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20 April 1964

Lobuloalveolar Differentiation in Mouse Mammary Tissues in vitro

Abstract. *Administration of lobuloalveolar mammogenic hormones (for a period insufficient to induce alveolar differentiation) to immature mice prior to cultivation of their mammary glands in chemically defined, hormone-supplemented media resulted in lobuloalveolar growth in vitro comparable to that of late pregnancy. No such growth occurred in cultures of mammary tissues from mice not treated with hormones. The use of tissues from hormone-treated donors might serve as a valuable tool for studies concerned with differentiation in vitro.*

The nature of the hormonal influence on the growth and differentiation of mammary glands in mice (*1*) and rats (*2*) has been established by studies of animals deprived of various endocrine glands. Such studies, however, do not permit precise delineation of the actions of hormones on mammogenesis, since the possible influence of the remaining endocrine glands or of other organismal effects cannot be eliminated. Methods in which organs are cultured in vitro offer an advantage in analyzing the role of hormones in growth and differentiation, provided successful cultivation of postnatal tissues can be attained in chemically defined media. Stimulation of secretion in cultures of mouse mammary lobules has been reported (*3*). Rivera (*4*) and Prop (*5*) induced some lobuloalveolar differentiation by cultivating mammary tissues of young mice in media containing serum and hormones, but a suitable method is still lacking for inducing such growth in a medium that is entirely chemically defined. Although Lasfargues (*6*) reported that mammary tissues of young adult mice showed slight lobular development when cultured in a defined medium containing only ovarian steroids as hormones, Rivera failed to obtain such growth in cultures of young adult mammary tissues (*4*) in media containing ovarian and adrenocortical steroids along with insulin. Thus, the induction in vitro, in defined media, of full lobuloalveolar development comparable to that seen in pregnancy has yet to be reported. The fulfillment of this condition, with which this report is concerned, may permit organ culture techniques to be applied to this and similar morphogenetic phenomena.

Three observations seemed pertinent to this problem. (i) Postnatal differentiation of mammary tissues of mice requires hormonal stimulation in vivo (*1*). (ii) Full lobuloalveolar development in mouse mammary glands is achieved in late pregnancy (*1*). (iii) Preliminary experiments indicated that hormone treatment for 15 to 20 days is required for lobuloalveolar differentiation to be induced experimentally in the mammary glands of immature mice. These findings suggest that mammary tissues of immature mice might require extended periods of exposure to hormones in vitro in order to undergo lobuloalveolar differentiation comparable to that seen in pregnancy. However, the period of survival of tissues in organ culture is often limited. As an alternative to merely increasing the period of culture, we therefore decided to "prime" the donor animals. To this end, immature mice received mammogenic hormones for a brief period of time, itself insufficient to initiate lobuloalveolar development. It seemed probable that mammary tissues from such mice would be more likely to undergo lobuloalveolar formation in vitro than tissues from mice not treated with hormones.

The watch-glass method of organ culture, as adapted by Chen (*7*) for liquid media, was used. The basal culture medium was synthetic medium MB 752/1 (*8*), routinely supplemented with penicillin G (35 μg/ml) and L-glutamine (350 μg/ml). The petri-dish culture chambers were incubated at 37°C in a humidified atmosphere of 95 percent O_2 and 5 percent CO_2.

One of the pair of second thoracic mammary glands from the following two types of donor animals was cultured. Intact 4-week-old BALB/cCrgl female mice which had received daily for 7 days subcutaneous injections of 1 μg of estradiol-17β (E-17β), 1 mg of progesterone, 50 μg of ovine mammotropin (MH), and 50 μg of bovine somatotropin (STH), prepared in distilled water [a combination of lobuloalveolar mammogenic hormones in vivo (*1*)]. Intact 3- and 4-week-old BALB/cCrgl female mice served as controls, having been either injected with distilled water for 7 days or subjected to no preliminary treatment. Whole mammary glands were cultured in any one of five types of media. Medium 1 contained no hormone supplements. Medium 2 was supplemented with insulin; medium 3, with insulin, E-17β, progesterone, and aldosterone. Medium 4 was supplemented with insulin, MH, and STH; and medium 5, with insulin, E-17β, progesterone, aldosterone, MH, and STH. The contents of media 2 and 3 will be referred to hereafter as "incomplete hormone supplements"; the contents of medium 4, as "complete hormone supplement." The method used for the addition of hormones to the synthetic medium was described previously (*4*). The concentration of hormones per milliliter of medium was: insulin, 5 μg; E-17β, 0.001 μg; progesterone, 1 μg; aldosterone, 1 μg; MH, 5 μg; and STH, 5 μg.

The medium was usually changed on the 3rd day of culture, and cultures were terminated at the end of 5 days. There were two exceptions to the basic experimental design. In one series of experiments, mammary tissues of 3-week-old control mice were cultured for 12 days. In another series, tissues from mice previously treated with hormones were cultured for 5 days with lobuloalveolar mammogens and then for 5 additional days with secretion-inducing hormones [either aldosterone, MH, STH, and insulin; or cortisol (8 μg/ml), MH, STH, and insulin (*9*)]. The assessment of cell viability, increased growth, and secretory stimulation was based on comparisons of stained whole-mount preparations and histologic sections of cultured glands with their uncultured contralateral controls.

The parenchyma of the second mammary glands of normal 3- to 4-week-old BALB/cCrgl females is organized into branching ducts with end buds of various sizes (Fig. 1*A*). Alveoli are absent. In similar mice injected with E-17β, progesterone, MH, and STH for 7 days (hormone-treated mice), the glands contain more numerous end buds, and possibly a few small alveoli (Fig. 1*C*).

After 5 days of culture, the mammary tissues appeared as follows. (i) In media unsupplemented with hormones, glands from both hormone-treated and control donors degenerated. (ii) In media containing incomplete hormone supplement, glands from both hormone-treated and control donors were only partially maintained. Degen-

erated areas were, however, more common in tissues from the control mice. (iii) In media containing complete hormone supplement, there was a distinct difference in response between tissues derived from the controls and from the hormone-treated donors. Glands from control animals showed no evidence of growth and resembled tissues cultured in media containing incomplete hormone supplement. In contrast, glands from hormone-treated animals displayed extensive lobuloalveolar development in culture, comparable to that seen in late pregnancy (Fig. 1*D*).

To determine whether mammary glands from control animals could show lobular development after longer periods in vitro, tissues of 3-week-old BALB/c mice were cultured in media containing complete hormone supplement for 12 days. Most of these explants contained degenerated parenchyma. A few, however, showed atypical focal hyperplasias (Fig. 1*B*). The addition of 10 percent blood serum from virgin BALB/c mice to the hormone-supplemented media did not alter these results.

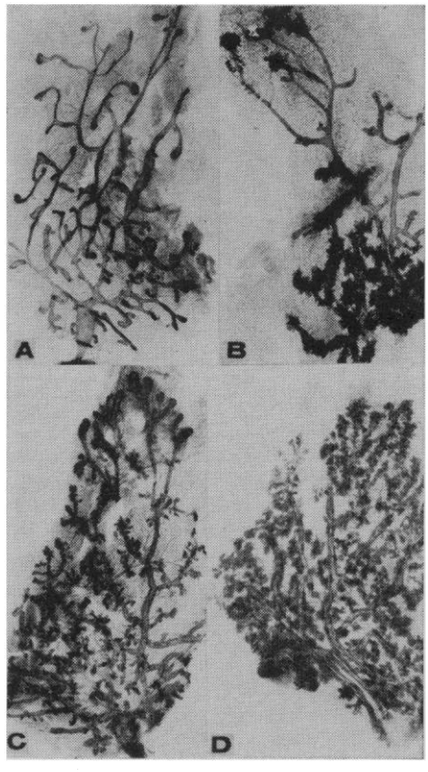

Fig. 1. All photographs show hematoxylin-stained wholemounts at (× 6). *A*, Mammary gland from 4-week-old female mouse. *B*, Mammary gland from 3-week-old control mouse after 12 days of cultivation in insulin, E-17β, progesterone, aldosterone, MH, and STH. The dense areas represent atypical foci of hyperplasia. *C*, Mammary gland from 4-week-old female treated with E-17β, progesterone, MH, and STH for 7 days before the gland was removed. *D*, Mammary gland contralateral to that in *C* after cultivation for 5 days in insulin, E-17β, progesterone, aldosterone, MH, and STH, showing extensive lobuloalveolar development.

Histologic evidence of highly active secretion (distended alveoli, intracellular secretory vacuoles) was seen in tissues from hormone-treated animals cultured for 5 days in complete hormone supplement followed by 5 additional days in secretion-inducing hormones. Tissues from hormone-treated mice cultured for 5 days in complete hormone supplement showed less secretion in the alveoli. This response affords additional evidence that the epithelial structures which grew in mammary cultures from hormone-treated animals behaved like the normal lobules characteristic of pregnancy, inasmuch as non-alveolar parenchyma does not respond to secretion-inducing hormones (*9*).

It appears that the treatment of donor animals with lobuloalveolar mammogens is necessary, under the conditions of culture used in our experiments, for extensive lobuloalveolar differentiation of the mouse mammary gland in defined, hormone-supplemented media. Mammary tissues from control mice did not show similar growth under these conditions. The effect of prior hormone treatment has also been observed in mammary tissues from 4-week-old mice from the strains C3H, C3Hf, and A/Crgl.

Our observations suggest that the hormone treatment in vivo that produced mammary tissues capable of undergoing lobular development in vitro involved the participation of the host in some as yet unknown way, since similar treatment of the glands in vitro was unable to produce tissues capable of growth. Once the tissue changes induced by treatment in vivo are established, however, the conditions for lobular development can be accurately defined.

The conclusion that hormone treatment prior to removal of the glands is necessary for lobular development in vitro is tentative, since appropriate alteration of the culture system might permit such differentiation of untreated tissues entirely in vitro.

It may be possible to use this combined in vivo–in vitro method for the analysis of other similar problems of differentiation. Thus, a lack of understanding of organismal factors initiating morphologic or physiologic changes in tissues need not prevent analyses in vitro of some steps in such transformations. Where the initiation of differentiation can be accomplished in vivo but not in vitro, much might still be learned by "triggering" the mechanism in vivo and studying the subsequent events in vitro.

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1 May 1964

Amitrole Translocation in Agropyron repens Increased by the Addition of Ammonium Thiocyanate

Abstract. *The mechanism for the enhancement of amitrole activity on quackgrass* (Agropyron repens) *by the addition of ammonium thiocyanate was studied. Absorption and translocation of C^{14}-amitrole after applications to foliage were determined by a direct count of the radioactivity in the wash solution and plant extracts. Regardless of the time of application, the addition of ammonium thiocyanate did not alter the amount of C^{14}-amitrole absorbed. Ammonium thiocyanate (5000 parts per million) greatly increased the amount of carbon-14 translocated. This increase may account for the greater herbicidal effectiveness of the mixture of amitrole and ammonium thiocyanate.*

Amitrole (3-amino-1,2,5-triazole) is an effective herbicide for the control of many species of perennial weeds because it is absorbed by the foliage and translocated throughout the plant (*1*). The herbicidal activity is enhanced by the addition of equimolar amounts of ammonium thiocyanate (NH_4SCN) at a concentration which is nonphytotoxic (*2*). Autoradiographic studies have