Europ. J. Cancer Vol. 17, No. 5, pp. 503–509, 1981
Printed in Great Britain

0014-2964/81/050503-06$02.00/0

The *In Vitro* Mammary Gland Response to Mammotropic Hormones in Mice with Different Mammary Tumorigenesis*

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Abstract—*Mammary gland DNA synthesis estimated by the* in vitro *incorporation of* [^{3}H]*thymidine in response to mammotropic hormones was compared between high and low mammary tumor strains of virgin mice (SHN and SLN). In SHN, mammary gland DNA synthesis when cultured in the medium containing insulin (I), aldosterone (A), estradiol-17β (E), progesterone, prolactin (PRL) and growth hormone (GH) showed a peak on* day 2 *of culture and declined thereafter. Quite the opposite was the case in SLN mammary glands. There was little strain-difference in mammary gland DNA synthesis when cultured for* 6 days *in the medium containing complete hormone mixture. However, DNA synthesis of SHN mammary glands cultured in the medium deficient in PRL was less than one-third of the control, whereas that of SLN glands was two-thirds of the control. Moreover, mammary gland DNA synthesis was decreased significantly by deficiency in GH or E in SHN strain only. In both strains, mammary gland DNA synthesis declined with an increasing dose of PRL when cultured in the medium containing I, A and PRL, which was associated with an activated secretory function. However, the changes were much more marked in SHN than in SLN. The results have demonstrated the higher dependency of SHN mammary glands than SLN glands upon mammotropic hormones, especially PRL. They further indicate that mammary gland potential for both growth and function is well reflected by mammary gland sensitivity to PRL.*

INTRODUCTION

WHILE there are two factors for mammotropic hormones to manifest their effects on mammary glands, the circulating levels of the hormones and mammary gland sensitivity to the specific hormones, the latter is more primary than the former [1]. SHN mice were higher than SLN mice in mammary gland sensitivity to prolactin (PRL) or ovarian steroid hormones associated with higher normal and neoplastic mammary growth; mammary growth in response to pituitary grafting [2] or to the subcutaneous injections of estrogen and progesterone (P) [3] was much better in SHN than in SLN. However, these *in vivo* studies have provided no evidence as to whether PRL secreted from the grafted pituitaries and the injected ovarian steroid hormones stimulate mammary growth directly or indirectly through their effects on the *in situ* ovaries and pituitary or both. The role of growth hormone (GH), which may be secreted from the pituitary grafts, has also not been evaluated. Furthermore, in the observation of whole-mount preparations employed in these studies [2, 3], no information is available on the dynamic aspects of mammary gland response which is of most importance for mammary tumorigenesis [4, 5].

In this paper, mammary gland DNA synthesis was compared *in vitro* between SHN and SLN strains of mice under the different hormonal conditions as a possible step to evaluate more precisely the significance of mammary gland sensitivity to mammotropic hormones in normal and neoplastic mammary growth.

MATERIALS AND METHODS

Animals

SHN and SLN strains of virgin mice which have been established in our laboratory [2] were used at the 37th and the 31st generations, respectively. Mice were weaned at 20–25 days of age, kept six per cage and

Accepted 12 November 1980.
*This work was supported partly by the grant-in-aid for Cancer Research, from the Ministry of Education, Science and Culture, Japan (No. 401041).

maintained in an animal room air-conditioned (24±0.5°C and 65–70% relative humidity) 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*. Each mouse was pretreated with daily subcutaneous injections of 1 μg estradiol-17β (E) and 1 mg P for 3 days beginning at 32–35 days of age.

Culture system of mammary glands

Mice were decapitated on the day after the last injection and the second thoracic mammary glands were cultured on Dacron rafts in Waymouth medium containing several combinations of hormones by the whole-gland culture method [6, 7]. The hormones used were as follows; insulin (I: Sigma Chem. Co., St. Louis, MO, U.S.A.) 5 μg/ml; aldosterone (A: Sigma Chem. Co.) 1 μg/ml; PRL (NIAMDD-Rat prolactin-B-1 in experiments I and II and NIH-P-SII in experiment III*) 5 μg/ml; GH (NIAMDD-Rat GH-B-5) 5 μg/ml; E (Sigma Chem. Co.) 1 ng/ml; P (Sigma Chem. Co.) 1 μg/ml. Usually, one gland from each strain was cultured in the same dish and there was randomization of two glands from each mouse to treatments.

In Experiment I, the glands were cultured in 3 ml medium containing complete hormone mixture for 0 to 10 days to examine the changes in mammary gland DNA synthesis with the duration of culture.

In Experiment II, mammary glands from both strains were cultured for 6 days in the medium deficient in either PRL, GH, E or P. The culture period of 6 days was chosen, since little strain-difference was observed in mammary gland DNA synthesis in the presence of complete hormone mixture in Experiment I.

In Experiment III, mammary glands were cultured for 6 days in the medium containing complete hormone mixture or I+A+PRL (5 μg/ml) or I+A+PRL (50 μg/ml).

In all experiments, the medium was changed every 3 days.

Mammary gland DNA synthesis

At the termination of culture, each gland was removed from the Dacron raft and placed in an Erlenmeyer flask containing 1 ml of medium with the same hormones as in the culture. 2.5 μCi [^{3}H]thymidine (5 Ci/mmole; The Radiochemical Centre, Amersham, U.K.) was added and the flasks were incubated for 2 hr under a constant gassing with 95% O_2–5% CO_2. After the incubation, the gland was put into approximately 10 ml acetone, shaken gently for several hours, left overnight, removed and kept in a desiccator for 24 hr. The dry, fat-free tissue was weighed and [^{3}H]thymidine incorporated into mammary gland DNA was determined by liquid scintillation counting as an index of the rate of DNA synthesis. All procedures were the same as detailed previously [8]. Radioactivity was expressed in terms of dpm/mg dry weight of the tissue, since dry weight (mg) and DNA content (μg) of the tissue were highly correlated ($r=+0.989$, $n=18$, $P<0.01$).

Histological observation

At the termination of culture, portions of some glands were fixed in Bouin's solution and used for histological observation in Experiment III.

Statistics

Statistical significance of difference was tested by Student's *t*-test.

RESULTS

Experiment I

The change in mammary gland DNA synthesis with the duration of culture is presented in Fig. 1. DNA synthesis in SHN female mice

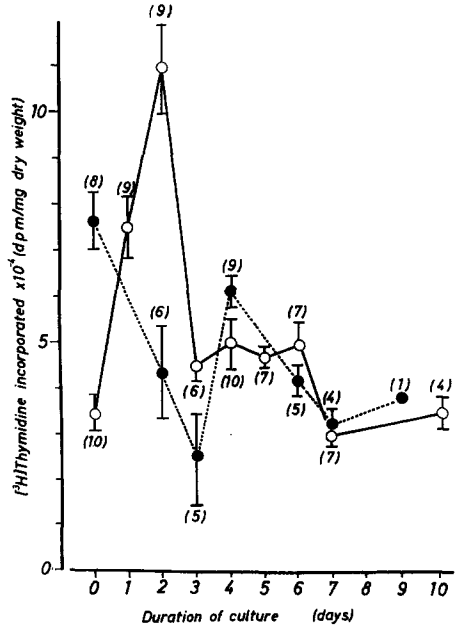

Fig. 1. Experiment I: *Changes with the duration of culture in* [^{3}H]*thymidine incorporation as an index of the rate of mammary gland DNA synthesis in SHN* (○) *and SLN* (●) *strains of female mice* (*means* ± *S.E.M.*). *The gland was cultured in Waymouth medium containing insulin* (5 μg/ml), *aldosterone* (1 μg/ml), *estradiol-17β* (1 ng/ml), *progesterone* (1 μg/ml), *prolactin* (5 μg/ml) *and growth hormone* (5 μg/ml). *On* day 0, *DNA synthesis was assayed without culture. The number of glands sampled is given in parentheses.*

*No difference in the activity between these preparations in both strains (compare the respective controls in Figs. 2 and 3).

was maximal on day 2 of culture, being approximately three times as high as the preculture level. The synthesis declined after 3 days, maintained it for some days and further decreased to the preculture level thereafter. The result is in accord with those of Banerjee [9] and Banerjee *et al.* [10] in which [^{3}H]thymidine incorporation or labeling index in mammary glands of BALB/c female mice cultured by the same procedure reached a maximum on days 2–3 of culture. On the other hand, quite the opposite was the case in SLN mammary glands; the preculture level was approximately twice as high in SLN mice as in SHN mice. The synthesis declined linearly, showed the lowest level on day 3 and rose significantly again after 4 days of culture.

Experiment II

As shown in Fig. 2, there was no significant strain-difference in the rate of mammary gland DNA synthesis of the controls cultured for 6 days in the medium containing complete hormone mixture.

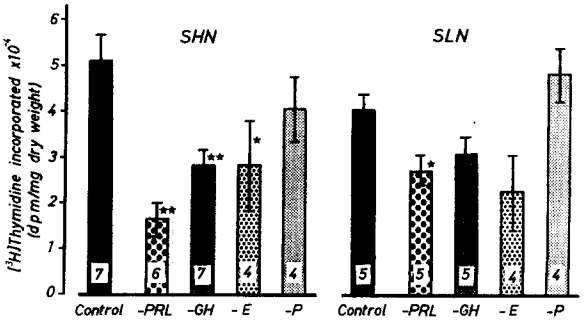

Fig. 2. Experiment II: *Effects of mammotropic hormones on* [^{3}H]*thymidine incorporation into mammary glands of SHN and SLN strains of female mice (means* $\pm$ *S.E.M.). The medium of the control contains insulin, aldosterone, estradiol-17β (E), progesterone (P), prolactin (PRL) and growth hormone (GH). The concentration of each hormone is the same as in Experiment* I. *The medium of the experimental group is deficient in either PRL, GH, E or P as indicated by the minus sign. The number in each column indicates the number of glands sampled. * and ** are different from the control at* $P<0.05$ *and* 0.02, *respectively.*

In SHN mice, DNA synthesis declined significantly in mammary glands cultured in the absence of PRL, GH or E, but not of P at doses employed. However, removal of PRL produced the greatest reduction in DNA synthesis; the rate of DNA synthesis in the PRL deficient group was about 32% of the control compared to about 55% in the group deficient in GH or E.

In SLN mice, mammary gland DNA synthesis was reduced significantly only in the absence of PRL to about 75% of the control, which was apparently higher than in SHN (32%).

Experiment III

In both SHN and SLN strains, DNA synthesis was high in the order of mammary glands cultured in the medium containing complete hormone mixture, glands in I+A+PRL (5 μg) and glands in I+A+PRL (50 μg) (Fig. 3). However, the rate of decline in the synthesis was more marked in SHN than in SLN; the difference between the former two groups was statistically significant only in SHN. While there were little strain-differences in mammary gland DNA synthesis when cultured in the medium containing complete hormone mixture, the synthesis of the other two groups was significantly lower in SHN than in SLN.

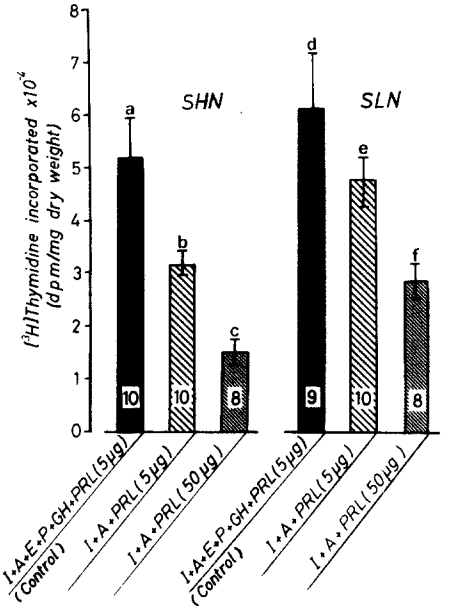

Fig. 3. Experiment III: *Effects of prolactin (PRL) on* [^{3}H]*thymidine incorporation into mammary glands of SHN and SLN strains of female mice (means* $\pm$ *S.E.M.). I, insulin; A, aldosterone; E, estradiol-17β; P, progesterone; GH, growth hormone. The concentration of each hormone is the same as in Experiments* I *and* II *except for* 50 μg *PRL. The number in each column indicates the number of glands sampled. a/b; a/c; b/c; b/e; d/f; e/f:* $P<0.01$. *c/f:* $P<0.05$.

Epithelial cells of mammary glands cultured in the medium containing complete hormone mixture were round and small and alveolar lumen was not formed in either strain (Figs. 4 A and B). On the other hand, in the glands cultured in the medium containing I+A+PRL, an increase in the formation of lobulo-alveolar system was observed and they contained colloidal substance in the lumina. Fat droplets were also seen in epithelial cells (Figs. 4 C–F). Whereas these secretory features were more marked in the higher PRL group in both strains (Figs. 4 C vs. E and D vs. F), mammary glands of SHN were much higher

than those of SLN in the activity (Figs. 4C vs. D and E vs. F).

DISCUSSION

Experiment I shows that there is a distinct difference between SHN and SLN mice in the pattern of response to mammotropic hormones, while the pattern of SHN mice is similar to that seen in BALB/c mice [9, 10]. These suggest the presence of strain-difference qualitative as well as quantitative in nature in mammary gland sensitivity to mammotropic hormones. In this respect, the apparent species- and strain-differences in the pattern of mammary gland response to PRL have been reported [11, 12].

In Experiment II, mammary gland DNA synthesis was significantly decreased when cultured in the medium deficient in either PRL, GH or E in SHN, but only in PRL in SLN. The results have demonstrated that SHN mice with a higher potential for both normal and neoplastic mammary growth [2] have higher mammary gland dependency upon mammotropic hormones than SLN mice and that PRL is essential in this process. Moreover, in Experiment III, SHN was found to be more responsive than SLN to PRL in mammary gland changes from a mitotic state to proliferative and functional states. All observations indicate that mammary gland potential for both growth and function is well reflected by mammary gland sensitivity to mammotropic hormones, especially PRL.

Singh *et al.* [13] examined morphologically the *in vitro* difference in mammary gland response to hormones between seven mouse strains with different mammary tumor potential and found that mammary gland sensitivity estimated by the minimal hormone requirement for lobulo-alveolar development appeared not to correlate with mammary tumor potential. The discrepancy between their and our results may partly be due to the difference in the index employed; the observation on the wholemount preparation is not always in accord with mammary gland DNA synthesis [12].

Acknowledgements—Our sincere thanks are due to Prof. M. R. Banerjee, Tumor Biology Laboratory, School of Life Sciences, University of Nebraska, Lincoln, Nebraska, U.S.A., and Prof. Y. Yokoyama and Dr. K. Ota, Department of Animal Breeding, Faculty of Agriculture, Nagoya University, Nagoya, Japan, for their invaluable suggestion. Technical help by Yuko Nakajima and H. Taniguchi is also acknowledged. Rat prolactin (NIAMDD-Rat prolactin-1), rat growth hormone (NIAMDD-Rat GH-B-5) and ovine prolactin (NIH-P-S11) were kindly donated by the Pituitary Hormone Distribution Program, NIAMDD, NIH, Bethesda, MD, U.S.A.

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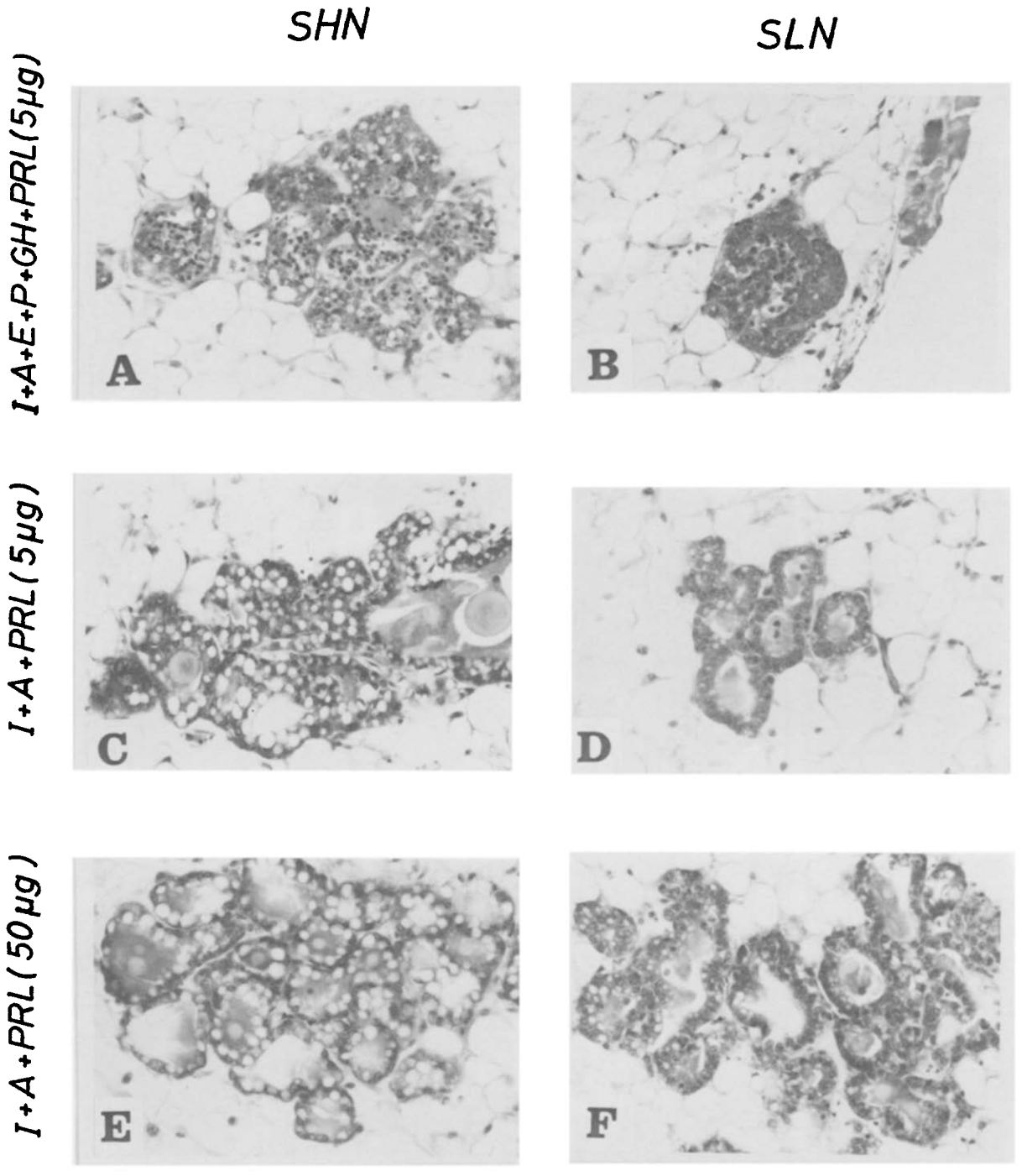

Fig. 4. *Experiment* III: *Representative histological features of mammary glands of each group in SHN and SLN strains of female mice (hematoxylin and eosin,* ×320). (A), (B): *Epithelial cells are round and small and alveolar lumen is not formed.* (C), (D): *The formation of lobulo-alveolar system is observed and colloidal substance and fat droplets are seen. The secretory activity in much higher in SHN* (C) *than in SLN* (D). (E), (F): *While the secretory activity increases in both strains, it is higher in SHN* (E) *than in SLN* (F).