

## HORMONAL REGULATION OF LOBULO-ALVEOLAR GROWTH, FUNCTIONAL DIFFERENTIATION AND REGRESSION OF WHOLE MOUSE MAMMARY GLAND IN ORGAN CULTURE

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(Received 17 May 1974)

### SUMMARY

The entire second thoracic mammary glands of 4-week-old BALB/c female mice primed with oestradiol plus progesterone were cultivated in organ culture medium containing the 'growth-promoting' hormone combinations: insulin, prolactin, growth hormone, oestradiol, progesterone and aldosterone or insulin, prolactin and aldosterone. Full lobulo-alveolar development was induced after 5–6 days of incubation and could be maintained for 15–16 days in organ culture in medium containing either hormone combination. After the initial 5–6 days in the 'growth-promoting' medium, subsequent cultivation of the glands in a medium with the 'lactogenic hormones', insulin, prolactin plus cortisol, led to accumulation of 'milk-like' secretory material in the ductal and alveolar lumina. Incubation of the lobulo-alveolar gland in medium with insulin alone for 7–9 days resulted in complete regression of the alveoli leaving only a ductal parenchyma. Incubation in insulin, prolactin, growth hormone or insulin plus the steroid hormones for 7–9 days led to considerable alveolar degeneration without a complete regression. The results indicate that both pituitary and steroid hormones are essential for development and maintenance of mammary alveoli; insulin can only sustain the basal ductal structure.

### INTRODUCTION

Animal tissue fragments incubated in the presence of plasma clot, embryo extract or chemically defined synthetic medium have been conventionally referred to as organ cultures. Similar cultures in synthetic medium have been extensively used in studies on the mechanisms of hormone action in the mammary gland (Elias, 1957; Mayne, Forsyth & Barry, 1968; Topper, 1970; Rivera, 1971, 1974; Turkington, Majumdar, Kadohama, MacIndoe & Frantz, 1973). Cultivation of the entire mammary gland in medium containing embryo extract and serum was initially attempted by Prop (1959). Subsequently, lobulo-alveolar development has been successfully induced in organ culture of the entire mammary gland of unprimed adult virgin mice maintained in medium containing hormones and serum (Prop, 1961, 1966) and of primed immature mice maintained in hormone-supplemented synthetic medium (Ichinose & Nandi, 1966). Since then the technique of culturing entire mammary glands of mice has been further improved with respect to optimum strain of mouse and gland used (Singh, DeOme & Bern, 1970; Banerjee, Wood & Kinder, 1973). The entire mammary

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gland organ culture is a useful tool in the study of the mechanisms of hormonal regulation of growth and functional differentiation uncomplicated by systemic factors. So far most cultures of entire mammary gland have been maintained for only about 5 days, and have been used for studying the growth of the lobulo-alveolar structures. The objectives of the present study were to investigate the hormonal regulation of lobulo-alveolar growth, functional differentiation and involution of the parenchyma during a longer period of culture.

## MATERIALS AND METHODS

### *Animals*

Four-week-old virgin BALB/c mice were primed daily for 9 days with s.c. injections of oestradiol (1  $\mu\text{g}$ ) and progesterone (1 mg) suspended in 0.9 % saline as described by Ichinose & Nandi (1966).

### *Culture methods*

Mice were killed by cervical dislocation, washed with 70 % ethanol and the second pair of thoracic glands, including the primary duct, were excised onto sterile dacron polyester rafts (Banerjee *et al.* 1973). Each excised gland on its raft was placed on a stainless steel grid contained in a Falcon plastic organ culture dish (Bioquest Inc.) with 1.0 ml Waymouth's (Waymouth, 1959) MB752/1 medium (Microbiological Associates) supplemented with 350.0  $\mu\text{g}$  glutamine/ml, 35.0  $\mu\text{g}$  penicillin/ml and various protein and steroid hormones (Table 1). Concentrations of the different hormones per ml of culture medium were: insulin (I), prolactin (Prl), growth hormone (GH), 5  $\mu\text{g}$  each; oestradiol, 0.001  $\mu\text{g}$ ; progesterone, aldosterone, 1  $\mu\text{g}$  each; cortisol, 5  $\mu\text{g}$ , as previously described by Ichinose & Nandi (1966) and Topper (1970). Incubation was carried out at 37 °C in a humidified chamber in an atmosphere of 95 % O<sub>2</sub> and 5 % CO<sub>2</sub> according to the standard procedure for mouse mammary gland organ culture (Rivera, 1971). The medium was renewed every 2–3 days. After the initial 5 days of incubation in the presence of 'growth-promoting' hormones, the glands were cultured in medium containing a 'lactogenic' hormone mixture for a further 5–6 days. Four different combinations of hormones were used to obtain regression of the lobulo-alveolar structures (Table 1).

Table 1. *Combinations of hormones needed to induce lobulo-alveolar growth, functional differentiation and regression of the mouse mammary gland in organ culture*

Hormones*	Biological activity
Insulin + Prolactin + Growth Hormone + Progesterone + Oestradiol + Aldosterone Insulin + Prolactin + Aldosterone	} Lobulo-alveolar growth
Insulin + Prolactin + Cortisol	} Secretory activity
Insulin	} Regression
Insulin + Growth Hormone + Prolactin Insulin + Progesterone + Oestradiol Insulin + Aldosterone	} Incomplete regression and poor maintenance of lobulo-alveolar structures

\* For concentrations of hormones see Materials and Methods. The hormone solutions used in the culture media were prepared and sterilized according to standard procedure (Ichinose & Nandi, 1966; Rivera, 1971). Sources of the hormones were as follows: prolactin (ovine, B grade, 1000 i.u./vial), aldosterone, cortisol, Sigma Chemical Co., St. Louis, Missouri; insulin (bovine, B-grade, 22.5–22.3 i.u./mg), oestradiol-17 $\beta$ , progesterone, Calbiochem, LaJolla, California; growth hormone (bovine, 1 U.S.P./mg), Miles Laboratories, Kankakee, Illinois.

*Morphological and histological preparation*

The gross morphology of the glands was studied using whole mounts prepared after fixation of the entire gland in modified Carnoy's solution (ethanol and glacial acetic acid, 3:1, v/v) for 2–4 h. The glands were stained with alum carmine, dehydrated in graded concentrations of ethanol and cleared in toluene. Glands subsequently used for histology were stored in toluene and whole mounts were stored in methyl salicylate. For histology, 5  $\mu\text{m}$  sections were stained with haematoxylin and eosin.

## RESULTS

At the start of culture, the morphology of the mammary gland was essentially ductal with some end-buds (Pl. 1, fig. 1). Consistent with earlier observations (Ichinose & Nandi, 1966), an extensive lobulo-alveolar development was induced in the glands after 5 days of incubation in serum-free medium supplemented with the hormones, I + Prl + GH + oestradiol + progesterone + aldosterone and similar lobulo-alveolar growth was present in glands incubated for 5 days in medium containing I + Prl + aldosterone (Pl. 1, fig. 2). The histology of the glands grown in the medium containing these two hormone combinations was similar (Pl. 1, fig. 2, inserts). However, the lumen of glands cultivated in I + Prl + aldosterone medium contained less secretory material and the epithelial cells appeared a little larger with vesicular nuclei. Continued incubation of the glands beyond 5–6 days in medium with either of the above two 'growth-promoting' hormone combinations failed to elicit further spatial growth of the lobulo-alveolar structures indicating that lobulo-alveolar development of the glands is mostly completed within the initial 5–6 days of organ culture. The mammary parenchyma at this time resembled that of the mid-pregnant animal. The lobulo-alveolar structures present in the gland after the initial 5–6 days of culture were mostly sustained when the glands were incubated in the presence of the 'growth-promoting' medium for another 10–12 days, with change of medium at 2–3 day intervals (Pl. 1, fig. 3). The histology (Pl. 1, fig. 3, inserts) of these glands was similar to that of glands after 5–6 days of incubation. After 18 days of incubation, however, occasional areas of necrosis were evident in the parenchyma distal to the primary duct and at present it is considered impractical to culture glands beyond 18 days.

In experiments involving induction of functional differentiation, the glands (after the initial incubation in the 'growth-promoting' medium for 5–6 days) were transferred to a medium containing the 'lactogenic' hormones I + Prl + cortisol. The glands incubated in this 'lactogenic' hormone medium for 5–6 days contained abundant 'milk-like' secretory material in the distended alveolar and ductal lumina (Pl. 2, fig. 4) suggesting a secretory function; whereas glands cultured in the 'growth-promoting' medium for the same period showed much less secretory activity (Pl. 2, fig. 5). Chemical analysis of the protein composition of the secretory material obtained in culture with 'lactogenic' hormones is in progress.

In the 'regression' experiments, lobulo-alveolar growth was induced in the glands by culture for 5–6 days in the 'growth-promoting' medium. Culture was then continued in medium with insulin alone for 6–7 days and as a result the lobulo-alveolar structures regressed completely (Pl. 2, fig. 6). The ductal arborization, however, was well preserved and the gland resembled the involuted mammary parenchyma of a parous animal 2 weeks after weaning. The histology of the regressed parenchyma in organ culture and *in vivo* was similar, showing disorganized areas of epithelial cells and well preserved ductal tissue (Pl. 2, fig. 6, inserts). The lobulo-alveolar gland in which secretory activity had been induced by incubation with 'lactogenic' hormones for 6 days followed by culture in medium containing insulin (for 5–6 days) also showed similar regression of the alveolar epithelium. The viability of the ductal

structure retained in the gland after regression of the alveoli in medium containing insulin was confirmed by its ability to produce ductal outgrowth after transplantation into 'gland-free' mammary fat-pads according to the procedure of DeOme, Faulkin, Bern & Blair (1959). Glands initially incubated in the 'growth-promoting' medium followed by 10 days of incubation in medium containing I+Prl+GH, I+progesterone+oestradiol or I+aldosterone showed poor maintenance of the lobulo-alveolar structures. Areas of alveolar degeneration and incomplete 'regression' were evident indicating that all three hormone combinations were inadequate for complete maintenance of alveolar structures. The results thus indicate that the biological cycle of growth, functional differentiation and regression of the mouse mammary gland can be mimicked in organ culture within a period of 2 weeks by incubating the entire gland in medium containing variable combinations of hormones (Table 1) and the culture can be maintained for a period of 15-17 days.

#### DISCUSSION

In the present study full lobulo-alveolar development of the mammary tissue of 4-week-old virgin BALB/c mice primed with oestradiol + progesterone was induced with hormones after 5 days of organ culture of the entire mammary gland, and the alveolar structures could be maintained in organ culture for 15-17 days. Two combinations of 'growth-promoting' hormones, I + Prl + GH + oestradiol + progesterone + aldosterone and I + Prl + aldosterone, were equally effective in promoting the development of lobulo-alveolar structures and also maintained these structures for an extended period. These results are in agreement with Ichinose & Nandi (1966) who also obtained full lobulo-alveolar development of the mammary gland on a synthetic medium containing I + Prl + GH + aldosterone, with or without ovarian steroids, during 5 days of organ culture. It is thus evident that a combination of insulin, prolactin and aldosterone, with or without the ovarian steroids, is adequate as a 'growth-promoting' medium in organ culture of the entire mammary gland and that the alveoli can be maintained in the same medium for at least 17 days.

The occurrence of 'milk-like' secretory material in the lobulo-alveolar gland after incubation in medium containing insulin + prolactin + cortisol is consistent with earlier reports that the same hormone combination stimulates lactation in the developed mouse mammary gland *in vivo* (Nandi, 1958), in whole glands cultured *in vitro* (Prop, 1961) and also milk protein synthesis in fragments of mid-pregnant mammary tissues in organ culture (Topper, 1970). Thus, it is reasonable that the 'milk-like' secretory material observed in the alveolar lumen of the cultured glands after incubation with insulin + prolactin + cortisol may reflect functional differentiation of the alveolar structures. Preliminary results of gel-electrophoretic analysis of a rennin, Ca<sup>2+</sup> precipitate from these glands indicate incorporation of radioactive amino acids into 'casein-like' protein.

Regression of alveoli in mammary glands initially incubated for 5 days in 'growth-promoting' medium followed by culture for an additional 7-10 days in a medium containing only insulin clearly demonstrates that insulin alone cannot maintain the lobulo-alveolar structures and that involution of the cultured gland ensues. Contrary to the contention that insulin is stimulatory to mammary epithelial cell proliferation *in vitro* (Topper, 1970; Turkington *et al.* 1973), the present results and those of other authors demonstrate that insulin alone cannot induce or support alveolar development in the mammary gland, but must be combined with steroid and anterior pituitary hormones (Prop, 1961; Ichinose & Nandi, 1966; Singh *et al.* 1970). This is consistent with our earlier report (Mukherjee, Washburn & Banerjee, 1973) that insulin alone cannot initiate the proliferation of cells, although it allows the completion of mitosis initiated *in vivo*.

It has been reported that fragments of mid-pregnant mammary gland can be maintained

in organ culture medium containing insulin only (Juergens, Stockdale, Topper & Elias, 1965). These cultures, however, were of very short duration, i.e. 2–5 days; incubation for an extended period probably would lead to alveolar regression. In fact, Rivera & Bern (1961) found that fragments of mammary gland from pregnant mice, cultured in medium containing insulin alone, undergo alveolar degeneration, but mammary explants from non-pregnant mice were maintained by insulin. These results are in agreement with the present finding that insulin acts as a 'permissive' hormone supporting the maintenance of the basal mammary structure, but not that of the alveolar epithelium. In conclusion, the present results indicate that organ culture of the entire mammary gland is a suitable model for the study of the mechanisms of hormone action during normal and neoplastic development of the mammary epithelium in culture.

We dedicate this paper to Professors H. A. Bern and S. Nandi as a token of our appreciation for their numerous contributions towards the understanding of the endocrine physiology of the mouse mammary gland. This work was supported by USPHS Grant Ca-11058 and a Contract (NIH-NO1-CP-33289) from the National Cancer Institute. We thank Miss Patricia Vandersall and Mr Wesley Rosenthal for technical and Mrs Naomi Windhorst for secretarial assistance.

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## DESCRIPTION OF PLATES

Whole mounts and histology of entire mouse mammary glands cultured *in vitro* with different hormone combinations.

## PLATE 1

Fig. 1. Whole mount of uncultivated gland from an oestradiol + progesterone pretreated mouse ( $\times 10$ ). Note the ductal architecture of the gland with end-buds

Fig. 2. Whole mount of a gland after organ culture in medium containing insulin + prolactin + aldosterone for 5 days ( $\times 7$ ). Note the extensive lobulo-alveolar structures. The upper left insert shows the histology of the same gland ( $\times 250$ ). Whole mounts of glands cultured in medium containing insulin + prolactin + GH + oestradiol + progesterone + aldosterone for 5 days showed similar morphology. Lower right insert shows the histology of one such gland ( $\times 250$ ).

Fig. 3. Whole mount of a gland after organ culture in medium containing insulin + prolactin + aldosterone for 16 days ( $\times 7$ ). Note that lobulo-alveolar structures predominate. The insert on left shows the histology of the same gland ( $\times 250$ ). The glands incubated in presence of insulin + prolactin + GH + oestradiol + progesterone + aldosterone were essentially similar and the insert on the right illustrates the histology of such a gland ( $\times 100$ ).

## PLATE 2

Fig. 4. Histology of a gland initially incubated in the 'growth-promoting' medium for 5 days followed by continued organ culture in medium containing insulin + prolactin + cortisol for 6 days. Note the abundant 'milk-like' secretory material in the distended lumina ( $\times 250$ ).

Fig. 5. Histology of a gland incubated in the 'growth-promoting' medium for 11 days ( $\times 250$ ). Note the absence of 'milk-like' secretory material in the lumina.

Fig. 6. Whole mount of a gland initially cultured in the 'growth-promoting' medium for 5 days, followed by organ culture in medium with insulin alone for 12 days ( $\times 7$ ). Note complete regression of the alveoli. The upper left insert shows the histology of the same gland ( $\times 100$ ) and the insert on the lower right illustrates the histology of the involuted mammary gland of a mouse 2 weeks after weaning ( $\times 100$ ). The overall architecture of the two parenchyma is similar.



