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# Human Prolactin Stimulates Estrogen Production by Feminizing Adrenal Neoplastic Cells (40012)

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*Introduction.* We have recently reported the establishment of a clonal line of human epithelial cells derived from a feminizing adrenal neoplastic gland (FANG) and designated them Fang-8 cells (1). In serial subculture, Fang-8 cells maintained the specific function of estrogen synthesis. Since normal adrenal functions are under the stimulatory control of adrenocorticotropin (ACTH) and possibly other pituitary hormones, we have carried out experiments to study the response of Fang-8 cells to possible stimulation by anterior pituitary hormones in culture.

*Materials and methods.* The cell culture environment was kept at 37°, 100% humidity, 5% $CO_2$–95% air. For replating, we transferred 5–10 × $10^4$ cells to each new Falcon 3003 petri dish (100 × 20 mm) and cultured them in 6–10 ml of regular medium [Matalon modified Eagle's medium (2) containing 10% each of calf and fetal calf sera]. To maintain the culture, medium was changed every 3 or 4 days. For experimentation, fresh medium was used at the beginning of experiments. If serum-free medium was used, the culture was limited to 24 hr or a shorter period of time. At the end of each experiment, the cells were harvested and washed three times with normal saline before cellular protein was determined by the method of Lowry *et al.* (3).

Estrogens secreted into medium were measured by radioimmunoassay technique (4) with or without Sephadex LH-20 column separation (5) for estrone ($E_1$) and estradiol-17$\beta$ ($E_2$). Fang-8 cells did not produce estriol ($E_3$) (1). When [$^{14}$C]acetate was added to medium as precursor, the radioactive estrogenic steroids secreted into medium were extracted alternately with ether and 1 *N* NaOH twice and then separated by the thin-layer chromatographic procedure (6). The identification of radioactive estrogens was achieved by precipitation with antisera specific to each steroid.

Pituitary hormones included human prolactin (hPRL) (73-4-27) which was generously donated by Dr. Henry Friesen, Manitoba, Canada; Cortrosyn (10 $\mu$g of $\beta^{1-24}$ACTH, equivalent to 1 USP unit), a product of Organon, Inc., West Orange, N.J.; and human GH (HS-2002F), human LH (LER-960), human FSH (LER-1575C), human thyrotropin (TSH-HS3), and rat PRL (I-1), all kindly supplied by NIAMDD, NIH, Bethesda, Md.

*Results.* Figure 1 shows the cell growth and estrogen production rates for the whole period of one subculture. In regular medium containing sera, cells started exponential growth on the third day after replating, as indicated by cellular protein. On the eighth day of subculture, cell growth began to decline and deceased cells floated in the medium. Cell growth was arrested completely after the 11th day, unless the surviving cells were immediately replated. Total estrogen production was correlated with cell protein during the subculture period. However, the $E_2/E_1$ ratio increased with the time of culture, from 0.33 on the second day to 2.6 on the eighth day. The synthesized estrogens were apparently secreted into medium constantly, as estrogen content inside the cells was always less than 0.1 pg/$\mu$g of cell protein, whereas medium content of estrogens might range from 2.8 to 8.8 pg/$\mu$g in 24 hr as calculated from data in Figs. 1A and B. When Fang-8 cells were cultured in serum-free medium for 24 hr, the cell growth was arrested and estrogen production was not halted, but was drastically reduced: The medium total estrogen content was 0.5–1.7 pg/$\mu$g (Fig. 1C). The



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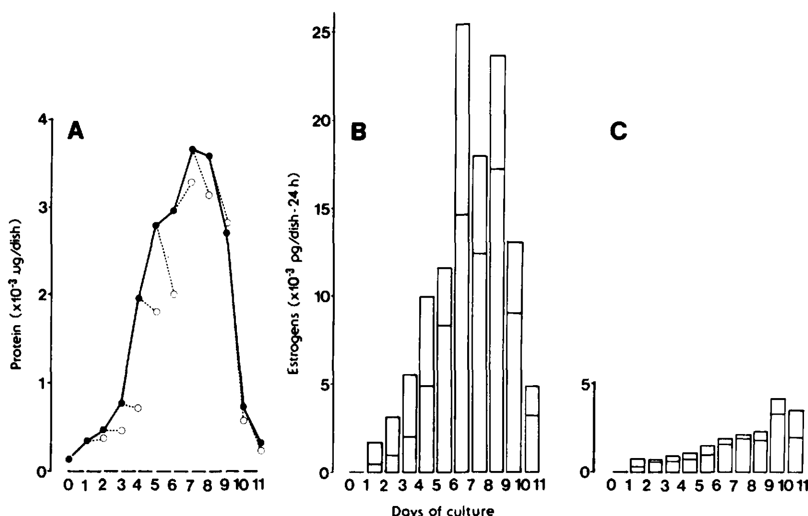


FIG. 1. (A) The growth curves of Fang-8 cells. Equal numbers of cells were replated to total 40 dishes on Day 0 and cultured in regular medium containing sera. From Day 1 on, four dishes were randomly taken daily during the following 10 days for 24-hr culture in 6 ml of fresh medium per dish: two dishes with regular medium containing sera (solid lines and circles) and the other two dishes with serum-free medium (dotted lines and open circles). The cells were harvested for protein determination. (B) The 24-hr production of $E_1$ (upper bar) and $E_2$ (lower bar) in medium, when cells were cultured in regular medium containing sera. (C) The 24-hr production of $E_1$ (upper bar) and $E_2$ (lower bar) in medium, when cells were cultured in serum-free medium. The values are the averaged means of two dishes.

$E_2/E_1$ ratio started at 0.35 on the second day and increased to 6.5 on the eighth day.

Fang-8 cells were able to synthesize radioactive estrogens from [$^{14}$C]acetate. In one experiment, Fang-8 cells on the fifth day after replating were cultured in regular medium containing sera and 1 µCi of [$^{14}$C]acetate per dish for various periods of time. The radioactivity incorporated into $E_1$ and $E_2$ increased with the time of exposure of Fang-8 cells to the isotope and amounted to 0.39 and 2.55 nCi at the end of 24-hr culture, respectively. The isolated radioactive $E_1$ and $E_2$ could be precipitated with excessive antisera coupled with a second antibody, up to 75 and 79%, respectively.

In regular medium containing sera, Fang-8 cells failed to be stimulated for estrogen synthesis by any single anterior pituitary hormone. However, using Fang-8 cells at their stage of exponential growth and cultured in serum-free medium, we could demonstrate a stimulatory effect of human PRL (1 µg/dish), as shown by the results in Table I. ACTH up to 10 µg, human GH up to 5 µg, and rat PRL up to 10 µg per dish failed to show a similarly stimulatory effect. Equivalent amounts of other human anterior pituitary hormones were ineffective. In a separate experiment, we further found that the stimulatory effect of human PRL on both $E_1$ and $E_2$ production by Fang-8 cells was a dose-dependent phenomenon (Table II). No similar dose–response relationship could be demonstrated with ACTH, hGH, and

TABLE I. EFFECT OF PITUITARY HORMONES ON ESTROGEN PRODUCTION BY FANG-8 CELLS CULTURED IN SERUM-FREE MEDIUM.[a]

| Hormone | Dose (µg/dish) | Cell protein (µg/dish) | Total estrogens in medium (pg/µg of protein in 24 hr) |
|---|---|---|---|
| None (control) | – | 1185 | 0.58 |
| hPRL | 1 | 1250 | 2.10 |
| ACTH | 10 | 1206 | 0.78 |
| hGH | 2 | 1191 | 0.82 |
| hLH | 1 | 1185 | 0.59 |
| hFSH | 1 | 1178 | 0.62 |
| hTSH | 1 | 1200 | 0.69 |
| rPRL | 1 | 1210 | 0.55 |

[a] Mean value of two dishes for 24-hr culture.

TABLE II. RELATIONSHIP BETWEEN DOSE OF HUMAN PRL AND ESTROGENS SECRETED BY FANG-8 CELLS INTO SERUM-FREE MEDIUM IN 24 hr.[a]

| Dose ($\mu$g/dish) | Cell protein ($\mu$g/dish) | $E_1$(pg/$\mu$g of protein) | $P^b$ | $E_2$ (pg/$\mu$g of protein) | $P^b$ |
|---|---|---|---|---|---|
| 0 (control) | 1445 ± 55 | 0.14 ± 0.04 | | 0.34 ± 0.05 | |
| 0.05 | 1430 ± 90 | 0.15 ± 0.05 | NS | 0.38 ± 0.06 | NS |
| 0.1 | 1440 ± 69 | 0.38 ± 0.14 | <0.01 | 0.53 ± 0.12 | <0.05 |
| 0.2 | 1480 ± 23 | 0.58 ± 0.06 | <0.001 | 0.95 ± 0.15 | <0.001 |
| 1.0 | 1466 ± 20 | 0.55 ± 0.10 | <0.001 | 1.20 ± 0.16 | <0.001 |

[a] Values are means ± 1 SD for four dishes.
[b] Compared with control group by Student's *t* test.

rPRL. The data in both tables also indicate that cell protein did not increase after the hormone treatment. The $E_2/E_1$ ratio remained constant after human PRL treatment (Table II). Thus, the action of human PRL on estrogen biosynthesis seemed to be specific, probably to affect selectively the steroidal biosynthesis by a mechanism involving some important enzymes but not the interconversion between $E_1$ and $E_2$.

When the time courses of estrogen production by Fang-8 cells cultured in serum-free medium with and without human PRL were compared, the response to the stimulatory effect of human PRL was found to be rapid and continuous (Fig. 2). By 3 hr of culture, the difference in medium $E_2$ content between PRL-stimulated and control cultures was significant ($P <$ 0.01). At the end of 24 hr, human PRL-stimulated cells secreted about three times the $E_2$ of the unstimulated culture. However, the $E_1$ production was stimulated to a lesser degree by human PRL.

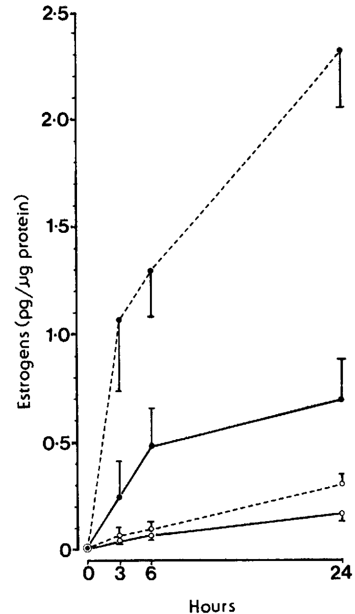


FIG. 2. Time courses of $E_1$ (open circles) and $E_2$ (solid circles) production by Fang-8 cells cultured in serum-free medium containing 0.2 $\mu$g of human PRL per dish (dotted lines) or no PRL as control (solid lines). The values are the means of four dishes and the vertical bars represent 1 SD.

*Discussion.* We were not totally surprised to notice that ACTH was ineffective in stimulating estrogen production by Fang-8 cells, as many adrenal tumors had failed to respond to ACTH (7). Millington *et al.* (8) recently reported that in primary tissue culture of a feminizing adrenocortical carcinoma, both ACTH and PRL stimulated the synthesis of estrogens and GH, LH, and ACTH were more effective than PRL in stimulating androgen synthesis. It is conceivable that being a clonal line, Fang-8 cells are a more specific type of cell culture than the mixed type of primary culture used by Millington *et al.* Furthermore, the difference between the two cultures could be accounted for if the two tumors were of entirely different types which nevertheless shared the characteristic of estrogen production. An earlier report by Mathur *et al.* (9) described another feminizing adrenocortical carcinoma which synthesized only estrone and $\Delta^5$-steroid sulfates *in vitro* but not estradiol-17$\beta$. There were several scattered reports published since the review by Gabrilove *et al.* (10), describing feminizing adrenal tumors. The steroidal and gonadotropin production by these tumors were obviously heterologous. The steroidogenic property of Fang-8 cells resembled that of the BeWo and Jar lines of pure trophoblastic cells

derived from human choriocarcinomas, as described by Pattillo *et al.* (11). The trophoblastic cells could synthesize estrogens from androstenedione and dehydroepiandrosterone, probably not from acetate.

The adrenal gland is one of the target organs of prolactin, as suggested by receptor study (12). There is no unequivocal evidence for secretion of estrogens by a normal adrenal gland (13). There is also only suggestive evidence that prolactin may alter sex steroidgenic pathways (14). Current efforts to define the relationship between prolactin and steroidogenesis are less successful. The results from our experiments clearly demonstrated that human prolactin had a stimulatory effect on estrogen synthesis by Fang-8 cells. This finding strongly indicates that such an action by prolactin may exist under pathological conditions. Vermeulen *et al.* (15) recently reported that a supraphysiological prolactin level might directly stimulate the adrenal cortex for increased production of DHEA-S and androgen. It is conceivable that the feminizing adrenal cortex cells have the capacity to utilize these steroids for estrogen biosynthesis subsequent to the stimulation of prolactin. The lack of effect of rat prolactin and human growth hormone may involve hormone receptor specificity and could be further investigated.

It should be noted, however, that human prolactin stimulated estrogen biosynthesis by Fang-8 cells when they were cultured with serum-free medium, which restricted cell growth and function (Fig. 1). It seems true that in order to demonstrate the stimulatory effect of a hormone in culture, the cells should be in a hormone-depleted state which will sensitize the cellular response. An excellent precedent was the stimulatory effect of thyroid hormones on the growth of $GH_1$ cells, which could not be documented, unless cells were cultured with medium depleted of thyroid hormone (16).

*Summary.* Using Fang-8 cells cultured in serum-free medium, we could clearly demonstrate a stimulatory effect of human prolactin on estrogen biosynthesis without concomitant stimulation of cell growth. The enzymic mechanism involved has not been defined. Human ACTH, rat prolactin, and other human anterior pituitary hormones were ineffective. This finding may bear some relevance to the pathological action of prolactin on the adrenal gland for sex steroidal biosynthesis.

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