CHARACTERIZATION OF TWO ARYL HYDROCARBON RECEPTOR (AHR) mRNA FORMS IN *SQUALUS ACANTHIAS* AND STAGE-DEPENDENT EXPRESSION DURING SPERMATOGENESIS

Marlies Betka, Alice Welenc, *Diana G. Franks, *Mark E. Hahn, and Gloria V. Callard
Department of Biology, Boston University, Boston MA 02215
*Department of Biology, Woods Hole Oceanographic Institution, Woods Hole MA 02543

Production and development of mature male gametes are essential for normal reproductive efficiency and survival of species. Recent epidemiological research and observations in wildlife have reported an increase in male reproductive disorders, including a decrease in sperm number (Carlsen et al., Environ. Hlth. Perspect.103:137-39, 1995). A possible link to environmental pollutants has been postulated, but clear cause-and-effect relationships remain to be established. Of over 60,000 chemicals in commercial use in the U.S. as industrial agents, pesticides, food additives, or therapeutic drugs, only a few have been tested for their effects on spermatogenesis (Paul & Himmelstein, Obst. Gyn. 71:921-38, 1988). Moreover, of the known or suspected spermatotoxicants, knowledge of mechanism is incomplete. What has hampered research in this area is the complex organization of the testis of common laboratory mammals and the lack of a valid in vitro spermatogenesis system. A program of research in this laboratory has identified the spiny dogfish shark *Squalus acanthias* as an advantagous alternative model (Callard et al., In: Boekelheide et al., eds., Comprehensive Toxicology, Vol. 10, Elsevier, NY, pp. 471-476, 1997). Although spermatogenesis is fundamentally conserved throughout the vertebrates, the organization of the testis of sharks and other squaliformes facilitates analysis of spermatogenesis stage-by-stage in vivo and in vitro: (a) each germ cell clone, plus cohort of stage-synchronized Sertoli cells, forms a discrete follicle-like unit (spermatocyst); (b) developing spermatocysts are arranged in maturational order across the diameter of the testis; and (c) germ cells rely on Sertoli cells alone for somatic cell support because Leydig and peritubular cells are absent at this phyletic level (Callard et al., In: Bartke, ed., Function of Somatic Cells in the Testis, Springer Verlag, NY, pp. 27-54, 1994).

To evaluate the utility of the shark testis model for toxicology studies, the focus of this project was the arylhydrocarbon receptor (AHR). The AHR is a ligand-activated transcription factor which, together with its dimerization partner (ARNT), binds to response elements in the regulatory region of target genes. Although its endogenous ligand is unknown, planar halogenated aryl hydrocarbons (HAH) such as the highly toxic 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and polychlorinated biphenyls (PCBs), flavonoids (BNF) and related compounds, are known to cause toxicity after binding and activation of AHR. Exposure to dioxins has been associated with decreased sperm production, altered testicular size and histology, impaired fertility and testicular cancer in humans and several animal species (Eskenazi & Kimmel, Environ. Hlth. Perspect. 103:143-5, 1995). Both AHR and ARNT have recently been identified in rodent testis (Roman et al., Toxicol. Appl. Pharmacol. 150:228-39, 1998). As a first step in determining which stages of spermatogenesis, physiological processes and genes are targeted by dioxin and dioxin-like compounds, we used a PCR cloning strategy to isolate two shark-specific AHR.

Total RNA was isolated from shark liver, brain, heart, muscle, epigonal organ, ovary and testis. Defined developmental stages were obtained from testicular cross-sections under a dissecting microscope and included: germinal zone (GZ), premeiotic (PrM), meiotic (M), and postmeiotic (PoM) regions containing germ cells in the gonocyte, spermatogonial, spermatocyte and spermatid stages of development, respectively. A zone of degeneration (ZD), which is positioned between PrM and M regions in early spring, was collected separately. ZD comprises a band of degenerate cysts containing Sertoli cells with phagocytized germ cell corpses, and reflects the wave of apoptosis that occurs at the end of the previous season's period of spermatogenic development. Degenerate primers were designed to target conserved amino acid sequences in the basic helix-loop-helix (bHLH) and Per-ARNT-Sim (PAS) domains of known vertebrate AHRs and used for polymerase chain reaction (PCR) amplification of cDNA (Karchner & Hahn, Mar. Environ. Res. 42:13-17, 1996). Two PCR products of predicted size were isolated and sequenced. Both had high overall sequence identity when compared to human AHR protein (59-68%). When compared to the two AHR forms previously identified in the killifish *Fundulus* (Karchner et al., J. Biol. Chem. 274:33814-24, 1999), one shark AHR was more closely related to killifish AHR1 (76% identity) and the other to killifish AHR2 (56% identity). The two shark AHR, designated AHR1 and AHR2, shared only 62% sequence identity with each other. Phylogenetic trees generated by Clustal W, Neighbor Joining algorithm and PAUP analysis showed that shark AHR1 grouped with mammalian AHR, but the position of shark AHR2 was less clear. It often occurred as a separate branch of the AHR gene tree, suggesting it is a third type or a highly divergent AHR2. The data imply that duplication of the AHR gene occurred in a common vertebrate ancestor of cartilagenous and bony fishes and possibly at other points in vertebrate evolution. RT-PCR analysis using shark AHR1- and AHR2-specific primers showed that both mRNA forms are expressed in most tissues, including brain, ovary, heart, muscle, and at lower levels in liver (Fig. 1A). Within the testis, distribution was stage-related but the two mRNAs covaried (Fig. 1B). Expressed levels were higher in immature

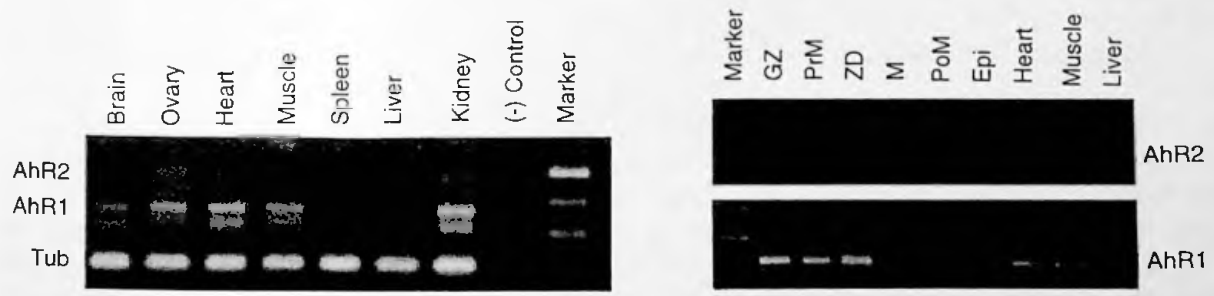

Figure 1. Tissue (A) and intratesticular (B) distribution of AHR1 and AHR2 mRNA variants as determined by RT-PCR analysis. GZ, germinal zone; PrM, premeiotic; ZD, zone of degeneration; M, meiotic; PoM, postmeiotic; Epi, epigonal tissue. In experiment A, gene specific primers for the two AHR genes and shark tubulin (Tub) were added to the reaction mix simultaneously. In experiment B, different primer sets were added to separate aliquots of the template.

(GZ and PrM) than in mature (M and PoM) regions, and band intensities were even greater in ZD regions where Sertoli cells predominate.

The data imply that persistent environmental HAH pollutants like dioxin may be able to perturb processes of spermatogenic development directly by virtue of their ability to bind to testicular AHR. Subsequent agonist or anatagonist actions would depend on the nature, binding affinity and concentration of a particular compound relative to the natural testicular AHR ligands. High AHR mRNA levels in ZD cysts, where germ cells are degenerate, imply that AHR are localized in Sertoli cells. If so, the stage-related pattern of AHR expression seen in shark testis (immature > mature) could be due in part to changing Sertoli:germ cell ratios as development progresses (e.g., 1:1 in GZ, 1:16 in M, and 1:64 in PoM spermatocysts), but direct visualization by in situ hybridization is required to confirm this interpretation. Mitosis and apoptosis are processes specific to GZ/PrM stages in shark testis and are key determinants of the number of clones advancing through all subsequent stages. Interestingly, both androgen and estrogen receptors are maximal in GZ and PrM regions of shark testis, implying that steroid-sensitive control points reside in these regions. Relatively high levels of AHR mRNA in the same regions are consistent with the proposed physiological functions of AHR in cell cycle control, developmental signaling, and modulation of growth factor and hormone-mediated signal transduction pathways. Although further studies are required to examine the role of AHR ligands in growth control processes during spermatogenesis, results to date illustrate the utility of the shark testis model for obtaining new information of general relevance to male reproductive toxicology. Supported by NIEHS P42 ES-07381 (Superfund Basic Research Center at Boston University)(GVC & MH), ES06272 (MH), and EPA R825434 (GVC). Contribution #10116 from the Woods Hole Oceanographic Institute.