Control of Cell Growth. I. Estrogen-Dependent Growth In Vivo of a Rat Pituitary Tumor

Cell Line

Joseph M. Sorrentino, Willis L. Kirkland, and David A. Sirbasku

SUMMARY—A subclone of rat pituitary tumor cells, designated GH3/C14, was isolated from the parent population of GH3 pituitary cells and was estrogen-dependent for growth in vivo. GH3/C14 cells inoculated into host animals formed tumors in intact females, estrogen-treated ovariectomized females, and estrogen-treated males, but not in untreated intact or castrated males, or untreated ovariectomized females. Exogenous treatment with estrogens promoted tumor formation in male animals. Radioimmunoassay of the average serum estradiol concentrations that support tumor growth in intact females, estradiol-treated intact females, and estradiol-treated intact males gave values of 41±4, 1,130±150, and 730±140 pg/ml, respectively. Tumor formation by GH3/C14 cells inoculated into male animals was not supported by either exogenous progesterone or hydrocortisone acetate. Further, these two steroid hormones had no significant effect on the estrogen-promoted growth in males or females. Exogenous testosterone treatment promoted tumor formation in males and ovariectomized females, but dihydrotestosterone was completely ineffective. Radioimmunoassay of the serum from tumor-bearing animals and the medium from the cells in culture showed that the cells produced growth hormone in vivo and in vitro but did not produce measurable amounts of luteinizing hormone or follicle-stimulating hormone. The growth of GH3/C14 cells in culture was examined in medium without added estrogen, and with estradiol added to the level of either the normal intact female rat or the estradiol-treated animals. No direct growth stimulation by estrogens could be detected in culture; the data suggested an indirect growth control mechanism.—J Natl Cancer Inst 56: 1149–1154, 1976.

Prolonged treatment of experimental animals with exogenous estrogens induces tumors of several tissues (1–9). In many of these instances, continued estrogen administration either stimulates or is essential for the growth of the transplanted tumor (1, 3, 5–9). One of the major barriers in the study of growth control of hormone-dependent tumors has been the difficulty in development of clonal cell lines that retain hormone dependence even after prolonged periods in culture and therefore permit extensive growth control studies under more defined conditions.

Here we describe the properties of an estrogen-dependent pituitary tumor cell line designated GH3/C14. This line was obtained as a subclone from the population of GH3 cells first established in culture by Tashjian et al. (10). The GH3 cell line was established from an MtTW5 tumor (11) that was induced by X-ray in a female WF rat. To obtain a stable, estrogen-dependent pituitary tumor cell line, we applied standard cloning techniques and thereafter examined the effect of several steroid hormones on tumor formation by GH3/C14 cells.

MATERIALS AND METHODS

Cultivation of cells.—The parent GH3 cell line produced rat growth hormone (RGH) and prolactin in vitro (12). The GH3/C14 cell line was obtained by standard low-density cloning techniques and by cloning after the formation of colonies in soft agar (13). The procedure is described in (14). The standard tissue culture medium used to grow GH3/C14 cells was Dulbecco's modified Eagle's medium (DME) (Grand Island Biological Co., Grand Island, N.Y.) as the high glucose concentration H-21 preparation; this was supplemented with 2 mM glutamine, 25 µg sodium ampicillin/ml, 121 µg penicillin/ml, 270 µg streptomycin/ml, 2.2 g sodium bicarbonate/liter, 12.5% horse serum (vol/vol), and 2.5% fetal calf serum (FCS) (vol/vol).

All sera used in these studies were obtained from Grand Island Biological Company and were used without heat inactivation. The cultures were maintained on Falcon tissue culture dishes at 37°C in a humid atmosphere of 95% air and 5% CO₂.

Animal experiment procedures.—Studies were done with 3- to 4-week-old WF rats from ARS-Sprague-Dawley, Madison, Wisconsin. The hypophysectomies were done by General Research Services Corporation, Albany, California. The rats were castrated 3 days before they were used in the experiments. Steroid hormones were administered as a single 25-mg pellet implanted sc in the left posterior cervical area. In all instances, the pellets consisted of a mixture of 90% steroid hormone (wt/wt) and 10% cholesterol and were prepared by compression of the powder mixture in a press obtained from Parr Instrument Company, Moline, Illinois. Pellets composed of any of the steroid hormones require at least 3 months to dissolve.

Radioimmunoassay procedures.—Total estrogens were extracted from serum samples with diethylether; the estratrien-17β-diol (estradiol) was separated from other estrogens by Sephadex LH-20 chromatography (15) and measured by the radioimmunoassay procedure of Emment et al. (16). This procedure measures only estradiol, and not 3-hydroxyestra-1,3,5(10)-tri-en-17-one (estrone) or estratrien-17β-triol (estradiol).
RGH was determined by the assay procedure described by Schneider et al. (17), whereas rat luteinizing hormone (RLH) and rat follicle-stimulating hormone (RFSH) were determined as described by Steinberger et al. (18).

RESULTS

Function of Serum in the Growth of GH3/C14 Cells in Culture

The growth of the GH3/C14 cells in culture was dependent to a marked degree on the serum used to supplement the medium. Text-figure 1 shows the growth of the cells in DME containing 5, 2.5, or 1% (vol/vol) FCS, yielding generation times of 30, 36, and 100 hours, respectively. Determination of the estradiol level in commercial serum (14) showed that medium containing 5% (vol/vol) FCS has an estradiol concentration of approximately 6 pg/ml. Text-figure 2 shows cell growth in DME supplemented with other sera. Growth in the presence of the standard combination of horse serum and FCS gives a doubling time of 36 hours, whereas growth in either 5% calf, or 5% sheep serum was considerably slower at 60 and 110 hours, respectively. Radioimmunoassay determinations of the average estradiol concentration of calf or sheep serum showed that the final concentration in the medium did not exceed 4 pg/ml (14).

The morphology of GH3/C14 cells growing in the standard DME containing the horse serum and FCS mixture is shown in figure 1. The cells grew as clusters that were either loosely attached to the dish surface, or floating freely as suspension colonies. This pattern of growth and morphology was not influenced by change in serum type or concentration.

Function of Estradiol in the Growth of GH3/C14 Cells In Vivo

The function of estrogens in tumor formation by GH3/C14 cells is described in table 1. Tumors did not form in normal or castrated males. In contrast, all males treated with exogenous estradiol developed large tumors. Other experiments (table 1) describe the effect of the endogenous female level of estrogen on tumor formation. All normal females developed tumors with an average mass of 2.9 g, whereas only 60% of the ovariectomized

<table>
<thead>
<tr>
<th>Animal groups*</th>
<th>Number with tumors/No. in group</th>
<th>Average tumor mass ±SE</th>
<th>Range of tumor masses, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal males</td>
<td>0/14</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Castrated males</td>
<td>0/6</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Normal males treated with estradiol</td>
<td>18/18</td>
<td>9.0±6.4</td>
<td>0.4-29.1</td>
</tr>
<tr>
<td>Normal females</td>
<td>25/25</td>
<td>2.9±2.6</td>
<td>0.3-10.0</td>
</tr>
<tr>
<td>Ovariectomized females</td>
<td>15/24</td>
<td>0.19±0.3</td>
<td>0.05-0.74</td>
</tr>
<tr>
<td>Normal females treated with estradiol</td>
<td>14/14</td>
<td>5.9±6.0</td>
<td>0.3-21.0</td>
</tr>
<tr>
<td>Ovariectomized females treated with estradiol</td>
<td>12/12</td>
<td>12.4±5.6</td>
<td>2.0-31.0</td>
</tr>
</tbody>
</table>

*3.6×10⁶ GH3/C14 cells were inoculated per animal. Tumors were collected for weighing 52 days after inoculation. Animals were exsanguinated by cardiac puncture, and the serum samples pooled for radioimmunoassay of estradiol content.
group had tumors (the average mass was 15 times less than that of tumors formed in normal females). Estradiol administration to ovariectomized animals caused formation of tumors that were an average of 60 times larger than those found in untreated ovariectomized hosts.

Radioimmunoassay of Serum Estradiol Levels

The serum estradiol concentrations of normal and estradiol-treated rats were determined by radioimmunoassay (table 2). The estradiol level that promotes tumor formation in normal females averaged 41 pg/ml and was within the range of female rat estradiol concentrations found by Butcher et al. (19). These investigators determined the plasma estradiol concentrations of normal female rats as a function of the estrous cycle. Their values ranged from a minimum of 17 pg/ml during metestrus to a maximum of 88 pg/ml at proestrus. The levels of estradiol in the serum of other animals groups studied are shown in table 2. Estradiol-treated males or estradiol-treated females have hormone levels about 20-30 times higher than do normal untreated hosts of the same sex.

Stimulation of Tumor Formation by Other Estrogens

Table 3 describes the results of tumor promotion by natural and synthetic estrogens. Exogenous administration of estrone, estradiol, and estriol were equally effective at promoting tumor formation in male rats.

Testosterone-Promoted Tumor Formation by GH3/C14 Cells

We presented evidence (table 1) that the normal male serum level of testosterone does not support the growth of GH3/C14 cells in vivo. However, in several experiments, we noted that marked elevation of serum testosterone by implantation of a pellet caused formation of large tumors in normal male and ovariectomized female rats (table 4). Male rats and ovariectomized females treated with exogenous testosterone developed significant tumors. To determine whether other androgens could stimulate tumor formation, we treated both normal males and ovariectomized females with dihydrotestosterone (DHT). Exogenous DHT was not effective in inducing tumor growth in normal males, and did not cause significant tumor formation in ovariectomized hosts (table 4).

To further clarify the testosterone stimulation of tumor growth, we measured the estradiol concentration in the serum of the testosterone-treated males. The results (table 2) showed an elevated estradiol concentration of 225±40 pg/ml in the serum of males treated with testosterone pellets. Production of a limited amount of plasma estrogen by the adrenal cortex is well known (20), and it is possible that elevated levels of testosterone increase this production. Testosterone is a known precursor of estradiol in the adrenal cortex, whereas DHT has not been identified as an estradiol precursor (20). However, from the data presented in this report, we cannot completely exclude the possibility that testosterone alone stimulates GH3/C14 cell growth in vivo.

Effects of Progesterone and Hydrocortisone Acetate on Tumor Formation by GH3/C14 Cells

Two other classes of steroid hormones known (8) to have marked negative effects on growth of estrogen-dependent tumors are progestins and corticosteroids. We investigated the effects of these types of steroids as sole growth-promoting agents and as antagonists of the estrogen-promoted tumor formation by GH3/C14 cells. hydrocortisone acetate was completely ineffective as a growth-promoting steroid in male rats. Further, simultaneous administration of estradiol and hydrocortisone acetate to male rats did not significantly affect the estrogen-promoted growth. Similar results were obtained when GH3/C14 cell growth in normal females was compared with that in hydrocortisone acetate-treated females. Progesterone did not support tumor growth in male rats, and when administered simultaneously with estradiol, did not influence significantly the estrogen-promoted growth.

Growth of GH3/C14 Cells in Hypophysectomized Rats

Tashjian and colleagues (12) showed that the parent GH3 cell line secreted RGH and prolactin in culture (12). We sought to determine whether the estrogens...
Table 5.—Radioimmunoassay determination of RGH, RLH, and RFSH in sera of normal and tumor-bearing rats

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Number of cells inoculated</th>
<th>Tumor-bearing animals</th>
<th>RGH, ng/ml b</th>
<th>RLH, ng/ml c</th>
<th>RFSH, ng/ml d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypophysectomized males</td>
<td>4.0 x 10⁴</td>
<td>No</td>
<td>1.0±0.5</td>
<td>20.7±8</td>
<td>119±36</td>
</tr>
<tr>
<td>Hypophysectomized males treated with estradiol</td>
<td>4.0 x 10⁴</td>
<td>Yes</td>
<td>620±50</td>
<td>16.2±4</td>
<td>105±45</td>
</tr>
<tr>
<td>Normal males treated with estradiol</td>
<td>4.0 x 10⁴</td>
<td>Yes</td>
<td>1,920±200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal males treated with estradiol</td>
<td>None</td>
<td>No</td>
<td>85±20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal males</td>
<td>4.0 x 10⁴</td>
<td>No</td>
<td>40±20</td>
<td>43±10</td>
<td>514±50</td>
</tr>
</tbody>
</table>

a Serum prepared from animal groups by cardiac puncture; all samples prepared by pooling serum from no less than 4 rats.

b Minimum sensitivity of assay; 0.2 ng RGH/ml serum.

c Minimum sensitivity of assay; 15 ng RLH/ml serum.

d Minimum sensitivity of assay; 60 ng RFSH/ml serum.

dependent subclone GH3/C14 also continues to produce RGH during in vivo and in vitro growth.

We examined the sera of hypophysectomized animals with GH3/C14 tumors for RGH, RLH, and RFSH by radioimmunoassay procedures (table 5). Two groups of hypophysectomized males were inoculated with equal numbers of GH3/C14 cells; one group was treated with exogenous estradiol, whereas the second group received no additional treatment. The group of hypophysectomized males treated with estradiol developed large tumors and also had serum RGH levels 600-fold greater than did the hypophysectomized males without tumors. Additional experiments with tumor-bearing male rats treated with estradiol showed RGH levels 22 times higher than those found in non-tumor-bearing normal males also receiving estradiol.

In contrast (table 5), the levels of RLH and RFSH in serum obtained from untreated hypophysectomized rats and tumor-bearing hypophysectomized animals were equal; this showed that such pituitary hormones are not produced in significant amounts by these cells growing in vivo.

To determine whether RGH secretion was continued by the cells under culture conditions, a radioimmunoassay comparison was made of RGH content of fresh tissue culture medium and of medium collected from the growing GH3/C14 cells after 4 days. Medium not previously used to grow cells gave an RGH concentration of approximately 6.5 ng/ml. This value presumably reflected the nonspecific cross-reaction of material in culture medium with antiglobulin to RGH. However, medium collected from growing cells contained 530±50 ng RGH/ml medium. We concluded that GH3/C14 cells continue the differentiated function of RGH secretion even under culture conditions. Attempts to estimate RLH and RFSH production in culture by radioimmunoassay procedures were unsuccessful, since the control medium contained unidentified components that yielded high blank values with the assay.

Estrogen Influence on the Growth of GH3/C14 Cells In Vitro

The data presented here describe the requirements for at least the normal female level of estrogen for growth of GH3/C14 cells in vivo. This concentration of estradiol measured in normal females averaged 41 pg/ml serum, but could vary with estrus and 17 to 88 pg/ml (19). Sera from animals treated with estradiol pellets gave values of 730–1,130 pg/ml. In contrast to these data, the cells were grown routinely in tissue culture medium containing a final concentration of 6 or less pg estradiol/ml. If estrogens were directly mitogenic for GH3/C14 cells, then growth in standard tissue culture medium was considerably suboptimal with respect to estrogens. Addition of estradiol to culture medium at a concentration equal to that of the pellet-treated rat should stimulate growth in vitro. Text-figure 3 shows the pattern of cell growth in medium prepared without added estrogen and in medium supplemented with either 1,000 pg estradiol or 1,000 pg estradiol/ml. Growth of GH3/C14 cells in medium containing either 5% calf serum or 5% FCS (vol/vol) was not influenced by either added estrogen.
Other concentrations of either estradiol or estriol (10,000 pg or 100 pg/ml) added to medium containing either calf serum or FCS also had no effect on growth.

**DISCUSSION**

Investigators studying the uptake of labeled sex steroids by another strain of GH3 cells (21) reported that weekly injections of estradiol valerate stimulated cell growth in male rats, and that these cells formed tumors in intact females but not ovariectomized females. However, the effects of other estrogens and steroid hormones on tumor formation were not explored further.

Evidence presented in this report shows that GH3/C14 cells grow in vivo in response to all of the estrogenic hormones tested, but not in response to nonestrogenic steroids. The only exception to this growth pattern appears to be testosterone, though we have shown (table 2) that exogenous testosterone administration to males was accompanied by an elevated level of serum estradiol. This estradiol elevation is presumably the result of testosterone influence of adrenal estrogen production.

From our data, we conclude that either estrogen per se or an estrogen-related control mechanism must be responsible for this selective pattern of GH3/C14 tumor cell growth. Text-figure 3 presents experiments demonstrating that cells grow with a generation time of 30 hours in culture medium supplemented only with 5% FCS, which has a final estradiol concentration of 6 pg/ml. Addition of either estradiol or estriol to the medium did not alter the growth rate. In addition, the GH3/C14 cells have been grown for 2 years in culture medium prepared with 12.5% horse serum and 2.5% FCS, which has a final concentration of 4 pg estradiol/ml (14).

Clearly, any of the serum-supplemented media used to grow the cells has an estradiol content well below that of the serum of a male rat. The most direct interpretation of these data is that estrogens are not directly mitogenic on these cells. In accompanying reports (14, 22) we continued the study of the growth requirements for GH3/C14 cells and identified hormones, other than estrogens, that are capable of promoting growth both in vivo and in vitro.

**REFERENCES**


(2) Allen E, Gardner WU: Cancer of the cervix of the uterus in hybrid mice following long-continued administration of estrogen. Cancer Res 1:359-366, 1941


(15) Hotchkiss J, Atkinson LE, Knobil E: Time course of serum estrogen and luteinizing hormone (LH) concentrations during the menstrual cycle of the rhesus monkey. Endocrinology 89:117-183, 1971


(18) Steinberger A, Chownbury M, Steinberger E: Effect of repeated replenishment of hypothalamic extract on LH and FSH secretion in monolayer cultures of rat anterior pituitary cells. Endocrinology 82:12-17, 1973

(19) Butcher RL, Collins WE, Fugo NW: Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17β throughout the 4-day estrous cycle of the rat. Endocrinology 94:1704-1708, 1974


FIGURE 1.—Morphology of the GH3/C14 cells growing in DME supplemented with 12.5% horse serum (vol/vol) and 2.5% FCS (vol/vol). × 100