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Estrogen-Independent Ovary Formation in the Medaka Fish, *Oryzias latipes*

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ABSTRACT—To investigate whether a female sex steroid, estrogen, acts as a natural inducer of female gonadal sex determination (or ovary formation) in the medaka fish, *Oryzias latipes*, the effects of an aromatase inhibitor and anti-estrogens on sexual differentiation of gonads were examined. We found that both drugs did not show any discernible effects on the genetically determined sex differentiation in both sexes. However, the aromatase inhibitor impaired the paradoxical effects of androgen (a male sex steroid), and the anti-estrogens inhibited the male-to-female sex reversal caused by estrogen. Treatments of the fertilized eggs with androgen disturbed the gonadal sex developments in both sexes, suggesting that sex steroid synthesis is detrimental to the gonadal sex developments in the medaka embryos. These results are consistent with the previous observation that sex steroids are not synthesized before the onset of gonadal sex differentiation, and suggest that ovary formation in the genetic females of the medaka fish is not dependent on estrogen.

INTRODUCTION

Gonadal sex determination is a process of choice between testicular and ovarian developments from the same primordial gonad. Sex determination in non-mammalian vertebrates may be fundamentally different from that of mammals, and is thought to be under the control of genetics and various environmental factors. In mammals, sex steroids (androgen and estrogen) are considered gonadal products synthesized as a result of gonadal sex differentiation, and they are believed to act in establishing the secondary sexual phenotype. However, this is clearly not the case in lower vertebrates, in which sex steroids can affect or reverse gonadal sex development if applied early enough during development. In chickens (Scheib, 1983) and turtles (Dorizzi *et al*., 1991), estrogens are synthesized by morphologically undifferentiated female gonads. Treatment with an aromatase inhibitor (which blocks the synthesis of estrogen from androgen) causes females to develop testes (Elbrecht and Smith, 1992; Richard-Mercier *et al*., 1995). Treatment with an anti-estrogen (which binds to estrogen receptors and compete with estrogens) does not disrupt but disturb ovarian development showing partial masculinization (some testicular appearance of female gonads) (Scheib, 1983; Dorizzi *et al*., 1991). The evidence is in favor of a role for estrogen in ovarian differentiation. However, it is not demonstrated unambiguously whether estrogen is identical with a natural sex inducer in female and whether there exists another, estrogen-independent pathway for ovarian develpoment, parallel to the estrogen-dependent one. In contrast, histological and ultrastructual studies in some fishes including medaka, *Oryzias latipes*, reveal that steroid hormone biosynthesis and steroid-producing cells appear after the completion of gonadal sex differentiation (Iwasaki, 1973; Takahashi and Iwasaki, 1973; Kagawa and Takano, 1979; Schreibman *et al*., 1982; van den Hurk *et al*., 1982; Kanamori *et al*., 1985). This suggests that gonadal sex determination does not depend on sex steroids in these fishes. However, pharmacological studies of these fishes using aromatase inhibitors and anti-estrogens have not been done, thus, the view remains an open question. We show here that both drugs have no discernible effects on genetically determined gonadal development of medaka fish, indicating that female sex determination of this fish is independent of estrogen.

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MATERIALS AND METHODS

We used the d-rR strain of medaka with orange-red (in body color) male (X^rY^R) and white female (X^rX^r) (Yamamoto, 1969). The genotype of sex can be judged by body color with more than 99% reliability (Yamamoto, 1969). Morphological sex differentiation of germ cells occurs at the time of hatching (Satoh and Egami, 1972; Hamaguchi, 1982). The fertilized eggs (1- or 2-day old after fertilization) were incubated in Yamamoto's salt solution at 25°C under artificial photoperiod of 14L:10D. The fry were then transferred to plastic aquaria and reared to adult by powdered Tetramin under the same condition for 3 months. Adult fish were dissected for sexing gonads under a

dissecting microscope.

RESULTS AND DISCUSSION

Treatment with aromatase inhibitor and androgen

Fertilized eggs were incubated in the presence of sublethal concentrations of an aromatase inhibitor, and after hatching fry were reared to adult by normal diet, dissected for sexing gonads. We could not detect any defects in both ovarian and testicular developments (Table 1): each sex developed normal size of ovary or testis which contained normal number of oocytes or sperms, respectively. The result is consistent with the notion that sex steroids are not synthesized in embryos and not involved in sex determination.

To examine whether exogenous androgen may disturb gonadal differentiation, eggs were incubated in the presence of methylandrostenediol (MA) (Table 1). Some genetic females sex-reversed to males with testes. This contrasts to the absence of sex-reversal by androgen treatment of chickens and turtles in which female embryos contain aromatase activity (Elbrecht and Smith, 1992; Richard-Mercier *et al*., 1995). Higher dosages of MA frequently produced fishes without visible gonads under a dissecting microscope, indicating the toxic action of androgen (or its metabolites) (Yamamoto, 1969). MA also sex-reversed from genetic males to females with ovaries. This was unexpected because oral administration of androgen does not cause the male-to-female sex reversal in medaka (Yamamoto, 1969). This paradoxical effect of androgen is probably the result of conversion of androgen to estrogen by aromatase present in male embryos because co-administration of aromatase inhibitor impaired the androgen effect. Another androgen, methyltestosterone, showed essentially the same results as MA did (data not shown). Taken together, androgen synthesis may be detrimental to genetically determined gonadal development in medaka embryos: in female embryos, even a low level of androgen could not be aromatized to estrogen and induced male gonadal development; and in male embryos, androgen could be aromatized to estrogen which induced female gonadal development.

Table 1. Aromatase inhibitor has no effects on gonadal differentiation. Eggs were incubated in Yamamoto's salt solution containing aromatase inhibitor (AI; Fadrozole; Novartis Pharma Inc.) and/or methylandrostenediol (MA) at the indicated concentrations (μg/ml), and after hatching fry were reared to adult by normal diet. Each value represents total number of fish carrying the indicated gonad from two or three independent experiments with similar results. O, ovary; T, testis; OT, ovotestis; and NF, gonads not found in abdominal cavity.

AI	MA	Orange-red (X^rY^R)				White (X^rX^r)			
		T	O	OT	NF	O	T	OT	NF
10	–	101	0	0	0	105	0	0	0
20	–	57	0	0	0	65	0	0	1
50	–	33	0	0	0	35	1	0	0
–	0.5	0	4	0	15	5	0	0	11
–	0.1	32	70	7	11	65	4	3	11
–	0.01	17	6	3	3	40	10	0	3
10	0.1	32	2	5	6	36	1	3	3
20	0.1	59	25	6	9	53	1	0	4
50	0.1	6	1	1	0	8	0	3	2

Treatment with anti-estrogens

We next examined for the effects of anti-estrogens on female gonadal differentiation (Table 2). It is well known that newly hatched fry of genetic males can be sex-reversed by oral administration of estrogen (Yamamoto, 1969), where gonadogenesis of the sex-reversed fry is morphologically the same as that of genetic female (Onitake, 1972). The sex reversal caused by oral administration of 17β-estradiol was completely inhibited by co-administration of anti-estrogen, tamoxifen, or partially by another anti-estrogen, ICI182780. We further examined for the effects of anti-estrogens on newly hatched fry destined to be female by incubating eggs in the presence of 17β-estradiol. Both anti-estrogens again impaired the male-to-female sex reversal. These results indicate that an estrogen receptor is involved in the estrogen-induced sex-reversal. However, each anti-estrogen could not affect normal ovarian development of genetic females at all. We also found that treatments of fertilized eggs with sublethal concentrations of anti-estrogens could not affect the genetically determined gonadal developments (data not shown). These results further support the conclusion that female sex determination in medaka is not dependent on estrogen. Recently, Miyata and colleagues (1999) reported the luck of detectable effects of an aromatase inhibitor on the gonadal sex differentiation in *Xenopus laevis*. They also found that expression of the aromatase gene was absent at the beginning of the estradiol-sensitive period and proposed that estradiol synthesis is not naturally involved in the gonadal sex differentiation.

If our conclusion is correct, one would expect that partial masculinization by anti-estrogens of female gonads in chickens and turtles could be explained by the presence of two parallel pathways for ovarian development: one is estrogen-dependent and another estrogen-independent. We assume that activation of estrogen receptor plays a key role in both pathways: one activator is estrogen and another activation mechanism may be similar to those recently proposed as ligand-independent activation of estrogen receptor (Power *et al*., 1991; Zwijsen *et al*., 1998). In medaka fish and *Xenopus* frogs, the estrogen-independent activation of estrogen receptor may be a primary pathway to the female gonadal development. In other lower vertebrates such as salmon (Piferrer *et al*., 1994), amphibia (Yu *et al*., 1993), and reptiles (Lance and Bogart, 1992), treatment with aromatase inhibitors causes the female-to-male sex reversal or disrupts normal ovarian developments, suggesting the importance of estrogen in the ovary formation in these animals. However, it should be noted formally that the findings with aromatase inhibitors only demonstrate the synthesis of sex steroid hormones during the sex determination period and the ability of androgens to cause female-to-male sex reversal in these animals but not the importance of estrogen as a natural sex inducer in the gonadal differentiation. It remains to be seen whether exogenous

Table 2. Anti–estrogen does not disturb female gonadal development. Newly hatched fry were reared to adult by Tetramin containing 17β-estradiol (E_2), tamoxifen (TAM), or ICI182780 (ICI; Zeneca Pharmaceuticals) at the indicated concentrations (μg/g of diet). Eggs were incubated in the presence of E_2 at the indicated concentration (μg/ml), and after hatching fry were reared to adult by Tetramin or the same diet containing TAM or ICI.

Egg	Oral after hatching			Orange–red (X^rY^R)				White (X^rX^r)			
E_2	E_2	TAM	ICI	T	O	OT	NF	O	T	OT	NF
–	5	–	–	47	0	1	0	32	0	0	0
–	10	–	–	7	9	23	0	19	0	0	0
–	20	–	–	0	106	0	0	105	0	0	1
–	50	–	–	0	21	1	0	22	0	0	0
–	10	100	–	29	6	6	0	17	0	0	0
–	10	1000	–	26	7	5	0	30	0	0	0
–	20	1000	–	42	19	11	0	45	0	0	0
–	20	2000	–	61	0	0	0	60	0	0	0
–	20	5000	–	49	0	0	0	51	0	0	0
–	20	–	2000	23	6	0	2	22	0	0	0
–	–	10	–	11	0	0	0	29	0	0	0
–	–	100	–	16	0	0	0	26	0	0	0
–	–	1000	–	22	0	0	0	43	0	0	0
–	–	2500	–	10	0	0	0	37	0	0	0
–	–	5000	–	24	0	0	0	55	0	0	0
–	–	–	1000	15	0	0	0	15	0	0	0
–	–	–	2000	29	0	0	0	37	0	0	1
0.2	–	–	–	2	89	0	1	75	0	0	0
0.2	–	2000	–	20	40	0	2	74	0	0	0
0.2	–	–	2000	8	19	0	2	34	0	0	0

estrogens can cause the male-to-female sex reversal at the same concentrations found in undifferentiated female embryos or whether anti-estrogens can disrupt female gonadal developments.

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