Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish

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Summary

Large numbers of apoptotic early diplotene oocytes were observed during the transition from ovary-like undifferentiated gonadal tissue to testes during sex differentiation in presumptive males of the zebrafish (Danio rerio). The percentage of terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL)-positive apoptotic oocytes in the gonads of presumptive males was approximately eight- to 12-fold higher than in genetic all-females. By 29 days post-hatching, all oocytes had disappeared from the gonads of presumptive males. In these males, we also observed apoptotic somatic cells in the ovarian cavity between 23 and 35 days post-hatching. Therefore, the disappearance of oocytes and the decomposition of the ovarian cavity caused by apoptosis during sex differentiation were male-specific events. In genetic all-females, apoptosis in a proportion of early diplotene oocytes was found in the undifferentiated gonads at 15–19 days post-hatching, probably as a result of programmed oocyte loss during ovarian development. These findings suggest that oocyte apoptosis is the mechanism of testicular and ovarian differentiation in zebrafish.

Key words: sex, differentiation, oocyte, apoptosis, testis, ovary, juvenile hermaphroditism, zebrafish, Danio rerio.

Introduction

During gonadal development in juvenile zebrafish, all individuals first develop undifferentiated ovary-like gonads, regardless of genotypic sex. In male zebrafish, the oocytes all disappear from the gonads by 30 days post hatching, and spermatocytes develop with testicular differentiation (Takahashi, 1977). In contrast, oocytes in ovaries grow to maturation. This phenomenon of having undifferentiated ovary-like gonads during the juvenile period is known as juvenile hermaphroditism (Takahashi, 1977). The present study focuses on the mechanisms of gonadal tissue differentiation and juvenile sex-reversal in zebrafish. The disappearance of oocytes within gonads as they change from ovary-like tissue to phenotypic testes is assumed to be caused by apoptosis (programmed cell death).

Cell death is an important feature of normal development and the maintenance of homeostasis (Cohen et al., 1992; Ellis et al., 1991; Granerus and Engstrom, 1996; Jacobson et al., 1997; Kerr et al., 1972). Cells undergoing apoptosis demonstrate nuclear/cytoplasmic condensation and membrane protrusions. These initial changes are followed by DNA fragmentation and subsequent encapsulation of these fragments into apoptotic bodies. To date, several studies have been conducted on apoptosis in gonadal tissue in fish and higher vertebrates.

In mammals, loss of oocytes occurs either directly through attrition, i.e. germ cell death, or indirectly through follicular atresia, i.e. somatic or granulosal cell death, and is associated with the time of normal or premature reproductive senescence in females (Tilly, 1996; Wise et al., 1996). In humans, approximately 10⁶ oocytes are initially present in the ovaries; however, as a result of apoptosis, fewer than 4×10⁵ oocytes are present by the time females reach puberty and fewer than 10³ oocytes remain in the years immediately preceding ovarian senescence (Wise et al., 1996). In the case of fish, such as rainbow trout (Oncorhynchus mykiss), apoptosis of preovulatory ovarian follicles is suppressed by the administration of partially purified salmon gonadotropin SG-G100, 17β-oestradiol and epidermal growth factor (Janz and Van Der Kraak, 1997).

In mammals, testicular germ cell apoptosis occurs normally and continuously throughout life. Overproliferation of early germ cells is tempered by selective apoptosis of the progeny...
Large numbers of spermatocytes undergo apoptosis in the testes of 4-week-old rats, whereas spermatogonia are the principal cell type undergoing apoptosis in adult rats. Massive testicular germ cell loss by apoptosis results from exposure to toxins (Richburg and Boekelheide, 1996), heat (Miraglia and Hayashi, 1993; Ohta et al., 1996) and radiation (Meistrich, 1993), as well as in response to treatment with chemotherapeutic compounds (Meistrich, 1993). In many of these situations, germ cells are known to undergo apoptosis, which indicates that a specific pathway is activated when the testicular environment cannot support spermatogenesis. Therefore, apoptosis plays an important role in the maturation of oocytes and testes in fish and other animals. However, it remains unclear whether germ cell apoptosis is involved in gonadal development and sex differentiation.

In this study, we identified apoptotic gonadal cells using in situ detection by the terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL) method to define the tissue- and cell-specific changes necessary for gonadal development and differentiation that are induced by oocyte apoptosis in juvenile zebrafish. Our findings suggest that oocyte apoptosis plays an important role in testicular differentiation in juvenile zebrafish.

**Materials and methods**

**Zebrafish**

Zebrafish (*Danio rerio*) were maintained on a 14h:10h light:dark cycle according to standard conditions (Westferfield, 1995). Each group of 20 larvae or juveniles was reared at 28.5 °C in 4-l freshwater tanks from 4 days post-fertilization with a diet of *Artemia salina* nauplii and commercial feed (Tetramine Baby; Tetra, Melle, Germany). Genetic all-females were produced by natural mating of wild-type females with gynogenetic, sex-reversed males. Gynogenetic diploid fish were produced by natural mating of wild-type females with a diet of *Tetramine Baby* (Tetra, Melle, Germany). The protocol was slightly modified by preparing ultraviolet-irradiated (880 J m–2 min –1) spermatozoa using an ultraviolet crosslinker (UVP, San Gabriel, CA, USA). The resulting gynogenetic fish was checked by DNA fingerprinting using tandem-repeat-primed polymerase chain reaction. The percentage of phenotypic females in each group was determined by histological observations of the gonads. Mixed-sex groups of wild-type fish were produced naturally by mating wild-type females and males. Total length and mass were measured, and tissue samples were collected from genetic all-females and wild-type fish at the indicated times post-hatching. Care and treatment of experimental animals received the approval of the Institutional Animal Care and Use Committee of the National Research Institute of Fisheries Science.

**Histological analysis and TUNEL staining**

To prepare tissue sections, fish were anaesthetized with 100 mg l⁻¹ tricaine methanesulphonate (Ardrich, Milwaukee, WI, USA), and tissue samples were collected. Gonads were fixed with Bouin’s solution, sectioned (4 µm thick) and stained with haematoxylin and eosin. For TUNEL staining, tissue samples were fixed in 4 % (w/v) paraformaldehyde in phosphate-buffered saline. Paraffin-embedded sections (5 µm cross sections) were used for in situ detection of DNA strand breaks in apoptotic cells (Gavrieli et al., 1992). After deparaffinization and rehydration, tissue sections were stained with terminal deoxynucleotidyl transferase (Roche, Wyningen, Germany) and fluorescein-12-2′-deoxuryridine-5′-triphosphate (Roche) in a humidified chamber at 37 °C for 1 h. Fluorescein-labelled apoptotic cells were identified by observation of both a fluorescent view in dark-field and a phase-contrast view in bright-field with a three-dimensional fluorescent microscope (R-400; Edge, Santa Monica, CA, USA). The sections were counterstained with haematoxylin and eosin, and photographed. Gonads from 20 fish per group at each stage were used for TUNEL staining. The percentages of TUNEL-positive oocytes were calculated by counting the number of TUNEL-positive apoptotic cells in the gonadal cells among six cross sections by cell type, according to Greier (1981).

**Statistical analyses**

Differences between percentages of oocytes and apoptotic oocytes in the gonadal cells between genetic all-females and wild-type fish were examined for statistical significance by calculating the mean ± S.E.M. and performing a one-way analysis of variance (ANOVA).

**Results**

**Generation of genetic all-females**

To investigate gonadal apoptosis during sex differentiation in juvenile zebrafish, genetic all-females and wild-type fish were used in this study. From 16 offspring of gynogenetic diploid individuals (15 males and one female), three groups of genetic all-females were obtained by natural mating of wild-type females with gynogenetic diploid males. Other groups were composed of 57.7–75.5 % females, which was slightly higher than the percentage of females in wild-type fish. The percentages of phenotypic females in genetic all-females and wild-type fish at 40 days post-hatching were 98.3±2.4 % (N=64) and 47.5±7.5 % (N=73), respectively. We measured the total length and body mass of genetic all-females (17.3±1.9 mm, 41.8±10.1 g; 40 days post-hatching) and wild-type fish (18.9±1.4 mm, 57.7±17.9 g; 40 days post-hatching) during sex differentiation and found that the growth of genetic all-females did not differ from that of wild-type fish at any stage.

**Sex differentiation patterns in zebrafish**

Gonadal development and sex differentiation in genetic all-females and wild-type fish were followed histologically according to the method of Takahashi (1977). Since no biochemical procedures for the determination of phenotypic sex
or genetic sex have been established for zebrafish, we compared the morphological features of gonadal tissues between genetic all-females and wild-type fish to characterize sex differentiation patterns from the undifferentiated ovary-like tissue to testes or ovaries. The ratio of perinucleolar oocytes in total germ cells was significantly different between the presumptive males and genetic all-females, indicating that sex differentiation began between 21 and 23 days post-hatching.

Wild-type fish showed two distinct morphological patterns of the gonads corresponding to presumptive males and presumptive females after 21 days post-hatching. We identified the phenotypic sex of each individual after 21 days post-hatching by counting the number of perinucleolar oocytes in the gonads, and found that the presumptive males had 17.3±2.7 % (N=10), the presumptive females had 42.3±6.2 % (N=10) and the genetic all-females had 44.3±3.6 % (N=10). Perinucleolar oocytes were not found at 17 days post-hatching. The percentages of perinucleolar oocytes were 8.0±3.4 % in the presumptive males and 10.1±3.7 % in the genetic all-females at 19 days post-hatching, showing no apparent difference between the two sexes.

**Apoptosis of early diplotene oocytes in the ovary-like tissue**

We identified apoptotic gonadal cells using in situ detection by the TUNEL method. The distribution patterns of early diplotene oocytes and apoptotic oocytes (cell diameter 6.4±0.2 μm, N=10) in undifferentiated gonads were indistinguishable between genetic all-females and wild-type fish by 20 days post-hatching. Many gonocytes and zygotene and pachytene oocytes were found at 10 days post-hatching. At this age, no apoptotic germ cells were found (Fig. 1A,B, see also Fig. 5). Early diplotene oocytes were found in ovary-like structures at 15 days post-hatching; the percentage of total gonadal cells was 19.5±4.5 % (N=10) in genetic all-females and 21.9±8.0 % (N=10) in wild-type fish (Fig. 1C). At this stage, only a few apoptotic oocytes (Fig. 1C,D, see also Fig. 5) were found in both genetic all-females and wild-type fish, and the percentage of total gonadal cells was 0.4±0.2 % (N=10) in the genetic all-females and 0.3±0.1 % (N=10) in the wild-type fish. The number of apoptotic oocytes was slightly larger by 17–19 days post-hatching (Fig. 1E–H, see also Fig. 5), and the percentage of gonadal cells was 0.8±0.2 % (N=10) in genetic all-females and 0.5±0.1 % (N=10) in wild-type fish.
Apoptosis of early diplotene oocytes in male gonads

To characterize the mechanism of male-specific changes during the gonadal transition from ovary-like tissue to testes, the degeneration of gonadal cells was examined by the TUNEL method. Oocytes in presumptive males of wild-type fish disappeared between 23 and 27 days post-hatching (Fig. 2A,C,E). Simultaneously, spermatogonia appeared near the degenerating oocytes, and large numbers of somatic cells were found in stroma along the periphery of the gonads at 23 days post-hatching (Fig. 2A); spermatocytes appeared at 27 days post-hatching (Fig. 2E). Testes were completely formed by 40 days post-hatching (Fig. 2G,H).

Most early diplotene, but only a few perinucleolar, oocytes were TUNEL-positive (Fig. 3A–D). The percentage of TUNEL-positive oocytes in gonadal cells was significantly higher in the presumptive males (0.78±0.27 %, N=10; P<0.05) than in the genetic all-females (0.16±0.01 %, N=10) at 21 days post-hatching (Fig. 4). In contrast, the percentage of early diplotene oocytes in the gonadal cells was similar in presumptive males (10.8±3.6 %, N=10) and genetic all-females (13.6±3.3 %, N=10) at 21 days post-hatching. The largest number of apoptotic early diplotene oocytes was observed in presumptive males between 23 and 25 days post-hatching. The percentage of apoptotic oocytes in gonadal cells was 0.9±0.2 % (N=10) at 23 days post-hatching (Figs 3A,B, 4) and 1.5±0.2 % (N=10) at 25 days post-hatching (Figs 3C,D, 4), respectively. The percentage of apoptotic oocytes in
Oocyte apoptosis in zebrafish gonadal cells in presumptive males was 7.8–12 times higher than in genetic all-females from 23 to 25 days post-hatching. At these stages, we frequently observed fragmented nuclei, which are typical of apoptotic cells, in the oocytes of presumptive males (Figs 3A,C, 4). The percentage of apoptotic oocytes in gonadal cells decreased (0.2±0.1 %, N=10) in presumptive males by 27 days post-hatching (Fig. 3E,F), and the testes had nearly matured by 29 days post-hatching (Fig. 4). Early diplotene oocytes were virtually absent from the gonads of presumptive males by 29 days post-hatching, and we found apoptotic somatic cells in the ovarian cavities, which decomposed and disappeared between 19 and 35 days post-hatching (Fig. 2C–F).

Apoptosis in testicular germ cells
Apoptotic spermatogonia were observed after 40 days post-hatching (Fig. 2G–J). Apoptotic spermatogonia and nucleus-condensed cells were located in the same cyst. Large numbers of apoptotic spermatogonia, spermatocytes and spermatids were found at 70 days post-hatching (Fig. 2I,J).

Apoptosis in ovarian gonadal cells
The pattern of differentiation from gonads to ovaries was characterized in genetic all-females. Developing perinucleolar oocytes were observed in the ovaries of genetic all-females, and ovarian connective tissue proliferated after 23 days post-hatching (Fig. 3A,C,E,G). Cortical alveolar oocytes and developing perinucleolar oocytes were found after 50 days post-hatching (Fig. 3G).

Large numbers of apoptotic somatic cells were observed in the ovarian connective tissue of genetic all-females after 23 days post-hatching (Fig. 3A–H). The numbers of these apoptotic somatic cells gradually increased in the connective tissue by 40 days post-hatching (Fig. 3C–F) and decreased at 70 days post-hatching (Fig. 3G,H). A few apoptotic somatic cells were found in the ovarian cavities of genetic all-females (Fig. 3B,D,F,H).

Discussion
Morphological and physiological juvenile hermaphroditism in zebrafish was found by 20 days post-hatching, as previously shown by Takahashi (1977). In the present study, we used genetic all-female lines produced by gynogenetic methods to observe the gonads during sex differentiation and to compare genetic all-females and wild-type fish.

Gynogenetic diploid founders were found to be male-rich (15 males and one female), suggesting that gynogenetic diploid males may have been sex-reversed from genetic females to
phenotypic males by the heat-shock treatment that immediately followed artificial fertilization during the gynogenetic procedure. The female-rich F1 progeny were obtained by natural mating of wild-type females with gynogenetic diploid males. At 40 days post-hatching, phenotypic females made up 98.3% of the F1 progeny of genetic all-females. By counting the percentage of perinucleolar oocytes in the total germ cells of male gonads, we determined that sex differentiation occurred between 19 and 23 days post-hatching in wild-type fish.

In this study, we observed apoptotic early diplotene oocytes during sex differentiation between 21 and 25 days post-hatching; the apoptotic oocytes disappeared rapidly by 29 days post-hatching. This pattern of oocyte apoptosis was male-specific and was found only in the early diplotene oocytes, but not in other types of germ cell. In addition, perinucleolar oocytes had disappeared from the gonads of presumptive males during sex differentiation and were not stained by the TUNEL method prior to their disappearance. Thus, the loss of these cells may be due to other cell death pathways that are not accompanied by DNA fragmentation. The disappearance of apoptotic oocytes from the male gonads was followed by the differentiation and development of spermatogonia, spermatocytes and spermatids. Such oocyte apoptosis is thus presumed to be male-specific, suggesting that prior apoptosis in early diplotene oocytes may trigger testicular differentiation.

At this stage, a proapoptotic signalling pathway specific to males may be present in the oocytes, and this pathway may be stimulated by sex-determining genes or by changes in oestrogen or thyroid hormone level.

A proportion of early diplotene oocytes showed apoptosis in the gonads of genetic all-females during the undifferentiated stage at 15–19 days post-hatching. Other germ cell types, such as gonocytes and perinucleolar oocytes, did not show apoptosis, and apoptotic early diplotene oocytes appeared only at this developmental stage. Such apoptosis in oocytes was rarely found in the gonads after 21 days post-hatching. In mammals, extensive oocyte loss by apoptosis in the ovary during puberty and ovarian senescence is well documented (Wise et al., 1996). The ovaries of female zebrafish at 15–19 days post-hatching probably undergo programmed oocyte loss during ovarian differentiation to eliminate unnecessary oocytes from the ovary.

Cytochrome P450 aromatase, an enzyme aromatizing testosterone to oestrogen, is known to regulate ovarian development in fish and other vertebrates (Chardard et al., 1995; Desvages et al., 1993; Desvages and Pieau, 1992; Young et al., 1983). In zebrafish, ovarian-type aromatase mRNA was

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**Fig. 4.** Percentage of TUNEL-positive oocytes in gonadal cells. Asterisks indicate significant (P<0.05) differences from genetic all-females (one-way ANOVA). Values are means ± s.e.m., N=10 fish.

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**Fig. 5.** Model for gonadal cell apoptosis. Typical cell types undergoing apoptosis during sex differentiation are illustrated. dph, days post-hatching. The width of each diamond represents the number of apoptotic gonadal cells.
localized in large vitellogenic follicles, but not in young small vitellogenic follicles (Chiang et al., 2001; Kishida and Callard, 2001; Kishida et al., 2001; Trant et al., 2001). Most developing oocytes expressed high levels of aromatase, whereas in male gonads that were changing from undifferentiated ovary-like tissue to testes, early diplotene oocytes expressed lower levels of aromatase than other cell types (D. Uchida, unpublished observations). In addition, the administration of the aromatase inhibitor fadrozole to zebrafish was found to induce oocyte apoptosis and sex reversal in genetic all-females (D. Uchida, unpublished observations). In chinook salmon (Oncorhynchus tshawytchesa), brief treatment with fadrozole during sex differentiation also caused genetic all-females to develop into phenotypic males (Piferrer et al., 1994). Kitano et al. (1999) suggested that low levels of aromatase regulate testicular differentiation in Japanese flounder (Paralichthys olivaceus). Miura et al. (1999) found that 17β-oestradiol stimulated the renewal of spermatogonial stem cells in the male gonad of Japanese eel (Anguilla japonica). It is therefore possible that oocyte apoptosis followed by spermatogenesis during sex differentiation in zebrafish may be induced by the depletion of aromatase and oestrogen.

In addition to genetic sex determination, environmental factors, such as heat stress, low pH, salinity, nutrition and exogenous oestrogen and androgen levels are known to influence the determination of phenotypic sex in fish (Kitano et al., 1999; Korpelainen, 1990; Nakamura and Takahashi, 1973; Shapiro, 1990; Yamamoto, 1953). Heat-induced germ cell deficiency was found in males and females of the South American atherinid fish Odontesthes bonariensis and Patagonia hatchery (Strüssmann et al., 1998). Zebrafish are also sensitive to environmental factors that may influence sex determination (Chan and Yeung, 1983), although the sex chromosomes in fishes have not yet been identified (Pelegri and Schulte-Merker, 1999). The inheritance pattern of genetic all-females used in this study suggests that the genetic sex of the undifferentiated gonads at 15–19 days post-hatching, suggesting a programmed loss of oocytes during ovarian development.

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