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Effects of Estradiol and Prolactin on Growth of Rat Mammary Adenocarcinoma Cells in Monolayer Cultures (39210)

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(Introduced by J. H. Weisburger)

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7,12-dimethylbenz(a)anthracene (DMBA)- induced rat mammary tumors are hormone-dependent. Extensive *in vivo* studies of this tumor model indicate that both prolactin and estrogen are required for tumor development (1-6), and it has been suggested that prolactin may act as the key promoter substance in mammary carcinogenesis (7). Paradoxically, high doses of estrogen inhibit growth of rat mammary tumors (3, 8). Meites *et al.* (9) showed that this inhibition could be counteracted by prolactin, and postulated that estrogen in large quantities blocked the "peripheral" action of prolactin. Pearson *et al.* (10) reported similar results.

It is difficult to study the mechanism by which prolactin and estrogen bring about their combined biological effects *in vivo* due to difficulty of monitoring their actions and interactions directly in the intact organism (4). Hormonal effects can be studied more effectively *in vitro* than *in vivo,* since the hormonal milieu can be more readily controlled. Hitherto, only organ culture experiments have been applied to the study of hormonal controls in mammary tissue. However, there are inherent disadvantages to organ culture experiments including short duration, individual variation, and limited amounts of material. Hence study of hormonal effects on growth are difficult to evaluate in a systematic manner. Accordingly, the present studies were designed to investigate the direct effects of prolactin and estradiol, alone and in combination, on the growth of rat mammary adenocarcinoma cells in monolayer cultures.

Materials and methods. The establishment of a pure epithelial cell line (AHF-RBA cells) derived from a DMBA-induced rat mammary adenocarcinoma and its characteristics have been described (11). The cells formed adenocarcinoma at the site of injection when newborn Sprague-Dawley rats were inoculated subcutaneously. The cells used were at 40-50th passages.

Charcoal extraction to remove steroids from fetal calf serum (Grand Island Biological Co., Grand Island, N.Y., Lot # C351019) was carried out by stirring the serum with 10 mg/ml Norit A (Sigma Chemical Co., St. Louis, Missouri) and 1 mg/ml Dextran T40 (Pharmacia, Uppsala, Sweden) (stock suspension in Dulbecco's saline) for 30 min at 55°, as described by Armelin and Sato (12). Total estrogen in the serum, measured by radioimmunoassay (13), was 228 pg/ml before and 12 pg/ml after charcoal extraction. The growth medium for routine maintenance consisted of Eagle's MEM supplemented with 10% fetal calf serum and glutamine.

Estradiol (Sigma Chemical Co., St. Louis, Missouri) was dissolved in absolute alcohol (1 mg/ml) and then diluted with normal growth medium to the final concentration indicated. Ovine prolactin (NIH-P-S11) (Hormone Distribution Program, NIA-MDD) was added to the medium directly. Media and supplements were prepared just prior to use.

The cells were plated by transferring an aliquot of trypsin-dispersed cells (about 50,000 cells) to petri dishes (60-mm petri dish, Falcon Plastics). Culture media containing hormones or alcohol were changed on Days 1, 4, and 7. At intervals, total protein in triplicate cultures was determined by the Lowry method as modified by Oyama and Eagle (14).

Results. Growth of rat mammary tumor cells in medium containing 1 or 10% serum from which steroids were removed by char-

coal extraction was similar to those grown in untreated serum (Table I). The cells grew faster in medium containing 10% serum than that grown in 1% serum.

Figure 1 represents the growth rate of cells in normal growth medium containing exogenous estradiol at concentrations ranging from 1 to 20 μg/ml. Estradiol inhibited growth of the cells in a dose-related manner: little or no effect at ≤1 μg/ml, inhibition at 5–10 μg/ml, and toxicity at 20 μg/ml. The cytotoxic effects of a high dose of estrogen include vacuole formation, rounding up and detachment from the surface of the culturing dish.

Figure 2 shows the effects of exogenous estradiol and prolactin alone and in combination on cell proliferation. A dose of 10 μg/ml of estradiol was selected for this purpose, since the growth inhibitory effect was pronounced at this concentration. It seems that exogenous prolactin alone, at concentrations <100 μg/ml had little or no effect on growth of the AHF-RBA cells. In addition, when prolactin at 20–40 μg/ml was added together with 10 μg/ml of estradiol, the inhibitory effect of estradiol on growth was not altered. However, when prolactin was increased to 50–100 μg/ml the inhibitory effect of estradiol was counteracted.

Discussion. Since mammary tumor growth was not affected by removal of estrogen from serum, it is assumed that the hormone present in commercial serum does not affect our experimental results. Our data indicate that estradiol, at a range of 12 pg/ml to approximately 1 μg/ml is sufficient to support mammary tumor cell growth. Further increases (>1 μg/ml) in exogenous

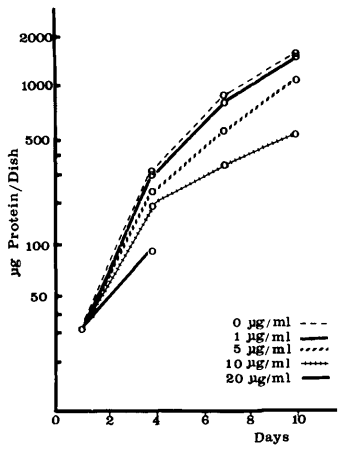

FIG. 1. The effects of estradiol on growth of rat mammary adenocarcinoma cells. Estradiol was added at medium changes at Days 1, 4, and 7. Each point represents an average of triplicate cultures.

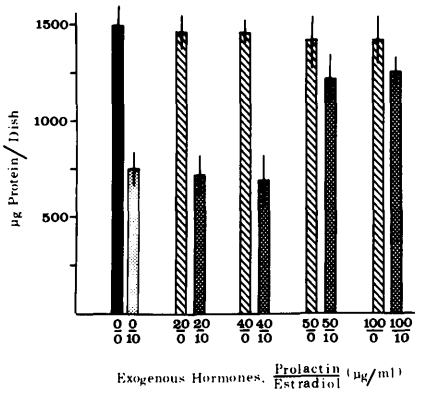

FIG. 2. Effects of estradiol and prolactin alone and in combination on growth of rat mammary tumor cells. The hormones were added to the cultures during medium changes at Days 1, 4, and 7. Total protein was determined after 10 days of culturing. Each bar represents mean ± SE.

TABLE I. GROWTH OF RAT MAMMARY TUMOR CELLS IN GROWTH MEDIUM CONTAINING UNTREATED FETAL CALF SERUM (FCS) AND CHARCOAL EXTRACTED FCS.

Medium	Growth rate (micrograms of protein per dish)[a]				
	Day 3	Day 4	Day 5	Day 6	Day 7
10% FCS	190 ± 50*	490 ± 50	670 ± 40	1150 ± 20	1160 ± 210
10% Charcoal Extracted FCS	190 ± 50	480 ± 50	660 ± 30	1070 ± 20	1100 ± 90
1% FCS	190 ± 50	340 ± 20	440 ± 20	630 ± 50	670 ± 30
1% Charcoal Extracted FCS	190 ± 50	310 ± 30	400 ± 30	560 ± 40	620 ± 90

[a] Protein value represents an average of triplicate cultures.
* Mean ± SD.

estradiol concentration depressed growth. Our findings are in agreement with studies by Welsch and Rivera (15) who reported that estrogen in pharmacological doses (5 μg/ml) inhibited, whereas physiological doses (0.0001–1 μg/ml) had no effect on, DNA synthesis in mammary tumor slices grown in synthetic medium. Estrogen in large doses is also known to inhibit mammary gland development (16) and lactation (4, 17) *in vivo*.

We demonstrated that exogenous prolactin alone failed to stimulate mammary tumor cell growth in monolayer cultures. Dao (5) also reported similar findings in *in vivo* and organ culture studies. In contrast, Welsch and Rivera (15) demonstrated that in short-term organ cultures of rat mammary tumors prolactin stimulated DNA synthesis. However, it is not known whether cell proliferation would ensue under such experimental conditions.

We have now found that prolactin at high concentrations counteracts the inhibitory effect of estrogen. As noted earlier, Meites *et al.* (4) and Pearson *et al.* (10) favor the notion that the tumor-inhibiting effect of large doses of estrogen *in vivo* might be due to a blocking of the peripheral action of prolactin. That is, estrogen acts indirectly by blocking the action of prolactin. Our results indicate, however, that estrogen at high concentrations has a direct cytotoxic effect on mammary tumor cells. Since prolactin is known to bind to the cell membrane (18), it could be that large amounts of prolactin might block the penetration of estradiol through the membrane, and thus prevent its cytotoxic action from being manifested intracellularly. However, this is unlikely, since Sasaki and Leung (19) recently demonstrated that prolactin increased the binding of estrogen to the receptor proteins intracellularly. Hence the mechanism by which prolactin counteracts the effect of estrogen remains to be elucidated.

Our studies demonstrated that the prolactin–estrogen balance present in fetal calf serum is suitable for growth and maintenance of rat mammary tumor cells in monolayer culture. However, when the prolactin: estrogen ratio is altered by exogenous addition of high doses of estrogen, cell growth is inhibited. The depressed state of growth evidently can be reversed by the addition of prolactin. Thus, our data suggest that prolactin and estrogen play a combined regulatory role in the growth of rat mammary tumor cells. The key appears to be the prolactin:estrogen ratio.

The results of our experiment indicate that, depending on relative concentrations, prolactin and estrogen can exhibit mutually antagonistic or synergistic effects on mammary tumor cell growth. Future studies will be directed toward elucidating the molecular basis of the changes reported here, such as those which occur at prolactin and estrogen receptor sites.

Summary. The effects of estradiol and ovine prolactin on the growth of a rat mammary adenocarcinoma cell line were investigated. The action of estradiol was dose-dependent. At ≤1 μg/ml it did not influence growth, at 5–10 μg/ml it was inhibitory, and at 20 μg/ml it was toxic. Prolactin alone had little effect on growth. However, the inhibitory effect of estradiol could be counteracted by a high concentration of prolactin. It appears, therefore, that the growth of cultured rat mammary adenocarcinoma cells can be regulated by the ratio of prolactin to estradiol: When the ratio is high growth is favored; when it is low, growth is inhibited.

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1. Huggins, C., Briziarelli, G., and Sutton, H. J., Exp. Med. **109,** 25 (1959).
2. Kim, U., and Furth, J., Proc. Soc. Exp. Biol. Med. **103,** 643 (1960).
3. Huggins, C., Cancer Res. **25,** 1163 (1965).
4. Meites, J., J. Nat. Cancer Inst. **48,** 1217 (1972).
5. Dao, T. L., *in* "Chemical Carcinogenesis, Part B" (O.P. Ts'o and J. A. DiPaolo, eds.), p. 503. Marcel Dekker, New York (1974).
6. McGuire, W. L., Chamness, G. C., Costlow, M. E., and Shepherd, R. E., Metabolism **23,** 75 (1974).
7. Furth, J., *in* "Prolactin and Carcinogenesis" (A. R. Boyns and K. Griffiths, eds.) p. 137. Alpha Omega

Alpha Publishing, Cardiff (1972).
8. Nagasawa, H., and Yanai, R., Intern. J. Cancer **8,** 463 (1971).
9. Meites, J., Cassell, E., and Clark, J., Proc. Soc. Exp. Biol. Med. **137,** 1225 (1971).
10. Pearson, O. H., Murray, R., Mozaffarian, G., and Pensky, J., *in* "Prolactin and Carcinogensis" (A. R. Boyns and K. Griffiths, eds.) p. 154. Alpha Omega Alpha Publishing, Cardiff (1972).
11. Cohen, L. A., Tsuang, J., and Chan, P. C., *In Vitro* **10,** 51 (1974).
12. Armelin, H. A., and Sato, G., *in* "Chemical Carcinogenesis, Part B" (O. P. Ts'o and J. A. DiPaolo, eds.) p. 483. Marcel Dekker, New York (1974).
13. Ferin, M., Warren, M., Dyrenfurth, I., Vande Wiele, R. L., and White, W. F., J. Clin. Endocrinol. Metab. **38,** 231 (1974).
14. Oyama, V. I., and Eagle, H., Proc. Soc. Exp. Biol. Med. **91,** 305 (1965).
15. Welsch, C. W., and Rivera, E. M., Proc. Soc. Exp. Biol. Med. **139,** 623 (1972).
16. Nagasawa, H., and Yanai, R. Endocrinol. Japon. **19,** 107 (1972).
17. Korsrud, G. O., and Baldwin, R. L., Canad. J. Biochem. **50,** 366 (1972).
18. Turkington, R. W., Cancer Res. **34,** 758 (1974).
19. Sasaki, G. H., Leung, B. S., Res. Commun. Chem. Pathol. Pharmacol. **8,** 409 (1974).

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