± 180	1330 ± 297
Tumor extract pH 4.5 µg protein/ml	2	120 ± 22	125 ± 45
	20	227 ± 38	277 ± 116
	100	403 ± 60	630 ± 177
Tumor extract : acetic acid µg protein/ml	0.5	130 ± 9	156 ± 20
	1	197 ± 20	148 ± 8
	2		
	10	140 ± 6	94 ± 19
	20		
	50	20 ± 2	4 ± 3
	1		

Y-1 cells and bovine adrenal cells were subcultured in microtiter wells (0.6 cm) in Ham's F-12 medium containing 10 % horse serum and 2.5 % fetal calf serum. After 18 to 20 hr medium was removed and cells were incubated in serum free medium for 72 hrs. The medium was removed and replaced by Ham's F-12 medium containing 0.005 % bovine serum albumin without (control) or with 10 % foetal calf serum or the indicated amounts of tumor extracts. Six hours later, [^{3}H] thymidine (0.2 µCi/well) was added; after an additional 18 hr-incubation [^{3}H] thymidine incorporation was determined as described in Materials and Methods. The results are expressed as % increase over control and represent the mean ± SD for triplicate wells of two different experiments. The [^{3}H] thymidine incorporation under control conditions was 112 ± 17 cpm/well and 158 ± 51 cpm/well for Y-1 and bovine adrenal cells respectively.

RESULTS

The adrenal weight of rats bearing MtT-F_4 tumor increases about ten fold (190 ± 15 vs 24 ± 2 mg per adrenal, n = 28) three to four weeks after transplantation, indicating that the rumor secretes factors which directly or indirectly stimulated adrenal growth. Indeed, crude tumor extracts prepared by both methods stimulated DNA synthesis of Y-1 and bovine adrenal cells (Table 1). With pH 4.5 extract maximal stimulation was obtained with between 20 and 100 µg of protein extracts/ml, and the stimulation varied from 30 to 50 % of that produced by 10 % FCS. On the other hand with acetic acid extracts maximal stimulation varied between 10 and 20 % of that produced by 10 % FCS, and this was observed with about 2 µg/ml. At higher concentrations the extract inhibited DNA synthesis. Since in several experiments the results obtained with bovine adrenal cells were more

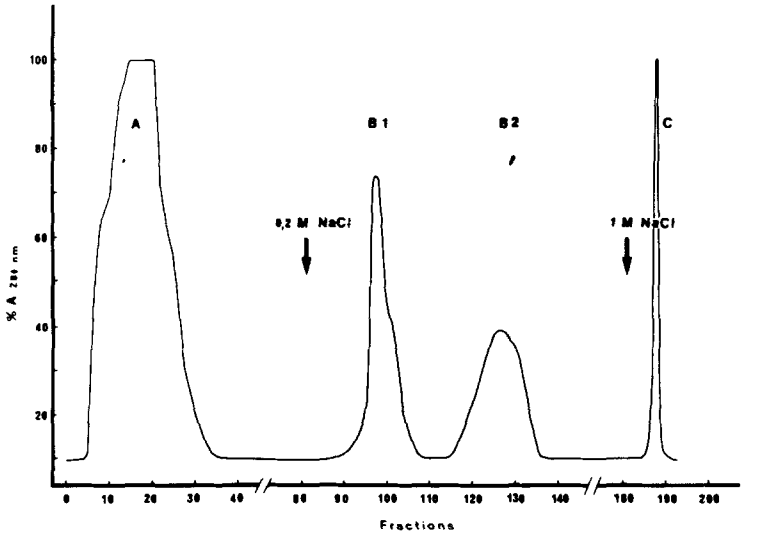

Figure 1. Elution pattern of pH 4.5 extract on carboxymethyl-Sephadex C-50 column. Two grams of protein dissolved in 10 mM sodium phosphate buffer pH 6 was applied to a column 2.7 x 30 cm. Fractions of 18 ml were collected. Fraction A represented unadsorbed proteins. The adsorbed proteins were eluted by increasing the NaCl concentration.

reproducible than with Y-1 cells, all the subsequent experiments were carried out using bovine adrenal preparations.

A typical elution pattern of the pH 4.5 extract on carboxymethyl Sephadex-C50 column is shown in Fig. 1. Fraction A, collected in the fall-through with 10 mM sodium phosphate (pH 6) contained most of the protein but was inactive. Elution with 10 mM sodium phosphate-0.2 M NaCl resulted in two clearly separated peaks named fractions B1 and B2 respectively. Elution with 1 M NaCl resulted in a fourth protein peak named fraction C. The elution profile of acetic acid extract was similar to that shown in Fig. 1 (data not shown).

The mitogenic activity of the four fractions from both extracts, pH 4.5 and acetic acid, of two experiments is given in Table 2. In most experiments fraction B1 from acetic acid extract displayed no significant mitogenic activity. In a few experiments some mitogenic activity for this particular fraction was observed, the values reported in Table 2 being the maximum observed. Fraction C was only active when prepared from pH 4.5 extracts, and indeed had inhibitory growth activity when prepared from acetic acid extracts, being actually cytotoxic at concentrations of 20 μg protein/ml or higher.

Since it has been shown that the MtT-F_4 tumors produce prolactin, growth hormone and ACTH (12, 13), we investigated whether the growth activity of fractions B_2 and C could be related to one of these hormones. Therefore the

TABLE 2

Effects of several fractions from MtT-F_4 rat pituitary tumor extracts and several hormones upon DNA synthesis of bovine adrenal cells

	pH 4.5 extract			Acetic acid extract			
	μg protein/ml			μg protein/ml			
	100	20	2	100	20	2	0.5
Crude extract	614 ± 154	286 ± 96	125 ± 45	4 ± 3	94 ± 19	148 ± 8	156 ± 20
Fraction A*	172 ± 42	119 ± 48	122 ± 22	139 ± 58	127 ± 42	132 ± 29	68 ± 8
Fraction B_1	225 ± 97	127 ± 53	68 ± 30	315 ± 30	177 ± 39	122 ± 41	105 ± 32
Fraction B_2	372 ± 166	259 ± 62	92 ± 16	451 ± 99	322 ± 82	261 ± 75	186 ± 45
Fraction C	581 ± 151	386 ± 96	224 ± 99	8 ± 6	29 ± 27	148 ± 27	98 ± 31

10 % FCS	1330 ± 297
Human growth hormone 1 μg/ml	69 ± 20
Rat prolactin 10 μg/ml	112 ± 17
FGF 100 ng/ml	450 ± 38
ACTH 10^{-7} M	32 ± 10

Quiescent cells were prepared by incubation for 72 hr in serum free medium. The medium was then removed and replaced by Ham's F-12 medium containing 0.005 % bovine serum albumin, without (control) or with the fractions or hormones indicated. [^{3}H] thymidine incorporation was determined as described in Materials and Methods. The results (mean ± SD of triplicate wells of two experiments) are expressed as % increase over the control which is arbitrary given as 100 % control value : 158 ± 51 cpm/well, 10 % FCS : 2105 ± 230 cpm/well.

* Fractions obtained after chromatography on carboxymethyl-Sephadex C-50 column (see Fig. 1).

TABLE 3

Protein, ACTH, prolactin and growth hormone content of several fractions of rat MtT-F_4 pituitary tumor

Fraction	pH 4.5 extract			Acetic acid extract		
	ACTH ng/mg	Prolactin µg/mg	GH µg/mg	ACTH ng/mg	Prolactin µg/mg	GH µg/mg
Crude extracts	8.8	5.3	11.9	22.1	< 0.04	< 0.04
Fraction A	0.12	7	1.2	0.04	< 0.04	< 0.04
Fraction B_1	0.28	14.3	26.5	1.70	< 0.04	< 0.04
Fraction B_2	1.7	0.3	1.5	6.67	< 0.04	< 0.04
Fraction C	26.7	0.05	0.15	134.4	< 0.04	< 0.04

Each extract was prepared from 26 g of a pool of dry powder (1.8 g and 0.52 g of protein for pH 4.5 and acetic acid extracts respectively) of rat pituitary tumor. The results are mean of two determinations.

hormonal content of the different fractions was determined (Table 3). Prolactin and growth hormone were detected in pH 4.5 extract but not in acetic acid extract. Both hormones were mainly located in fractions A and B. On the other hand, ACTH was present in both extracts and located mainly in fraction C. These results suggested that the growth activity of both extracts and fractions B_2 and C (from pH 4.5 extract) cannot be related to their prolactin and growth hormone content. This was confirmed by the fact that neither rat prolactin nor human growth hormone were able to stimulate adrenal DNA synthesis (Table 2). On the other hand the inhibitory growth effects of crude acetic acid extracts and fraction C could be due in part to their content in ACTH.

The stimulatory effect of these different fractions on cAMP and steroids produced by bovine adrenal cells is shown in Table 4. At the concentrations used fraction C and fraction B2, from pH 4.5 extracts, had steroidogenic activity without modification of cAMP production. This is not surprising since low concentrations of ACTH are able to stimulate steroidogenesis without significant changes in cAMP production (22-24). Crude acetic acid extracts had also some steroidogenic activity, as well as fractions A and B_2. The steroidogenic activity of the former could be due to the cAMP content of that fraction (≃ 20 pmoles/20 µg protein), but that of fraction B_2 could not be related either to its content of cAMP (≃ 0.3 pmoles/20 µg protein) or to that of ACTH (Table 3). In spite of the high content of ACTH in fraction C (Table 3) its inhibition of the steroidogenic effect is probably due to the toxicity of this fraction.

TABLE 4

Effects of several fractions from MtT-F_β rat pituitary tumor extracts on cAMP and steroid production by bovine adrenal cells

			pH 4.5 extract 20 μg/ml		Acetic acid extract 20 μg/ml	
	Cortisol ng/day	**cAMP** pmoles/2 h	**Cortisol** ng/day	**cAMP** pmoles/2 h	**Cortisol** ng/day	**cAMP** pmoles/2 h
Control	83 ± 23	4.8 ± 0.9				
ACTH 10^{-8} M	785 ± 175	78 ± 14				
Crude extract 20 μg protein/ml			92 ± 13	6.9 ± 1.4	177 ± 47*	8.3 ± 1.4
Fraction A "			66 ± 11	7.3 ± 1.6	359 ± 42*	25 ± 3*
Fraction B_1 "			97 ± 15	6.1 ± 1.1	81 ± 10	5.1 ± 0.9
Fraction B_2 "			131 ± 19*	4.9 ± 0.7	371 ± 57*	11 ± 1.2
Fraction C "			134 ± 10*	6.2 ± 1.5	52 ± 9	5.1 ± 0.5

Bovine adrenal cells were subcultured in 3.5 cm petri dishes and in Ham's F-12 medium containing 10 % horse serum and 2.5 % foetal calf serum. After 18 h medium was removed and cells were incubated in Ham's F-12 medium containing 0.5 % horse serum and 50 μg/ml of human low density lipoprotein for 24 h. Medium was again removed and replaced by the same fresh medium without or with the indicated tumor fractions. Two hours later an aliquot was removed for measurement of cAMP. After an additional 22 h incubation the medium was removed for measurement of cortisol. Mean ± SD of triplicate determination of two dishes.

* $p < 0.05$ compared to control.

DISCUSSION

The increased adrenal weight of rats bearing MtT-F_4 that we have found was similar to that reported by other investigators (14-16). This increase in adrenal weight, which correlates with an increase on adrenal DNA content (16), has been related to increased ACTH secretion by the tumor (12, 13, 15). However, the adrenal weight of rats receiving pharmacological doses of ACTH (25 μg twice per day of $ACTH_{1-24}$ depot for three weeks) was far less (85 ± 10 mg/adrenal) (unpublished results) than that of rats bearing the tumors. This suggests that the tumor may be producing some factors able to stimulate adrenal growth. Prolactin and growth hormone could be the agents responsible, since the tumor secretes these two hormones, but our results (Tables 2 and 3) suggest that this is not the case. However the situation could be different in vivo, since the effect of growth hormone is mediated by somatomedins.

The mitogenic activity of fraction C from pH 4.5 extract could be due to FGF. The methods that we have used to prepare the crude extract and the carboxymethyl-Sephadex C-50 column are similar to the first steps used by Gospodarowicz to prepare FGF from bovine pituitary (17). Like FGF, the mitogenic activity of fraction C disappeared following treatment with 1 M acetic acid and heating (25). Moreover, FGF has mitogenic activity on bovine adrenal cells (8, 19). However, purification of fraction C is required to confirm that the latter contains FGF.

The growth activity of fraction B_2 cannot be related to any of the known factors secreted by MtT-F_4 tumors, namely prolactin, growth hormone and ACTH. Since the tumor secretes peptides similar to β-LPH (26) it is likely that pro-ACTH/lipotropin macromolecules are synthesised (27). If this was so, then the mitogenic activity of fraction B_2 could be due to one of the ACTH/lipotropin family of peptides. In favour of this hypothesis is the fact that some peptides of this family, like fraction B_2, are resistant to heating and 1 M acetic acid. However, in gel filtration most of the mitogenic activity of fraction B_2 has a molecular weight about 45000 daltons, which is higher than that of pro-ACTH/lipotropin precursors of rat pituitary (≃ 32000 daltons) (27, 28). It should be noted that bovine pituitary seems to contain, in addition to FGF, another adrenal growth factor which in a carboxymethyl-Sephadex C-50 column was eluted with about 0.2 M NaCl (29).

In conclusion, the present results demonstrate that the rat pituitary tumor MtT-F_4 contains at least two adrenal growth factors, which probably explain the adrenal hyperplasia of rats bearing the tumor. Since both factors seem to be present in the normal pituitary gland, one could postulate that some insight into mechanisms involved in regulation of adrenocortical growth might be provided by completely purifying these factors and then determining how their secretion is regulated.

ACKNOWLEDGEMENTS

The authors thank Mrs. P. Bourcet, Mr. O. Pascal and Ms. C. Delore for performing the radioimmunoassays of prolactin, GH and ACTH, Dr. M. Donaldson for his editorial help and Ms. J. Bois for her faithful secretarial assistance. This work was supported by INSERM grant ATP n° 82 79 114 and Ligue Nationale Contre le Cancer.

REFERENCES

1. Masui, H., and Garren, L.D. (1970) J. Biol. Chem. 245, 2627-2632.
2. Saez, J.M., Morera, A.M., and Gallet, D. (1977) Endocrinology 100, 1268-1275.
3. Imrie, R.C., Ramaiah, T.R., Antoni, F. and Hutchison, W.C. (1965) J. Endocrinol. 32, 303-312.
4. Masui, H., and Garren, L.D. (1971) Proc. Natl. Acad. Sci. 68, 3206-3210.
5. Ramachandran, J., and Suyama, A.T. (1975) Proc. Natl. Acad. Sci. 72, 113-117 (1975).
6. Weidman, E.R., and Gill, G.N. (1977) J. Cell Physiol. 90, 91-104.
7. Gill, G.N., and Weidman, E.R. (1977) J. Cell Physiol. 92, 65-76.
8. Hornsby, P.S., and Gill, G.N. (1977) J. Clin. Invest. 60, 342-352.
9. Morera, A.M., and Saez, J.M. (1980) Exptl. Cell Res. 127, 446-450.
10. Gill, G.N., Hornsby, P.J., Ill, C.R., Simonian, M.H., and Weidman, E.R. (1978). In Serio, M. (ed.), Endocrine Function of the Human Adrenal Cortex, Academic Press, London, pp. 207-228.
11. Furth, J. (1955) Recent Prog. Hormone Res. 11, 221-255.
12. Bates, R.W., Milkovic, S., and Garrison, M.M. (1962) Endocrinology 71, 943-948.
13. Lis, M., Guerinot, M.C., and Chretien, M. (1975) Endocrinology 96, 739-743.
14. Ueda, G., Takizawa, J., Moy, P., Marolla, F., and Furth, J. (1968) Cancer Res. 28, 1963-1975.
15. Milkovic, S., and Bates, R.W. (1964) Endocrinology 74, 617-626.
16. Stowe, N.W., Whitley, T.H., Ney, R.L., and Steiner, A.L. (1977) Biochim. Biophys. Acta 497, 690-701.
17. Gospodarowicz, D. (1975) J. Biol. Chem. 250, 2515-2520.
18. Buonassisi, V., Sato, G., and Cohen, A.I. (1962) Proc. Natl. Acad. Sci. 48, 1184-1190.
19. Gospodarowicz, D., Ill, C.R., Hornsby, P.J., and Gill, G.N. (1977) Endocrinology 100, 1080-1089.
20. Harper, J.M., and Brooker, G. (1975) J. Cyclic Nucleot. Res. 1, 207-218.
21. Steiner, A.L., Parker, C.P., and Kipnis, A.M. (1972) J. Biol. Chem. 247, 1106-1113.
22. Beall, R.J., and Sayers, G. (1972) Arch. Biochem. Biophys. 148, 70-76.
23. Mackie, C., Richardson, M.C., and Schulster, D. (1972) FEBS Letters 23, 245-348.
24. Saez, J.M., Evain, D., and Gallet, D. (1978) J. Cyclic Nucleot. Res. 4, 311-321.
25. Gospodarowicz, D., Bialecki, H., and Greenburg, G. (1978) J. Biol. Chem. 253, 3736-3743.
26. Bourassa, M., Scherrer, H., Pezalla, P.D., Lis, M., and Chrétien, M. (1978) Cancer Res. 38, 1568-1571.
27. Eipper, B.A., and Mains, R.E. (1980) Endocrine Reviews 1, 1-27.
28. Paquette, T.L., Herbert, E., and Hinman, M. (1979) Endocrinology 104, 1211-1214.
29. Armelin, M.C.J., Gambarini, A.G., and Armelin, H.A. (1977) J. Cell Physiol. 93, 1-9.