Studies on Hormone Production by Human Fetal Pituitary Cell Cultures[1] (34720)

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Hormone elaboration by certain mammalian cells cultured *in vitro* was first demonstrated approximately 30 years ago (1). Repetitive confirmation of these early studies has been made with a variety of endocrine tissues and cell culture systems (2–10).

Conventional cell culture systems have long been recognized as generally yielding dedifferentiated cells despite their maintenance of one or more of a variety of functions for a time. Furthermore, it has been demonstrated that the ease of establishing cultures is directly related to the state of the tissue of origin, *e.g.*, the least number of difficulties are encountered in the establishment of cultures from embryonic or malignant tissues.

Prior reports have stressed the role played by cell dissociation (11), culture surface (12), kinetics of gas diffusion (13, 14), and physiologic steady-state in the successful culture of differentiated liver cells (11, 15, 16). This preliminary report demonstrates the levels of hormone elaboration in thin-film culture systems of cells of endocrine origin under conditions similar to those employed in the cultures of liver cells.

Materials and Methods. Human fetal pituitary glands were obtained aseptically within 0.5 to 2 hr of therapeutic abortion by hysterotomy. The technique of cell cultures employed was described in detail previously (11). Briefly, the cultures were initiated by rinsing in 808–600 salt solution and mincing the tissue to provide a crudely dissociated cell suspension. The cell inoculum achieved was of heterogeneous composition, *i.e.*, discrete cells and cell clumps. The cells and cell clumps were suspended in 1 ml of culture media and then implanted in T-30 flasks previously coated with calf collagen (11). The culture medium used was NCTC 109 containing 10–20% calf serum. The entire tissue preparative procedure was performed as quickly as possible (less than 15–20 min) to avoid the deleterious effects of anoxia (13, 14). The culture flasks were placed on the rocker tray and were gassed while incubated in a horizontal position for 1 hr. Cell attachment was rapid and secure. Following this, the culture media volume was adjusted to 8 ml. (only 4 ml in the first four cultures) and the tray was inserted in the rocker incubator system (16). It was slowly rocked from the stationary home position (2 min, 20 sec) of a 25° angle through a 50° arc and back in 40 sec. The pH control was achieved by continuous gassing with 2 or 5% CO_2 in air with the appropriate bicarbonate level incorporated in the medium (12). The preset pH was 7.4 and the incubation temperature was 36.5°. The media were changed twice weekly; and harvested culture medium fluid was stored in a freezer at −20°. Samples were thawed periodically for various assay procedures. Coverslips were embedded in the collagen on the bottom of the flask. At various intervals, the coverslips were removed and stained for cytological verification (11).

Human growth hormone (HGH) in the culture media was assayed by radioimmunoassay using the double antibody system of Schalch and Parker (17). Growth hormone activity was measured by rat tibial epiphysis bioassay (19) in a limited number of culture media.

[1] This investigation was supported by Public Health Service Research Grant No. C-5834 from the National Cancer Institute and by Grant No. ES 00030-05 from the United States Public Health Service.

TABLE I. The Elaboration of Human Growth Hormone (mμg/flask/day).

Elapsed time (days)	Subgroup:	A: 5% CO_2; 4-ml media vol				B: 5% CO_2; 8-ml media vol					
	Age of fetus (weeks):	13	17	18	20	8	9	15	16	22	
	Culture no.:	Hu-3	Hu-10	Hu-2	Hu-4	Hu-17	Hu-21	Hu-24	Hu-15	Hu-18	
	Flask no.:									11	12
0–3		173			226		a. 25		7627		
4–6			260	112				448		7000	
7–9			213			a. 3		224			10,000
10–12			150					120			
13–15		62	197		173			56	2850	9850	
16–18		43	150				a. 25	33			11,333
19–21			197	70						6500	13,250
22–24									1360	5000	10,480
25–27		46			115						8900
28–30				68			25			3200	7000
31–33		26				6				1400	3400
34–36											
37–39											
40–42				44						1150	2500
43–45									550		
46–48											
49–51										1000	1733
52–54											
55–57									290		
58–60											
61–63										500	1100
64–66				20							
67–69											
70–72									100	667	1067
73–75											
76–78											
79-81											
148–150					135						

Radioimmunoassay methods were used for the measurement of luteinizing hormone (LH) (19) and thyrotropin (TSH) (20) in the culture media.

Results. Table I presents the daily rates of human growth hormone (HGH) released into the culture media during the course of incubation of cultures initiated from 16 human fetuses. The data were arranged in order of the age of the fetus into three subgroups. The culture conditions in these subgroups differed in two respects. The first variable was the use of 5 or 2% CO_2 in air in gassing the media. The second variable was the use of 4 ml of culture media/flask in the first four pituitary cultures and 8 ml of culture medium in the rest. The medium was changed twice weekly.

Certain trends can be appreciated even in these limited data. Four ml of culture media appear to be severely limiting, as reflected in the very low daily rates of HGH release. It also appears evident that the daily rate of HGH produced by the anterior pituitary cells *in vitro* is related to the age of the donor. High rates of HGH—3500 mμg, or more —are not observed in any instance of cultures initiated from fetal tissue of 14

C: 2% CO_2; 8-ml media vol											
12–14	12		12–14		14	14		20		24	
Hu-25	Hu-31		Hu-28		Hu-33	Hu-32		Hu-26		Hu-30	
	1	2	1	2		1	2	11	12	1	2
	2347	2400			2133	3195	1893				
100			379	1024				7200	10,200	17,600	8880
98	990	996	200	592	1030	1100	800	7467	6133	13,333	5280
72	547	667	1200	374	747	533	453	5240	3680	7400	3200
	200	300	80	232	560	340	390	4080	3200	5866	3280
	245	368			600	565	400	980	640	3800	1640
	216	184	110	172	440	340	340	533		3040	2213
			133	173	426	339	387			1573	746
			174	134				630			
			149	165		450	444	640	112		
						333	379	540	900		
								287			493
								90	10,600		464
								53			458
											320
								200			293
								227			
								533			344
								276			587
								290			240
								233			
								220			
								216			
								100			
								47			

weeks of age or less. Maximum daily rates—7000 mμg, or more—are observed only in cultures derived from fetal pituitaries of 16 weeks or older when 5% CO_2 is employed, and 20 weeks in the instance of 2% CO_2. The significance of the role of CO_2 remains to be elucidated.

In each human rocker culture established, the daily rate of elaboration of HGH was reduced after a variable period of time. In some instances, the initial rate of production appeared to be maintained for nearly 3 weeks (Hu-18—F-11, F-12) before the level began to fall. In others (Hu-26, Hu-30, Hu-15), the reduction in rate appeared to begin more promptly—within 1 week. In some instances the rate of elaboration of HGH dropped to a stable level which was maintained for periods of from 2 to 3 months (Hu-30, Hu-18).

Measurement of the cumulative total amount of growth hormone released into the media during a prolonged period of observation was attempted on cultures Hu-26 and Hu-30. In the two flasks from Hu-26, 201 μg of HGH was present over a period of 35 days (Flask 1), and 72 days (Flask 2), and in the two flasks of Hu-30, 295 μg in 29 days (Flask 1), and 94 μg in 57 days (Flask 2).

Growth hormone was not detected in control culture media or in culture media har-

vested from liver, thyroid, or adrenal cell cultures initiated from the same fetuses as were the pituitaries.

The biological activity of growth hormone released in the culture media was assessed by the rat tibial epiphysis bioassay (18). Culture media with growth hormone activity ranging from 7 to 11.33 μg/ml were pooled and were injected intraperitoneally daily for 4 days into hypophysectomized immature female rats. One, 3, 5 ml of fresh culture media were injected in a similar fashion as a control. A definite increase in tibial epiphyseal width (more than 40 μ) was observed in the groups receiving the higher doses of the pooled culture media (Table II).

Leuteinizing and thyrotropic hormones were tested for and were found in the culture media from Hu-30. As in the case of growth hormone, there was a progressive decline in the level of these two hormones (Fig. 1).

Generally, there appeared to be an uncertain correlation between the rates of cellular growth, morphologic appearance, and level of hormone elaboration. Cellular growth resembled explant outgrowth from the larger clumps of cells and tissue since only on occasion did it appear that isolated cells formed true colonies. The cells contained in the sheets appeared to be of several types, the majority being both granular and pleomorphic. Periodic acid Schiff (PAS) positive granular cells as well as extensive mitotic figures were identified during the active phase of growth. The growth appeared to be rapid initially, and then to reach a plateau wherein further sheeting and mitosis did not occur.

Discussion. This study was carried out as part of an overall program to study the physiology and growth *in vitro* of differentiated mammalian cells which are representative of the cells in their tissue of origin. Pituitary cells are especially suitable for such a study because of their ability to produce hormones which may be used as metabolic or functional tags and because of the availability of extremely sensitive and precise radioimmunoassay methods for quantitative assessment. Our initial experience, reported herein, was limited to human pituitaries because of

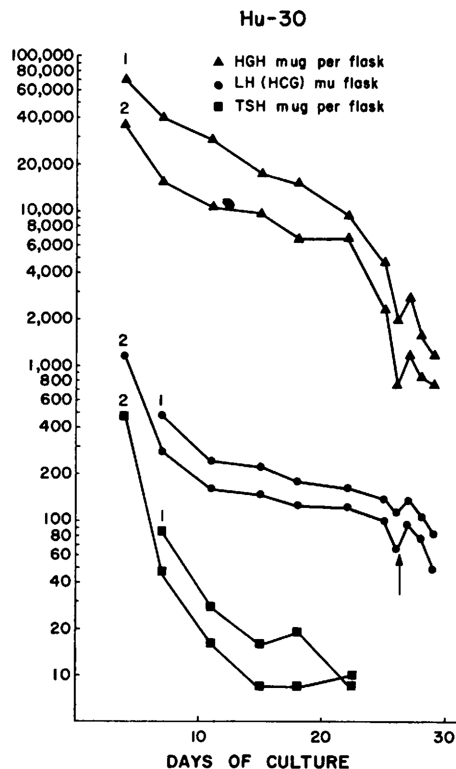

FIG. 1. Levels of HGH, leuteinizing hormone and thyrotropin in the two flasks of Hu-30.

TABLE II. Rat Tibial Epiphysis Bioassay of Growth Hormone in Pooled Pituitary Culture Media.

Group	Culture madia (ml/injection)	Av width of tibial epiphysis (μ) (2 tibias/animal) Mean	Range	No. of animals
Control	1, 3, and 5	135	119–154	6
Group I	1	153	142–170	3
II	3	175	164–190	3
III	5	195	168–205	3

the availability of radioimmunoassay to measure human growth hormone. Two unsuccessful attempts were made to grow pituitary cells from adult pituitaries obtained at necropsy. Subsequently, only pituitaries obtained from fetuses delivered by hysterotomy were used.

One of the cell cultures described in this study (Hu-4) continued to produce growth hormone for 150 days before the culture was discontinued. Many of the other cultures were lost through contamination or laboratory accident while they were still actively growing and producing hormones. With the exception of culture Hu-4, there was a progressive decline in the level of hormones in the culture media. A possible contributing factor to this decline is the lack of stimuli for hormone production similar to those which take place *in vivo*. There is abundant experimental evidence demonstrating that the hypophysis can function normally *in vivo* only if its anatomical connections are intact (21). Another interpretation is that the decline in hormone level in the culture media reflects a decline in functional capacity of the cultured cells because of failure to supply them with essential nutritional and environmental needs. Except for two brief reports (22, 23) and the present communication, cell and tissue cultures of nonneoplastic pituitary cells produced growth hormone for brief periods of 3–33 days only. Conventional cell culture systems display varying degrees of anoxia (13, 14). The role of pH and pCO_2, nutritional factors, the role of serum components and the incorporation of hormones into the culture environment may be equally vital to the role of oxygen (11). Thus, it seems imperative to establish optimum physiologic conditions prior to final assessment of the role of hypothalamic releasing factors and other hormones.

The emphasis in this investigation has been to study the morphologic and functional changes that occur in sustained primary cultures rather than to attempt, at this time, to establish cell lines via continuous subcultures. A seriously limiting aspect has been the difficulty of obtaining fresh human fetal tissue. This, in turn, has limited the number of cultures that could be established. We have since initiated studies employing calf pituitary tissues on organs wherein replicate cultures are readily attained, thus permitting the establishment of more definite experimental protocols for critical analysis.

This study confirmed the work of others in demonstrating the capacity of human fetal pituitaries to produce hormones. There was unequivocal demonstration of the presence of growth hormone, thyrotropin, and luteinizing hormones. Tests for production of other hormones have not yet been made. Pituitary cells from 8- to 9-week-old fetuses produced small amounts of growth hormone, which is in accord with the data of Kaplan and Grumbach (24) who found growth hormone on direct assay of extracts of human pituitaries as early as the 71st day of pregnancy.

Summary. Growth hormone (HGH) elaboration in culture media of 16 human fetal anterior pituitary cell cultures was studied using the rocker incubator system. HGH was measured by radioimmunoassay technique. HGH was demonstrated in all culture media from human fetal pituitaries but no such activity could be demonstrated from media obtained from other tissue cultures. There was a general correlation between the age of the fetus and the amount of HGH elaborated. In each of the cultures established, the daily elaboration of HGH was reduced after a period of time ranging from 1 to 4 weeks. The biologic activity of the hormone was confirmed in one sample by bioassay. Generally, there appeared to be no correlation between the rate of cellular growth, morphologic appearance, and level of hormone elaboration. The growth appeared to be rapid initially and then to reach a plateau. Luteinizing hormone and thyrotropic hormone were tested for and were found in the culture media of one pituitary.

The authors express their appreciation to the National Pituitary Agency, Endocrinology Study Section, and to the National Institute of Arthritis and Metabolic Diseases for providing the purified human growth hormone, the antihuman growth hormone serum, the purified human thyrotropic hormone (TSH) and anti-TSH serum.

1. Gey, G. O., Seegar, G. E., and Hellman, L. M.,

Science **88**, 306 (1938).

2. Jones, G. E. S., Gey, G. O., and Gey, M. K., Bull. Johns Hopkins Hosp. **72**, 26 (1943).
3. Waltz, H. K., Tullner, W. W., Evans, V. J., Hertz, R., and Earle, W. R., J. Nat. Cancer Inst. **14**, 1173 (1954).
4. Guillemin, R., and Rosenberg, B., Endocrinology **57**, 599 (1955).
5. Florsheim, W. N., Imagawa, D. T., and Greer, M. A., Proc. Soc. Exp. Biol. Med. **95**, 664 (1957).
6. Larson, E., and McLimans, W. F., Rep. Nat. Found. Infantile Paralysis, March, 1958, Amer. Chem. Soc. Meet., October, 1959.
7. Thompson, K. W., Vincent, M. M., Jensen, F. C., Price, R. T., and Shapiro, E., Proc. Soc. Exp. Biol. Med. **102**, 403 (1959).
8. Brauman, J., Brauman, H., and Pasteels, J. L., Nature (London) **202**, 1116 (1964).
9. Kobayashi, T., Kobayashi, T., Kigawa, T., Mizuno, M., and Amenomori, Y., Endocrinol. Jap. **8**, (3), 223 (1961).
10. Tashjian, A. H. Jr., Yasumura, Y., Levine, L., Sato, G. H., and Parker, M. L., Endocrinology **82**, 342 (1968).
11. McLimans, W. F., *in* "Axenic Mammalian Cells Reactions" (G. L. Tritsch, ed.), p. 307. Dekker, New York (1969).
12. McLimans, W. F., Mount, D. T., Bogitch, S., Crouse, E. J., Harris, G., and Moore, G. E., Annals N. Y. Acad. of Sci. **139**, (1), 190 (1966).
13. McLimans, W. F., Crouse, E. J., Tunnah, K. V., and Moore, G. E., Biotechnol. Bioeng. **10**, 725 (1968).
14. McLimans, W. F., Blumenson, L. E., Tunnah, K. V., and Moore, G. E., Biotechnol. Bioeng. **10**, 741 (1968).
15. Harris, G., Mount, D. T., McLimans, W. F., Tunnah, K., Scheele, S., and Moore, G. E., Biotechnol. Bioeng. **8**, 489 (1966).
16. Tunnah, K. V., McLimans, W. F., and Moore, G. E., Biotechnol. Bioeng. **10**, 698 (1968).
17. Schalch, O. S., and Parker, M. L., Nature (London) **203**, 1141 (1964).
18. Greenspan, F. S., Li, C. H., Simpson, M. E., and Evans, H. M., Endocrinology **45**, 455 (1949).
19. Wilde, C. E., Orr, A. H., and Bagshame, K. D., Nature (London) **205**, 191 (1965).
20. Utiger, R. D., J. Clin. Invest. **44**, 1277 (1965).
21. Reichlin, S., N. Engl. J. Med. **275**, 600 (1966).
22. Tashjian, A. H., Jr., Yasumura, Y., Levine, L., and Sato, G. H., Endocrine Soc. Program of the 48th Meet., June 27, 1966.
23. Kohler, P. O., Chrambach, A., and Ross, G. T., Excerpta Medica Int. Congr. Endocrinol. 3rd, Mexico, D. F., June 30-July 5, p. 72 (1968).
24. Kaplan, S. L., and Grumbach, M. M., Excerpta Medica, Int. Symp. Growth Hormone, Milan, Italy, Sept. 11–13, p. 51 (1967).

Received Dec. 17, 1969. P.S.E.B.M., 1970, Vol. 134.