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Prolactin and Growth Hormone Synthesis and Thymidine Incorporation in Dissociated Rat Pituitary Tumor Cells (42219)

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Abstract. The effect of *in vivo* diethylstilbestrol (DES) treatment on the MtT/W15 transplantable pituitary tumor was examined in dissociated pituitary cells by measuring the rate of incorporation of [^{3}H]thymidine into DNA and the synthesis of prolactin (PRL) and growth hormone (GH) as assessed by the rate of incorporation of [^{3}H]leucine. MtT/W15 transplantable pituitary tumors from rats treated for 3 weeks with DES showed significant reduction in the extent of [^{3}H]thymidine incorporation compared with tumor cells from untreated rats (2231 ± 182 vs 172 ± 17 dpm/10^5 cells; n = 3). In addition, tumor cells from DES-treated rats showed a significant increase in GH synthesis compared with tumor cells from untreated rats. In contrast to these findings, dissociated pituitary cells from non-tumor-bearing rats given 10 mg DES in Silastic tubing for 3 weeks showed a three-fold increase in PRL synthesis compared to cells from untreated control rats (29.3 ± 1.5 vs 10.0 ± 0.9% of total radioactivity in gel; n = 3. There was also a four-fold increase in the rate of [^{3}H]thymidine incorporation after DES-treatment in non-tumor-bearing rats (695 ± 114 vs 178 ± 13.9 dpm/10^5 cells; n = 3). These results indicate that DES inhibits MtT/W15 pituitary tumor cell proliferation, while stimulating synthesis of GH.

Chronic administration of estrogen is associated with hyperplasia of pituitary prolactin (PRL) cells and development of pituitary tumors in the rat (1–6). Furth *et al.* (7) produced several transplantable PRL-producing tumors, including the MtT/W15 and $MtTF_4$ tumors by DES treatment. Recent reports from our laboratory and by others have indicated that estrogens can inhibit the growth of the MtT/W15 transplantable rat pituitary tumors (8–10). The inhibitory effects of estrogens on growth of the $MtTF_4$ transplantable tumor have also been reported (11–13). Our recent analysis of the effects of diethylstilbestrol (DES) on the MtT/W15 tumor by immunohistochemistry and radioimmunoassay of serum hormone levels showed that DES produced an increase in GH-producing cells while decreasing PRL-producing cells and inhibiting tumor growth (10). The pituitary of the animals with MtT/W15 tumors developed pituitary PRL-cell hyperplasia concomitantly (10). The present study describes the analysis of PRL and GH synthesis *in vitro* and incorporation of [^{3}H]thymidine into DNA in dissociated cells from transplantable MtT/W15 pituitary tumors after 3 weeks of DES treatment. These results were compared with those seen in pituitary gland tissues from non-tumor-bearing rats after DES treatment. The aim of this work was to determine what changes in hormone synthesis are associated with inhibition of tumor growth.

Materials and Methods. *Animals.* Wistar Furth (40 days old) female rats were obtained from Harlan–Sprague–Dawley (Madison, Wis.). Animals were housed in a controlled 12-hr light, 12-hr dark environment and fed *ad libitum.*

Chemicals and other materials. Dow–Corning Silastic tubing and medical adhesive (silicone type A) were obtained from Metzger Medical (Brookfield, Wis.). [^{3}H]leucine (60 Ci/mole) and [^{3}H]thymidine (15.6 Ci/mole) were purchased from New England Nuclear (Boston, Mass.). Nonradioactive DES, leucine, and other chemicals were obtained from Sigma Chemical Company (St. Louis, Mo.). Culture media, sera, antibiotics, and trypsin were obtained from GIBCO (Grand Island, N.Y.). Sterile plastic wares were obtained from Flow Labs (McLean, Va.). Glass-fiber filters Whatman GFA and scintillation fluid were purchased from Fisher Scientific Company (Detroit, Mich.). Deoxyribonuclease was obtained from Amersham (Arlington Heights, Ill.).

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Implants. DES-containing implants were prepared as previously reported (6, 10). Briefly, Silastic tubings (inside diameter 0.198 cm, outside diameter 0.3175 cm) were plugged at one end with Silastic medical adhesive and then filled with 10 mg DES dissolved in absolute ethanol. After the alcohol evaporated, the other end was plugged and allowed to dry for 1 day. The Silastic tubes were placed in PBS–0.1% BSA overnight before implanting sc into the posterior neck area of 40-day-old non-tumor-bearing rats for 3 weeks or into rats with palpable MtT/W15 tumors for 3 weeks. Control rats received empty Silastic tubes.

To obtain tumors, rats were given 2-mm^3 tumor transplants at 40 days of age and they subsequently developed palpable tumors measuring 1–2 cm in maximum diameter by 70 days of age. Rats with palpable tumors subsequently received DES implants. Animals were sacrificed by decapitation.

Tissue dissociation. Pituitary tissues were collected aseptically and minced with a sterile scalpel blade in Dulbecco's modified Eagle's minimal essential medium (DMEM). Four pooled pituitaries from normal and DES-treated rats and two MtT/W15 tumors were used per group. Cell dissociation was done as previously reported (14). After rinsing, the pieces were dispersed in 0.25% trypsin in DMEM for 45 min at 37°C with gentle stirring. After mechanical dissociation with a Pasteur pipet the cell suspension was washed by centrifugation in DMEM with 10,000 U penicillin, 10,000 μg/ml streptomycin and 25 μg/ml fungizone supplemented with 2.5% fetal calf and 15% horse serum. The final cell suspension was counted in a hemocytometer and cells were diluted to a range of 1 to 5×10^5 cells/ml for subsequent studies. Viability was greater than 95% when tested with trypan blue.

Protein synthesis. After cell dissociation the dispersed cells were incubated with leucine-free DMEM for 2 hr and then the cells were incubated in duplicate dishes with 12.5 μCi [^{3}H]leucine/ml DMEM in a volume of 0.5 ml over 95% air–5% CO_2. The final concentration of leucine was made 30 μM by adding unlabeled leucine. Incubation was done for 4 hr at 37°. After incubation the medium was removed and the cells were rinsed with an ice-cold solution containing 0.15 *M* NaCl, 10 n*M* sodium phosphate (pH 7.4) and 1 m*M* leucine. Cells were counted with a hemocytometer and then homogenized in 200 μl of 0.1 *M* Tris (pH 7.3), 8 *M* urea, and 2% (wt/vol) sodium dodecyl sulfate (SDS) and stored at −20° until analyzed.

Analysis on SDS gels. Aliquots of the homogenate were thawed, heated at 90°C for 5 min, and then analyzed on SDS gels under reducing conditions containing 12.5% polyacrylamide using a Tris–glycine buffer system as described by Mauer and Gorski (15), except that 1.5 × 160 mm slab gels and the buffer systems of Laemmli (16) were used. PRL and GH standards (5 μg) from the NIADDK were added to each sample. Electrophoresis was done at 7 mA/gel. After electrophoresis, gels were stained for 30 minutes in a solution of 0.2% (wt/vol) Coomassie brilliant blue-R-250 in 50% (vol/vol) methanol and 7.5% vol/vol acetic acid solution. The destaining solution was changed daily for 4–5 days which helped to eliminate free, nonincorporated [^{3}H]leucine from the gels. Each lane of the gel was sliced into 2.0-mm sections and dissolved overnight in 1 ml of 20% H_2O_2 at 60°C. Samples were counted in 5 ml of Scintiverse Universal LSC cocktail in a Beckman LS7500 scintillation counter with an efficiency of 25–35%. The percentage of radioactivity in PRL and GH was determined by counting the radioactivity in the appropriate band and comparing it to the total counts in the gel.

Thymidine incorporation. After 2 hr of preincubation the pituitary cells in DMEM, 12.5 μCi of [^{3}H]thymidine was added to each dish, mixed, and incubated for 4 hr at 37° under 5% CO_2, 95% air as described by Peebles *et al.* (17). The culture medium was removed and cells were harvested and washed in PBS, pH 7.2, three times. After washing, the cells were counted in a hemocytometer then 200 μl of 50% TCA was added. Samples were stored at −20°C and subsequently filtered through GFA glass filters by vacuum. Each sample was then mixed twice with 3.0 ml 5% TCA and filtered, followed by 3 washes with 5 ml of 5% TCA. Aliquots of 100 μl were treated with 5 μl of deoxyribonuclease I (100 ng/ml) for 2 hr at 37°C, pH 7.5, in Tris buffer. After addition of 10 μl of 0.3 m*M* EDTA, samples were filtered and washed as before. Filters were counted in 5.0 ml of Scintiverse Universal LSC cocktail in a Beckman LS7500

Scintillation Counter with a 25–35% efficiency.

Immunohistochemistry. Slices of MtT/W15 tumor tissues from untreated rats and rats treated with DES for 3 weeks were fixed in buffered Formalin and prepared for immunohistochemical staining with PRL and GH antisera prepared in rabbits and monkeys, respectively (NIADDK). Immunohistochemical staining with the avidin-biotin peroxidase system and cell counts were done as previously reported (6, 10).

Statistical analysis. Statistical analysis was done with the Student t test. A P value of <0.05 was statistically significant.

Results. *Effects of DES on MtT/W15 tumors and on pituitaries.* DES produced an inhibition in the growth of MtT/W15 tumors during 3 weeks of treatment, while tumors from untreated rats continued to increase in size. Tumors from untreated rats weighed between 15 and 25 g, while rats receiving DES-treatment had small tumors which weighed between 0.50 to 0.90 g. Non-tumor-bearing rats with DES implants on Day 40 had an increase in pituitary gland weight which was about three times greater than control pituitary weights (7.6 ± 0.57 mg vs 23.6 ± 0.99 mg), respectively. Immunohistochemical staining and cell counts showed 66 ± 1.2% PRL cells and 24 ± 1.0% GH cells in tumors from untreated rats (n = 4) and 21 ± 4.7% PRL cells and 63 ± 4.3% GH cells in tumors from rats treated with DES for 3 weeks (n = 6).

PRL and GH synthesis. Incubation of cells with [^{3}H]leucine led to incorporation of tritium into PRL and GH. A SDS–gel electrophoresis of cell extracts from a MtT/W15 tumor is shown in Fig. 1. Although many protein bands were present after Coomassie blue staining, one major PRL and one major GH band co-migrated with the NIH standards for PRL and GH. Analysis of the radioactivity extracted from the gel indicated that the PRL and GH bands contained a significant amount of the total radioactivity. After 3 weeks of DES treatment the MtT/W15 tumors showed a significant increase in the rate of [^{3}H]GH synthesis compared to MtT/W15 tumors, from untreated rats (Table I). The surprising effects of DES treatment in rats with MtT/W15 tumors were further evaluated by examination of PRL and GH synthesis in pituitaries from non-tumor-bearing rats treated with DES for 3 weeks also. In pituitaries from untreated rats, GH was the major protein fraction identified. After 3 weeks of DES treatment there was a marked increase in PRL synthesis and a relative decrease in GH synthesis (Table I).

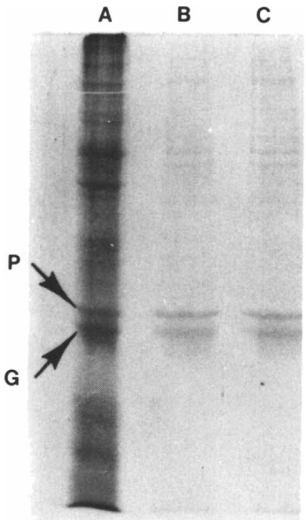

FIG. 1. SDS polyacrylamide gel electrophoresis on a slab gel of cell extracts from MtT/W15 mammosomatotropic tumor. Gel electrophoresis on 12.5% acrylamide was run under reducing conditions. Gels were sliced into 2-mm pieces and the radioactivity was extracted and counted. Extracts of 10^7 cells are shown in A and 10^6 cells in B and C. Purified rat PRL and GH from the NIADDK were used as protein standards.

Dissociated cells from the MtT/W15 tumor had the highest rate of [^{3}H]thymidine incorporation into DNA, while the rate of incorporation of radioactivity in tumor cells after DES treatment was significantly decreased (Table II). Treatment of aliquots of the TCA precipitates with deoxyribonuclease resulted in a 90% decrease in radioactivity after filtration, indicating that the [^{3}H]thymidine was incorporated into DNA. When pituitaries from non-tumor-bearing rats treated with DES for 3 weeks were incubated with [^{3}H]-thymidine there was a significantly higher rate of incorporation of radioactive label into the pituitary cells after DES treatment compared to pituitary cells from untreated rats.

Discussion. This study shows that the rate of [^{3}H]thymidine incorporation into dissociated pituitary cells, which is a measure of

TABLE I. EFFECT OF DES TREATMENT ON PROLACTIN AND GROWTH HORMONE SYNTHESIS BY DISSOCIATED RAT PITUITARY CELLS

Cell source	DES treatment	PRL synthesis: Percentage of total radioactivity	PRL synthesis: dpm/10^5 cells	GH synthesis: Percentage of total radioactivity	GH synthesis: dpm/10^5 cells
MtT tumor	−	6.7 ± 1.4	602 ± 220	2.4 ± 0.4	178 ± 72
	+	7.4 ± 0.4	528 ± 33	13.5 ± 1.0[a]	967 ± 49[a]
Pituitary	−	10.0 ± 0.9	2616 ± 340	12.3 ± 0.9	3175 ± 293
	+	29.3 ± 1.5[b]	9515 ± 762	6.0 ± 0.9[b]	1959 ± 336

Note. Dissociated cells were incubated for 4 hr with media containing [^{3}H]leucine, then homogenized and run on SDS–polyacrylamide gels under reducing conditions as described under Materials and Methods. The radioactivity recovered with PRL and GH standards was expressed as a percentage of the total radioactivity recovered from the gel. Values are from three separate experiments with duplicate samples. The pituitary cells were from non-tumor-bearing rats. Values are means ± SE for three experiments.

[a] $P < 0.001$ comparing tumors from untreated rats to tumors from rats treated with DES.

[b] $P < 0.001$ comparing pituitaries from DES-treated non-tumor-bearing animals.

cell proliferation, is inhibited in the MtT/W15 tumor by DES treatment. In addition, this study also suggests that the inhibitory effect of DES on growth of the MtT/W15 tumor is associated with an increase in GH production. However, several other possibilities must be considered in interpreting these results. Since the DES was administered for 3 weeks *in vivo* it is not known if the effects of DES treatment resulted directly from this estrogen, from a possible metabolite of DES, or if the effects resulted from other interactions in the intact animal. *In vitro* studies with cell culture are needed to show a direct effect of DES on GH and PRL synthesis and on the rate of [^{3}H]thymidine incorporation in the MtT/W15 tumor. Another problem in the interpretation of these results is that of the specific activity of radioisotopes in different cell populations. We cannot exclude the possibility that there was a differential rate of radiolabel uptake into different cells and/or different concentrations of substrates in the various cell populations. The results of the *in vitro* protein synthesis experiments are probably related to the distribution of the various cell types in the tumors before and after DES treatment. Immunohistochemical studies of the tumor with and without estrogen treatment showed a change in the percentage of immunoreactive PRL and GH cells with an increase in GH cells which agrees with the results of the protein synthesis experiments. Our previous analysis of serum hormone levels of PRL and GH in MtT/W15 tumor-bearing rats also showed a relative increase in serum GH levels after 3 weeks of DES treatment (10).

The complexity of proteins produced by the transplantable MtT/W15 tumor was reported by MacLeod *et al.* (18). In the present study one major band of PRL and of GH was seen after gel electrophoresis of protein extracts from the MtT/W15 tumor. MacLeod *et al.* also observed that pituitary glands obtained

TABLE II. EFFECT OF DES TREATMENT ON [^{3}H]THYMIDINE INCORPORATION IN THE MtT/W15 PITUITARY TUMOR AND IN PITUITARY CELLS FROM DES-TREATED ANIMALS

Cell source	DES treatment	[^{3}H]Thymidine dpm/10^5 cells
MtT tumor	−	2231 ± 182[a]
	+	172 ± 17.1
Pituitary	−	178 ± 13.9
	+	695 ± 114[b]

Note. Values are means ± SE for three experiments. Dissociated cells were incubated for 4 hr in media containing [^{3}H]thymidine, then precipitated with 50% trichloroacetic acid, filtered, and the radioactivity counted as described under Materials and Methods. Values are from three separate experiments with duplicate samples. The pituitary cells were from non-tumor-bearing rats.

[a] $P < 0.001$ comparing MtT tumors to MtT tumors from animals treated with DES.

[b] $P < 0.001$ comparing normal to DES-induced tumors from non-tumor-bearing animals treated with DES.

from rats bearing transplantable tumors weighed less than control pituitaries, indicating that the large amount of PRL produced by the tumors probably inhibited the growth of the animal's own pituitary (18).

The direct effects of estrogens on PRL cell proliferation in the normal pituitary *in vivo* and *in vitro* have been characterized extensively (5, 19). Other workers have also noted an inhibitory effect of estrogen on the proliferation of transplantable pituitary tumors (8–10, 13), but a relative increase in GH synthesis has not been previously reported. Lamberts *et al.* (9) noted a decrease in pituitary PRL levels after estrogen treatment in rats with 7315a tumors but they did not demonstrate changes in the GH or ACTH levels in the tumors. The effects of estrogens on inhibiting tumor growth has also been observed *in vitro* in the GH_4C_1 pituitary tumor cells. Amara and Dannies (20) and Kiino and Dannies (21) reported a concentration-dependent inhibition of GH_4C_1 cell proliferation. The increased production of GH in a cell line that normally produces both GH and PRL was reported by Tashjian *et al.* (22) who noted that glucocorticoids increased GH production in a clonal strain of a PRL and GH-producing cell line. This finding is similar to the effects of DES on MtT/W15 tumors *in vivo*.

Other workers have not found an inhibitory effect of estradiol on the growth of the MtT/W15 tumors (23). Winneker and Parsons reported that weekly injections of 600 ng/g body wt of 17β-estradiol stimulated growth of MtT/W15 tumors (23). Since we (10) have found an inhibitory effect of estrogens on MtT/W15 tumor growth, including an inhibition of PRL production, this suggests that the route and frequency of estrogen administration as well as the dosage are some of the factors that are critical in determining the effects of estrogen on tumor growth. Furth studied mammotropic (MtT) tumors extensively and his group produced the MtT/W15 tumor (11, 24, 25). He noted that the rate of growth of the MtT tumors was roughly proportional to the quantity of estrogen administered (24) and that even autonomous MtT tumors had retained responsiveness to estrogen. The maximal growth of autonomous MtT Strain F6 was seen in rats receiving one-tenth or less of the DES dose required to induce maximal proliferation of the pituitary (25). Our results using pharmacologic doses of DES [i.e., 10 mg of DES in Silastic tubes probably release about 45 μg/day of estrogens (5)] indicate that very high doses of estrogens can lead to inhibition of tumor growth.

The results of these studies indicate that DES has an inhibitory effect on the growth of the MtT/W15 transplantable pituitary tumor causing a decreased rate of incorporation of thymidine into DNA and an increased rate of synthesis of GH. DES also has a stimulatory effect on normal pituitary cell proliferation with increased PRL production and on thymidine incorporation in rat pituitaries from non-tumor-bearing animals. The mechanism by which DES and other drugs produce an increase in the rate of GH synthesis is not known. The MtT/W15 tumor can thus be used as a model to examine the regulation of expression of PRL and GH by steroids.

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