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Effects of Progesterone on Normal and Preneoplastic Mammary Development in Mice in Relation to Prolactin and Estrogen*

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Abstract—*As a possible step to evaluate the role of progesterone in normal and neoplastic mammary development with special reference to its relation to prolactin and estrogen, the effects of alteration of circulating level of progesterone by prostaglandin ($PGF_{2\alpha}$ or PGE_2) or an analogue of gonadotropin releasing hormone (TAP-144) on precancerous mammary hyperplastic alveolar nodules (HAN) were studied in female mice. Plasma levels of prolactin and progesterone were assayed by radioimmunoassay and estrogen level was estimated by the uterine weight.*

Daily injections of PGE_2 (50 μg × 2) or TAP-144 (10 μg) for 20 days *resulted in the increase in the rate of HAN formation associated with the elevation of circulating level of progesterone and no alteration of prolactin and estrogen. Furthermore, in ovariectomized, pituitary grafted mice given progesterone pellets and estrone as drinking water, the rate of HAN formation was found to depend upon progesterone level in the circulation. All observations indicate that elevated progesterone acts stimulatorily on HAN formation under no difference in the circulating levels of prolactin and estrogen.*

INTRODUCTION

WHILE there are several works on the role of progesterone in mammary tumorigenesis, its carcinogenicity is still far from conclusion [1–3].

One of the principal participations of prostaglandins in endocrine organs is the alteration of progesterone secretion from the ovary through their ovulation-inducing, luteotropic or luteolytic effects [4]. On the other hand, gonadotropin releasing hormone (GnRH) primarily stimulates the pituitary gonadotropin secretion and would secondarily modify the ovarian progesterone secretion.

In this paper, the effects of chronic treatment with prostaglandins ($PGF_{2\alpha}$ and PGE_2) and an analogue of GnRH on the growth of normal mammary glands, the formation of precancerous mammary hyperplastic alveolar nodules (HAN) and the secretion of prolactin, progesterone and estrogen were studied as a possible step to evaluate the role of progesterone in mammary tumorigenesis and its relationship to prolactin and estrogen. The effect of chronic administration of progesterone on HAN formation was also examined.

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MATERIALS AND METHODS

Animals

Three to 4 month-old SHN strain of female mice with mammary tumor virus [5] were used. They were kept six per cage in Teflon cages (15 × 30 × 12 cm) with wood shavings, maintained in an animal room that was air-conditioned (24 ± 0.5 °C and 65–70% r.h.) and artificially illuminated (14 hr of light from 5.00 a.m. to 7.00 p.m. and provided with a commercial diet and tap water *ad libitum*.

Treatment

Experiment I. Experimental mice received s.c. injections of 50 μg $PGF_{2\alpha}$ or PGE_2 (Upjohn Co., Kalamazoo, Mich., U.S.A.) dissolved in 0.05 ml physiologic saline twice a day for 20 days and once on the morning of

the 21st day. The control mice were given vehicle only.

Experiment II. Daily doses of 500 and 10 μg of an analogue of GnRH, TAP-144 [(D-Leu^6, des-Gly-NH_2^{10}, pro-ethylamide^9)-GnRH; Takeda Chemical Industries, Ltd., Osaka, Japan] [6, 7] were dissolved in 0.05 ml physiologic saline and each was injected i.p. for 20 and 50 days in experiments II_a and II_b, respectively. The injection was started to 16 weeks of age in experiment II_a and at 12 weeks in experiment II_b so that mice were killed at the similar ages in both experiments. The controls received vehicle only during the respective periods.

Experiment III. Mice were bilaterally ovariectomized. Each mouse was simultaneously given a single pituitary graft under the kidney capsule and s.c. implantation of progesterone [P(1)] or cholesterol only (C) or the mixture of both at the ratio of 1:10 [P(1/10)]. In all groups, the weight of pellet was 30 mg. All mice received estrone as drinking water at the concentration of 0.1 μg/ml. After 40 days of treatment, they were bled and killed.

Body weight change and estrous cycles (*experiments* I *and* II)

Body weight was measured at the beginning and at the end of experiment and the percent change in the weight was calculated. Vaginal smears were checked every morning in all mice throughout the experiments in experiments I and II_a and during the last 10 days in experiment II_b.

Uterine weight and ovarian histology (*experiments* I *and* II)

Mice were bled from posterior vena cava under the light ether anesthesia. After bleeding, mice were killed by decapitation and uterus was immediately removed, weighed and expressed in terms of mg/100 g body wt. Uterine weight was employed as the index of estrogen level in the circulation, because of the insufficient amounts of plasma samples, since blood was collected within 1.5 min in total to avoid possible influence of ether on prolactin secretion. Ovaries were fixed in Bouin's solution, embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin.

Plasma level of progesterone (*experiments* I, II *and* III)

Plasma progesterone level was assayed by radioimmunoassay. Progesterone fraction extracted with ether was dissolved in phosphate buffer and an antigen [progesterone-1,2,6,7-^{3}H (New England Nuclear Corp., Boston, Mass., U.S.A.)] and anti-progesterone-3-BSA rabbit serum (Teikoku Hormone Mfg. Co. Ltd., Tokyo, Japan) were added. After incubation overnight at 4°C, free and bound steroids were separated by dextran-coated charcoal and the radioactivity of the supernatant was counted by a scintillation counter.

Plasma level of prolactin (*experiments* I, II *and* III)

Plasma prolactin level was assayed by radioimmunoassay using the kit supplied by Dr. W. P. VanderLaan, La Jolla, Calif., U.S.A. Plasma samples mixed with anti-mouse prolactin rabbit serum were incubated for 24 hr at 4°C. Then, ^{125}I-labeled mouse prolactin and anti-rabbit gamma globulin were added after further 48 and 72 hr of incubation, respectively. After final 24 hr incubation, the mixture was centrifuged and the radioactivity of the precipitate was counted by a gamma counter [8].

Normal and preneoplastic mammary gland growth (*experiments* I, II *and* III)

Bilateral third thoracic mammary glands were used for wholemount evaluation and checked at 10 × magnification. The degree of growth of normal end-bud system was rated from 1 to 7 in increments of 1 [9] and the mean of the rating in the bilateral glands was employed for the representative value for each animal. The number of HAN was counted and the value for each mouse was expressed in terms of the sum of the numbers in the bilateral glands. The mathematical mean of the major two diameters of each HAN was also calculated as an index of the size.

Statistics

Significance of difference between groups in each parameter was evaluated by Duncan's multiple range test [10] except for plasma progesterone level in experiment III which was evaluated by the Kruscal–Wallis and Mann–Whitney U-tests [11].

RESULTS

EXPERIMENT I

Body weight change, estrous cycle, uterine weight and ovarian histology

The percentage changes in body weights during the experiment were 0.0 ± 0.1, -6.8 ± 0.8 and $-2.8 \pm 1.4\%$ in the control, mice given $PGF_{2\alpha}$ and mice receiving PGE_2, respectively.

Continued diestrous phase of the estrous cycle, which is one of the characteristics in SHN mice [12], was shortened and alternatively estrous and metestrous phases were more frequent by treatment with $PGF_{2\alpha}$ and PGE_2 (Fig. 1).

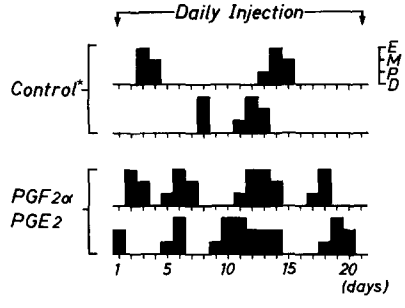

Fig. 1. Representative patterns of estrous cycles of SHN female mice treated with prostaglandins $F_{2\alpha}$ and E_2 ($PGF_{2\alpha}$ and PGE_2) (experiment I).
**See Materials and Methods for details of each treatment.*

While there was no difference in the uterine weight between mice given $PGF_{2\alpha}$ (383 ± 22 mg/100 g body wt) and mice receiving PGE_2 (428 ± 31 mg), both experimental groups were significantly higher than the control (325 ± 15 mg) in the weight (Fig. 2 C).

No apparent differences in the histological structures of ovaries were found between groups; in both experimental groups and the control, ovaries were normal and contained both follicles and corpora lutea at various stages of development.

Plasma level of progesterone (Fig. 2 A)

Plasma progesterone level of mice given PGE_2 (16.8 ± 1.3 ng/ml) was significantly higher than those of mice treated with $PGF_{2\alpha}$ (5.2 ± 0.6 ng/ml) and the control (9.7 ± 2.1 ng/ml), between which no significant difference was seen.

Plasma level of prolactin (Fig. 2 B)

There were little differences between groups in plasma prolactin level at autopsy.

Normal mammary gland growth (Fig. 2 D) and HAN formation (Fig. 2E)

Whereas development of normal end-bud system was similar in all groups, the number of HAN was significantly higher in mice receiving PGE_2 (29 ± 4) than in mice given $PGF_{2\alpha}$ (16 ± 4) and the control (18 ± 3).

Average size of HAN was different little between groups.

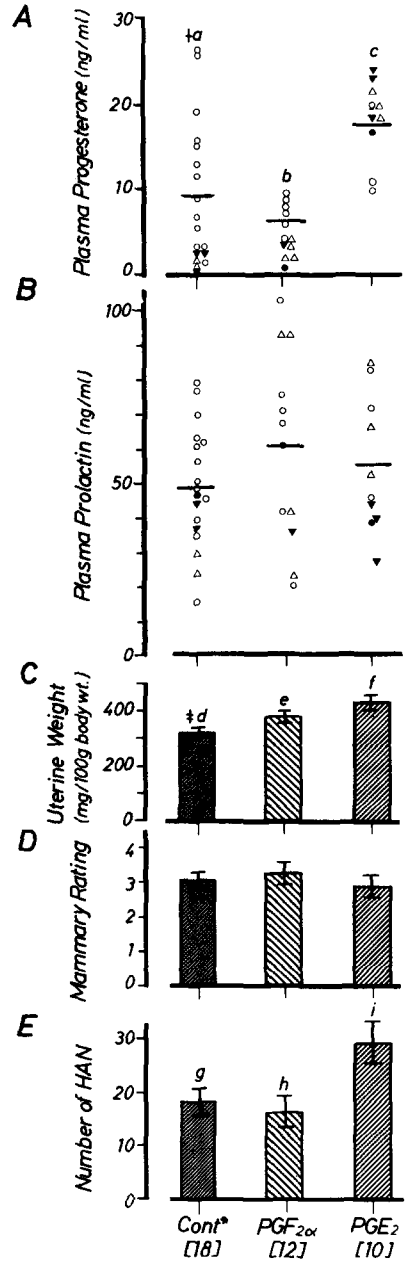

Fig. 2. Effects of prostaglandins ($PGF_{2\alpha}$ and PGE_2) on plasma levels of progesterone and prolactin, uterine weight as an index of circulating estrogen level, mammary rating and number of mammary hyperplastic alveolar nodules (HAN) in intact SHN female mice (experiment I).
**See Materials and Methods for details of each treatment. Number of estimates is indicated in the parentheses.*
†(○) Diestrus; (●) proestrus; (▼) estrus; (△) metestrus.
‡Means + S.E.M.
Significance of difference: d/e; g, h/i: $P<0.05$; a, b/c; d/f: $P<0.01$.

EXPERIMENT II

Body weight change, estrous cycle, uterine weight and ovarian histology

Body wt increased or changed little, but never decreased by the treatment with TAP-144 in both experiments II_a and II_b.

In experiments II_a, the cycles of mice treated with 500 or 10 μg TAP-144 showed further prolonged diestrous phases occasionally interrupted by estrus (Fig. 3A). In experiment II_b, all mice receiving 500 μg TAP-144 showed only persistent estrous vaginal smears during the last 10 days checked. The cycles of mice given 10 μg TAP-144 were little different from those of the control (Fig. 3B).

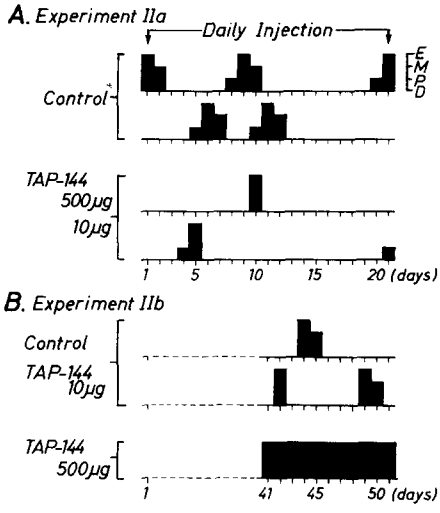

Fig. 3. Representative patterns of estrous cycles of SHN female mice treated with an analogue of gonadotropin-releasing hormone (TAP-144) (experiment II).

**See Materials and Methods for details of each treatment. Vaginal smears were checked for total period and the last 10 days in experiments II_a and II_b, respectively.*

There were little differences between groups in the uterine weight in experiment II_a (Fig. 4C). On the other hand, in experiment II_b, the uterine weight of mice given 500 μg TAP-144 (541 ± 15 mg) was significantly higher than those of mice treated with 10 μg TAP-144 (250 ± 11 mg) and the control (263 ± 25 mg) (Fig. 4C).

In both experiments II_a and II_b, the ovaries of the control mice were consisted of both follicles and corpora lutea at various stages of development (Fig. 5A). The ovarian structures of mice given 10 μg TAP-144 in experiment II_b were essentially similar to those of the controls. Meanwhile, corpora lutea and follicles were predominant in ovaries of mice receiving 10 μg TAP-144 in experiment II_a (Fig. 5B) and in ovaries of mice treated with 500 μg TAP-144 in experiment II_b (Fig. 5C), respectively.

Plasma level of progesterone (Fig. 4A)

In experiment II_a, treatment with 10 μg TAP-144 (11.4 ± 1.6 ng/ml) significantly elevated plasma progesterone level when compared to treatment with 500 μg TAP-144 (5.8 ± 1.2 ng/ml) and the control (4.9 ± 1.3 ng/ml).

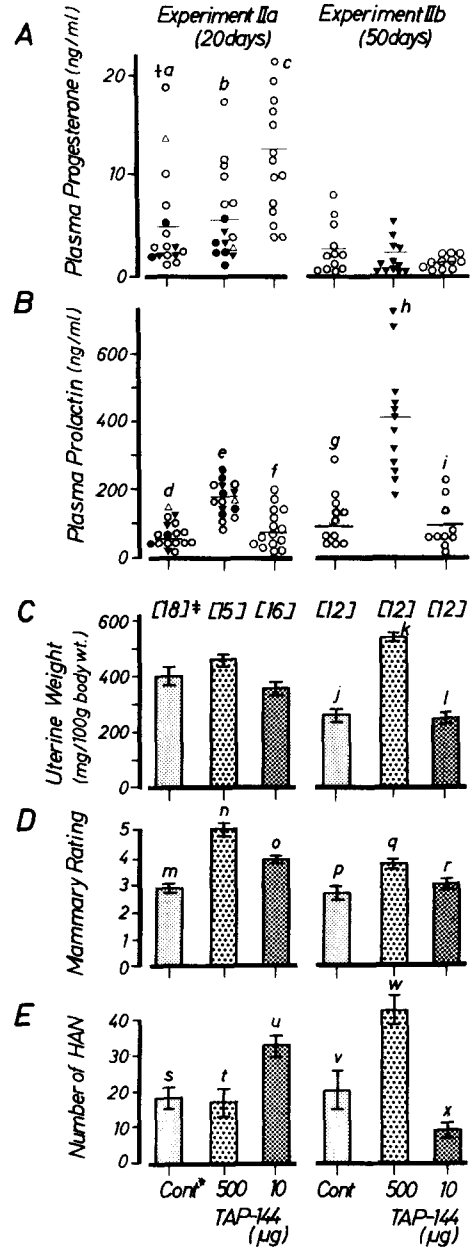

Fig. 4. Effects of an analogue of gonadotropin releasing hormone (TAP-144) on plasma levels of progesterone and prolactin, uterine weight as an index of circulating estrogen level, mammary rating and number of mammary hyperplastic alveolar nodules (HAN) in intact SHN female mice (experiment II).

**See Materials and Methods for details of each treatment*

†(○) *Diestrus;* (●) *proesterus;* (▼) *estrus;* (△) *metestrus.*

‡*Means* ± S.E.M.

Significance of difference: e/d, f: n/o: $P<0.05$: *a, b/c: h/g, i:k/j, l: m/n, o: p/q, u/s, t: w/v, x:* $P<0.01$.

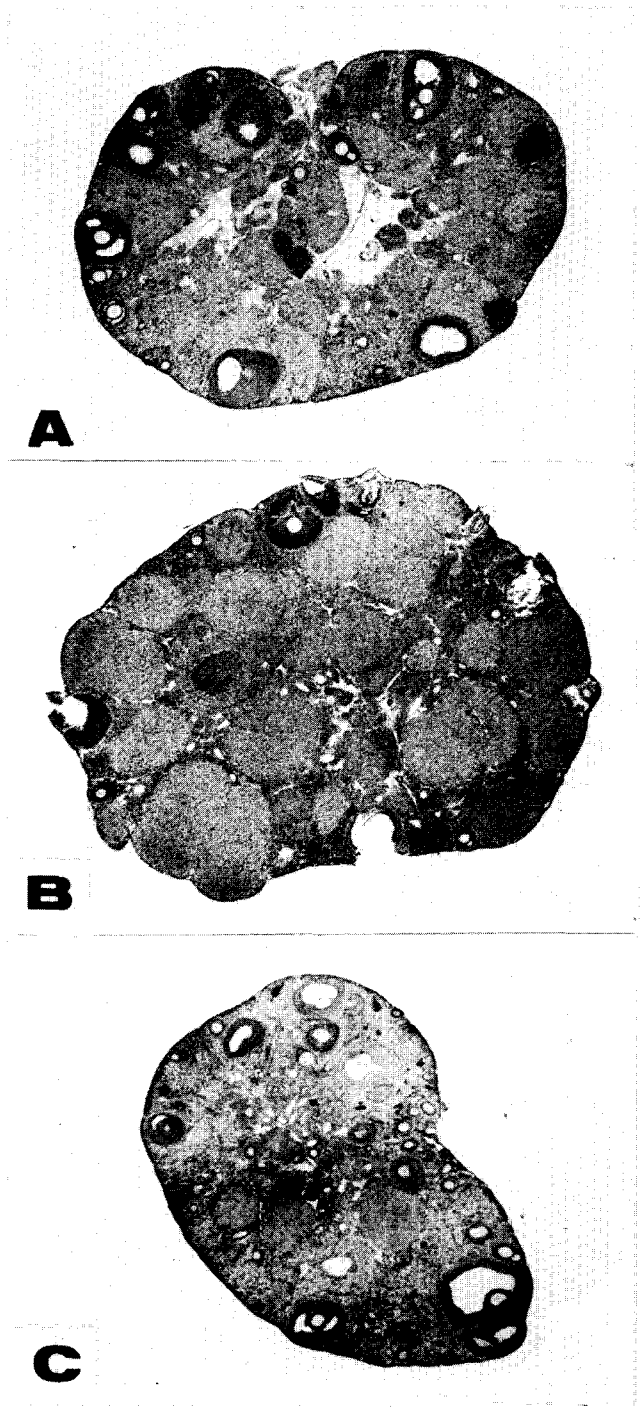

Fig. 5. *Representative ovarian structures of intact SHN female mice treated with TAP*-144 (*experiment* II). *Hematoxylin–eosin;* × 20.

(*A*) *Controls* (*experiments* II_a *and* II_b), *mice receiving* 500 μg *TAP*-144 *for* 20 days (*experiment* II_a) *and mice given* 10 μg *TAP*-144 *for* 50 days (*experiment* II_b). *Consisted of both follicles and corpora lutea at various stages of development.*

(*B*) *Mice receiving* 10 μg *TAP*-144 *for* 20 days (*experiment* II_a). *Corpora lutea are predominant.*

(*C*) *Mice receiving* 500 μg *TAP*-144 *for* 50 days (*experiment* II_b). *Follicles are predominant.*