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Effects of Fetal Brain Extract on the Growth and Differentiation of Rat Pituitary Anlage Cells (39840)[1]

HIROSHI ISHIKAWA, MASATAKA SHIINO, AND EDWARD G. RENNELS

Department of Anatomy, The University of Texas, Health Science Center at San Antonio, San Antonio, Texas 78284

It is an established concept that anterior pituitary cells differentiate from the epithelial cells of Rathke's pouch which develops in the fetus as an invagination of oral ectoderm. Since the anterior pituitary gland is already established by about the 15th day of fetal life in the rat, we have attempted to determine if the anlage cells are uncommitted or committed before 13 days of fetal age.

In studies utilizing encephalectomized fetuses Daikoku and co-workers (1) demonstrated cytodifferentiation of anterior pituitary cells. However, there is not good agreement among investigators (2-7) concerning the effects of releasing hormones or other substances on the cytodifferentiation of Rathke's pouch epithelial cells. As all of the above investigations were performed at a comparatively late stage of fetal development (after 16 days) it is probable that the anlage cells may have already been genetically committed to become specific types of anterior pituitary cells. Consequently, we have employed younger fetuses (11-13 days) in our experiments in order to study the effects of a fetal brain extract on the growth and differentiation of anterior pituitary cells. We have successfully established normal anterior pituitary cell lines from the anlage cells by growing cells in the presence of fetal brain extract, but adult median eminence extract was not effective in promoting the differentiation of cells.

Our results indicate that Rathke's pouch anlage cells require certain neural substances in fetal brain extract which are necessary for the differentiation of anterior pituitary cell lines, and it is postulated that Rathke's pouch cells at 11-13 days of fetal life have not yet developed *receptors* to adult neural substances such as those present in adult median eminence extracts.

Materials and methods. Rats of the Wistar-Imamichi strain were used in this study. Small pieces of tissue which included Rathke's pouch epithelium of 11 to 13-day-old fetuses were immersed in Rinaldini's solution (8) containing 2% rat serum (RBS solution) and 0.1% trypsin. After 15-30 min, the supernatant solution was decanted, and dissociation of the cells was carried out using the procedures of Steinberg (9) and Hopkins and Farquhar (10). Several NRR (normal rat Rathke) cell lines were established from dissociated cells by adding lyophilized fetal brain extract (FBE) (1 μg/ml) to growth medium (85% Ham's F10 medium and 15% fetal calf serum). Lyophilized material from median eminence extract (MEE) of adult rats and FBE was obtained by the method of Kuroshima *et al.* (11). Fibroblasts remaining in primary cultures of cells were removed using Tashjian *et al.'s* method (12) or by use of a micromanipulator equipped with a microglass tube (50 μm in diameter).

In order to establish clonal strains of multipotential progenitor cells, one NRR cell line (NRR-5) was cultured with MEE-(1 μg/ml) added to the growth medium. Clonal strains were established by the following procedure: Single cells picked up at random from NRR-5 cell cultures were grown in 0.55% agar solution dissolved in growth medium by using the feeder layer method in a modified Rose chamber. After clones were established, they were cultured and grown to confluence in 60 × 13-mm Falcon dishes under humidified atmosphere of 5% CO_2, 5% O_2, and 90% air.

After 4 days of culture, the conditioned

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media from the NRR cell lines and from the cell clones were analyzed by radioimmunoassay (RIA) for the six anterior pituitary hormones using the method of Berson and Yalow (13) for adrenocorticotrophic hormone (ACTH), the method of Niswender *et al.* (14) for prolactin, the method of Daane and Parlow (15) for follicle stimulating hormone (FSH) and luteinizing hormone (LH), the method of Odell *et al.* (16) for thyroid stimulating hormone (TSH), and the method of Birge *et al.* (17) for growth hormone (GH).

Several growth parameters of the NRR cell lines were studied. Standard methods were used to measure doubling time, plating efficiency, and cell viability (dye exclusion). The karyotype of each cell line was determined by the method of Rothfels and Siminovitch (18).

Results. In these studies, eleven NRR cell lines were established from Rathke's pouch epithelium of 11- to 13-day-old fetuses by supplementing the growth medium with fetal brain extract (FBE). In the absence of FBE or when adult rat MEE was substituted for FBE, the epithelial cells did not grow and the NRR cell lines could not be established. Furthermore, when the FBE was made from the brains of fetal rats 16 days of age or older, it was not effective in promoting the growth of the pituitary anlage cells. We presume that some factor must be present in the brains of fetal rats younger than 16 days which is necessary for the growth and development of pituitary anlage cells *in vitro*. This factor appears not to be present in the brains of older fetal rats or in the median eminence of adult rats. After the NRR cell lines were established, however, the addition of adult rat MEE was effective in promoting cell proliferation and growth. Thus, cell proliferation in the NRR-5 cell line was enhanced by the addition of adult rat MEE to the growth medium. A 5 μg/ml dosage of MEE was more effective than a 1-μg/ml concentration in promoting cellular proliferation (Fig. 1).

The NRR cell lines produced no detectable anterior pituitary hormones when cultured in growth medium alone. However, after the NRR cell lines were cultured for 4 days in growth medium containing adult

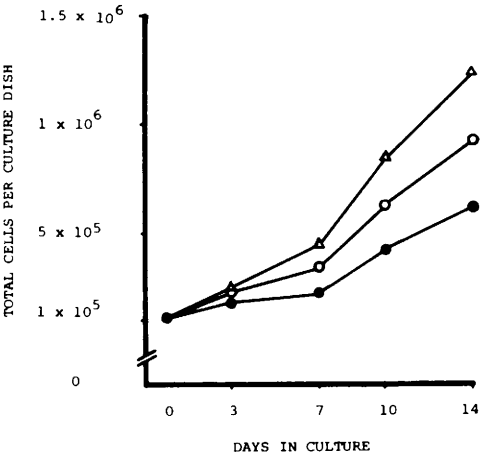

FIG. 1. The effect of a lyophilized preparation of adult rat median eminence extract on cell proliferation in the NRR-5 cell line. The mean number of cells per culture dish is shown for control cells grown in growth medium alone (●——●) and for cells grown with the addition of either 1 (○——○) or 5 μg/ml (△——△) of rat medium eminence extract to the medium. Each curve represents mean cell counts obtained from seven culture dishes. The standard error of the mean for each point on the graph was approximately 1% of the mean.

MEE (1 μg/ml), the conditioned media were shown by radioimmunoassay to contain from one to six anterior pituitary hormones (Table I).

One NRR cell line, NRR-5, produced detectable quantities of all six hormones. In the medium of NRR-11, five hormones (ACTH, GH, prolactin, FSH, and LH) were detected. NRR-1 and NRR-14 cell lines produced ACTH, GH, and prolactin. Similarly, FSH, LH, and TSH were detected in NRR-4; prolactin and ACTH in NRR-6; prolactin and LH in NRR-8; ACTH and LH in NRR-9; and NRR-10 and NRR-12 cell lines produced TSH only.

The doubling time, plating efficiency, and viability (dye exclusion) of all 11 cell lines were similar as shown in Table II. The karyotype of all of these cell lines was found to be diploid. The diploid chromosome number was found in 85–95% of the cells examined. The karyotype is stable and appears normal as indicated in Table III.

From the NRR-5 cell line, we derived 65 clonal strains of single cell origins. Conditioned media from 56 of these clonal strains have been analyzed for hormone production

TABLE I. HORMONE CONCENTRATIONS IN THE CONDITIONED MEDIA OF NRR CELL LINES.

Cell lines	ACTH[d] (ng)	GH[b] (ng)	PRL[c] (ng)	FSH[d] (ng)	LH[e] (ng)	TSH[f] (ng)
NRR 1	4.5 ± 0.6*	9.4 ± 1.5	11.2 ± 3.6	ND**	ND	ND
NRR 4	ND	ND	ND	7.6 ± 0.9	5.4 ± 0.6	11.8 ± 3.7
NRR 5	3.7 ± 0.7	7.4 ± 0.9	13.8 ± 2.7	8.9 ± 0.7	13.1 ± 2.4	25.3 ± 4.5
NRR 6	0.9 ± 0.1	ND	6.2 ± 1.1	ND	ND	ND
NRR 8	ND	ND	14.2 ± 5.6	ND	7.8 ± 0.9	ND
NRR 9	8.2 ± 0.9	ND	ND	ND	6.4 ± 0.8	ND
NRR 10	ND	ND	ND	ND	ND	15.7 ± 4.2
NRR 11	5.2 ± 0.7	4.6 ± 1.2	8.4 ± 0.9	4.6 ± 0.5	7.2 ± 0.8	ND
NRR 12	ND	ND	ND	ND	ND	13.6 ± 5.2
NRR 14	5.1 ± 0.4	8.2 ± 1.0	11.4 ± 2.6	ND	ND	ND
NRR 15	2.6 ± 1.2	1.5 ± 0.4	2.8 ± 3.2	2.3 ± 0.4	4.5 ± 0.7	13.6 ± 4.8

[a] In terms of Corticotropin A, minimum detectable level was 80 pg/ml.
[b] In terms of NIAMDD-rat GH-I-2, minimum detectable level was 1.2 ng/ml.
[c] In terms of NIAMDD-rat prolactin-RP-1, minimum detactable level was 5 ng/ml.
[d] In terms of NIAMDD-rat-FSH-RP-1, minimum detectable level was 2 ng/ml.
[e] In terms of NIH-LH-S17, minimum detectable level was 4 ng/ml.
[f] In terms of NIAMDD-rat-TSH-RP1, minimum detectable level was 8 ng/ml.
* Mean SE: the value is represented as nanograms per milligram of cell protein.
** ND = nondetectable.

TABLE II. PARAMETERS OF CELL GROWTH OF NRR CELL LINES.[a]

Cell lines	Plating efficiency (%)	Viability [Dye exclusion] (%)	Doubling time (days)
NRR 1	<1	90	2.3
NRR 4	<1	93	2.0
NRR 5	<1	92	3.5
NRR 6	<1	94	2.2
NRR 8	<1	91	1.7
NRR 9	<1	92	2.1
NRR 10	<1	94	2.4
NRR 11	<1	93	2.0
NRR 12	<1	91	2.3
NRR 14	<1	90	2.5
NRR 15	<1	92	2.7

[a] These cell lines were cultured in growth medium. These parameters of cell growth of NRR cell lines were calculated using 1×10^5 cells/ml at the 18th passage.

TABLE III. KARYOTYPE[a] OF NRR CELL LINES[b] DERIVED FROM RAT RATHKE'S POUCH EPITHELIUM.

Number of chromosomes	40	41	42	43	44
NRR 1	2	2	94	1	1
NRR 4	1	0	95	3	1
NRR 5	0	3	94	2	1
NRR 6	2	4	90	2	2
NRR 8	0	6	91	3	0
NRR 9	2	3	89	3	3
NRR 10	1	3	92	1	3
NRR 11	2	3	88	5	2
NRR 12	3	4	87	5	1
NRR 14	1	3	92	3	1
NRR 15	3	5	85	4	3

[a] Rothfels and Siminovitch's method (18) was used in this study.
[b] Chromosomes were counted in metaphase spreads of 100 cells from each cell line.

by radioimmunoassay. Eleven clones among the 56 secreted GH, prolactin, and ACTH; 13 clones, GH and prolactin; two clones, GH and ACTH; 19 clones, prolactin only; nine clones, GH only; and two clones, ACTH only (Table IV).

Discussion. By supplementing the growth medium with FBE we have established eleven NRR cell lines from Rathke's pouch epithelial cells of fetal rats of 11–13 days gestation. However, substitution of adult rat MEE for FBE did not permit the establishment of any Rathke's pouch epithelial cell lines. After the NRR cell lines were established, it was found necessary to supplement the growth medium with adult rat MEE in order to obtain any hormone production. Sixty-five clonal strains were then derived from the NRR-5 cell line, one of the NRR cell lines which secreted six kinds of anterior pituitary hormones into the culture medium when the medium was supplemented with adult rat MEE. By radioimmunoassay it was determined that 56 clones among the 65 clonal strains produced anterior pituitary hormones. About 34% of these clones produced only prolactin, and 16% of the clones produced GH only. Similarly, 23% of the clones produced GH and prolactin, 4% produced GH and ACTH, 20% produced ACTH, GH, and prolactin, and 4% produced ACTH only. These results suggest

TABLE IV. HORMONES[a] PRODUCED BY 65 CLONAL STRAINS[b] DERIVED FROM NRR-5 CELL LINE.

Clone number	Hormones	Clone number	Hormones	Clone number	Hormones
1B2	G P	4A2	G P A	5E4	G P
1B4	G	4C2	P	5E5	
1D7		4C3	G P	5F5	G P A
1E11	G P	4C4	P	5F7	G A
1F7	G	4C6	G P A	5F10	P
1G4	P	4D5	G	5F12	
1F2	G	4E5	G P	5G3	P
2A8	G P A	4E6		5H2	G P
2A9	G P A	4E10	P	5H5	P
2B7	G A	4F2	G P	6B7	G P
2B8	P	4F7	G P	6B10	G
2B10	P	4F11	P	6C7	P
2C6	G P A	4G7	G P A	6C8	G P A
2C11		4G8	P	6C9	G
2D2	P	5A6	G P A	6D3	G
2D6	G P	5A10	P	6E6	P
2D8	P	5B8	A	6E9	G P A
2D10	P	5B11		6F6	G
2D12	G	5C2		6G6	P
2E6	G P	5C9	G P	6H11	G P
2E10	G P A	5D11		6H12	
2G12	A	5E2	P		

[a] G = growth hormone, P = prolactin, A = ACTH.

[b] Hormone content of conditioned media was determined by radioimmunoassay. Standards and minimum detectable levels are given in footnotes (*a–f*) to Table 1.

that by 11–13 days of development some pituitary anlage cells are committed cells, while others are multipotential cells (uncommitted cells). Furthermore, it appears from our results that the growth and differentiation of the anlage cells *in vitro* depends on the availability of some unknown factor(s) in FBE. In our experience the fetal brain extract was essential for the establishment of the NRR cell lines. It is suggested that at this early stage of development some factor(s) in FBE (which is not present in MEE) may induce the formation of receptors for the hypothalamic hormones.

Gash *et al.* (19) reported that Rathke's pouch tissue from fetal rats of 11 days gestation did not develop into functional anterior pituitary cells after implantation into the hypothalamus of hypophysectomized adult female rats, and most of the anlage invariably produced neoplasms when they were obtained from 12-day-old fetuses. They also stated that cephalic mesenchymal cells are necessary for development of pituitary anlage cells into the anterior pituitary cells. In fact, the dissociated epithelial cells of Rathke's pouch may always contain mesenchymal cells which are removed with the epithelial cells when collecting the material. We could not estimate the effect of mesenchymal cells on cytodifferentiation of anlage cells in our experiment, but it is quite likely that some mesenchymal cells were included with the dissociated anlage cells.

Daikoku *et al.* (5) and Chatelain (7) in their encephalectomy experiments stated that pituitary anlage cells were autodifferentiated into anterior pituitary cells without any hypothalamic control. However, they employed 16-day fetuses, and it is generally known that the rat pituitary anlage cells are already differentiated into anterior pituitary cells and thus are genetically committed cells at 16 days of fetal age (20). The discrepancy between their findings and ours may be due to age differences in the fetuses employed in the two experiments. Furthermore, it is very difficult to determine whether or not their pituitary anlage cells were autodifferentiated into anterior pituitary cells in organ culture (1) since the organ culture must have contained not only Rathke's pouch epithelium but also cephalic mesenchymal cells and nerve cells which were impossible to remove.

Summary. Using dissociated Rathke's pouch epithelial cells from 11- to 13-day-old fetal rats as starting material, we have successfully established 11 NRR (normal rat Rathke) cell lines. It was necessary to supplement the growth medium with a rat fetal brain extract in order to induce cell growth and differentiation. These NRR cell lines produced no detectable anterior pituitary hormones unless adult rat median eminence (MEE) extract was added to the culture medium. Following the addition of MEE to the culture medium the NRR cell lines continued to produce from one to six anterior pituitary hormones. The NRR cell lines all show a normal diploid karyotype and have exhibited good growth characteristics through 18 cell passages. From one of the NRR cell lines (NRR-5) 65 clones of normal diploid anterior pituitary cells have been established. Fifty-six of these clones have been examined for hormone production, and each clone examined has been shown to produce one, two, or three hormones.

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