GROWTH CHARACTERISTICS IN MAMMARY GLAND TISSUE CULTURES FROM MICE OF A NONCANCER LINEAGE (D) ASSOCIATED WITH THE ADDITION OF ANDROSTERONE AND ESTRADIOL 17$^{\beta}$ TO THE NUTRITIVE MEDIUM

E. N. Nikiforova

Pathomorphological Laboratory (Head–Corresponding Member of the AMN SSSR Prof. M. F. Glazunov) of the Institute of Oncology (Director–Active Member of the AMN SSSR A. I. Serebrov) Acad. Med. Sci., USSR, Leningrad

(Presented by Active Member of the Acad. Med. Sci., USSR A. I. Serebrov)
Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 49, No. 6, pp. 92-97, June, 1960
Original article submitted May 6, 1959

In the present work we have undertaken the problem of observing the behavior of mammary gland tissue in cultures in which female or male sex hormones have been added to the fluid medium.

A. A. Maximov [4], in 1925, first cultivated mammary gland tissue from rabbits with the addition of fragments of a yellow body; he did not obtain positive results, inasmuch as the yellow body degenerated very early. F. Elias [2] cultivated the mammary gland tissue of pregnant mice, and established, as we did [1], that the growth of epithelium is observed only when the experiment is performed within the first half of the pregnancy. With the addition to the cultures of estrone, progesterone, cortisone, growth hormone, and mammatropic hormone, the most prolonged epithelial growth was noted only in association with the simultaneous action of the growth hormone plus the mammatropic hormone. All the remaining hormones, both in combination and individually, were ineffective in this regard. E. Lasfargues [3], using the same subject with the addition of serum from the unbilical cords of humans, observed epithelial growth up to 30 days; with normal serum the cultivation duration was considerably less. V. Monesi [5], cultivating adenocarcinoma of the mammary gland of the mouse in the presence of estrone, progesterone, and testosterone, noted a stimulatory action on the part of estrone and progesterone, and a strongly depressant effect from testosterone.

METHOD

In the experiments we used 40 mice (20 virgins and 20 parous) of noncancer lineage (D), from 2 to 6 months of age. In the first series of experiments we introduced androsterone into the fluid nutritive medium, while in the second we used estradiol 17$^{\beta}$. For the seeding we took thoracic and abdominoinguinal mammary glands, divided them up, and seeded them in 6 flacons, 4 pieces in each; 3 of the flacons served as the control. The nutritive medium consisted of a mixture of heparinated chicken plasma, Henk's solution, an extract of 10-day-old chicken embryos, and group IV (AB) human serum. In the flacons with the experimental cultures we added the hormones, diluted in six drops of nutritive medium: estradiol 17$^{\beta}$–0.05γ and androsterone–0.1γ. The fluid nutritive medium was replaced after 4-5 days; along with this we added the hormone to the flacons each time in the same quantity and dilution. The cultures were studied from the moment of seeding up to 45 days–alive and in the fixed and strained preparations. Fixation was carried out with 10% formalin, Zenker-formol, and Carnu's solution. A portion of the culture was imbedded in celloidin for serial sections. The total cultures and sections were stained with Karatstsa's hematoxylin, iron hematoxylin by the method of Yasvoin, azure eosin, Mallory's, van Gieson's, and Sudan III.

RESULTS

Mammary Gland Cultures from the Virginal Mice. In the control series we seeded 240 fragments from 20 animals. Growth of the epithelium was obtained only in 2 cultures from one animal, while growth of connective tissues was observed in 212 cultures from 19 mice. The epithelium began to grow on the 4th day. Its membranes did not attain large proportions; they had a multilayered structure, which, toward the periphery, thinned out and in the border zone became unilayered. The cells were uniform elongated forms, and the oval nuclei at the periphery of the membrane were coarse. Despite the fact that mitoses and amitoses were observed in the epithelium in the first days of growth, the dimensions of the membranes increased by only a small degree. Dystrophic changes were already noted on the 13th day of explantation. Growth of the connective tissue occurred on the 2nd day. At the end of the 4th day the growth zone surrounded the fragment from all sides; numerous mitoses were observed in it. The nuclei of the fibroblasts differed little from each other in form

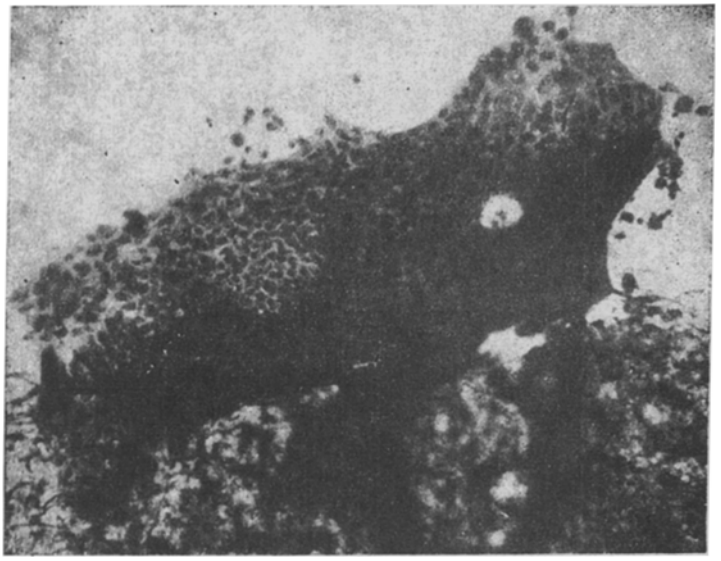

Fig. 1. Twelve-day old total culture of mammary gland epithelium, from virginal mice of a noncancer lineage (D), with androsterone added. Over-all view of the growth zone. Two replacements of the fluid phase. Karatstsa's hematoxylin. Magnification: ocular 7×, object. 8×.

and size. We were able to cultivate the connective tissue for 25 days.

One hundred and twenty fragments from 10 mice were seeded with the addition of androsterone. Epithelial growth was observed in 9 cultures from 4 animals, while connective tissue growth was obtained in 103 cultures from all the animals. The epithelium began to grow on the 2nd day. Initially, there appeared multilayered membranes and projections from the uniform cells, which, subsequently, expanded into a unilayered membrane of closely joined cells, forming an even border at the periphery (Fig. 1). Mitoses were noted in the growth zone from the first days of cultivation. The majority of the cells had an elongated form and an oval nucleus. Beginning with the 6th day amitoses were encountered as well as mitoses; there appeared polymorphic cells, differing considerably from one another in their dimensions. Their nuclei also differed in form and size and contained from 1 to 6 coarse nucleoli. Fine fat droplets were noted in the cytoplasm, beginning with the 7th day; uniting, they formed a single coarse droplet which deformed the nucleus. By the 12th to 14th day the number of mitoses decreased, dystrophic changes gradually developed, and, despite replacement of the fluid nutritive medium and trimming, the epithelium perished. The duration of cultivation was 17 days.

Just as in the case of the control, we were able to cultivate the connective tissue for a longer period of time than we could the epithelium. The effect of androsterone on the tissue was manifested by the appearance of a large number of bi- and trinucleated cells.

One hundred and twenty fragements from 10 animals were seeded with the addition of estradiol 17^{β}. Epithelial growth was obtained in 12 cultures from 4 animals, while connective tissue growth was noted in 102 cultures from all the animals. The epithelial growth began on the 2nd day, and on the 8th to 10th day the membranes had attained larger proportions than in the series with androsterone. The intercellular spaces were considerably wider, and, thus, the membranes had a looser structure (Fig. 2). The border of the growth zone was always irregular, and bore projections of various forms and lengths; the cells here were flattened, and they became coarser and adopted a diverse form. Polymorphism of the cells and nuclei, and differences in their size, were very markedly manifested. If in the series of experiments using androsterone the smallest cell differed from the largest by a factor of 3, then in the experiments with estradiol this factor was equal to 6-7. An analogous difference was also noted in the dimensions of the nuclei. In this series the epithelial growth was more intensive than in the series of experiments using androsterone; mitoses and amitoses were more frequently observed, and the growth zone was wider. As usual, fat appeared in the cytoplasm on the 6th-7th day. Dystrophic changes occurred in several cells on the 16th-18th day; we were able to carry a portion of the culture for 23 days.

Growth of the connective tissue was accompanied by the appearance of bi-, tri-, and pentanucleated fibroblasts. It was possible to cultivate the connective tissue for 35 days.

Mammary Gland Cultures from the Parous Mice. In the control series we seeded 240 fragments from 20 mice. Epithelial growth was noted in 11 cultures from 4 animals, while connective tissue growth occurred in 180 cultures from all the mice. The epithelium began to grow on the

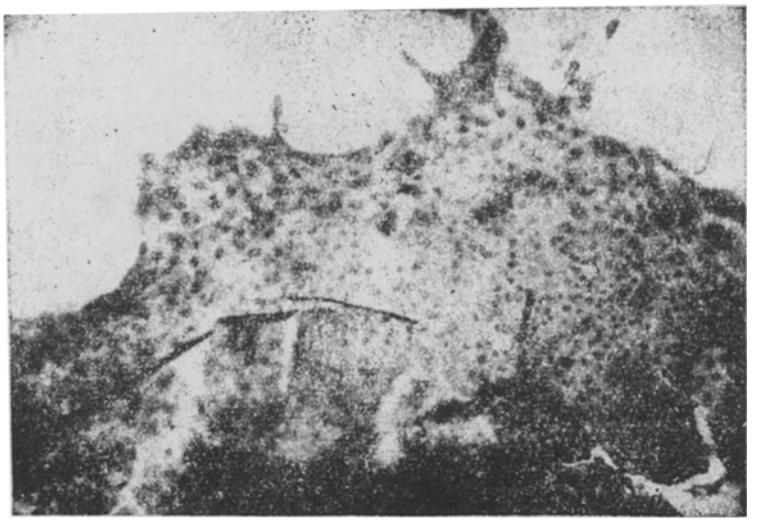

Fig. 2. Ten-day old total culture of mammary gland epithelium, from virginal mice of a noncancer lineage (D), with estradiol 17^{β} ad-added. Over-all view of the growth zone. Two replacements of the fluid phase. Karatstsa's hematoxylin. Magnification: ocular 7×, object. 40×.

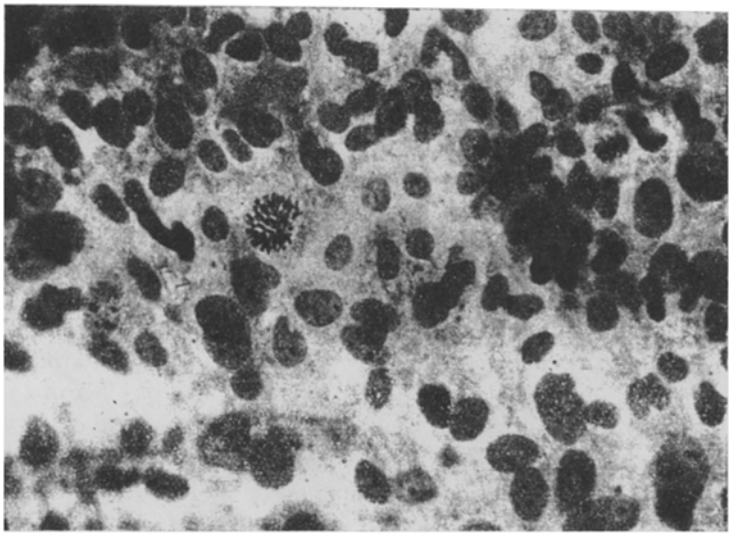

Fig. 3. Nine-day old total culture of mammary gland epithelium, from parous mice of lineage (D), with estradiol 17^{β} added. Nuclear polymorphism. Mitoses. Two replacements of the fluid phase. Glandular hematoxylin. Magnification: ocular 7×, object. 40×.

3rd-4th day; multilayered and unilayered membranes formed on the 5th to 6th day. Mitoses were encountered rather rarely in them. The form and size of the cells and nuclei underwent insignificant variation. The epithelial growth zone did not attain large proportions. Fat appeared in several cells on the 6th to 7th day. Growth and multiplication of the epithelium was observed up until the 16th to 18th day. On the 20th to 25th day the epithelium perished. Connective tissue growth was more rapid than epithelial growth; mitoses were encountered in it rather frequently. The cells were fundamentally similar to one another, and had a fusiform shape. The connective tissue grew for 30 days.

One hundred and twenty fragments from 10 animals were seeded with the addition of androsterone. Epithelial growth was obtained in 5 cultures from 3 animals, while connective tissue growth was noted in 94 cultures from all the animals. Growth of both tissues began on the 2nd day. The epithelium formed membranes and projections with densely distributed cells. Growth intensity was greater than

Data on the Cultivation of Mammary Gland Tissue from Mice of a Noncancer Lineage (D)

Group of mice	Control			Androsterone			Control			Estradiol 17β		
	seeded	growth obtained		seeded	growth obtained		seeded	growth obtained		seeded	growth obtained	
		epitheli-um	connec-tive tissue		epitheli-um	connec-tive tissue		epitheli-um	connec-tive tissue		epitheli-um	connec-tive tissue
	A/B	B/C	B/C	A/B	B/C	B/C	A/B	B/C	B/C	A/B	B/C	B/C
Virginal	120/10	2/1	105/9	120/10	9/4	103/10	120/10	—	107/10	120/10	12/4	102/10
Parous	120/10	6/2	88/10	120/10	5/3	94/10	120/10	5/2	92/10	120/10	7/3	99/10

Notes: 1. Cultures with simultaneous growth of epithelium and connective tissue were classified in the group of cultures with epithelial growth. 2. A: number of fragments; B: number of animals; C: number of cultures.

in the cultures of the control series, and the corresponding growth was wider. Mitoses were encountered constantly, from 1 to 2 per visual field; amitoses were also observed along with them. Nuclear and cellular polymorphism was markedly manifested only in spots; the nuclei sometimes attained very large proportions. The majority of nuclei had an oval form: they contained up to 8 coarse nucleoli,, varying in shape and size. Cells with 2-4 nuclei appeared in the growth zone on the 8th to 10th day. Beginning with the 7th day fat appeared in the cytoplasm, initially in the form of droplets which united into a singly coarse drop and deformed the nucleus. We were able to cultivate the epithelium for up to 20 days.

The connective tissue grew more actively than in the control. Many mitoses and amitoses were observed in it, and also cells, in large number, containing from 2 to 6 nuclei. The duration of cultivation was 40 days.

One hundred and twenty fragments from 10 animals were seeded with the addition of estradiol 17$^{\beta}$. Epithelial growth was observed in 7 cultures from 3 animals, while connective tissue growth was noted in 99 cultures from all the mice. The epithelium and connective tissue, as in the other series of experiments, began to grow on the 2nd day. A peculiarity of the series of experiments with estradiol appeared to be the presence of epithelial membranes with loosely distributed cellular elements. Polymorphism of the cells and nuclei and variation in their dimensions were manifested very markedly (Fig. 3). Almost in every culture cells were encountered with 3-4-6 nuclei or with one very coarse nucleus. The nucleoli, numbering from 1 up to 10, were coarse and variable in form. Sometimes rod-shaped nucleoli seemed to divide the nucleus into pieces. In cultures of this series a very large number of mitoses were observed; in a single visual field it was possible to see up to five. Often 2-4 polar mitoses were encountered. Sometimes the width and the structural characteristics of the membranes in the same culture were dissimilar. Along with loosely structured, unilayered portions there were also dense, multilayered parts. The dimensions of the cells and nuclei in the latter were considerably smaller than in the loose membranes, the polymorphism weakly manifested, and the multinucleated cells encountered as an exception. Mitoses and amitoses were observed more rarely. Multipolar mitoses were absent. Beginning with the 6th day fat appeared, as usual, in the cytoplasm of several of the cells.

Degenerative changes in this series occurred later. We were able to cultivate the epithelium for up to 25 days.

The connective tissue grew more energetically than with the addition of androsterone. Mitoses and multinucleated cells also were more numerous. The duration of cultivation was 45 days.

The results of our investigations are presented in the table. If one is orientated by the percent of growing epithelial cultures in relation to the number of fragments of mammary gland tissue seeded and the number of animals from which fragments were taken, i.e. by the output of

cultures, then the effect of the action of androsterone and estradiol 17^{β} emerges with sufficient conclusiveness only in the group of virginal mice. In this group in two control series (total number of mice–20, from which 240 fragments were seeded) only 2 cultures from one animal grew out. At the same time, in the series of experiments with androsterone (10 mice, 120 fragments) 9 cultures from 4 mice grew out, and in the series with estradiol 17^{β}, 12 cultures from 4 of the 10 animals successfully grew.

In the group of parous mice the addition of androsterone and estradiol 17^{β} did not show any effect on the number of growing cultures as compared with the control. Along with this, the addition of androsterone and estradiol 17^{β} reflected itself in principally the same manner on the intensity of growth and culture morphology of both the virginal and parous mice, the only difference being that the changes were manifested considerably more clearly in the experiment with estradiol 17^{β}. The action of both hormones, in comparison with the control, made itself apparent in the quantitative increase in mitoses, the multipolarity within them (possible also amitoses), in the formation of multinucleated cells, in the polymorphism of the cells and nuclei, and in the characteristics of the membrane: it was significantly wider in the series of experiments with estradiol 17^{β} than in the control or in the series with androsterone.

As far as the connective tissue from the mammary gland was concerned, the number of growing cultures was similar in both the control and the experimental series, and in both the virginal group and the parous group of animals. However, here the action of androsterone and estradiol 17^{β} was expressed in the appearance of a large number of multinucleated cells, especially in the experiments with estradiol 17^{β}.

Thus, the addition of androsterone or estradiol 17^{β} to cultures of mammary gland tissue bears evidence in favor of the feasibility of their direct morphogenetic activity on the epithelium and connective tissue of the mammary gland. This action is apparent in both a fortification of the process of mitotic division and in its disruption, which is attested by the presence of pathological mitoses and multinucleated cells in the cultures. In this regard, estradiol 17^{β} is more active than androsterone.

SUMMARY

The author cultured the tissues of the mammary gland obtained from 40 mice (aged from 2 to 6 months) of noncancer lineage (D). Androsterone was introduced into the fluid nutritive medium in the first series of experiments, and estradiol 17^{β} – in the second. The effect of the action of androsterone and estradiol 17^{β} was sufficiently convincing only when culturing the tissues of the virgin mice (according to the number of the growing epithelial cultures). The effect of androsterone and estradiol on the growth intensity and the morphology of the epithelial cultures was reflected in a principally similar way both in the virgin and parous mice, the only difference being that these changes were more clear-cut in the experiment with estradiol 17^{β}. The effect of the hormones was manifest in the increased number of mitoses, including multipolar (possible also of amitoses), in the formation of the multinuclear cells, the polymorphism of the cells and nuclei, the greater width of the membrane in the series of experiments with estradiol 17^{β} (in comparison with control and the series of experiments with androsterone). The number of the growing connective tissue cultures of the mammary gland were the same both in the control and in the experimental series, in the group of virgin and in the group of parous animals. Here the effect of estradiol 17^{β} and androsterone was manifested in the appearance of a large number of multinuclear cells, especially in the experiment with estradiol 17^{β}.

LITERATURE CITED

1. E. N. Nikiforova, Trudy Leningradskogo In-ta Onkologii Akad. Med. Nauk SSSR 2, 139 (1958).
2. F. F. Elias, Science 126, 842 (1957).
3. E. Y. Lasfargues, Anat. Rec. 127, 117 (1957).
4. A. Maximov, Arch. Path. Anat. 256, 813 (1925).
5. V. Monesi, Tumori 42, 334 (1956).