Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*)

(androgen/testis/Sertoli cell/fish/organ culture)

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ABSTRACT The importance of gonadotropins and androgens for spermatogenesis is generally accepted in vertebrates, but the role played by specific hormones has not been clarified. Under cultivation conditions, male Japanese eels (*Anguilla japonica*) have immature testes containing only premitotic spermatogonia, type A and early-type B spermatogonia. In the present study, a recently developed organ-culture system for eel testes was used to determine *in vitro* effects of various steroid hormones on spermatogenesis. After 9 days of culture in serum-free, chemically defined medium containing 11-ketotestosterone (10 ng/ml), a major androgen in male eels, type A and early-type B spermatogonia began mitosis, producing late-type B spermatogonia. After 18 days, zygotene spermatocytes with synaptonemal complexes appeared, indicating that meiosis had already started by this time. In testis fragments cultured for 21 days, round spermatids and spermatozoa were observed with spermatogenic cells at all stages of development. Addition of 11-ketotestosterone to the culture medium also caused a marked cytological activation of Sertoli cells. No other steroid hormones tested had such stimulatory effects. These results, together with our earlier observations, suggest the following sequence for the hormonal induction of spermatogenesis in eel testes; gonadotropin stimulates the Leydig cells to produce 11-ketotestosterone, which, in turn, activates the Sertoli cells leading to the completion of spermatogenesis. This is, thus, an example of an animal system in which all stages of spermatogenesis have been induced by hormonal manipulation *in vitro*.

The formation of sperm, spermatogenesis, is an extended process that begins with the proliferation of spermatogonia and proceeds through the extensive morphological changes that convert the haploid spermatid into a mature, functional spermatozoon. Although it is generally accepted that the principal stimuli for vertebrate spermatogenesis are pituitary gonadotropins and androgens, the specific role played by individual hormones has not been clarified (1–4). A number of factors complicating *in vivo* investigations of the mechanisms involved in the spermatogenesis can be eliminated in *in vitro* organ (5, 6) and cell (7–10) culture systems in which the direct effects of various factors, including hormonal influences, upon the spermatogenic cells and testes can be investigated.

Under conditions of cultivation, male Japanese and European eel have immature testes containing only premitotic spermatogonia, type A and early-type B spermatogonia (11–15). It has been reported that in both species a single injection of exogenous human chorionic gonadotropin induces all stages of spermatogenesis *in vivo* (12, 14, 15). This injection also caused an increase in plasma levels of 11-ketotestosterone (12, 15). Thus, the eel testis provides an excellent

system for studying the mechanism by which spermatogenesis is regulated. In the present study, we have used a recently developed organ culture system for eel testes to demonstrate completion of spermatogenesis *in vitro* from premitotic spermatogonia by the addition of 11-ketotestosterone to the medium.

MATERIALS AND METHODS

Animals. Male cultivated Japanese eels (*Anguilla japonica*) (180–200 g in body weight) were purchased from a commercial eel supplier. They were kept in circulating fresh-water tanks with a capacity of 500 liters at 20°C.

Organ Culture Techniques. The culture techniques used were a modification of Trowell's method (16). Freshly removed eel testes were cut into 1 × 1 × 0.5 mm pieces, which were placed on floats of elder pith (pretreated with 100% ethanol, autoclaved, dried, and covered with a nitrocellulose membrane) in 24-well plastic tissue-culture dishes (Costar). They were then cultured for 3–36 days at 20°C in humidified air. The basal culture medium consisted of Leibovitz L-15 medium supplemented with 1.7 mM proline, 0.1 mM aspartic acid, 0.1 mM glutamic acid, 0.5% bovine serum albumin fraction V (Sigma), retinol at 50 µg/liter, bovine insulin at 1 mg/liter, and 10 mM Hepes, adjusted to pH 7.4 with 1 M NaOH. The medium was changed every 3 days. Steroids were first dissolved in ethanol and then diluted with the medium.

Microscopy. Testis fragments before and after culture were fixed in a 2% (wt/vol) paraformaldehyde/2% (wt/vol) glutaraldehyde mixture in 0.1 M cacodylate buffer at pH 7.4, postfixed in 1% osmium tetroxide in the same buffer, and embedded in epoxy resin according to standard procedures. Sections, 1-µm thick, were stained with toluidine blue for light microscopic examination. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by using a JEOL 100-CX transmission electron microscope operated at 80 kV.

The following two criteria were used to evaluate the effect of various culture conditions on testicular fragments. First, five random toluidine blue-stained 1-µm-thick plastic sections from each of five cultured testis fragments originating from five eels were examined, and the number of cysts containing each germ cell type was counted. Results were expressed in percent of cysts of a particular germ cell type per total cysts observed. Cysts of the following five germ cell types were distinguished and counted: (i) type A spermatogonia and early-type B spermatogonia, (ii) late-type B spermatogonia, (iii) primary and secondary spermatocytes, (iv) spermatids, and (v) spermatozoa. Isolated type A spermatogonia, or groups of two cells surrounded by Sertoli cells, were counted as cysts.

FIG. 1. Light micrographs of toluidine blue-stained 1- μ m sections of testes before culture (A) and cultured in medium without steroid hormones for 36 days (B). (Bars = 10 μ m.)

Second, the areas of nuclei in Sertoli cells and the area of mitochondria in Leydig cells were measured on electron micrographs of 5 Leydig cells and 30 Sertoli cells, sampled at random from each five cultured fragments, by using an interactive image analysis system IBAS-1 (Kontron, Munich). These parameters were used as indicators of the growth and development of each cell type.

Statistics. Results were expressed as means and SEMs. Changes over time were measured by two-way analysis of variance, and differences in means within each group were measured by paired student's *t* tests.

RESULTS

Structures of Control Testes Before and After Cultivation. Spermatogenesis in teleost fish takes place within testicular cysts formed by Sertoli cells. Within each cyst, germ-cell development occurs synchronously (3, 17, 18). In the cultivated male eel, type A and early-type B spermatogonia are

the only germ cells present in the testis (Fig. 1A). Type A and early-type B spermatogonia are primitive spermatogonia that have not begun to proliferate. They are morphologically similar, with many clear homogeneous spherical mitochondria with clear matrices; the few mitochondrial cristae observed were oriented obliquely or roughly parallel to the mitochondrial wall (Fig. 2A). Type A spermatogonium occurs singly, each cell being almost completely surrounded by Sertoli cells. Early type B spermatogonia form a cyst of two or four germ cells surrounded by Sertoli cells. Several type A spermatogonia and cysts of early-type B spermatogonia form seminal lobules that are spread out in an irregular connective-tissue framework. Most of these spermatogonia are at mitotic rest, although mitotic metaphase and anaphase are observed occasionally.

Sertoli cells enclosing spermatogonia have irregular nuclei containing some electron-dense areas and a relatively narrow cytoplasm with poorly developed organelles (Fig. 2A). Leydig cells occur in the interstitial tissue and have round or oval nuclei containing some electron-dense areas. Their mitochondria are generally round or oval in form but can be irregularly elongated in some cases. Mitochondrial cristae are usually indistinct. The endoplasmic reticulum of Leydig cells is poorly developed (data not shown).

Testes were cultured in the basal medium without steroid hormones for periods up to 36 days. Under this culture condition, the testicular architecture was well maintained for at least 36 days; there were still only type A and early-type B spermatogonia in cultures after 36 days (Fig. 1B).

Effects of 11-Ketotestosterone on Induction of Mitosis in Spermatogonia *in Vitro*. The first experiment was conducted to examine whether 11-ketotestosterone induces mitosis *in vitro*. Testes removed from eels were cultured for 15 days in medium with or without various concentrations of 11-

FIG. 2. Electron micrographs of spermatogenic cells in testes before culture or cultured in medium with 11-ketotestosterone at 10 ng/ml. (A) A type A spermatogonium surrounded by Sertoli cells (S) before culture; (B) A spermatocyte with synaptonemal complexes (arrowhead) cultured for 18 days; spermatids (C) and a spermatozoon (D) with 9 + 0 flagellum (Inset) cultured for 21 days. (Bars: A–D, 1 μ m; Inset, 0.1 μ m.)

FIG. 3. Effect of various steroid hormones on induction of mitosis *in vitro*. Testis fragments were cultured in medium with various steroid hormones for 15 days. C, control; KT, 11-ketotestosterone; HT, 11 β -hydroxytestosterone; T, testosterone; DHT, 5 β -dihydrotestosterone; DHA, dehydroepiandrosterone; A, androsterone; AD, androstenedione; F, cortisol.

ketotestosterone (0.01, 1, 10, and 100 ng/ml). The appearance of late-type B spermatogonia in cysts was used as the criterion of mitosis. Late-type B spermatogonia had dense and heterogeneous nuclei. The mitochondria had a darker matrix and were smaller and more elongate than those of type A and early-type B spermatogonia. Concentrations of 10 and 100 ng/ml were almost equally effective; mitosis occurred in 50–60% of cysts. The concentration of 10 ng/ml corresponds to that in the plasma of maturing male eels receiving a single injection of human chorionic gonadotropin (12). In contrast, the lower two concentrations were without effect.

Effects of Various Steroids on Induction of Mitosis in Spermatogonia *in Vitro*. The next experiment was conducted to determine the effectiveness of eight different steroid hormones (11-ketotestosterone, 11 β -hydroxytestosterone, testosterone, 5 β -dihydrotestosterone, dehydroepiandrosterone, androsterone, androstenedione, and cortisol) on induction of mitosis *in vitro*. Testis fragments were cultured in medium containing one of these steroids at a dose of 10 ng/ml for 15 days. Fig. 3 shows that active mitosis occurred within the cultured testes only when 11-ketotestosterone was added to the medium. Although a slight stimulation was observed with 11 β -hydroxytestosterone and testosterone, this may have been from the conversion of these steroids to 11-ketotestosterone by endogenous enzymes.

FIG. 4. Light micrograph of toluidine-blue 1- μ m section of testis cultured in medium with 11-ketotestosterone at 10 ng/ml for 36 days. (Bar = 10 μ m.)

FIG. 5. 11-Ketotestosterone-induced spermatogenesis *in vitro*. Testis fragments were cultured in medium with 11-ketotestosterone at 10 ng/ml for various periods up to 36 days. GA, type A spermatogonium; E-GB, early-type B spermatogonium; L-GB, late-type B spermatogonium; C, spermatocyte; T, spermatid; Z, spermatozoon.

Effects of 11-Ketotestosterone on Spermatogenesis *in Vitro*.

We then examined the effects of 11-ketotestosterone on spermatogenesis *in vitro*. Sequential changes in testicular germ cells and somatic cells were investigated in cultures for periods up to 36 days in the presence of 11-ketotestosterone at 10 ng/ml. Nine days after the start of culture with 11-ketotestosterone, spermatogonia began mitotic division, producing late-type B spermatogonia. Zygotene spermatocytes of the meiotic prophase occurred in testes cultured for 18 days (Fig. 2B). After 21 days, spermatids and spermatozoa were observed for the first time (Fig. 2C and D). These spermatids had small, round, and heterogeneous nuclei. Each spermatozoon possessed a crescent-shaped nucleus. On the caudal end of the base of the nucleus, a flagellum with a 9 + 0 axonemal structure was attached (Fig. 2D). After 36 days, all stages of germ cells were present (Fig. 4) in the following percentages (Fig. 5): type A and early-type B spermatogonia, $5.6 \pm 1.9\%$; late-type B spermatogonia, $32.4 \pm 6.1\%$; spermatocytes, $38.6 \pm 6.6\%$; spermatids, $15.2 \pm 1.5\%$; and spermatozoa, $8.2 \pm 1.9\%$.

Treatment with 11-ketotestosterone also induced a marked cytological activation in Sertoli cells (Fig. 6A and B). In Sertoli cells after 6 days in culture with 11-ketotestosterone, the area of Sertoli cell nuclei increased significantly compared with those of initial controls and controls cultured in medium without 11-ketotestosterone for 6 days (Fig. 7A and B). The cytoplasm became filled with organelles including Golgi apparatus, smooth endoplasmic reticulum, and free ribosomes. These active features of Sertoli cells were maintained in culture for up to 36 days. In contrast, the morphology of Leydig cells remained unchanged throughout the experimental period.

DISCUSSION

Because cultivated male Japanese eel have immature testes containing only type A and early-type B spermatogonia, appearance of any spermatogenic cells beyond these stages indicates the definite proof of new formation in culture. By using a newly developed organ culture system for eel testes, we obtained evidence that 11-ketotestosterone, a major androgen in teleosts, can induce the entire process of spermatogenesis *in vitro* from premitotic spermatogonia to sper-

FIG. 6. Electron micrographs of Sertoli cells of eel testis explants cultured in medium alone (A) or with 11-ketotestosterone at 10 ng/ml (B) for 6 days. GA, type A spermatogonium; S, Sertoli cell. (Bars = 1 μ m.)

matozoa within 21 days. The time required for the progression of premitotic spermatogonia to spermatozoa in cultured testis fragments was similar to the *in vivo* timing determined by Miura *et al.* (12). Furthermore, the structures of spermatozoa developed in culture were comparable to those of the spermatozoa of Japanese eel obtained after a single injection (12) or repeated injections of human chorionic gonadotropin (11). Due to the unavailability of mature eggs of the Japanese eel (19), however, we could not determine whether spermatozoa obtained from the cultured testes are capable of fertilizing eel eggs and supporting normal embryonic development.

It has been reported that in some teleosts the major testicular and circulating androgens are the 11-oxygenated metabolites of testosterone. The first identification of 11-ketotestosterone was by Idler *et al.* (20) in plasma of sockeye salmon (*Oncorhynchus nerka*), and since then it has been demonstrated in the males of numerous teleost species (21, 22). In the cultivated Japanese eel, human chorionic gonadotropin injection markedly increased the serum levels of 11-ketotestosterone but not of testosterone as early as 1 day after injection; thereafter high levels were maintained throughout the period of spermatogenesis (12). Similarly, eel testes produced a large amount of 11-ketotestosterone in response to gonadotropin *in vitro* (12). Furthermore, our recent studies with thin-layer chromatography have also revealed that 11-ketotestosterone is the major metabolite of 17 α -hydroxyprogesterone and testosterone in testis frag-

ments of human chorionic gonadotropin-injected eels (T.M. and Y.N., unpublished work). Thus, it is concluded that 11-ketotestosterone is the major androgen produced in eel testes during spermatogenesis.

In this study, Leydig cells remained inactive throughout 11-ketotestosterone-induced spermatogenesis *in vitro*. Our earlier studies showed that eel Leydig cells became activated after a single injection of human chorionic gonadotropin, with ultrastructural features characteristic of active steroid hormone production, such as the occurrence of mitochondria with developed tubular cristae and the development of smooth endoplasmic reticulum. This activation of the Leydig cells was accompanied by a marked increase in plasma levels of 11-ketotestosterone (12). Thus, the major role of Leydig cells during spermatogenesis in the eel is probably to produce 11-ketotestosterone in response to gonadotropin, which, in turn, initiates spermatogenesis.

Although numerous *in vivo* studies have suggested the important role of androgens in vertebrate spermatogenesis (1–3), there have been, to our knowledge, no *in vitro* studies to directly show the involvement of androgens in this process. Our results indicate that 11-ketotestosterone induces and maintains all stages of spermatogenesis, including spermatogonial proliferation, meiotic division, and spermiogenesis. Six related androgens were found to be without effect or to have minimal effects. This, then, is an example of an animal system in which all stages of spermatogenesis, from premitotic spermatogonia to spermatozoa, have been induced by androgen manipulation *in vitro*. Risley *et al.* (23), using *Xenopus* testes, in which full spermatogenesis existed at the time of explantation, were able to develop spermatogonia to sperm *in vitro*. In their study, exogenous testosterone was not essential for the maintenance of spermatogenesis; however, the involvement of endogenous androgens produced by the cultured testis fragments cannot be eliminated.

It is unclear whether 11-ketotestosterone directly affects spermatogenic cells or whether their mechanism of action requires Sertoli cells. In this respect, it is of great interest that the first morphological changes induced in testis fragments by 11-ketotestosterone were seen in Sertoli cells. Subsequently, the proliferation of spermatogonia, meiosis, and spermiogenesis were observed, with the continuous presence of active Sertoli cells throughout the period of spermatogenesis. These results suggest that the action of 11-ketotestosterone on spermatogenesis is mediated through the action of Sertoli cells. Because active Sertoli cells did not possess cell organelles typical of steroid production, it is unlikely that the role of Sertoli cells on spermatogenesis is to produce another steroid hormone in response to 11-ketotestosterone. Spermatogenesis in vertebrates occurs in the testes in a complex

FIG. 7. Morphometric analysis of Leydig cells (A) and Sertoli cells (B) in testes before culture (IC) and cultured in medium alone (C) and with 10 ng/ml 11-ketotestosterone at 10 ng/ml (KT) for 6 days.

cellular environment and in close association with Sertoli cells. Although exactly how the Sertoli cells may assist spermatogenic cell development is not yet clear, a number of specific functions have now been ascribed to the Sertoli cells, including secretion of nutrients and mechanical support for germ cells (6, 24, 25). An important role for Sertoli cells in the regulation of mammalian spermatogenesis is also suggested by the demonstration of Sertoli cells as targets for follicle-stimulating hormone and testosterone (26). Therefore, it is important to determine the role of Sertoli cells during 11-ketotestosterone-induced spermatogenesis in the eel. Using the cell separation techniques, we have recently succeeded in isolating both premitotic spermatogonia and Sertoli cells from immature eel testes (T.M. and Y.N., unpublished work). Studies of spermatogonia cultured in medium containing 11-ketotestosterone with or without Sertoli cells provide insights into the role of Sertoli cells in androgen-induced spermatogenesis and may help identify Sertoli cell secretory products that influence spermatogenesis.

In conclusion, the results of the present *in vitro* studies, together with our earlier findings *in vivo*, suggest that hormonal induction of spermatogenesis in eel testes is via gonadotropin stimulation of Leydig cells to produce 11-ketotestosterone. In turn, 11-ketotestosterone activates Sertoli cells to stimulate premitotic spermatogonia and complete spermatogenesis. The testis of the cultivated Japanese eel, combined with the techniques of organ and cell culture, should facilitate investigations of the hormonal regulation of spermatogenic cell differentiation and development in vertebrates.

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