

Role of epithelial cells and programmed cell death in *Hydra* spermatogenesis

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Summary

Spermatogenesis in higher animals is a tightly regulated process, in which survival and death of sperm precursor cells depends on the presence of somatic cells in gonads. In the basal metazoan *Hydra* spermatogenesis takes place in anatomically simple testes and in the absence of accessory structures. *Hydra* sperm precursors are derived from interstitial stem cells. Here we show that large numbers of sperm precursors in testes of *Hydra vulgaris* undergo programmed cell death (apoptosis) and that ectodermal epithelial cells phagocytose the apoptotic sperm precursors. This is surprising since so far no evidence has been reported that epithelial cells are directly involved in germ cell differentiation in *Hydra*. We propose that, similar to Sertoli cells in mammals, in *Hydra* epithelial cells support and perhaps even control spermatogenesis.

Key words: apoptosis, *Hydra*, phagocytosis, spermatogenesis, epithelial cells

Introduction

The cnidarian *Hydra* reproduces asexually through formation of buds. Although budding is the primary mode of propagation, all *Hydra* species have been observed to go through occasional sexual phases. Onset of egg and sperm differentiation is triggered by environmental signals. Under laboratory conditions, sexual differentiation of *Hydra* is induced by low temperatures or starvation. Germ cells in *Hydra* derive from multipotent interstitial stem cells (Bosch and David, 1986) which aggregate in the intraepithelial space to form ovaries and testes (Tardent, 1985). Male “gonads” are made of ectodermal epithelial cells which form conical swellings at the surface of the body column in which spermatogenesis occurs. Spermatogenesis is initiated by local accumulations of interstitial cells within the intercellular spaces of ectodermal epithelial cells. As these clusters of spermatogonia grow they lift the

stratum of the epithelial cells, which then forms the protruding outer wall of the testis (Tardent, 1974). Between the mesoglea and the outer epithelial wall, the various cell layers represent the succession of the various stages of spermatogenesis (Brien, 1966). Clusters of accumulated interstitial cells synchronously undergo meiotic divisions and the subsequent process of spermiogenesis (Munck and David, 1985). While the differentiation pathway as well as the structure of the mature spermatozoa is very similar in *Hydra* and higher metazoa, nothing is known about the temporal and spatial signals controlling spermatogenesis.

In higher animals germ cell proliferation and differentiation is regulated by a complex network of endocrine and paracrine signals. Apoptosis, also known as programmed cell death, is involved in maintaining homeostasis of each germ cell type (reviewed in Print and Loveland, 2000). Molecular components of the apoptotic machinery have been identified (Jacobson et al.,

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1997). Invention of the apoptotic machinery can be traced back in evolution from vertebrates to *Hydra*. Cikala et al. (1999) have shown that *Hydra* genes encoding caspase proteases are closely related to caspase genes in worm and man. Apoptosis in *Hydra* appears to have the same morphological features as apoptosis in higher animals and presumably is used to eliminate "excess" cells, e.g., in starving polyps (Bosch and David, 1984; Cikala et al., 1999). Apoptosis also plays a key role in *Hydra* oogenesis since the primary oocyte seems to induce apoptosis in immature nurse cells (Miller et al., 2000) and engulfs and integrates them as apoptotic bodies ("pseudocells", Kleinenberg, 1872; "shrunken cells", Zihler, 1972; Honegger et al., 1989) into the ooplasm to fulfill its nutritional needs.

In order to determine whether apoptosis is also involved in spermatogenesis, males of *Hydra vulgaris* were analyzed. Here we show that differentiation of interstitial cells into sperm is accompanied by massive programmed cell death. We also present evidence that the ectodermal epithelial cells act as phagocytes to remove apoptotic sperm precursors and, therefore, appear to play an important role in *Hydra* spermatogenesis.

Materials and methods

Animals

Experiments were carried out with *Hydra vulgaris* strains AEP and PA2. The strains were obtained from Dr. Ulrich Technau, Darmstadt, and maintained as stock culture as described (Martin et al., 1997). Sexual differentiation was induced by starving the animals for up to two weeks.

Histological analysis and detection of programmed cell death (apoptosis)

For histological analyses of sperm differentiation, polyps were fixed in 4% formaldehyde overnight at 4 °C. Polyps were relaxed in 2% urethane in *Hydra* medium for 2 min prior to fixation. Fixed animals were embedded in "Historesin" (Leica Instr. GmbH, Heidelberg, Germany) according to the manufacturers protocol. 3–5 µm thick sections were prepared according to standard procedures and stained with methylene blue/borax/basic fuchsin.

Hydra cells undergoing apoptosis were detected by three methods. (1) Animals were stained in acridine orange, a fluorescent dye that is widely used to specifically highlight apoptotic cells in a variety of organisms including *Hydra* (e.g.: Delic et al., 1991; Foster et al.; 1998; Cikala et al., 1999). Animals were stained essentially as described (Cikala et al., 1999) by incubating living polyps in 0.1 µM acridine orange for 10 min.

Thereafter the animals were washed several times with hydra medium, mounted on glass slides in a drop of 2% urethane and examined with a Zeiss Axiophot epifluorescence microscope attached to a Sony 3CCD colour video camera. (2) DNA fragmentation as indicator of apoptosis was detected by TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine-digoxigenin triphosphate nick-end labeling). Both whole hydras fixed in formaldehyde as well as maceration preparations (David, 1973) were washed with PBST (PBS, 0.2% Tween-20) and PBS followed by equilibration for 1 hr in TdT buffer (TdT-FragEL DNA fragmentation kit, Amersham Pharmacia Biotech). The terminal dUTP transfer reaction was performed using the TdT enzyme from the same kit (diluted 1:20 with TdT buffer) and 1 µM Dig-dUTP (Roche) for 2 hrs at 37 °C. The reaction was stopped by incubation in 1 mM EDTA in PBS at 65 °C for 2 hrs followed by washing with PBS. Detection of digoxigenin was carried out according to standard procedure using an antidigoxigenin antibody coupled with alkaline phosphatase and phosphatase reaction with NBT/BCIP as a substrate. (3) For identification of epithelial cells containing phagocytic vacuoles and apoptotic bodies maceration preparations were stained by the Feulgen method as described previously (Bosch and David, 1984).

Results

Morphological features of *Hydra* spermatogenesis

Spermatogenesis in *Hydra* takes place in ectodermal intraepithelial spaces along the body column in the absence of specialized gonadal epithelia (Tardent, 1985). There are no accessory gonadal structures such as associated follicle cells and ducts for the storage and/or release of the gametes. Figure 1 shows the differential localization of the differentiating sperm precursors in *Hydra* testis. Large spermatogonia with interphase nuclei form the basal layer adjacent to the mesoglea. More distally, large primary spermatocytes and smaller secondary spermatocytes with clearly visible condensed chromosomes undergo meiosis. Postmeiotic spermatids and spermatozoa are restricted to the middle region and the most distal germ layer. Ectodermal epithelial cells form not only the envelope of the conical testis in which spermatogenesis occurs, but are also found between the developing sperm precursors (Fig. 1B–D). Epithelial protrusions seem to divide the gonad into separate chambers with peripheral chambers containing mainly spermatogonia and spermatocytes and central regions mainly occupied by differentiating spermatids and spermatozoa (Fig. 1C and D). Due to the long epithelial protrusions which stretch from the surface towards the mesoglea (Fig. 1C and D), interstitial sperm

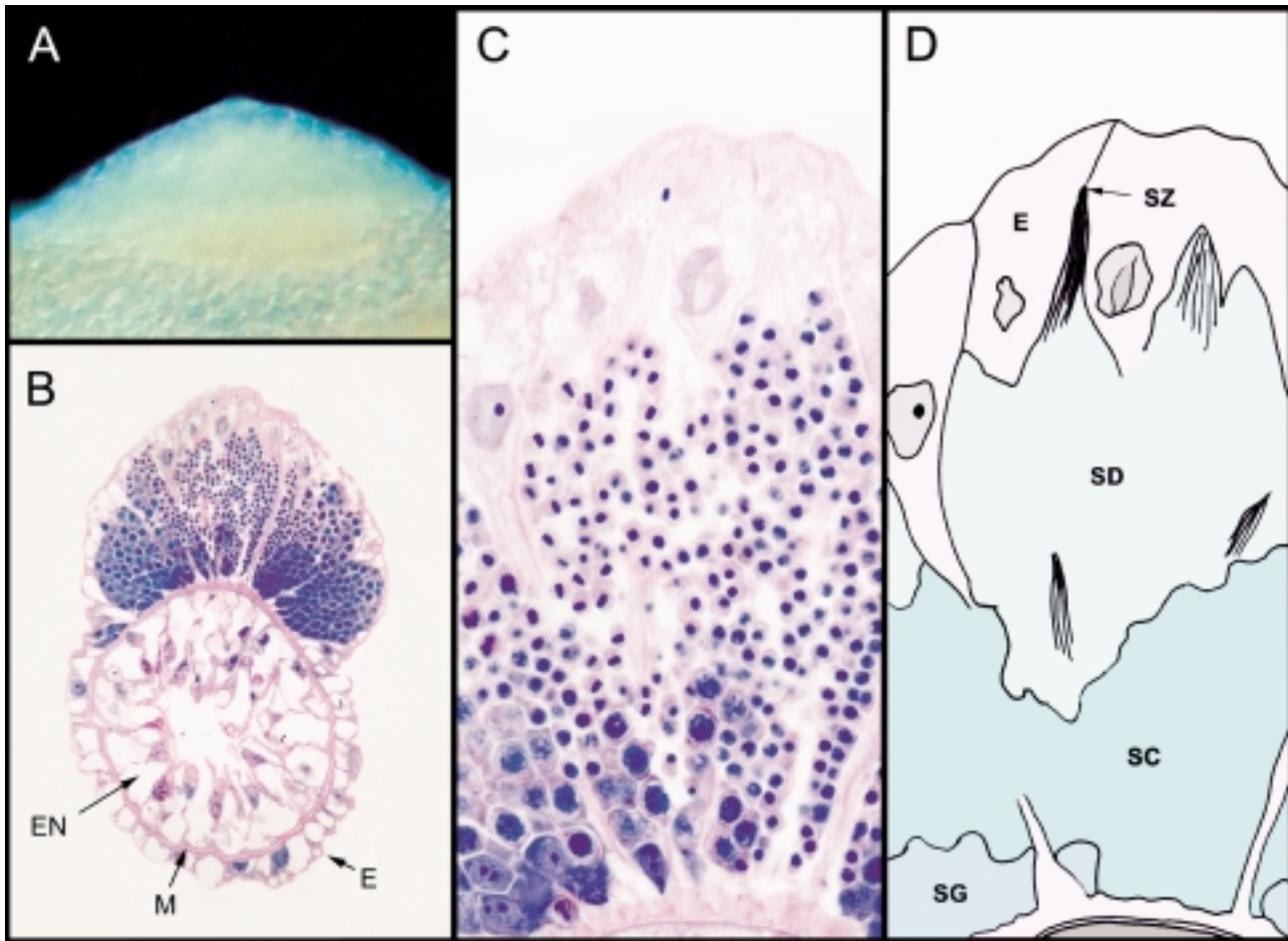


Fig. 1. The anatomy of the testis in *Hydra vulgaris*. (A) Testis viewed under phase-contrast illumination; (B) Methylene blue/basic fuchsin/borax stained section of mature testes. Abbreviations: E, ectoderm; EN, entoderm; M, mesoglea; (C) higher magnification ($\times 1000$) of the testis shown in (B). (D) Schematic diagram illustrating the close relationship between epitheliomuscular cells and male germ cells of all developmental stages. Abbreviations: SG, spermatogonia; SC, spermatocytes; SD, spermatids; SZ, spermatozoa; E, ectodermal epitheliomuscular cell.

precursor cells seem to be in close contact to epithelial cells all along their differentiation pathway.

Apoptosis in *Hydra* testes

Hydra cells undergoing apoptosis can be identified by various methods (see Material and methods). Staining mature male *Hydra vulgaris* polyps with acridine orange reveals large number of apoptotic cells within the testes (Fig. 2B and E). Acridine orange positive cells are not restricted to distinct regions of the testis (Fig. 2E) suggesting that sperm precursor cells undergo programmed cell death at various stages of spermatogenesis. In male polyps taken from the stock culture before the onset of sexual differentiation, little or no

signs of cell death were found in the body column (data not shown); in foot, head and tentacle tissue we observed a slight increase in the number of acridine orange positive cells, which is consistent with previous observations (Cikala et al., 1999) and the fact that hydra cells are continuously displaced towards the extremities. To support the view that acridine orange positive cells within the testes undergo programmed cell death, DNA fragmentation was analyzed in male polyps by a TUNEL (Gavrieli et al., 1996) assay. Figure 2C and F show that within each testis numerous nuclei are stained. Interestingly, most of the stained nuclei are located in the peripheral parts of the testes (Fig. 1F) and therefore most likely represent rather early stages of spermatogenesis.

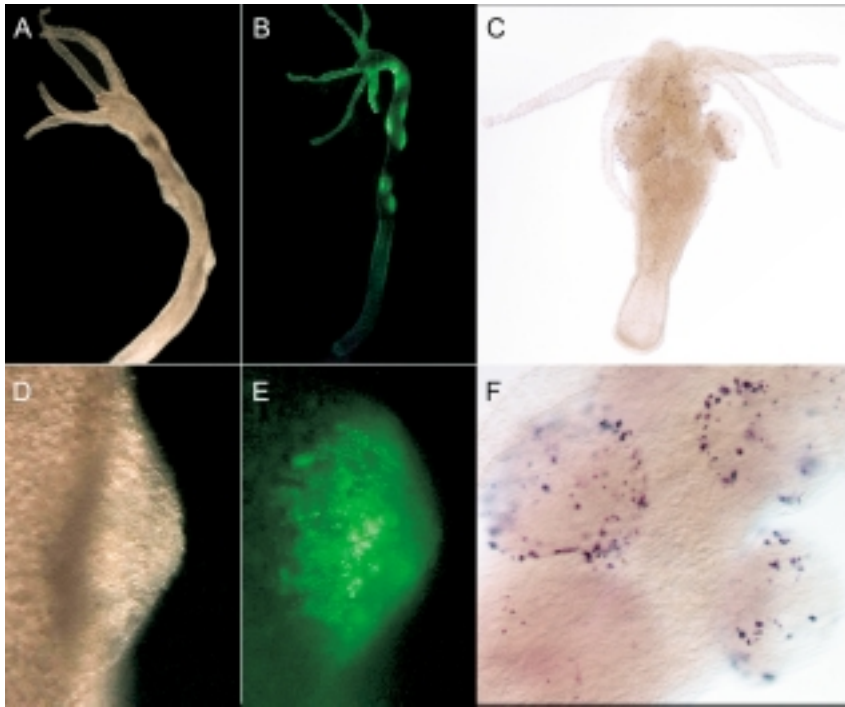


Fig. 2. Large numbers of cells undergo apoptosis in male *Hydra vulgaris*. (A) Sexually mature male *Hydra vulgaris* polyp; (B) male polyp stained with acridine orange. Signs of massive cell death (green staining) appear in all the testes; (D and E) higher magnification of one of the testes shown in (B). Signs of massive cell death are detectable throughout the whole testes; (C) Whole-mount detection of DNA fragmentation in male *Hydra vulgaris* by the TUNEL method; (F), higher magnification of the polyp shown in (C) demonstrating numerous TUNEL positive nuclei at the periphery of each testis.

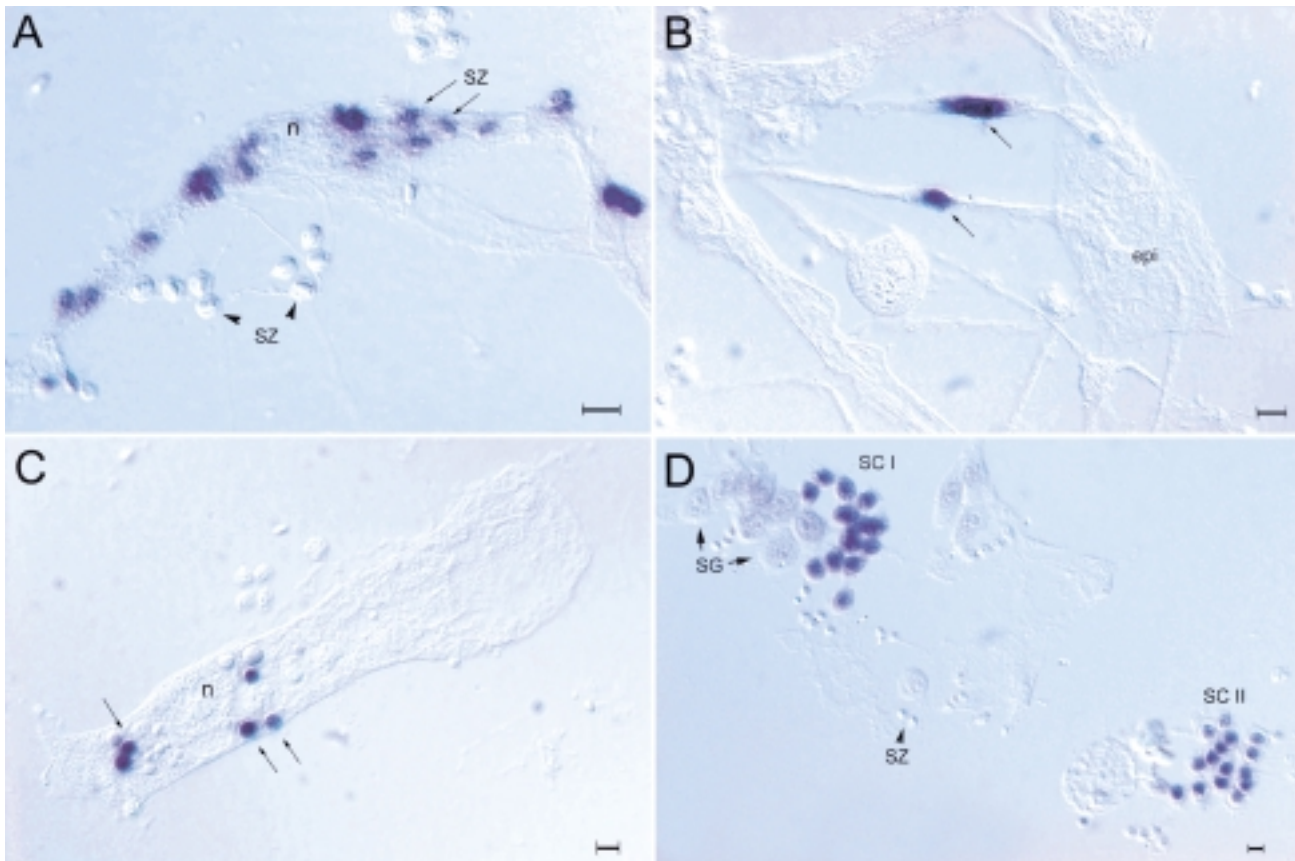


Fig. 3. Sperm precursors undergo apoptosis at various stages of spermatogenesis. (A–E) TUNEL stained maceration preparation of sexually mature *Hydra vulgaris* male. (A) TUNEL positive spermatozoa (arrows) enclosed in phagocytic vacuoles of an epithelial cell. SZ, spermatozoa; n, nucleus of the epithelial cell. Arrowheads indicate TUNEL negative spermatozoa. (B) Apoptotic nuclei in long protrusions of ectodermal epithelial cells (epi). (C) Epithelial cell containing phagocytosed sperm precursors other than spermatozoa; arrows indicate apoptotic bodies; n, nucleus of the epithelial cell. (D) Apoptotic spermatocytes prior to engulfment. SG, spermatogonia; SC I, primary spermatocytes; SC II, secondary spermatocytes; SZ, spermatozoa. Scale bar corresponds to 2 μ m.

Cell types undergoing programmed cell death

To identify the cell types undergoing apoptosis during spermatogenesis, maceration preparations of sexually mature male polyps were subjected to the TUNEL assay. As shown in figure 3, TUNEL positive cells can be found either as single cells (Fig. 3D) or in vacuoles within epithelial cells (Fig. 3A–C). Out of 236 TUNEL positive nuclei analyzed, 17 nuclei (7%) were identified as nuclei of single cells, mostly spermatogonial cells and spermatocytes (Fig. 3D). The remaining 219 TUNEL positive nuclei (93%) were found to be enclosed in phagocytic vacuoles within 93 epithelial cells. When enclosed within a phagocytic vacuole, the cell type corresponding to a TUNEL positive nucleus is more difficult to identify. According to their size and nuclear morphology, however, at least some of the nuclei (54/219) appear to belong to spermatozoa (Fig. 3A). TUNEL positive spermatozoa were found in only a few (10/93) epithelial cells. The remaining TUNEL positive cells appear to represent earlier stages of spermatogenesis. The observation that spermatozoa are phagocytosed during spermatogenesis was confirmed by Feulgen staining (see below, Fig. 4D).

Epithelial cells act as phagocytes and remove apoptotic sperm precursors

Identification of apoptotic sperm precursor cells reveals that these cells are engulfed and eliminated by ectodermal epithelial cells. Figure 3A–C indicates TUNEL

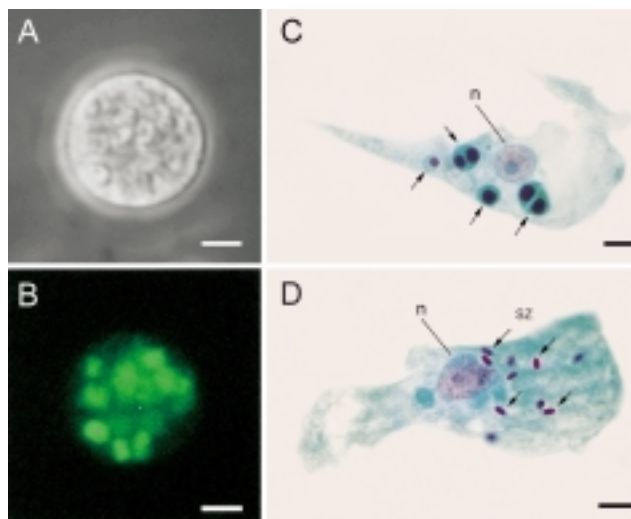


Fig. 4. Ectodermal epitheliomuscular cells function as phagocytes and eliminate apoptotic sperm precursor cells. (A) Epithelial cell after pronase treatment viewed under phase contrast; (B) The cell shown in (A) stained with acridine orange. (C) Feulgen stained epithelial cell containing several apoptotic bodies (arrows); n, nucleus of the epithelial cell. (D) Feulgen stained epithelial cell containing spermatozoa (arrows); n, nucleus of the epithelial cell. Scale bar corresponds to 3 μ m.

positive nuclei within phagocytic vacuoles of epithelial cells. While most of the engulfed nuclei are located in the central part of the epithelial cell close to the epithelial nucleus (Fig. 3A and C), TUNEL stained nuclei occasionally can also be found in long epithelial protrusions (Fig. 3B) indicating that the epithelial cells which form the supporting pillars for the protruding testes (see Fig. 1B–C) are able to phagocytose differentiating sperm cells even over long distances.

To support the apoptosis data obtained by the TUNEL assay, male polyps were also examined by staining with acridine orange and Feulgen (see Materials and methods). When testes were dissociated by pronase and stained with acridine orange, about 5 % of the ectodermal epitheliomuscular cells contained large numbers of acridine orange positive vacuoles (Fig. 4A and B). Analysis of Feulgen stained preparations (Fig. 4C and D) provides additional evidence that *Hydra* ectodermal epitheliomuscular cells serve as phagocytes to eliminate apoptotic sperm precursors.

Discussion

In mammalian spermatogenesis, germ cell death is used to remove defective sperm precursor cells (Yin et al., 1998) and to maintain a critical cell number ratio in the testis (Rodriguez et al., 1997). Here we present evidence that this function of apoptosis appears to be conserved across the animal phyla by showing that apoptosis also occurs during spermatogenesis in *Hydra*, a member of the most basal eumetazoan animal phylum Cnidaria.

Detection of cell death during spermatogenesis

By Feulgen and acridine orange staining large numbers of sperm precursor cells could be observed which apparently undergo apoptosis in testes. Apoptosis in testes was confirmed by using a TUNEL assay which identified nuclei with clear signs of DNA fragmentation, a hallmark of apoptosis. Interestingly, the use of the three independent methods to detect apoptotic sperm precursors revealed significant differences. TUNEL staining revealed fewer apoptotic sperm precursors than acridine orange staining (compare figure 2B and E with figure 2C and F). This could indicate that in *Hydra* acridine orange identifies all cells and cell fragments which are contained in phagocytic vacuoles while only the TUNEL assay specifically labels cells that are undergoing apoptosis, independent of whether the cells are in vacuoles or not. Alternatively, consistent with the idea that DNA fragmentation is a rapid process, this difference in the staining pattern could indicate that the acridine orange positive phase of apoptosis is longer than the TUNEL positive phase.

Function of programmed cell death during spermatogenesis in *Hydra*

In contrast to oogenesis, cell death and phagocytotic activity has never before been observed in *Hydra* spermatogenesis. What is the function of programmed cell death during spermatogenesis in *Hydra*? Sperm precursors in contrast to growing oocytes do not feed on adjacent cells to reach their final size. Since the function seems not to be related to nutrition, there are two major roles for cell death during spermatogenesis. First, as massive amounts of sperm are produced in each testis, apoptosis of sperm precursors and their removal by epithelial cells may be used as “quality control” to eliminate defective sperm. Second, programmed cell death may also be used to achieve the precise homeostasis of each germ cell type in the mature *Hydra* testis. Apoptosis may contribute to maintain a critical cell number ratio between differentiating spermatogonia and epitheliomuscular cells. Since sexual maturation in *Hydra* can be induced by starvation (at least in certain periods of the year) and since starvation can induce apoptosis (Bosch and David, 1984), one could ask if apoptosis is the relevant signal for sexual maturation. This seems unlikely for two reasons. First, apoptosis can be detected only in already well-developed testes and not before onset of spermatogenesis. Second, the use of caspase inhibitors such as caspase-1 inhibitor II (Ac-Y-V-A-D-cmk) can not prevent spermatogenesis reproducibly (Kuznetsov and Bosch, data not shown). Experimental evidence for the precise function of apoptosis during spermatogenesis in *Hydra* could now be obtained by silencing of genes (Lohmann et al., 1999) which are critical for the execution of apoptosis.

Epithelial cells play an active role in *Hydra* spermatogenesis

Hydra as member of the basal eumetazoan phylum Cnidaria has a limited number of different cell types. Ectodermal epithelial cells are multifunctional cells and as “epitheliomuscular cells” are responsible for motility. In addition, epithelial cells also appear to control morphogenesis (Bosch, 1998) as well as the phagocytic removal of “excess” cells (Bosch and David, 1984; Fujisawa and David, 1984; Bosch and David, 1986). Until now there was no evidence that epithelial cells were also actively involved in spermatogenesis, which was thought to be an autonomous function of interstitial stem cell differentiation. The role of ectodermal epitheliomuscular cells in spermatogenesis was confined to forming the outer wall of the testes (Tardent, 1985). In contrast to that view, our data clearly show that ectodermal epithelial cells play an active role during spermatogenesis as phagocytes.

Recent evidence from genetically modified mice indicates that control of male germ cell apoptosis is mediated by signals derived from the Sertoli cells (reviewed in Print and Loveland, 2000). Based on our observations it is tempting to speculate that in *Hydra* testes the epitheliomuscular cells, which are closely adjacent to the developing spermatozoa (Fig. 1), function like Sertoli cells by controlling the microenvironment of the developing interstitial sperm precursor cells and sequestering the sperm precursor populations into environmentally distinct compartments. The view that epithelial cells in *Hydra*, in addition to their role as phagocytes, produce specific factors which are necessary for interstitial cell differentiation is supported by the recent observation (Takahashi et al., 2000) that *Hydra* epithelial cells directly influence the nerve cell differentiation pathway by secreting PW peptides.

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References

- Bosch, T.C.G. and C.N. David. 1984. Growth regulation in *Hydra*: Relationship between epithelial cell cycle length and growth rate. *Dev. Biol.* 104: 161–171.
- Bosch, T.C.G. and C.N. David. 1986. Immunocompetence in *Hydra*: Epithelial cells recognize self-nonsel and react against it. *J. Exp. Zool.* 238: 225–234.
- Bosch, T.C.G. 1998. *Hydra*. In: Cellular and Molecular Basis of Regeneration: from Invertebrates to Humans (P. Ferretti & J. Géraudie, eds). Wiley & Sons Ltd., Sussex. ISBN 0471-97271-1.
- Brien, P. 1966. *Biologie de la reproduction animale*. Masson and Cie, Paris.
- Cikala, M., B. Wilm, E. Hobmayer, A. Böttger and C. N. David. 1999. Identification of caspases and apoptosis in the simple metazoan *Hydra*. *Curr. Biol.* 9: 959–962.
- David, C.N. 1973. A quantitative method for maceration of *Hydra* tissue. *Wilhelm Roux's Archiv Entwicklungsmech. Org.* 171: 259–268.
- Delic, J., J. Coppey, H. Magdelenat, and M. Coppey-Moisan. 1991. Impossibility of acridine orange intercalation in nuclear DNA of the living cell. *Exp. Cell. Res.* 194: 147–154.

- Foster, J.S. and M.J. McFall-Ngai. 1998. Induction of apoptosis by cooperative bacteria in the morphogenesis of host epithelial tissues. *Dev. Genes Evol.* 208 (6): 295–303.
- Fujisawa, T. and C.N. David. 1984. Loss of differentiating nematocytes induced by regeneration and wound healing in *Hydra*. *J. Cell. Sci.* 68: 243–255.
- Gavrieli, Y., Y. Sherman, and S.A. Ben-Sasson. 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119: 493–501.
- Honegger, T.G., D. Zurrer and P. Tardent. 1989. Oogenesis in *Hydra carnea*: a new model based on light and electron microscopic analyses of oocyte and nurse cell differentiation. *Tissue Cell* 21: 381–393.
- Jacobson, M.D., M. Weil and M.C. Raff. 1997. Programmed cell death in animal development. *Cell* 88: 347–354.
- Kleinenberg, N. 1872. *Hydra*. Eine anatomisch-entwicklungsgeschichtliche Untersuchung. Engelmann, Leipzig.
- Lohmann, J. U., I. Endl, and T.C.G. Bosch. 1999. Silencing of developmental genes in *Hydra*. *Dev. Biol.* 214: 211–214.
- Martin, V.J., C.L. Littlefield, W.E. Archer and H.R. Bode. 1997. Embryogenesis in hydra. *Biol. Bull.* 192: 345–363.
- Miller, M.A., U. Technau, K. Smith and R.E. Steele. 2000. Oocyte development in *Hydra* involves selection from competent precursor cells. *Dev. Biol.* 224: 326–338.
- Munck, A.C. and C.N. David. 1985. Cell proliferation and differentiation kinetics during spermatogenesis in *Hydra carnea*. *Roux. Arch. Dev. Biol.* 194: 247–256.
- Print, C.G. and K. L. Loveland. 2000. Germ cell suicide: new insights into apoptosis during spermatogenesis. *BioEssays* 22(5): 423–430.
- Rodriguez, I., C. Ody, K. Araki, I. Garcia and P. Vassalli. 1997. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *EMBO J.* 16: 2262–2270.
- Takahashi, T., O. Koizumi, Y. Ariura, A. Romanovitch, T.C.G. Bosch, Y. Kobayakawa, S. Mohri, H.R. Bode, S. Yum, M. Hatta and T. Fujisawa. 2000. A novel neuropeptide, Hym-355, positively regulates neuron differentiation in *Hydra*. *Development* 127 (5): 997–1005.
- Tardent, P. 1974. Gametogenesis in the genus *hydra*. *Amer. Zool.* 14: 447–456.
- Tardent, P. 1985. The differentiation of germ cells in *Cnidaria*. In: *The Origin and Evolution of Sex*. Alan R. Liss, Inc., pp 163–197.
- Yin, Y., B.C. Stahl, W.C. DeWolf and A. Morgenthaler. 1998. p53-mediated germ cell quality control in spermatogenesis. *Dev. Biol.* 204: 165–171.
- Zihler, J. 1972. Zur Gametogenese und Befruchtungsbiologie von *Hydra*. *Wilhelm Roux's Archiv.* 169: 239–267.