Molecular Analysis of Bivalve Tumors: Models for Environmental/Genetic Interactions

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An increase in both the numbers and types of tumors found in finfish and shellfish has been noted in the past several decades. In many cases, while the increase in tumor incidence can be correlated with increases in aquatic toxicant levels, causality cannot be definitively proven. One recent epidemiologic investigation identified the prevalence of gonadal cancers as high as 40% in softshell clams (Mya arenaria) in Maine and 60% in hardshell clams (Mercenaria spp.) from Florida. A second study of these same geographic areas identified human mortality rates due to ovarian cancer as significantly greater than the national average. The rise in mortality rates in humans correlated with the increased use of herbicides in these areas as well as with the appearance of significant numbers of gonadal tumors in the clams. Studies were initiated in our laboratory to examine the molecular basis of these neoplasms in bivalves. NIH3T3 transfection assays were used to examine DNA isolated from these molluscan tumors for the presence of activated oncogenes. DNAs isolated from advanced tumors in both species were able to transform NIH3T3 cells and induce tumors in athymic mice. Studies are now underway to identify the gene(s) detected by these assays and also to examine the molecular mechanisms of toxic response of herbicide-exposed clams. — Environ Health Perspect 102(Suppl 1):81-83 (1994)

Key words: chemical carcinogenesis, reproductive tumor, transfection, oncogenes, Ah receptor, dioxin, herbicide, gonadal tumors

Introduction

Recent ERL/N (U.S. EPA Environmental Research Laboratory, Narragansett) field surveys identified epizootic germinomas in three geographically distinct populations of softshell clams (Mya arenaria) in eastern Maine (1). Investigation into the etiology of these tumors revealed that all three locations had been subjected to herbicide exposure. 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 forestry and in the culture of blueberries (1). 2,4-D and 2,4,5-T are not known to be potent carcinogens. However, 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 (2). Histologically similar tumors were reported in hardshell clams (Mercenaria spp.) taken from the Indian River, Florida (3). This estuarine system drains a wide area of citrus groves and receives substantial domestic and agricultural runoff. The suggested association between herbicides and molluscan gonadal neoplasms was strengthened by critical laboratory studies of bivalves that linked rapid uptake of chemical carcinogens, mutagenic activity, and tumor formation (4-6).

Additional reports support the hypothesis that exposure to environmental toxicants may contribute to tumor induction. Bullhead catfish taken from lakes near Indian River exhibited a variety of neoplasms of epidermal origin (7). Seminomas have been reported in military working dogs exposed to herbicides in Vietnam (8). More recently, a positive correlation was found between the incidence of malignant lymphomas in dogs and their owners' use of phenoxyacetic acid herbicides on their lawns (9). Furthermore, an EPA survey of cancer mortality rates in the United States indicated that the mortality rate due to ovarian and other reproductive organ cancers in human females from Washington County, Maine, and near Indian River was significantly higher than the national average (10). These are the same geographic areas in which the tumor-bearing clam populations are located. Together, these similar toxicologic effects at different phylogenetic levels suggest that herbicides, and/or the contaminating TCDD, may contribute to tumor formation.

These data are correlative and suggest that more detailed studies are warranted. The degree to which exposure to environmental toxicants induces cancer has long been debated and in most cases, is impossible to prove. One approach is a comparative study of the basic underlying mechanisms of chemical carcinogenesis. Recent advances in molecular techniques have shown that many of these mechanisms are evolutionarily conserved (11).

To address this hypothesis, we initiated investigations of the molecular mechanisms of tumor formation correlated with environmental exposure to herbicides in the two bivalve species. We approached this problem from two directions. First, we examined DNA from clam tumors for the presence of activated oncogenes using DNA transfection. Our second approach was to address the possible molecular mechanisms of herbicide toxicity. Since the symptoms observed in polycyclic aryl hydrocarbon/dioxin-exposed animals seem to arise from the interaction of these compounds with the Ah receptor (12), we focussed on identifying and characterizing an analogous system in the clam.

DNA Transfection Analysis

Softshell clams (M. arenaria) and hardshell clams (Mercenaria spp.) were collected from Dennysville, Maine, and the Indian River, respectively. Tissue sections were fixed in Helly's fixative and the histopathologic analysis determined as previously described (13). Tissue for DNA extraction was rapidly frozen in liquid nitrogen and stored
at −70°C. DNA was prepared by Quick-
Dounce homogenization as reported earlier
(14). DNAs from both cell species were
co-transfected with the plasmid pSV₂neo
into NIH3T3 cells using a calcium-phos-
phate precipitation procedure (14, 15)
modified from that of Graham and van der
Eb (16). Stable transfectants were selected
by growth in media containing G418
(Genetecin; Gibco/BRL). G418-selected
cells were pooled and assessed for transfor-
mation/tumorigenicity in three assays: stan-
dard focus assay, colony selection assay and
the nude mouse assay (17). Foci and
colonies were picked and expanded. High
molecular weight DNAs isolated from
transformed cells were used to initiate a sec-
ond transfection cycle as described previ-
ously (15). These data were confirmed by
repeating the secondary transfection cycle,
the results of which are presented in
Table 1. DNA isolated from cells which
were transfected with DNA from an
advanced tumor in Mercenaria spp. and by
both an early and an advanced tumor from
M. arenaria were able to transform
NIH3T3 cells in a secondary cycle of trans-
fec tion. This confirms our studies reported
previously (15). In addition, cells from the
same pool (M. arenaria advanced tumor
and Mercenaria spp. early tumor) were able
to produce colonies in the colony selection
assay when grown in the presence of low
(0.1%) serum (data not shown). Only
DNA from cells which were originally
transfected with M. arenaria early tumor
were able to induce tumors in nude mice
within a short latent period (one of two
mice at 6 weeks post-injection).

The results of our preliminary studies
presented above indicate that clon tumor

| Table 1. Secondary transfection of NIH3T3 cells with clon tumor DNA* |
|-----------------------------|-----------------------------|-----------------------------|
| DNA source                  | Standard focus assay,       | Cells plated in DMEM + Dex  |
|                            | no. foc/plate               |                             |
| Mya arenaria                |                            |                             |
| Reference animal            | 3                          | 0                           |
| Early tumor                 | 70                         | 7                            |
| Advanced tumor              | 33                         | —                            |
| Mercenaria spp.             |                            |                             |
| Reference animal            | 20                         | 1                            |
| Early tumor                 | 0.5                        | —                            |
| Advanced tumor              | 90                         | 6                            |

Abbreviations: DMEM, Dulbecco's modified eagle medium; Dex, dexamethasone. DNA isolated from primary transfectants was used in a second transfection cycle. Twenty micrograms of DNA were transfected per each plate. Cells in the standard focus assay were plated half in DMEM and half in DMEM + Dex of the addition of dexamethasone. Data from nude mouse experiments are discussed in the text. Results of the primary transfection assays and a previous secondary transfection cycle have been described earlier (15).

DNA is able to transform NIH3T3 cells. Although the identity of this gene(s) has not yet been determined, the data suggest that it is a highly conserved sequence since the gene is active in a mammalian transfection system. If our hypothesis is correct, that herbicide/dioxin exposure may be involved in form of some gonadal tumors by a mechanism conserved in different species, and that the identification of this gene would provide an important clue to this puzzle. Little is known about the molecular events in tumorigenesis in marine invertebrates. In women, however, ovarian carcinoma is one of the leading causes of death due to cancer (18) and its molecular basis is an active field of investigation. Activation of proto-oncogenes (K-ras, H-ras, c-my, HER-2/neu) and mutated alleles of the p53 tumor suppressor gene have been detected in ovarian tumors and ovarian tumor cell lines (19–21). The identification of the transforming genes in bivalves would enable us to compare their molecular mechanisms with those known in humans.

Identification of a Bivalve Ah Receptor

Studies are also underway to identify the bivalve analog of the Ah receptor and to define the role that it may play in these tumors. The Ah receptor has been identified in a number of vertebrate species including humans, rodents and trout (22). A number of genes which affect cellular growth and differentiation are in some way affected by interactions with the Ah receptor. Our pre-
liminary results of photoaffinity ligand-
binding assays (Brown et al., unpublished) suggest that Mercenaria possess cytosolic proteins which specifically bind a dioxin analog (23). The presence of an Ah recep-
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receptor.

The outcome of these studies awaits fur-
ther investigation. The role of herbicides in
the etiology of these tumors will be exam-
ined in controlled laboratory exposures. We are continuing to investigate the role of suppressor genes and activated oncogenes in the bivalve gonadal tumors as well as the possible link to the transcriptional activa-
tion activity of the Ah receptor.

REFERENCES

1. Gardner GR, Yevich PP, Hurst J, Thayer P, Benyi S, Harshbarger JC, Pruell RJ. Germinomas and teratoid siphon anomalies in softshell clams, Mya arenaria, environmentally exposed to herbicides. Environ Health Perspect 90: 43–51 (1991).

2. Schmidt KE. Dioxin's other face: portrait of an "environmental hormone." Science News 141:24–27 (1992).

3. Heselman DM, Blake NJ, Peters EC. Gonadal neoplasms in hardshelled clams, Mercenaria spp., from the Indian River, Florida: occurrence, prevalence and histopathology. J Invert Pathol 52:436–446 (1988).

4. Pittenger CA, Buikema AL Jr, Horner SG, Young RW. Variation on tissue burdens of polycyclic aromatic hydrocarbons in indigenous and relocated oysters. Environ Toxicol Chem 4:379–387 (1985).

5. Pittenger CA, Buikema AL Jr, Falkingham JO III. In situ variations in oyster mutagenicity and tissue concentrations of polycyclic aromatic hydrocarbons. Environ Toxicol Chem 6:51–60 (1987).

6. Gardner GR, Yevich PP, Harshbarger JC, Malcolm AR. Carcinogenicity of Black Rock Harbor sediment to the eastern oyster and trophic transfer of Black Rock Harbor carcinogens from the blue mussel to the winter flounder. Environ Health Perspect 90:53–66 (1991).

7. Harshbarger JC, Clark J. Epizootiology of neoplasms in bony fish of North America. Sci Total Environ 94:1–32 (1988).

8. Hayes HM, Tarone RE, Casey HW, Hussell DL. Excess of seminomas observed in Vietnam Service U.S. military working dogs. J Natl Cancer Inst 82:1042–1046 (1990).

9. Hayes HM, Tarone RE, Cantor KP, Jessen CR, McCurnin DM, Richardson, RC. Case-control study of canine malignant lymphoma: positive association with dog owner's use of 2,4-dichlorophenoxyacetic acid herbicides. J Natl Cancer Inst 83:1226–1231 (1991).

10. Riggan WB, Creason JP, Nelson WC, Manton KG, Woodbury MA, Stallard E, Pellein AC, Beaubier J. U.S. Cancer Mortality Rates and Trends, 1950–1979, Vol 4: Maps. EPA/600/ 1–83/013e, U.S. Environmental Protection Agency, Health

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Effects Research Laboratory, Research Triangle Park, NC (1987).

11. Yuspa SH, Poirier MC. Chemical carcinogenesis: from animal models to molecular models in one decade. Adv Cancer Res 50:25–70 (1988).

12. Roberts L. Dioxin risks revisited. Science 251:624–626 (1991).

13. Gardner GR. Chemically induced histopathology in aquatic invertebrates. In: Pathobiology of Marine and Estuarine Organisms (Couch JA, Fournie JW, eds). Boca Raton, FL: CRC Press, 1993:359–391.

14. Van Beneden RJ, Henderson KW, Blair DG, Papas TS, Gardner HS. Oncogenes in hematopoietic and hepatic fish neoplasms. Cancer Res (Suppl) 50:5671s–5674s (1990).

15. Van Beneden RJ, Gardner GR, Blake NJ, Blair DG. Implications for the presence of transforming genes in gonadal tumors in two bivalve mollusk species. Cancer Res 53:2976–2979 (1993).

16. Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adeno virus 5 DNA. Virology 52:456–467 (1973).

17. Blair DG, Cooper CS, Oskarsson MK, Eader LA, Vande Woude GF. New method for detecting cellular transforming genes. Science 218:1122–1125 (1982).

18. Barber HRK. New frontiers in ovarian cancer diagnosis and management. Yale J Biol Med 64:127–141 (1991).

19. Berchuck A, Kamei A, Whitaker R, Kerns B, Olt G, Kinney R, Soper JT, Dodge R, Clarke-Pearson DL, Marks P, McKenzie S, Yin S, Bast RC. Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. Cancer Res 50:4087–4091 (1990).

20. DiCioccio RA, Piver MS. The genetics of ovarian cancer. Cancer Invest 10:135–141 (1992).

21. Mazars R, Pujol P, Maudelonde T, Jeanteur P, Theillet C. p53 Mutations in ovarian cancer: a late event? Oncogene 6:1685–1690 (1991).

22. Lorenzen A, Okey AB. Detection and characterization of [3H]2,3,7,8-tetrachlorodibenzo-p-dioxin binding to Ah receptor in a rainbow trout hepatoma cell line. Toxicol Appl Pharmacol 106:53–62 (1990).

23. Poland A, Glover E, Kende AS. Stereospecific high affinity binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin by hepatic cytosol. J Biol Chem 251:4936–4946 (1976).