BIOMARKERS, 1999, VOL. 4, NO. 6, 485–491

Potential alterations in gene expression associated with carcinogen exposure in *Mya arenaria*

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Gonadal cancers in soft-shell clams (*Mya arenaria*) have been found at high prevalences (20–40%) in populations in eastern Maine. The aetiology of these tumours is unknown. We hypothesized that gene expression would be altered in gonadal tumours and that examination of gene expression patterns would provide some information as to the mechanism of tumour development. To investigate this hypothesis, we initiated a broad search for differentially expressed genes using differential display polymerase chain reaction (dd-PCR) to compare RNA from tumour and normal gonadal tissue. We identified two classes of genes whose expression may be altered in the gonadal tumours: genes involved in biosynthetic processes and genes with possible roles in signal transduction. We also investigated the hypothesis that environmental contaminants, such as tetrachlorodibenzo-*p*-dioxin (TCDD), may play a role in the development of these tumours. To investigate this hypothesis, we performed a short-term exposure of *M. arenaria* to [^{3}H]TCDD. Tissues were sampled up to 2 weeks after a 24-h exposure to 10 pptr or 2000 pptr of [^{3}H]TCDD in the water. Using dd-PCR, we identified potential alterations in expression of genes associated with cell proliferation: heparan sulphate proteoglycan, E3 ubiquitinating enzyme and p68 RNA helicase/initiation factor eIEF-4A. There were no observable histopathological alterations in gonadal or gill tissue from exposed animals. These results suggest possible early changes in gene expression indicative of environmental exposures.

Keywords: inducible gene expression, biomarker, bivalve, dioxin.

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Introduction

Epidemiological observations of soft-shell clams (*Mya arenaria*) from eastern Maine revealed unusually high prevalences of gonadal cancers (germinomas; Gardner *et al*. 1991). The only known common denominator for the sites where the clams were found was contamination of the watersheds by significant amounts of herbicides from silviculture, agriculture and roadside maintenance. The predominant herbicides used were the phenoxyacetic acid derivatives dichlorophenoxyacetic acid (2,4-D) and trichlorphenoxyacetic acid (2,4,5-T). The environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD/TCDD/dioxin) was identified as a by-product contaminant in the synthesis of 2,4,5-T. There is increasing evidence that chronic exposure to herbicides may play a role in tumorigenesis in many phylogenetically-divergent species such as catfish (Harshbarger and Clark 1990) and dogs (Hayes *et al*. 1991). As part of a larger investigation, we initiated experiments to assess the potential role of TCDD in the

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Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 1999 Taylor & Francis Ltd
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aetiology of gonadal neoplasia in *M. arenaria*. We hypothesized that in order for an environmental contaminant to produce gonadal pathology, the gonad must be exposed to the contaminant and that an early effect of exposure would be changes in gene expression.

In order to initiate a broad search for dioxin-responsive genes, we adapted the differential display-polymerase chain reaction (dd-PCR) method of Liang and Pardee (1992). This method detects relative differences in gene expression between populations, such as tumour-bearing clams and normal gonadal tissue or contaminant-exposed animals vs controls. dd-PCR does not require prior knowledge of the genes. Using differential display polymerase chain reaction (dd-PCR), we hoped to uncover novel and familiar biomarkers of TCDD exposure in a marine bivalve (Rhodes and Van Beneden 1996a, b).

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Methods

Analysis of naturally occurring tumours

Clams were collected from the Cobscook Bay region in eastern Maine where tumour prevalences have been determined as 20–40%. Samples of gonadal tissues were taken for histopathology in order to determine sex and confirm the presence or absence of tumour tissue. Slides were prepared by the University of Maine Diagnostic Laboratory. RNA was isolated from gonadal tissue by the method of Chomczynski and Sacchi (1987) and used in dd-PCR as described below.

Laboratory exposure studies

M. arenaria were exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-[1,6- ^{3}H] ([^{3}H]TCDD; 40 Ci mmol^{-1}, Radian Corp., Austin, TX) at the Environmental Protection Agency Laboratory in Narragansett, RI as described previously (Rhodes *et al*. 1997). Briefly, four exposure groups of 50 animals per tank were used: (1) control; (2) solvent control (250 ppm acetone); (3) 10 pptr [^{3}H]TCDD; and (4) 2000 pptr [^{3}H]TCDD. The animals were treated for 24 h under static, non-aerated conditions. Ten animals per exposure group were sacrificed at five post-exposure periods: 0 h (immediately at the end of the exposure period), and at 12 h, 24 h, 48 h and 2 weeks post-exposure. Tissue samples were taken from the gill, digestive gland, foot and gonad; ^{3}H levels were measured by liquid scintillation counting. A sample of gonad was prepared for histological examination.

dd-PCR

To assay for changes in gene expression, we used dd-PCR (Liang and Pardee 1992, Liang *et al*. 1992), modified as described previously (Rhodes and Van Beneden 1996a). RNA was isolated (Chomczynski and Sacchi 1987) from gill, a site of direct contact with water-borne TCDD, and gonad, a potential target for pathology. Differentially-expressed cDNA fragments were cloned and sequenced either by the Sanger dideoxy-mediated chain termination method using Sequenase 2.0 (US Biochemical, Cleveland, OH), or by cycle sequencing double-stranded templates with dye-labelled dideoxynucleotide terminators at the University of Maine Automated DNA Sequencing Facility. Database similarity searches were performed using the BLAST algorithm (Altshul *et al*. 1990, Gish and States, 1993).

Results

Differential gene expression in gonadal tumours

RNA was examined from 29 individual clams (16 with tumours; 13 with normal gonads). Twenty cDNA fragments were found which were amplified in animals with the gonadal neoplasm. The sequences of these cDNAs were compared with sequences in GenBank; those which had significant predicted amino acid identity (Poisson probability $< 10^{-17}$) are reported in Table 1 (see also Rhodes and Van Beneden 1996b, 1997).

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Table 1. Matches to the predicted amino acid sequences of dd-PCR products from *M. arenaria* gonadal neoplasms[a]

Gene	Poisson probability
L6 ribosomal protein (human, yeast)	$\leq 5.9 \times 10^{-34}$
Fibroblast growth factor receptor (FGFR; chicken)	$\leq 1.5 \times 10^{-27}$
Glycyl-tRNA synthetase (human, silkworm)	$\leq 2.5 \times 10^{-27}$
cGMP phosphodiesterase (cow)	$\leq 2.2 \times 10^{-25}$
Fibrillarin (human, mouse, yeast)	$\leq 9.6 \times 10^{-23}$
S19 ribosomal protein (human, rat, *Drosophila*)	$\leq 2.4 \times 10^{-17}$

[a] Modified from Rhodes and Van Beneden (1996b). The expression of these genes is increased in gonadal tumours relative to normal gonadal tissue as determined by dd-PCR. These are candidates for further study. Further characterization of S19 expression in *M. arenaria* and confirmation by Northern blotting has been described previously (Rhodes and Van Beneden 1997).

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Differential gene expression in TCDD-exposed clams

The temporal patterns of [^{3}H]TCDD concentration were strikingly different among the organs examined and have been described previously in detail (Rhodes *et al*. 1997). Elimination of [^{3}H]TCDD from the gill began immediately after exposure and continued over the 2-week sampling period. In contrast, gonadal concentrations of [^{3}H]TCDD were low immediately following the exposure period and steadily increased in both treatment groups. By 2 weeks post-exposure, concentrations of [^{3}H]TCDD were highest in the gonad, suggesting that the gonad may serve as a long-term reservoir of TCDD.

In vertebrates, response to TCDD exposure has been best characterized by the induction of CYP1A1 gene transcription (Stegeman and Hahn 1994, Whitlock *et al*. 1996). The regulation of other dioxin-responsive genes is less well understood (Dong *et al*. 1997). In order to screen for a wide range of up or down-regulated genes, alterations in mRNA levels associated with TCDD exposure were determined by dd-PCR. A total of 14 differentially-amplified gene fragments were identified from tissues collected at the 2-week sampling period. Three of these had excellent predicted amino acid identity ($p < 10^{-7}$) to sequences in protein databases (table 2). These include: (1) mRNA for an extracellular matrix protein (heparan sulphate proteoglycan) amplified in gills of control animals; and two cDNA fragments amplified in TCDD-exposed animals, (2) a regulator of RNA processing/translation (RNA helicase/translation initiation factor eIF-4A) and (3) a regulator of protein degradation (E3 ubiquitinating enzyme).

Discussion

Differential gene expression in gonadal tumours

Genes whose expression was altered in the gonadal tumour samples fell into two categories. The first includes those involved in biosynthetic pathways such as the gene for ribosomal protein S19. Elevated protein synthesis is associated with rapidly dividing cells and may contribute to proliferation of tumour cells. In humans, increased expression of S19 has been found to be a marker for colon carcinoma (Kondoh *et al*. 1992). Increased expression of other ribosomal proteins has been observed in colorectal tumours, malignant haemopoietic neoplasms, rectal, stomach, breast, and bladder cancers (Pogue-Geile *et al*. 1991, Shimbara *et*

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Table 2. Matches to the predicted amino acid sequences of dd-PCR products from *M. arenaria* exposed to ^{3}H-TCDD[a]

Gene	Source of RNA	Expression pattern in exposed clams	Poisson probability
Heparan sulphate proteoglycan (human, yeast)	Gill	Down-regulated	$\leq 7.0 \times 10^{-8}$
E3 ubiquitinating enzyme (human)	Gill	Up-regulated	$\leq 7.4 \times 10^{-53}$
p68 RNA helicase/initiation factor eIF-4A (human, yeast, *Drosophila*)	Gonad	Up-regulated	$\leq 3.0 \times 10^{-34}$

[a] Modified from Rhodes *et al.* (1977).
These genes are candidates for those whose expression may be altered by exposure to ^{3}H-TCDD. Tissues were harvested 2 weeks post-exposure.

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al. 1993, Chassin *et al.* 1994). A neoplastic phenotype induced by ribosomal proteins, however, has not yet been demonstrated. Therefore, enhanced S19 mRNA levels in gonadal neoplasms of *M. arenaria*, are probably a result, rather than a cause, of the neoplastic phenotype.

The second class of elevated mRNAs includes those genes with sequence identity to signal transduction genes, which includes clam sequences with amino acid identity to the fibroblast growth factor (FGF) receptor and a cGMP-activated phosphodiesterase (see table 1 and Rhodes and Van Beneden 1996b). Expression of the human FGF receptor has been used as a biomarker for human pancreatic cancers (Korc 1998). The cGMP-activated phosphodiesterase has a role in signal transduction pathways (Degerman *et al.* 1997).

Differential gene expression in TCDD-exposed clams

Although there has been considerable work done on biotransformation enzymes (Livingstone 1991), little is known about other bivalve genes which may be responsive to environmental exposure. Using dd-PCR, three gene fragments were identified with strong identity ($p < 10^{-7}$) to genes in the protein data bases (table 2). The first, a cDNA with sequence identity to heparan sulphate proteoglycan, was down-regulated in gills of exposed animals. Down regulation of hevin, an antiadhesive extracellular matrix protein, has been reported in human metastatic prostate adenocarcinoma and transformed prostate epithelial cell lines (Nelson *et al.* 1998). In *M. arenaria*, focal areas of hyperplasia and lamellar fusion in the gills have been observed in clams collected from a TCDD-contaminated site in New Jersey estuary and after laboratory exposure to TCDD (Brown 1992). Changes in expression of extracellular matrix components may represent an early stage of cell damage.

A second messenger RNA up-regulated in gonads of animals sampled 2 weeks post-exposure showed sequence identity to the gene for p68 RNA helicase/translation initiation factor eIF-4A (Ford *et al.* 1998). This protein is involved in unwinding RNA secondary structure which suggests an increase in RNA processing or translation, even though no visible histopathologic changes were observed. A third messenger RNA from gill was similar to an E3 ubiquitinating enzyme that recognizes the tumour suppressor protein p53 (Huibregtse *et al.*

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1995). These enzymes mediate the ligation of ubiquitin to proteins, targeting them for degradation. Decreased *p53* protein levels affect a critical checkpoint in the cell cycle (King *et al.*, 1996). The identification of these genes potentially regulated by TCDD is consistent with its role as a tumour promoter. Further studies into the relationship of changes in gene expression, pathological alterations and tissue-specific uptake of TCDD will help to define any causal association between this environmental contaminant and its biological effects.

Application of these techniques to the development of new biomarkers for bivalves

Bivalves have historically been important sentinel species for water quality. Adults are long-lived and sessile, with a filter-feeding strategy that exposes them to both suspended and soluble compounds. Since bivalves exhibit low mono-oxygenase activity, these compounds are slowly eliminated and therefore tissue burdens can serve as integrative indices of bioavailable contaminants (Livingstone *et al*. 1989, Stegeman and Lech, 1991). Extensive investigations have focused on the development of biomarkers for environmental exposures (Ringwood *et al*. 1999). Classic examples of differential gene expression in response to changes in environmental parameters include the stress proteins (Wu 1995). Organisms respond to elevated temperatures and to chemical and physiological stresses by an increase in the synthesis of this family of proteins. Metallothioneins have also been used as biomarkers for heavy metals. Metallothionein is a thiol-rich protein that has been well characterized for its ability to bind and sequester heavy metal cations, free radicals and other reactive toxicants. (Roesijadi and Robinson 1994).

Usefulness of gene expression changes as biomarkers for environmental exposure has been revolutionized and expanded by recent advances in molecular biology, including PCR-based techniques. These advances have greatly impacted research in toxicological pathology. Recent innovative methods utilizing PCR technology have been developed to detect mutations in neoplastic cells, to study biomarkers of genetic susceptibility, genes involved with carcinogen metabolism, to detect gene mutations, to find novel genes induced by chemical exposure and to characterize and quantitate gene expression (Malarkey and Maronpot 1996). These new and evolving techniques offer tremendous potential in providing new insights into the molecular basis of toxicity and in the development of new biomarkers.

Differential display has been used extensively to detect differentially-expressed genes. However, this technique has numerous disadvantages including the low reproducibility of displayed bands, isolation of false positive and labour-intensive verification by Northern blots. Modifications to dd-PCR appear almost weekly in the literature which aid in reproducibility, efficiency of techniques (see, for example, Yoshikawa *et al*. 1998). Genes have been identified by dd-PCR whose expression is altered by exposure to toxic chemicals (Dong *et al.* 1997, Lu *et al.* 1997).

The correlation of differential gene expression with exposure of an organism to environmental contaminants may provide another class of molecular biomarkers. Pathological changes in many species are frequently linked by common molecular pathways which regulate cell growth, cell differentiation and cell death. A comparative approach would allow determination of similarities and differences in the toxic response in different animal models and enable us to make a rational extrapolation to assess environmental and human health.

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Acknowledgements

We thank Dr B. Beal and D. Clifford for help in collecting animals. We are indebted to L. L. Clepper, Dr D. J. Brown, D. Borsay, M. Berthiaume and S. Roderick for their assistance with the exposure study. This research was supported in part by NIH grant R01 RR08774.

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