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Environmental Effects and Aquatic Organisms: Investigations of Molecular Mechanisms of Carcinogenesis

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Cancers of the reproductive system are among the leading causes of mortality in women in the United States. While both genetic and environmental factors have been implicated in their etiology, the extent of the contribution of environmental factors to human diseases remains controversial. To better address the role of environmental exposures in cancer etiology, there has been an increasing focus on the development of nontraditional, environmentally relevant models. Our research involves the development of one such model. Gonadal tumors have been described in the softshell clam (Mya arenaria) in Maine and the hardshell clam (Mercenaria spp.) from Florida. Prevalence of these tumors is as high as 40% in some populations in eastern Maine and 60% in some areas along the Indian River in Florida. The average tumor prevalence in Maine and Florida is approximately 20 and 11%, respectively. An association has been suggested between the use of herbicides and the incidence of gonadal tumors in the softshell clam in Maine. The role of environmental exposures in the development of the tumors in Mercenaria in Florida is unknown; however, there is evidence that genetic factors may contribute to its etiology. Epidemiologic studies of human populations in these same areas show a higher than average mortality rate due to cancers of the reproductive system in women, including both ovarian and breast cancer. The relationship, if any, among these observations is unknown. Our studies on the molecular basis of this disease in clams may provide additional information on environmental exposures and their possible link to cancer in clams and other organisms, including humans. — *Environ Health Perspect* 105(Suppl 3):669–674 (1997)

Key words: comparative carcinogenesis, gonadal tumor, *Ah* receptor, dioxin, bivalves, TCDD, nontraditional models

Introduction

Twenty-five years ago, President Richard Nixon signed into law legislation that created the National Cancer Act and made the eradication of cancer a national priority. This landmark legislation promoted both basic research and its clinical applications, established the first cancer centers and cancer control programs, and created programs to increase public awareness of cancer. During the past 25 years, we have made tremendous progress toward defining the molecular basis of cell growth, cell death and differentiation, and how the deregulation of these normal processes contributes to cell transformation. In 1996, the annual appropriation for the National Cancer Institute was $2.25 billion, a significant increase from the $200 million allotted in 1971 at the inception of the program. Yet, in spite of the great strides we have achieved in basic research, approximately 40% of Americans will develop cancer in their lifetime and one in four of us will die of cancer. Breast cancer rates in particular continue to increase, so that now a woman in the United States has a one in eight chance of developing the disease. By the turn of the century, cancer is predicted to be the leading cause of death in the United States (*1*).

The difficulty in understanding and treating cancer lies in its complex nature. Cancer is not single disease. In humans alone, there are more than 100 different cancers that affect more than 35 different organs. Carcinogenesis is a very complex, multistep process involving both external factors imposed by the environment, and the intrinsic genetic background of the individual. The degree to which exposure to environmental toxicants induces cancer has long been a subject of extensive debate. In most cases, direct causality has been difficult, if not impossible, to prove. In spite of the difficulty in addressing these questions, there is a growing recognition that human health and ecosystem health are closely linked. One approach to understanding this process is to examine the basic underlying mechanisms of chemical carcinogenesis. Rapid technological advances in recombinant DNA analysis have had a tremendous impact on basic research on the molecular mechanisms of chemical carcinogenesis (*2*). For obvious ethical reasons, it is impossible to do controlled laboratory studies on human subjects in order to determine directly their sensitivity to potential carcinogens. By doing studies in phylogenetically diverse species, we are better able to understand which mechanisms are conserved and which are species specific. This provides a much more rational basis from which to infer human risk assessment.

The comparative approach has been of enormous importance in understanding all aspects of human health. Sea urchins have long been used to illustrate developmental processes. The fruit fly *Drosophila* has served as a model organism from the heyday of classical genetics through the advent of molecular biological techniques, where understanding of regulation of the genes involved in development and differentiation have provided many clues to understanding their counterparts in higher organisms, including humans (*3*). Unraveling the elegant and complicated regulation of the cell cycle owes much to the study of the surf clam (*Spisula solidissima*) cyclin proteins (*4*). More recently, information obtained from the

ciliate *Tetrahymena* led to the discovery of telomerase and the solution of the chromosomal end-replication problem (*5*)—information that has had enormous impact on the study of aging and cancer. The fields of comparative carcinogenesis and toxicology have benefitted from numerous studies of a diverse array of aquatic organisms. The very potent human carcinogen aflatoxin B_1 was first described as a mycotoxin in moldy fish food and a causative agent of hepatocarcinogenesis in trout (*6*). Clearly, comparative biology is fundamental to our understanding of the unity and diversity of function of living organisms.

Since the 1960s, epidemiological research of aquatic animals has demonstrated a correlation between the incidence of tumors and environmental exposure to chemicals (*7,8*). Tumor prevalence has reached alarming proportions in English sole (*Parophrys vetulus*) in certain areas of Puget Sound (*9*), winter flounder (*Pseudopleuronectes americanus*) in the Boston Harbor (*10*), brown bullhead catfish (*Ictalurus nebulosus*) in the Black, Niagara and Buffalo Rivers (*11,12*) and Atlantic tomcod (*Microgadus tomcod*) in the Hudson River (*13*). It is important to emphasize that although exposure to chemical carcinogens is highly correlated with tumor incidences, no direct evidence of the role of chemical exposure in the etiology of these tumors exists.

We have summarized here the investigation of the molecular mechanism of gonadal neoplasms in two marine bivalve species. These bivalves represent important model organisms that are in direct contact with inshore waters and sediments and thus are excellent candidates for sentinel species. The initial descriptions of these tumors were made about 20 years ago (*14,15*); however, their etiology is still unknown. Both chemical exposure and genetic factors have been investigated. In the early to mid 1970s, results of field surveys by the Environmental Research Laboratory of the U.S. Environmental Protection Agency (U.S. EPA) in Narragansett, Rhode Island, suggested a possible association between herbicide contamination and epizootic germinomas in three geographically distinct populations of softshell clams (*Mya arenaria*) in eastern Maine (*16*). In three collections from the Hobart Stream near the Moosehorn Wildlife Refuge in eastern Maine, tumor prevalence averaged 32%. Investigation into the etiology of these tumors revealed that all three locations in Maine had been subjected to herbicide

exposure. No other source of contaminants was found. Significant quantities of Tordon 101 (picloram), 2,4-D (2,4-dichlorophenoxyacetic acid), and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) had been used in these areas in blueberry culture, road maintenance and forestry (*16*). While 2,4-D and 2,4,5-T have exhibited low toxicity in mammalian assays, TCDD (dioxin; 2,3,7,8-tetrachlorodibenzo-*p*-dioxin), a by-product contaminant from the synthesis of 2,4,5-T, has been described as one of the most toxic environmental contaminants known (*17*).

Gonadal neoplasms have also been reported in hardshell clams (*Mercenaria* spp.) taken from the Indian River in Florida (*18*). The Indian River estuary is a poorly flushed, 200-mile long estuarine area that receives drainage from central Florida agricultural areas. This estuarine system drains a wide area of citrus groves and receives substantial domestic and agricultural runoff. The average tumor prevalence in this area was 11%, although in some areas, it was greater than 60%. The cause of the high incidence of gonadal tumors in this area is unknown.

While the hypothesis that herbicide exposure may have a role in the etiology of the gonadal tumors in clams is intriguing, it is also controversial, and at this time we have no direct evidence to support it. There are numerous other factors that alone, or more likely in combination, may contribute to this disease. Possible genetic contributions to the disease in *Mercenaria* have been addressed by Bert et al. (*19*). In the region where a notably high incidence of gonadal neoplasm was reported by Hesselman et al. (*18*), two species (*M. mercenaria* and *M. campechiensis*) occur sympatrically (*20*). Hybridization occurs readily between them and hybrids may be distinguished by allozyme patterns (*20*), mitochondrial genotypes (*21*), and shell morphology (*20,22,23*). Gonadal tumors occurred 2 to 3 times more often in the hybrid animals relative to either parental species, which led Bert et al. (*19*) to propose that hybridization in *Mercenaria* may have disrupted genetic mechanisms that are involved in cell growth and differentiation but not the regulation of cellular proliferation. These observations do not rule out the possibility that the hybrids may be more susceptible to environmental contaminants.

Additional reports suggest that herbicide-associated neoplasia is not limited to poikilotherms. Seminomas have been detected in military working dogs believed to have been exposed to herbicides in

Vietnam (*24*). A second study (*25*) showed a positive correlation between the incidence of malignant lymphomas in dogs and their owners' use of herbicides containing 2,4-D on their lawns. In other studies, rhesus monkeys exposed to chronic, low levels of TCDD were found to have an increased rate of endometriosis (*26*). Exposure to TCDD was directly correlated to the incidence of endometriosis and the severity was dose dependent. Preliminary studies of female veterans who served in the Vietnam War indicated a higher than expected mortality rate from pancreatic and uterine cancer (*27*). Furthermore, a U.S. EPA survey of cancer mortality rates in the United States indicated that the mortality rate due to ovarian cancer in human females from Washington County, Maine, and near Indian River, Florida, was significantly higher than the national average (*28*). These are the same geographical areas in which the tumor-bearing clam populations are located. Human mortality due to breast cancer is also elevated in these areas (*28*). It is premature to draw any conclusions from these collective observations. Further investigation is needed. One approach to test this hypothesis would be comparative studies of the molecular mechanisms of toxicity of TCDD and other halogenated aromatic hydrocarbons (HAH).

TCDD and other polyhalogenated aromatic hydrocarbons are man-made compounds that are now ubiquitous, low-level environmental contaminants. The widespread occurrence largely results from the use of contaminated herbicides, high-temperature combustion processes, and chlorine bleaching of wood products. Although most sources of dioxin have been identified and eliminated, these are very stable compounds that can remain in sediments for many years. Animals living in these sediments are readily exposed to the dioxins and other compounds that may be bound to them, some by external exposure and others by ingestion during filter feeding.

Laboratory animals exposed to dioxin exhibit extreme variations in sensitivity as well as a wide range of symptoms including decreased appetite, immunosuppression, chloracne, teratogenicity, carcinogenicity, immunotoxicity, and death (*29*). The molecular basis for the toxicity and carcinogenicity of TCDD in vertebrates has been extensively investigated (*30–33*). In 1976, a clue to the mechanism of these responses was provided by the discovery of the *Ah* receptor (aryl hydrocarbon receptor, AhR), a high-affinity cytoplasmic receptor that

binds dioxin and other related HAHs (*34*). Most of the symptoms observed in animals exposed to HAH are believed to arise from their interaction with this receptor (*35*). The inactive cytoplasmic AhR is bound to two molecules of the stress protein HSP90. Binding of a ligand to the AhR results in the dissociation of the HSP90 molecules and the association of at least one other protein, ARNT (*Ah* receptor nuclear translocator). The transformed receptor complex is translocated to the nucleus where it binds to specific dioxin-responsive elements upstream of specific genes where the receptor complex functions as a transcriptional activator. The best understood target gene is the activation of the cytochrome P4501A1 gene (*CYP1A1*) (*36,37*). Studies of the cloned mouse AhR confirm its role as a transcriptional activator (*38*). Recent studies of AhR-knockout mice have provided some clues to the physiological role of the AhR (*39*). Almost half of the AhR-deficient mice (ahr$^{-/-}$) died at birth. The survivors had decreased numbers of lymphocytes in their spleen and lymph nodes and their livers were half the size of those of normal mice. This suggests that the AhR may play an important role in the development of both the liver and the immune system. However, the long-sought endogenous ligand has not yet been described for this receptor.

It is not yet clear which specific alterations in gene expression mediated by the AhR complex are responsible for toxicity and carcinogenicity. There is not an absolute requirement for *CYP1A1* or *CYP1A2* expression for HAHs to cause a toxic response in rodents (*40*). Furthermore, in invertebrates, exposure to HAHs elicits a relatively low level of *CYP1A1* expression (*41*). Consequently, there has been great interest in identifying other target genes (*40*). Particular interest has been focused on the expression of genes that are involved in cell growth and differentiation, such as epidermal growth factor (*42*), interleukin-1β, and plasminogen activator inhibitor-2 (*43*). However, there is no direct evidence as yet that any of these genes are responsible for the toxic effects of dioxin and related HAHs.

The mechanisms by which the AhR complex might cause cancer are not well understood. In liver and skin cancer studies, TCDD acts as a potent tumor promoter with little or no initiating activity (*44,45*). The role of cell proliferation in chemical carcinogenesis has been well established. Cellular proliferation signals

in the liver and many other tissues are mediated by the epidermal growth factor (EGF) receptor (*50*). Livers of TCDD-exposed rats show a dose-dependent decline in the maximal binding of EGF (*51*). Further evidence exists that implicates receptor-mediated processes as having critical roles in the carcinogenic effects of many chemicals (*46*). Laboratory studies have demonstrated that TCDD is a multisite carcinogen in several animal models (*32,47*). TCDD is negative in short-term genotoxicity tests and does not appear to form DNA adducts (*48*). Dioxin modulates the expression of genes in a wide array of pathways including receptors (estrogen, glucocorticoid, EGF), hormones, transforming growth factor, tumor necrosis factor, proteins in intermediary metabolism, biotransformation enzymes, inflammatory factors, interleukins, and protooncogenes (*49*). The ability of dioxin to elicit carcinogenic effects probably requires the interaction of multiple proteins. Our understanding is further complicated by the cell specificity and hormonal regulation of dioxin effects. TCDD exposure is associated with an increased incidence of liver tumors in female but not male rats (*44*). These and other studies suggest that estrogens play a major role in TCDD-mediated hepatocarcinogenesis.

The AhR has been identified in a number of vertebrate species including humans, rodents, and fish (*52,53*). Hahn et al. (*54*) reported that AhR activity, as measured by photoaffinity labeling of specific proteins by a dioxin analog, was found in a number of different species of marine fish, but was not present in lower fish (hagfish or sea lamprey) or in the nine invertebrate species that they examined. The detection of an analogous dioxin receptor system in an invertebrate would suggest conservation over a long evolutionary time period. Studies are underway in our laboratory to determine whether *M. arenaria* and *M. mercenaria* have AhR-like proteins and, if so, to define the role that they may have in the etiology of the gonadal tumors.

Methods

Studies were initiated in our laboratory to determine whether the clams possessed an AhR system analogous to that reported in vertebrates. For these studies, cytosols were prepared from *M. mercenaria* from several different tissues, including gonadal tissue from both males and females (*55*). Cytosol fractions were labeled with the photoaffinity analog, 2-azido-3-[^{125}I]-iodo-7,8-dibromo-dibenzo-*p*-dioxin ([^{125}I]N$_3$Br$_2$DpD) by the

method of Poland et al. (*56*) with minor modifications as described previously (*55*). Precipitated samples were run on sodium dodecyl sulfate–polyacrylamide gels (*57*) and subjected to autoradiography.

A Western blot was prepared using photoaffinity-labeled cytosolic proteins from female *Mercenaria* gonadal tissue (*55*). Twenty micrograms of the labeled proteins were fractionated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and 12% Tris–glycine continuous gels (Novex, San Diego, CA). The separated proteins were transferred to polyvinylidene fluoride membrane (Millipore, Bedford, MA) and incubated with a polyclonal antibody (17743) directed against the mouse AhR-DNA binding region (gift of G Clark). Antibody binding was detected with the chemiluminescent ECL Western blotting detection system (Amersham, Arlington Heights, IL).

In addition to *M. mercenaria*, cytosols were assayed from eleven species of marine invertebrates that represented six different phyla: Cnidaria, Mollusca, Annelida, Arthropoda, Chordata, and Echinodermata (*58*). When possible, cytosols were prepared from gill and gonad, since the previous experiments with *M. mercenaria* indicated that the highest levels of expression of dioxin-specific binding proteins occurred in these tissues. *Aiptasia pallida* and *Amaroucium* sp. cytosols were prepared from the whole animal, because of the difficulty in dissecting out specific tissues. All animals (with the exception of *M. arenaria*, which were from Maine) were collected in Beaufort, North Carolina. The protein concentrations of the cytosols were determined using the Pierce Protein Detection System (Rockford, IL) with bovine serum albumin as the standard. The cytosols were photoaffinity labeled with [^{125}I]N$_3$Br$_2$DpD in the presence or absence of a cold competitor, tetrachlorodibenzofuran (TCDF), using conditions that had been developed for *M. mercenaria* (*55*).

Results

In *M. mercenaria*, photoaffinity labeled gonadal cytosols yielded a number of radiolabeled bands. There were only two bands, however, that appeared consistently and that were significantly diminished by inclusion of the competing ligand. The molecular weights of these two proteins were determined by SDS–PAGE to be 39 and 28 kilodaltons (kDa). A third, specifically labeled 52-kDa band was observed most frequently in samples collected during

the summer months. Comparisons of photoaffinity-labeled cytosols from male and female gonads indicated that expression of the 28-kDa protein was approximately the same in both sexes in all tissues examined. However, female gonadal cytosols had significantly more of the 39 kDa protein than did males. Oocytes, which are impossible to completely remove from the gonadal stroma, express high levels of the 39-kDa protein but very little of the 28-kDa protein, which may account for this difference (55). In addition, immunoblotting of photoaffinity-labeled proteins from female *M. mercenaria* gonadal tissue detected a single band that comigrated with the 52-kDa affinity-labeled protein (Figure 1).

Previous studies that investigated tissue distribution of these proteins in *M. mercenaria* indicated that both the 28- and 39-kDa proteins were expressed to different degrees in all tissues examined. Gill cytosol had the highest levels of both TCDD-binding proteins. The kidney contained predominantly the 28-kDa protein.

Proteins specifically labeled with $[^{125}I]N_3Br_2DpD$ were also found in cytosols prepared from soft-shell clam

Figure 1. Immunoblot using the mouse 17743 antibody directed against the putative DNA binding region of the murine AhR. Twenty micrograms of photoaffinity labeled cytosolic proteins isolated from gonadal tissue from a female *Mercenaria mercenaria* were loaded per lane and separated by SDS–PAGE using a Tris–glycine continuous gel. The gel was then transferred to a polyvinylidene membrane. (A) Autoradiograph. Plus and minus signs indicate the presence or absence, respectively, of excess cold TCDF during photoaffinity labeling. The three arrows on the left side indicate the positions of proteins that were specifically labeled by $[^{125}I]N_3Br_2DpD$. The estimated molecular masses of these proteins are 52, 39, and 28 kDa. (B) Western blot of the same membrane using antibody 17743 directed against the putative DNA binding region of the murine AhR. A single band was detected which comigrated with the photoaffinity labeled 52-kDa protein.

(*M. arenaria*), eastern oyster (*Crassostrea virginica*) and blue crab (*Callinectes sapidus*) (Table 1). In all species that we examined, the expression of the TCDD-binding proteins appeared to be tissue specific. Expression of the specifically labeled 35-kDa protein was observed in both the gill and gonad cytosols for *M. arenaria*. In *C. virginica*, the specifically labeled protein appeared to be expressed at a higher level in the gill cytosol than in the gonad. In *C. sapidus*, the 33-kDa protein that was specifically photoaffinity labeled was only seen in the hepatopancreas. Inclusion of proteinase inhibitors during cytosol preparation did not appear to affect the amount of protein that specifically bound the TCDD photoaffinity ligand.

Discussion

Analogous dioxin-binding proteins that have been reported in vertebrates span a wide range of sizes (mammals, 95–135 kDa; fish, 105–146 kDa), all much larger than the proteins identified from invertebrates in our laboratory. The small size of these invertebrate TCDD-binding proteins and their tissue-specific expression could explain why they had not been recognized previously (54).

At this time, the distribution of these proteins among invertebrate species is difficult to explain from an evolutionary perspective. They are expressed in phylogenetically divergent species, from mollusks to crustaceans, all of which are classified as protostomes. We observed no TCDD-binding proteins in invertebrates from the deuterostome branch of coelomates (which gave rise to the fishes and mammals that have AhRs) or in more primitive animals. Furthermore, TCDD-binding proteins were detected in only three of the four bivalve mollusks that we examined and only in the hepatopancreas of the blue crab, and not in the gill. We can speculate that the larger vertebrate AhR evolved separately from the TCDD-binding proteins that we observed in the mollusks and the crustacean. Based on the photoaffinity binding data, we would have to assume a path of convergent evolution that resulted in the invertebrate and vertebrate proteins that have in common the ability to bind TCDD. Without knowledge of the identity of the invertebrate proteins or clues to their biological function, we can only speculate as to their possible role, if any, in TCDD toxicity. We know from these studies in our laboratory that they are cytosolic proteins that bind a dioxin analog. We do not know whether they function in a manner analogous to the vertebrate AhR to mediate HAH toxicity, act as transport proteins, or
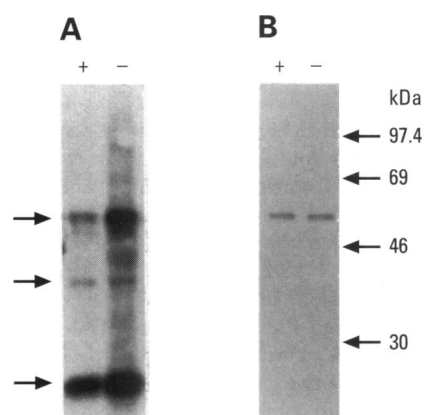
Table 1. Photoaffinity labeling of cytosolic proteins in some invertebrate species.

Species	Tissue	Gender	Approximate mw, kDa
Hardshell clam, *M. mercenaria*	Gill Gonad	Female	28, 39, 52 28, 39, 52
Softshell clam, *Mya arenaria*	Gill Gonad	Female	35 35
Ribbed mussel, *Geukensia demissa*	Gill Gonad	Female	ND
Eastern oyster, *Crassostrea virginica*	Gill Gonad	Female	34 34
Knobbed whelk, *Busycon carica*	Dig. gland Gill Gonad	Female	ND ND ND
Brown anemone, *Aiptasia pallida*	Whole	Unknown	ND
Tube worm, *Chaetopterus variopeatus*	Digestive tract Gonad	Mix	ND
Blue crab, *Callinectes sapidus*	Gill Hepatopancreas	Female	ND 33
Purple sea urchin, *Arbacia punctulata*	Gonads Oocytes		ND ND
Sea pork, *Amaroucium* sp.	Whole	Unknown	ND
Rough sea squirt, *Styela plicata*	Viscera	Unknown	ND

ND, not detected. Data summarized from Brown et al. (58).

perhaps have another biological function. Our photoaffinity-labeling studies with *M. mercenaria* suggest that the proteins share some homology with the ligand-binding epitope of the mouse AhR. The different tissue distribution of the *M. mercenaria* proteins suggest that they may perform different functions.

So what can animals without breasts tell us about breast cancer in humans? Recent studies of the breast cancer associated (BRCA) genes and other genes suggest some genetic linkage between breast and ovarian cancer (*59*). Women in families with heritable breast cancer associated with mutations in the *BRCA1* gene also are at greater risk for the development of ovarian carcinoma. Ovarian carcinoma is now one of the leading causes of death due to cancer

among women (*60*), and the molecular basis of these tumors is now an active field of investigation. Numerous studies have investigated the roles of activated oncogenes and tumor suppressor genes in the etiology and prognosis of human ovarian tumors (*61*). Far more controversial is the role of environmental factors and their contribution to breast and ovarian cancer (*62*). Aquatic organisms have long been used as sentinels to detect changes in the environment. As we learn more of mechanisms through which they respond to environmental carcinogens, we will be better able to understand how we might most effectively use this knowledge to evaluate human and environmental health risk.

Our preliminary results have now generated as many questions as they have

answered. In our laboratory, we are investigating the clam gonadal neoplasm as a model system that we hope will help us understand some of the complex interactions of environmental exposures. In addition to the photoaffinity-labeling studies discussed here, investigations of the molecular mechanisms of gonadal tumor formation in the two bivalve species are proceeding along several lines in our laboratory (*63*). We are examining the role of differential gene expression using differential display polymerase chain reaction (*64*), the role of the *p53* tumor suppressor gene, population genetics of the Maine softshell clams, and, of course, we are pursuing the identity and biological function of the dioxin-binding proteins.

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