INFLUENCE OF OVARIAN EXTRACT UPON THE SEX
DIFFERENTIATION OF TERMINAL CREST IN THE
TESTIS OF *TRITURUS PYRRHOGASTER* (BOIE)

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The present author reported in a previous paper (Hanaoka, 1960) that when a piece of testis of *Triturus pyrrhogaster* is implanted into the ovarian cavity of an adult female, the columnar cells of peritoneal epithelium which cover the surface of the terminal crest are transformed into primordial germ cells provided with large bilobed nuclei. In advanced stages of sex differentiation, some of these modified primordial germ cells, as well as those which were nested in the germinal tissue under columnar epithelium, developed into large oogonia containing vesicular nuclei and yolk granules.

It has been established in various forms of amphibians that the development of female germ cells is brought about by proper treatment with estrogenic hormones. In *T. pyrrhogaster*, Uchida and Hanaoka (1949) reported that subcutaneous injections of estrogenic hormone induced the formation of oviform germ cells in the regenerated testes of incompletely castrated males.

Concerning the modes of oviform germ cell differentiation in the testis, however, there are several important differences between 2 groups of germ cells, one group implanted in the ovary and the other group subjected to estrogen injection. Types of the oviform germ cells in estrogen treated animals were as follows:

- 1) Oviform germ cells of a type smallest in size and greatest in number, intermingling with normally differentiated spermatogonia or spermatozoa in seminal tubules.
- 2) Oviform germ cells irregular in shape and rather great in number, forming small groups in seminal tubules.
- 3) Oviform germ cells intermediate in size and rather small in number, in connective tissue between the seminal tubules.
- 4) Oviform germ cells rather rarely found in follicle cells, among stroma cells of irregularly regenerated testis.

Oviform germ cells of these 4 types were evenly distributed through the testicular tissue in which the fundamental structure remained almost unchanged.

In the testis implanted into the ovarian cavity, on the other hand, seminal tubules containing spermatogonia degenerated sooner or later. Development of new seminal tubules was suppressed and some of germ cells in the primordial

germinal tissue of the terminal crest transformed into oviform germ cells which were individually enveloped within follicle cells.

On the basis of these observations, the author conceived the idea that the ovary of the mature female releases certain substances which affect the sex differentiation of the terminal crest of the testis in a different manner from that of ordinary estrogenic hormone. For the purpose of providing the hypothesis, pellets of crude extract of ovaries of adult females were implanted into the testes and histological differentiation of the primordial germinal tissue in the terminal crest was observed.

METHODS

The extract of ovary was prepared by the following method. Fresh ovaries excised from adult females were suspended in Holtfreter's solution, 3 times as great in vol., and homogenized in a glass homogenizer kept in ice water. The homogenate obtained was centrifuged at a low temperature for 30 mins. at 7800 r.p.m. and the granular precipitate which was presumably derived from mature eggs was separated. The supernatant was then lyophilized and the residual soft hygroscopic substance was used as an extract. 73.1 g of fresh ovaries collected from 100 mature females yielded 5.7 g of crude extract. Before implantation into testis the extract, added with a few drops of distilled water, was made into small pellets each containing 0.03 g of the ovarian extract.

The experimental animals were divided into 2 groups. In the 1st group the animals received the implantation of ovarian extract into the testes on both sides. In the 2nd group, which served as the control to the 1st one, the pellets containing a mixture of crystalline estrogenic substance (Estrone, Teikoku Hormone Mfg. Co., Ltd. or Diethylstilbestrol=Robal, Chugai Pharmaceutical Co., Ltd.) and corn starch were implanted into testes in a similar way to the 1st group. Each pellet contained 0.015 g of estrogenic substance. After the operation, 4 or 5 animals were dissected every fortnight and their gonads were preserved in Bouin's solution. For microscopic observations the sections were cut by routine paraffin method in 8 μ thickness and stained with Mallory's triple stain.

RESULTS

First group The pellets of the ovarian extract implanted into testes remained for several weeks as distinct clots among seminal tubules near the terminal crest. After 6 or 8 weeks they were invaded by a large number of stroma cells which had migrated from surrounding tissues and the peripheral regions of the grafts became disintegrated into small drops which contained several empty vacuoles (Fig. 1-A). Afterwards, these drops, intermingling with stroma cells, streamed out from the original site of implantation into narrow spaces between the seminal tubules and in some animals beads of an oily substance which stained yellow with orange G were observed running through the testis for 10 or more weeks after the implantation of the extract (Fig. 1-B).

The influence of ovarian extract implanted into testis was observed at first in the modified development of primordial seminal tubules in the terminal crest.

In early stages of normal development of seminal tubules the primordial germ cells with large bilobed nuclei are always surrounded, in small groups, by

Fig. 1. A; a part of ovarian extract disintegrated by the invasion of stroma cells.
B; a stream of ovarian extract between the seminal tubules

several follicle cells which have small ovoidal nuclei stained dark yellow with orange G. Lying between the germ cells there are also a few small somatic cells which presumably give rise to Sertoli cells in the mature tubules. As the histological development of testis proceeds, the germ cells increase in number and differentiate into primary spermatogonia while the follicle cells come to form a part of the external wall of the seminal tubules.

In the testis implanted with ovarian extract, on the other hand, the primordial germ cells were much decreased in number and excessive development of the sterile seminal tubules in the terminal crest were observed (Fig. 2). A small number of primordial germ cells which remained outside of the seminal tubules

Fig. 2. Sterile seminal tubules in the terminal crest

Fig. 3. Sections of sterile seminal tubules with abnormally located germ cells (A. B) and normal seminal tubules (C).

were individually enclosed within the follicle cells. Figure 3 shows the sterile and non-sterile tubules, *i.e.*, the germ cells are located only outside (Figs. 3-A and B) and inside (Fig. 3-C) of the seminal tubules. Surrounding the sterile seminal tubules there were thick layers of fibrous loose connective tissue. In advanced stages of sex differentiation, however, the fibrous substance in the terminal crest gradually disappeared and seminal tubules were found in a large empty space under the columnar epithelium.

The sterile seminal tubules survived longer than the fibrous connective tissue. Ten or more weeks after the operation, however, distinct signs of degeneration began to appear, and the primordial germ cells which were enveloped by follicle cells were pushed against the connective tissue wall lining the cavities due to the degeneration of central core of the terminal crest (Fig. 4).

Differentiation of oviform cells was one of the most conspicuous features in the histological changes of the terminal crest. These oviform germ cells appeared in 5 specimens among 8 which were preserved 6 or 8 weeks after the implan-

Fig. 4. Cavities produced by the degeneration of seminal tubules

tation of ovarian extract. Each testis contained 2 or 4 oviform germ cells of intermediate sizes and with a single exception, they were individually enveloped within a small number of follicle cells. Their nuclei were large and vesicular and in some of them the cytoplasm contained several small vacuoles in the periphery although the deposits of yolk granules were observed in none of them. It is of special interest to note that in some cases a distinct gradient of sex modification was observed along the long axis of the terminal crest. Figure 5 represents a typical example of this type of sex modification. On the left side of Figure 5-A, nearest to the graft, one can find several sterile seminal tubules

Fig. 5. Gradient in sex modification in the terminal crest. B, C, and D represent respectively the histological structures near b, c, and d in A.

embedded in stroma cells (b). An oviform cell which is enveloped within follicle cells is differentiated in the middle part of the terminal crest (c). Some of the primordial germ cells on the right side of the oviform germ cells are individually enveloped within follicle cells, but in the adjacent region the germ cells appear always in groups and show the normal development of seminal tubules (d). The right end of Figure 5-A continues to the central part of the testicular lobes.

After 10 weeks the oviform germ cells in the terminal crest began to degenerate and in a specimen kept for until 12 weeks after the operation, only a rem-

Fig. 6. Modified differentiation of seminal tubules in the terminal crest of both operated and unoperated lobes. B, C, D, E and F represent the histological structure of terminal crest of B, C, D, E and F respectively in A. O; oviform germ cell, G; graft of ovarian extract.

nant of disintegrated vesicular nucleus was observed in a seminal tubule.

The modified development of seminal tubules was frequently observed not only in the terminal crests adjacent to the operated lobe but also in those which were at some distance from the site of implantation of ovarian extract. Figure 6-A represents schematically one example of this. This testis consisted of 4 small lobes each of which was provided with a terminal crest at the anterior apex, and the ovarian extract was implanted in the 2nd lobe. It was naturally expected that the development of seminal tubules in the terminal crest on both sides of the operated lobe should be modified in a striking degree. The changes in the terminal crest of the 3rd and 4th lobes, however, were none the less conspicuous and several oviform germ cells were found in the terminal crest of the 4th lobe. Undoubtedly, the influence of ovarian extract was transmitted through the 3rd and 4th lobes to the terminal crest at the apical end of the testis.

In spite of these severe modification of sex differentiation in the terminal crest, the differentiation of spermatogonia in the central part of each lobe was little affected by the ovarian extract. The spermatogonia in the tubules which were immediately adjacent to the graft multiplied quite normally and the spermatogenesis proceeded as in the intact testis. Although a small number of residual spermatogonia were observed in the connective tissue between the seminal tubules, none of them become differentiated into oviform germ cells.

Second group Pellets of crystalline estrogenic substance and corn starch were rather rapidly disintegrated in the testis and, sooner or later, replaced by a network of connective tissue. In animals dissected a few weeks after the operation, small clots of starch granules which stained in a bluish tinge with aniline blue were observed in the operated lobes but entirely disappeared soon after. Remaining connective tissue was perforated with many pores which are irregularly squarish shapes. These pores are presumably the space occupied by crystalline granules of estrogenic substance which had been contained in the graft and was dissolved in ethanol and other solvents during the preparation of microscopical slides (Fig. 7).

Fig. 7. Connective tissue with empty spaces made by dissolution of crystalline estrone

The implantation of crystalline sex hormone exerted a striking destructive influence upon the general differentiation of germ cells in the testis. In the seminal tubules containing only a small number of young spermatogonia, on the contrary, the degeneration was relatively slight, and consequently, the subsequent regeneration of the testicular tissue was possible.

The development of primordial seminal tubules in the terminal crest was more or less retarded. However, the occurrence of sterile tubules was far less conspicuous and many primordial germ cells were yet observed among the stroma cells. They were always accompanied with a small number of follicle cells and some of them were rather larger than normal size. The influence of the implantation of estrogenic substance was restricted only to the terminal crest connected directly with the graft-bearing lobes, while the tubules of the non-graft-bearing lobes, developed as in intact testes.

In one case removed 8 weeks after operation, differentiation of oviform germ cells was observable, *i.e.* 2 cells of the intermediate size, accompanying spermatogonia, were found in a common follicular layer derived from the wall of seminal tubules. Oviform germ cells of this type closely resembled to those produced by the subcutaneous injection of estrogenic substance.

DISCUSSION

Since the discovery that the free martin in cattle is produced by the action of male sex hormone from a co-twin, the concept of hormonal control of sex differentiation has been widely adopted in vertebrate embryology. Recent experiments performed on birds and mammals have, however, revealed that the hormonal theory does not apply at least to some animals of these classes.

In a series of experiments on chick embryo, Wolff and Haffen (1952) used the technique of culture *in vitro* and reported that the reversing activity of ovarian explants to the juxtaposed testes was restricted only to the parts of the testis near ovary. Therefore, the active substance is probably not transmitted by the general blood stream.

In mammals Jost (1946) jointed 2 rabbit embryos by sectioning 1 hind leg of each and coupling the wounds to form a parabiotic twins. In another series of experiments, the same author implanted testicular tissue subcutaneously into female embryo (Jost, 1947). Both of these trials, however, yielded negative results. Macintyre (1956), on the other hand, found that 3 weeks implantation of embryonal ovaries together with testes to the adult rat kidney, resulted in remarkable inhibition of the ovarian cortex and excessive development of medullary tubules while in ovaries without testes, these modification of sex differentiation did not occur. Similar observations were also made on rabbit by Halyoke (1956).

In parabiotic twins of salamanders and newts, it has been well established that inhibitive actions against the differentiation of opposite sexes are transmitted by the general blood stream (Witschi, 1936 and 1937; Hanaoka, 1942). This rule, however, appears to meet with some conflict in frog in which the antagonistic action between the testicular and ovarian differentiation varies according to the distances between the gonads of parabiotic twins, the most striking disturbance

of ovarian differentiation appearing in the region where the ovaries and testes lie nearest to each other (Witschi, 1930). On the basis of these observations, he concluded that the testes of a male co-twin affect the ovaries of the female by the production of an inductive substance which spreads by intercellular diffusion.

The relationship between inductive substance and sex hormone, however, has never been made clear. Recent investigators on amphibians repeatedly reported that treatment with appropriate doses of estrogenic substances produced complete reversal of sex differentiation at least in some forms of frogs and salamanders (Chang and Witsch, 1955; Gallien, 1954 and 1956). These results, however, do not lead to the conclusion that the estrogenic hormone is identical with inductive substance for sex differentiation.

Matured testis of *T. pyrrhogaster* consists in most cases of several small lobes each of which is provided at its anterior end with a permanent embryonal germinal tissue. Following the release of spermatozoa in the breeding season, the evacuated seminal tubules in the central region of each lobe degenerate and the development of new seminal tubules in the terminal crest occurs every year. In a sense, therefore, embryonal development of testicular tissue takes place continuously through the reproductive periods of the male and this presumably makes the differentiation of oviform germ cells in the testis of adult male easier than in other forms of amphibians. Indeed, Uchida and Hanaoka (1949) found that the subcutaneous injections of estrogenic substance in the incompletely castrated male brought about the development of oviform germ cells in the regenerated testis.

It must be noted, however, that the modes of oviform germ cell differentiation in the testis treated with estrogenic substance differ in many important points from those produced by the implantation of testis into the ovarian cavity (Hanaoka, 1960). In the former case the fundamental structure of the regenerated testis was not altered to any conspicuous degree and most of the oviform germ cells were formed within the confines of the seminal tubules. In the latter case, on the contrary, the host ovary suppressed the histological development of seminal tubules in the terminal crest and a rather small number of oviform germ cells which proceeded to more advanced stages of differentiation appeared individually enveloped within follicular tissue of stroma cells. In other words the administration of estrogenic substance produced mainly the modification of germ cell differentiation in seminal tubules while the implantation of the testis into the ovarian cavity affected the histological development of seminal tubules which was secondarily followed by the development of oviform germ cells.

The results of the present experiments clearly indicate that the matured ovaries of *T. pyrrhogaster* contain a certain substance which affects the sex differentiation of the terminal crest of testis in a different manner from that of estrogenic substance already known, and, although the modification produced by the implantation of ovarian extract does not coincide in every detail with what was observed in the testis implanted into the ovarian cavity, it is highly probable that the sex modification observed in both groups of experimental animals are due to a common factor which should be regarded as an inductive substance.

Chemical analysis of ovarian extract used in the present experiment is now proceeding in this laboratory. According to personal information from Professor

Takikawa of the Department of Physiological Chemistry in this institute, the ovarian follicle of rat contains a certain steroid substance which differs in its chemical mode of occurrence from estrogenic substance found in the blood stream (in press). Such a finding seems to suggest that the ovarian follicles of the newt contain also some chemically and/or biochemically different estrogens highly inductive in sex differentiation.

SUMMARY

Crude extract of ovaries of *Triturus pyrrhogaster* was implanted into the testis of the adult male and observations were made on the histological differentiation of terminal crest at the apex of the testis. In the control animal pellets of a mixture of crystalline estrogenic substance and starch were grafted into the testis in a similar way as in the 1st group.

The implantation of ovarian extract brought about a striking suppression of development of seminal tubules in the terminal crest followed by the differentiation of oviform germ cells. These feminized germ cells appeared outside the seminal tubules and each of them was individually enveloped within the follicle cells derived from the walls of the seminal tubules. The modification of sex differentiation was observed not only in the terminal crests of the operated lobes but also in those which lie far from the site of implantation.

Crystalline sex hormone, on the other hand, exerted a severe destructive influence upon the germ cell differentiation in the testis but the differentiation of primordial seminal tubules was less conspicuously affected and oviform germ cell differentiation was detected only in a single specimen in which 2 cells accompanied by several small spermatogonia were formed in a common follicular tissue derived from the cells of the wall of seminal tubules.

These results, taken together with those of previous experiments, show that the ovary of *T. pyrrhogaster* produces an inductive substance which affects the sex differentiation of the terminal crest in a manner different from that of estrogenic hormones already known.

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