IN SITU END-LABELING OF FRAGMENTED DNA IN APOPTOTIC GERM CELLS IN THE SPINY DOGFISH (*SQUALUS ACANTHIAS*) TESTIS

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Due to a cystic mode of spermatogenesis and a simple diametrical arrangement of succeeding germ cell stages, the dogfish shark testis is advantageous for studying spermatogenesis (for review see, Callard et al., In: *Function of Somatic Cells in the Testis,* Bartke A. (ed.), Springer, NY. p.27-54, 1994). After the winter period of spermatogenic inactivity, or following hypophysectomy of spermatogenically active animals, a band of degenerating spermatocysts (ZD) appears between premeiotic (PrM, stem cells/spermatogonia) and meiotic (M, spermatocyte) stages. This has been interpreted as resulting from withdrawal of hormonal support. As part of a program of research designed to identify factors and mechanisms that regulate production and development of male germ cells, we have shown that apoptosis (programmed cell death, PCD) is the form of degeneration occurring in PrM spermatocysts, which leads to their accumulation in ZD (Callard et al., *Develop. Genetics* 16:140-147, 1995). In the same study, we observed that apoptosis was limited to PrM and ZD regions and affected spermatogonia, not Sertoli cells; however, Sertoli cells of cysts in ZD contained phagocytized germ cell corpses (apoptotic bodies). Although our initial attempts to study putative regulators relied on biochemical analysis of internucleosomal fragmentation products on agarose gels (DNA "ladders"), this method is not sufficiently sensitive or quantifiable, nor does it allow observation of single cells or substages. Previously, we described a procedure for visualizing the condensed nuclei characteristic of apoptosis in living spermatocysts using acridine orange (AO) as a vital stain (McClusky et al., *Bull. MDIBL* 35:96-97, 1996). Here we verify results obtained with the AO technique by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay (Gavrieli et al., *J. Cell Biol* 199:493-501, 1992). TdT labels blunt ends of double-stranded (ds) DNA breaks, the predominant type of DNA fragmentation during apoptosis, and thus is able to distinguish PCD from the randomly fragmented DNA generated during necrosis.

Cross-sections (≈0.5 mm thick) of testes from *Squalus acanthias* were fixed in 10% neutral buffered formalin for 24 hr and paraffin-embedded. Sections (8 µm) were affixed to poly-L-lysine coated slides and processed using the protocol of the In Situ Cell Death Detection Kit (Boehringer Mannheim) with fluorescein as a reporter. Briefly, deparaffinized sections, were treated with proteinase K (20 µg/ml) for 20 min at 37° C before application of the TUNEL reaction mixture (50 µl, containing 5 µl TdT enzyme and 45 µl nucleotide labeling solution). After incubation for 60 min at 37° C and washing, sections were mounted with Fluoromount G. Positive-stained controls entailed DNase I-treatment (1 µg/ml) for 10 min at room temperature, followed by proteinase K treatment. Negative-stained controls entailed substitution of TdT enzyme with water in the TUNEL reaction mixture. Sections were viewed and images recorded using an Olympus Epifluorescent Microscope and Hamamatsu CCD videocamera attached to a Power Macintosh computer.

As shown in Fig. 1a-f, spermatocysts in different developmental stages were distinguishable under the fluorescent microscope by their size, light reflectance properties, and the presence or absence of a lumen. Fluorescein-labeled spots varying in size and brightness, were rare in the germinal zone (stem cells, spermatogonial generations$_{1-4}$; Fig. 1a), but increased progressively in abundance in more mature PrM cysts (spermatogonial generations$_{5-13}$; Fig. 1b). Specific TUNEL labeling was eliminated by excluding TdT from the reaction mix (Fig. 1c). Cysts in advanced stages of degeneration (ZD) had intense, diffuse TUNEL labeling (Fig. 1d). M-stage cysts, even when present in the same section as ZD, were unlabeled (Fig. 1d). Although TUNEL-positive spots were never seen over cysts in postmeiotic stages (PoM, spermatids; Fig. 1f), labeled cells,

presumed to be macrophages, were often present in the interstices at this and other developmental stages (Fig. 1a, 1f; arrows). Prior treatment of TUNEL-negative M-stage cysts with DNase I led to intense labeling of all nuclei, demonstrating that the stage-specificity of the reaction is due to intranuclear DNA cleavage, and not to differential access of reagents (Fig. 1e). The position of fluorescent spots in PrM cysts in Fig. 1b suggests labeling of both germ cells (double arrowhead) and Sertoli cells (arrowhead), which are, respectively, peripheral and adluminal at this developmental stage. However, examination of adjacent hematoxylin and eosin stained sections indicated that TUNEL-positive cells are spermatogonia.

Results show that the stage- and cell-specificity of apoptosis with the TUNEL in situ labeling technique is essentially the same as that obtained with AO vital staining, and is consistent with our earlier biochemical and structural data. Whereas AO has utility for testing direct effects of regulators and toxicants on apoptosis in cultured cysts in vitro, TUNEL will allow us to analyze the process in a normal physiological context after experimental manipulation of animals in vivo.
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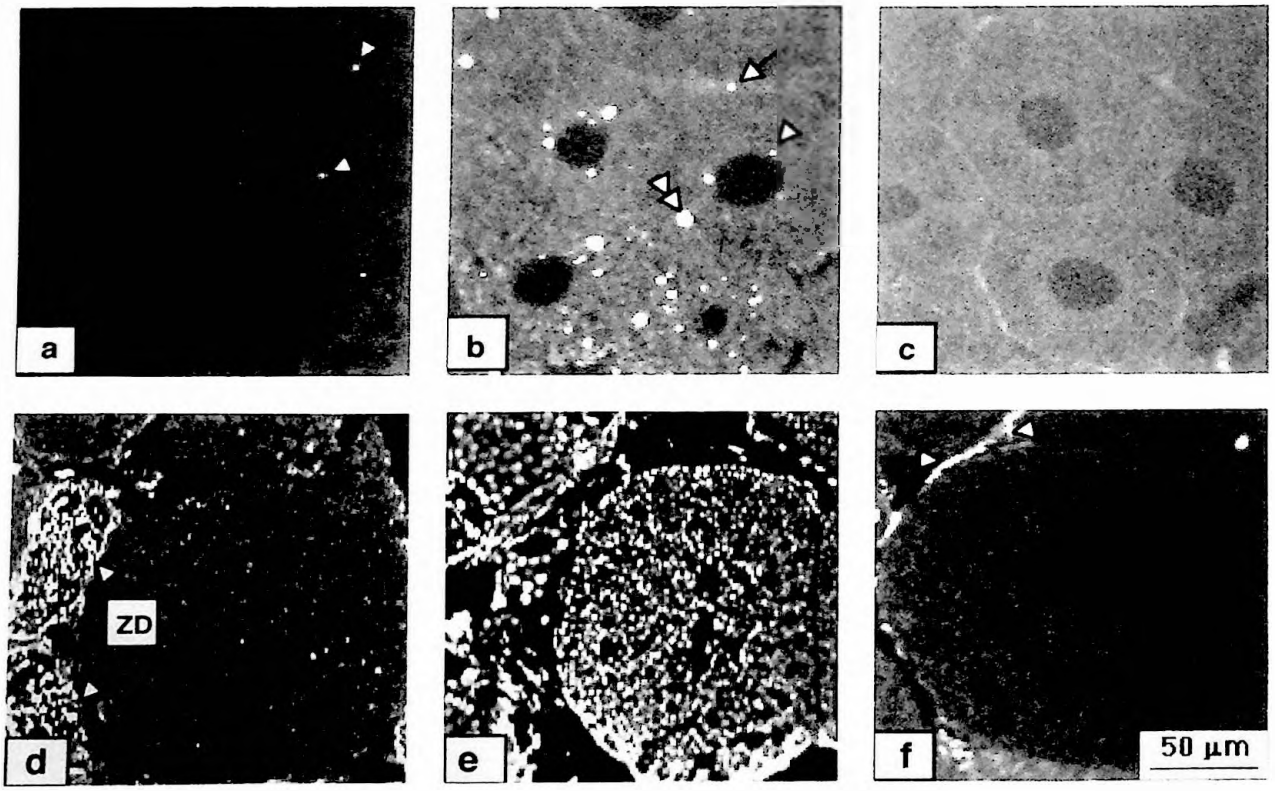

Figure 1. Fluorescent light micrographs of dogfish shark testis showing TUNEL-labeling of apoptotic spermatocysts. (a) Germinal zone with TUNEL-negative immature PrM cysts and TUNEL-positive interstitial cells (arrows). (b) TUNEL-positive mature PrM cysts, showing adluminal (arrowhead) and peripheral (double arrowhead) labeled nuclei and TUNEL-positive interstitial cell (arrow). (c) Unlabeled mature PrM cysts (no Tdt enzyme; negative control). (d) TUNEL-stained ZD- and adjacent -unstained M-stage cysts. (e) M-stage cyst pretreated with DNase I to cleave DNA (positive control). (f) TUNEL-negative PoM cyst and TUNEL-positive interstitial cells (arrows).