ANALYSIS OF THE APOPTOTIC FORM OF PROGRAMMED CELL DEATH (PCD) DURING SPERMATOGENESIS IN SPINY DOGFISH (SQUALUS ACANTHIAS)

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Due to a cystic mode of spermatogenesis and a simple diametrical arrangement of succeeding germ cell stages in the testis of the shark, this organ is ideal for studying spermatogenesis and its regulation stepwise through development (see review, Callard et al., In: Function of Somatic Cells in the Testis, Bartke A., ed., Springer, NY, p.27-54, 1994). The apoptotic form of programmed cell death (PCD) has been identified as a mechanism for specifically regulating the premeiotic (PrM=stem cell/spermatogonial) cyst population in shark testis (Callard, et al. Develop. Genetics 16:140-147, 1995). Germ cells but not Sertoli cells are affected all-or-none in a given cyst, but Sertoli cells subsequently phagocytize germ cell remnants. The winter period of spermatogenic inactivity or hypophysectomy induces a zone of degenerating cysts (ZD) between PrM and meiotic (M) stages. Biochemical analysis shows extensive internucleosomal DNA fragmentation (DNA "ladders" on agarose gels), an early marker of PCD, in PrM and ZD stages only, in vivo and in vitro, but this method is not sufficiently sensitive for routine regulatory and toxicological studies. Here we describe a procedure for visualizing PCD in living spermatocysts using acridine orange (AO) as a vital stain. AO has been reported to label condensed chromatin and apoptotic bodies characteristic of cells undergoing PCD during Drosophila embryogenesis, and also labels corpses of apoptotic cells after phagocytosis by macrophages, but necrotic cells are never labeled (Abrams et al. Development 117, 29-43, 1993).

Razor blade-cut cross-sections (≈500 μm thick) of freshly dissected testes or isolated PrM or ZD cysts were cultured in basal medium for 0-7 d as previously described (DuBois and Callard, J. Exp. Zool. 258, 359-372, 1991) with/without various additives. AO (5 μg/ml) was added for the final 30 min of culture. Tissues were washed twice with fresh medium and viewed using an Olympus IMT-2 inverted epifluorescence microscope (502 nm excitation and 526 nm emission maxima) coupled to a Hamamatsu 2400 CCD video camera controlled from a Power Macintosh computer by using the NIH-Image (version 1.57) software package. An AG-5 frame grabber (Scion), which converted the analog video signal into digital values was installed in the computer. Images were captured, averaged, and analyzed or stored for later analysis. In both tissue slices and dispersed spermatocyst cultures, a subset of cysts displayed punctate green or yellow fluorescent spots, as previously described for cells undergoing apoptosis in Drosophila. The presence of apoptotic germ cells in AO-positive cysts was verified by electron microscopy (not shown).

Results indicate that the cysts undergoing apoptosis or nearing the end of the apoptotic process can be identified in both tissue cross-sections and dispersed cyst cultures. Initial data show that the percentage of cysts labeled are affected by stage of development, time in culture, and in vivo or in vitro exposure to added regulators (e.g., steroids) or spermatotoxicants (e.g, cadmium). The technique of AO labeling will allow study of cellular and molecular mechanisms controlling programmed cell death during male germ cell development and is amenable to future analysis by confocal imaging. Supported

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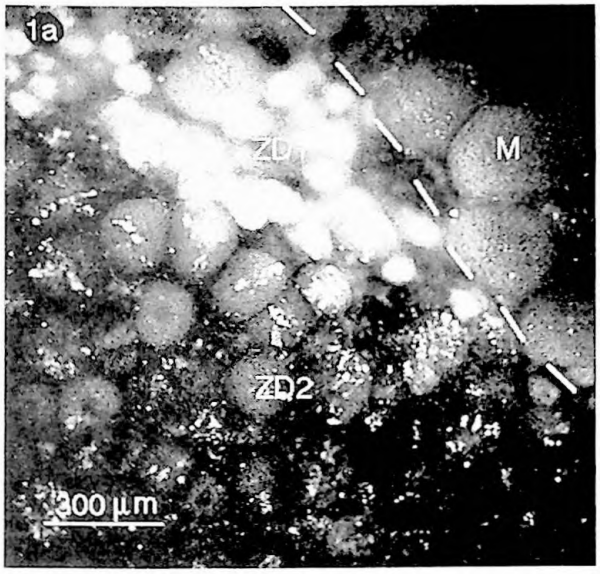

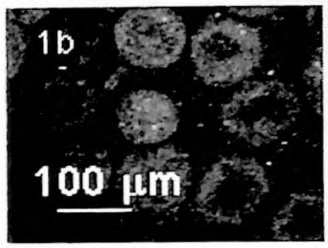

Figure 1. Fluorescence microscopic images of apoptotic cysts in testicular cross section. (a) Spermatogenic development proceeds from lower left to upper right. Note intense labeling of shrunken cysts in ZD_1, indicating an advanced stage of apoptosis and cyst degeneration. Yellow fluorescence suggests AO labels phagocytized germ cell corpses present in Sertoli cells. Late-stage PrM cysts in adjacent ZD_2 had punctate green fluorescent spots, indicating an earlier stage of germ cell apoptosis. Dotted line indicates boundary of PrM(ZD) and M stages. M-stage cysts were never labeled. (b) Small early-stage PrM cysts normally positioned out of the field of view in (a) were mostly unlabeled. Although AO facilitated visualization of cell nuclei in healthy cysts, especially those with mature spermatids undergoing condensation during spermiogenesis, this form of labeling was easily distinguished from labeling of apoptotic cells.

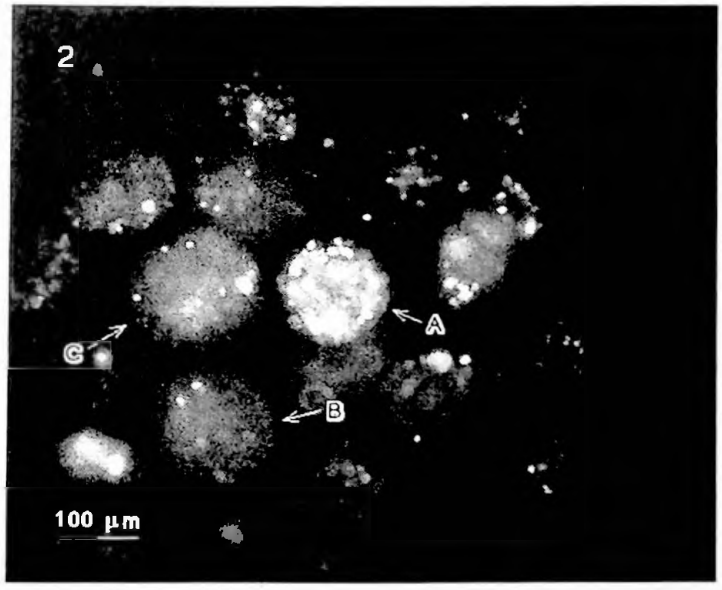

Figure 2. Isolated PrM cysts after 7 days of culture in basal medium. Eighty percent of cysts in this culture were AO-positive. Cyst A which has large yellow spots may represent an advanced stage of apoptosis characteristic of ZD cysts. Cysts B and C have only a few punctate yellow spots and may represent earlier stages of apoptosis.